Chapter 4

Electron Tomography and Correlative Approaches in Platelet Studies

Kasia B. Engberts, Cor Seinen, Willie J. C. Geerts, and Harry F. G. Heijnen

Abstract

Blood platelets play a central role in the arrest of bleeding and the development of thrombosis. Unraveling the complex processes of platelet biogenesis from megakaryocytes, platelet adhesion, aggregation, and secretory responses are important topics in the field of hemostasis and thrombosis. Analysis of the ultrastructural changes that occur during these processes is essential for understanding the rapid membrane dynamics and has contributed substantially to our present knowledge of platelet formation and functioning. Recent developments in real-time imaging, correlative light and electron microscopy imaging (CLEM), and 3D (cryo) electron microscopy and tomography offer exciting opportunities to improve studies of the platelet adhesive responses and secretion at the ultrastructural level in a close to native environment. In this chapter we discuss and illustrate cryo preparation techniques (high-pressure freezing, vitrification), correlative LM and EM workflows, and 3D cryo-electron tomography that we apply in our current research projects.

Key words TEM, Immuno-EM, Fixation and cryo-immobilization, High pressure freezing EM tomography, Cryo-EM, Correlative light and electron microscopy (CLEM)

Abbreviations

EM	Electron microscopy
ET	Electron tomography
CLEM	Correlative light and electron microscopy
FS	Freeze substitution
HPF	High-pressure freezing
LN2	Liquid nitrogen
CCD	Charge coupled device
3D	Three-dimensional
vWF	von Willebrand factor
SIRT	Simultaneous iterative reconstruction technique

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Terminology

Missing Wedge	Missing information due to limited tilt angles
Contouring	Manual drawing of contour lines in slices of a tomogram
Tomogram	Computed 3D volume reconstruction of a specimen by using multiple projection images

1 Introduction

Platelet biogenesis, platelet adhesion, aggregation, and the secretory responses are complex processes. The unraveling of these processes is an important goal for researchers in the field of hemostasis and thrombosis. Structural and ultrastructural information of platelets is essential for the understanding of the rapid membrane dynamics that occur during these events (see ref. 1 and citations therein for a recent overview). Transmission electron microscopy (TEM) methods have contributed enormously to our present knowledge of the platelet ultrastructure and remain an essential tool to provide insight into the mechanisms of these membrane dynamics. Due to the short wavelength of electrons, TEMs have a much higher resolution, i.e., the ability to observe two closely positioned organelles as discrete structures, than light microscopes (LM). For comparison, the *x*-*y* resolution of the human eye, the light microscope, the scanning electron microscope, and the transmission electron microscope are in the order of >0.2 mm; >0.2 μm; >1 nm; and >0.2 nm respectively.

Conventional TEM approaches use ultrathin sections (60–70 nm) of usually chemically fixed and plastic-embedded cell and tissue samples to study the subcellular structure and dynamics of organelles. These methods have been applied in numerous platelet and megakaryocyte (MK) studies [2–12]. Although still valuable, conventional TEM approaches provide only ultrastructural images of the cells with limited resolution in the *Z*-axis. Furthermore, the relative low fixation speed and harsh fixation protocols (e.g., glutaraldehyde and OsO_4) induce ultrastructural artifacts [13, 14], and the embedding in epoxy resins limits antibody access and thus detection of the molecular distribution of proteins [15]. The cryosectioning and immunogold labeling technology (IEM) as developed by Tokuyasu and the group of Slot and Geuze [16, 17] overcomes this latter problem and is now an accepted and widely used technique for locating molecules in their subcellular context with nanometer resolution. IEM has been perfected since its first use and now surpasses classical resin TEM techniques, combining optimal membrane preservation with high labeling sensitivity [18].

Over the past decade, real-time imaging and 3D electron tomography (ET) have increasingly replaced conventional light microscopy and immunofluorescence microscopy (LM and IF) and

2D transmission electron microscopy, respectively. In addition, fast freezing technologies (vitrification, high-pressure freezing, HPF) have become available to arrest cellular membrane dynamics within milliseconds, thereby providing a physiological “snapshot” of the cell without the artifacts produced by chemical fixation [19–21]. Fast immobilization methods, combined with (cryo) TEM tomography and 3D reconstruction allow the snap-frozen structures to be reconstructed into 3D models [22–24]. This led to the development of new approaches that combine light microscopy imaging with high-resolution (cryo) electron microscopy (correlative light and electron microscopy, CLEM) [25, 26]. CLEM utilizes the two complementary visual techniques, enabling visualization of dynamic cellular processes by LM/IF with direct coupling to high-resolution ultrastructural imaging representing these cellular events. This chapter describes the preparation methods for entire platelet vitrification, HPF for platelets and whole bone marrow, and methods for (cryo)correlative imaging and electron tomography. In addition, the future direction of modern EM technologies is discussed.

2 Materials

The different sample preparation procedures that we currently use in our research projects are depicted schematically in Figs. 1 and 2. Figure 1 shows the classical chemical fixation procedures and the high-pressure freezing technology (HPF). These methods are applicable for cells in suspension (isolated platelets and cultured MKs) as well as whole bone marrow. Fixation is followed by either plastic embedding (lane A), or by cryosectioning and immunogold labeling (Tokuyasu method, lane B). The preparation method for HPF-FS is shown in lane C. Figure 2 shows two CLEM procedures to study adherent platelets. The protocols are given below.

2.1 Human Platelets

1. 0.1 M sodium citrate anticoagulant.
2. Acid/citrate/dextrose anticoagulant: 85 mM sodium citrate, 71 mM citric acid, and 111 mM D-glucose.
3. Prostacyclin (Cayman Chemical, USA): stock 25 μL aliquots of 10 μg/mL stored at −80 °C (*see* **Note 1**).
4. Cell analyzer for assessment of mean platelet volume (MPV) (e.g., Abbott Cell-Dyn 1800).
5. 0.1 M TRIS-buffered saline (TBS, pH 9.0): stored as frozen 225 μL aliquots.
6. Modified Tyrode buffer: 129 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$ (pH 6.5 and pH 7.3).

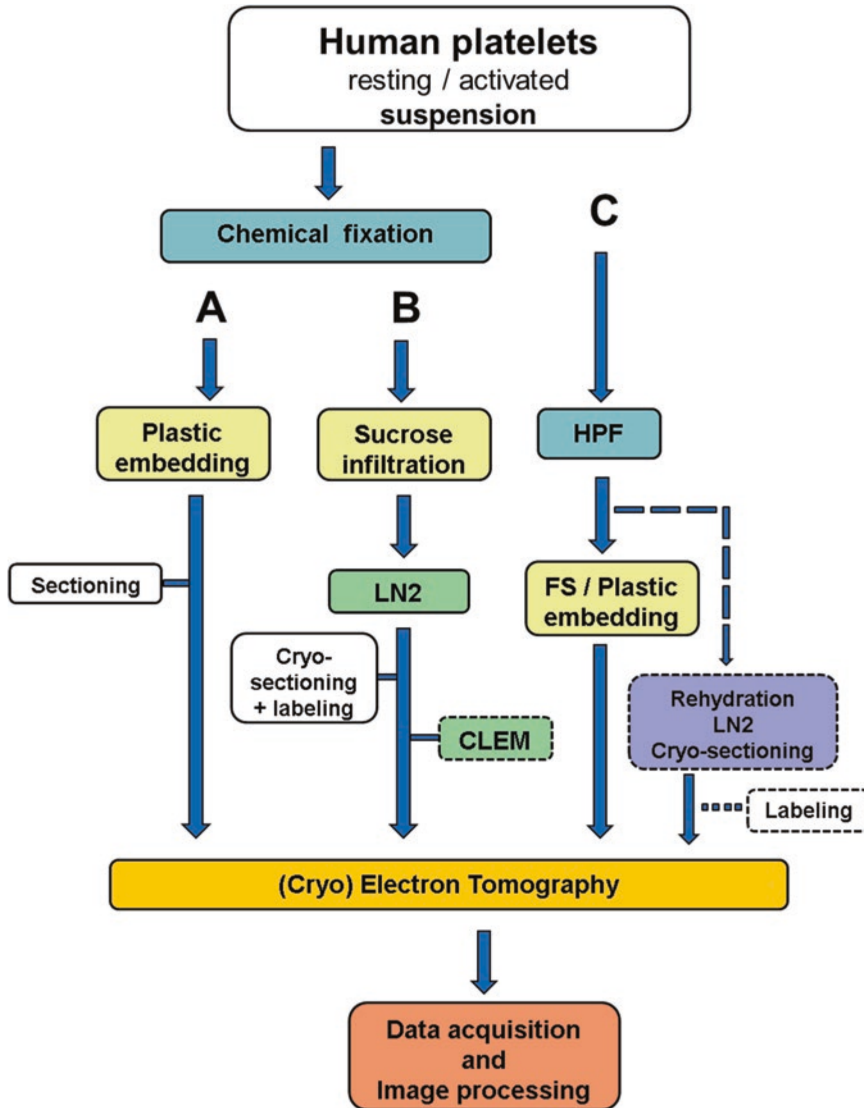

Fig. 1 Schematic overview of the preparation procedures for resting and activated platelets in suspension. (**a**) Classical chemical fixation followed by plastic embedding. (**b**) Cryosectioning and immunogold labeling (Tokuyasu method). (**c**) HPF-FS. Note that chemical fixation and HPF-FS can also be applied to the study of cells in whole bone-marrow or isolated MKs. For details of the procedures see text

2.2 Megakaryocytes and Other Marrow Cells

1. 8- to 10-week-old Balb/c mice (*see* **Note 2**).
2. Instrumentation for perfusion: fixation (refer to Chapter 14 this volume).
3. Dissection instruments for removal of femurs.
4. 0.1 M sodium cacodylate buffer. Stock solution: 0.4 M (8.56 g Na-cacodylate in 100 mL Milli-Q® water) (*see* **Note 3**).

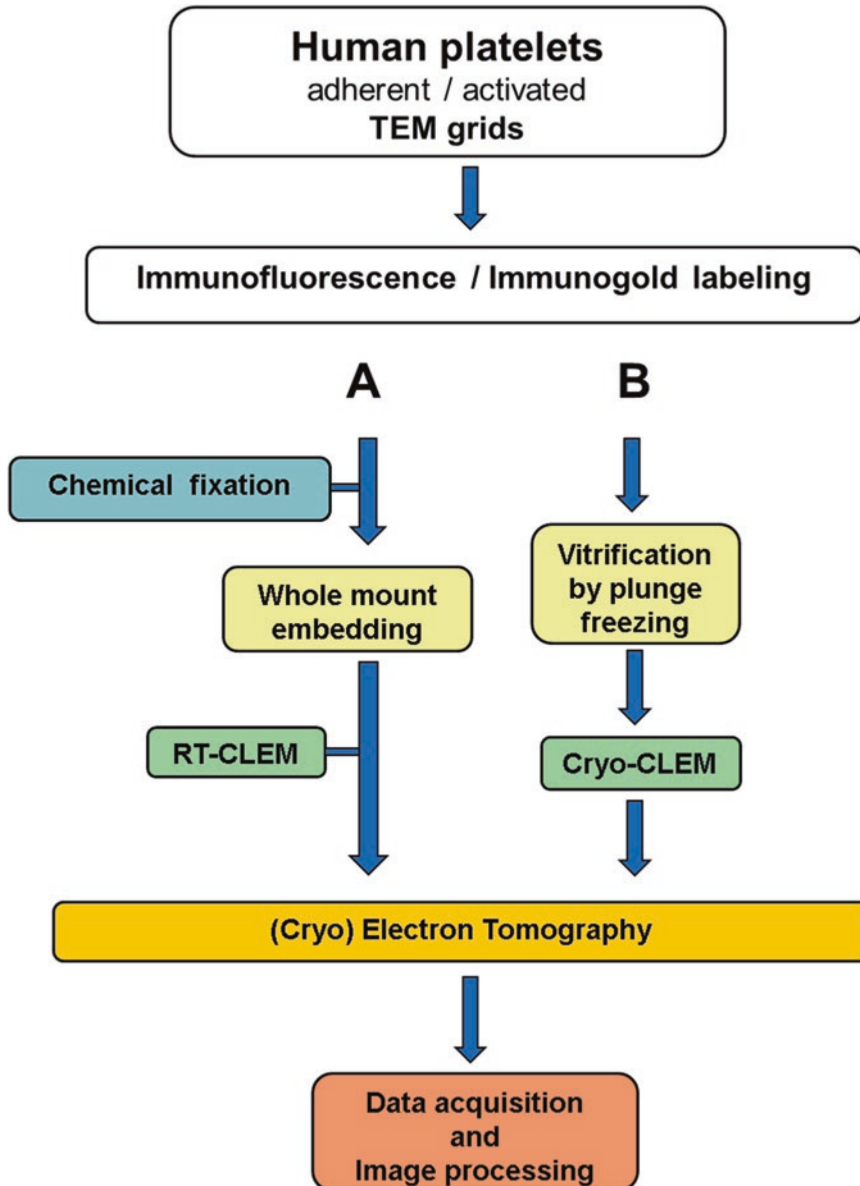

Fig. 2 Schematic overview of two CLEM procedures used for studying platelet adhesion to a physiological substrate. (**a**) whole mount room temperature CLEM. (**b**) cryo-CLEM procedure. For details of the procedures see text

2.3 Resin Embedding and Sectioning

1. Graded series of ethanol (70%, 90%, 96%, 100% in Milli-Q® water).
2. 1,2-propylene oxide.
3. Epon-812 embedding resin.
4. Flat embedding molds (clear silicone, e.g., EMS Cat#70900).
5. Ultramicrotome using a diamond knife.

6. Formvar and carbon-coated 200 mesh copper grids (Agar Scientific, Essex, UK).
7. Uranyl acetate: 0.5% in Milli-Q® water.
8. Lead acetate: 3% in Milli-Q® water.

2.4 Tokuyasu Method

1. 0.2 M phosphate buffer (pH 7.4): 9.5 mL 0.2 M NaH_2PO_4 and 40.5 mL 0.2 M Na_2HPO_4 (pH adjusted to 7.4 by the two components).
2. Fixative solution: 2% paraformaldehyde (PFA) and 0.2% monomeric EM-grade glutaraldehyde (GA) in 0.1 M phosphate buffer.
3. PHEM buffer: 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM $MgSO_4$, adjusted to pH 7.0 with 5 M KOH.
4. 12% gelatin dissolved in PHEM buffer at 37 °C.
5. Sucrose-based cryoprotectant solution: 2.3 M sucrose in 0.1 M phosphate buffer (pH 7.4).
6. Small pins suitable for securing 1 mm^3 gelatin blocks.
7. Any type of Whatman filter paper (to remove excess sucrose).
8. Liquid nitrogen storage system.
9. A cryo-ultramicrotome: e.g., Ultracut-S, Leica Microsystems, Vienna, Austria.
10. Mixture of 2% methylcellulose and 2.3 M sucrose (Subheading 2.4, **item 5**).
11. If carrying out simultaneous immunohistochemistry for CLEM: antibodies tagged with fluorophores and/or 10 nm protein A gold (Cell Microscopy Core, UMCU, Utrecht, the Netherlands (http://www.cellbiology-utrecht.nl/products.html).
12. 2% uranyl acetate or 2% uranyl oxalate. Both are dissolved in Milli-Q® water with the pH adjusted to 7.0 with 25% NH_4OH.
13. Mixture of 1.8% methylcellulose and 0.3% uranyl acetate in Milli-Q® water.

2.5 High Pressure Freezing

1. HPF equipment is available from several providers (Leica Microsystems, Vienna, Austria; Baltzers, Liechtenstein). We have used the Leica EMPACT2 high pressure freezer and the Leica EM AFS2 freeze substitution apparatus.
2. Flat HPF specimen carrier (0.2 mm deep and 1.2 mm diameter carrier).
3. 20% human albumin serum (HAS) solution: 20% w/v in Hepes-Tyrode solution + glucose 1 mg/mL.
4. Cryo substitution apparatus (AFS, Leica Microsystems).
5. 1.5 mL micro tubes.

6. Acetone-based substitution medium (95, 90, 80, and 70% (v/v) acetone in Milli-Q® water).
7. Additional fixatives. We prefer a mixture of 0.5% glutaraldehyde (GA), 0.25% uranyl acetate (UA), 1% OsO_4 (osmium) and 3% H_2O in anhydrous acetone (Merck).
8. PHEM buffer (*see* Subheading 2.4, **item 3**).
9. 50% and 30% (v/v) acetone in PHEM buffer.
10. Low melting point agarose (2%) in 0.1 M phosphate buffer.

2.6 Whole Mount Protocol

1. EM grids: either 200 mesh carbon-coated formvar grids (Agar Scientific, Essex, UK), gold quantifoil grids (R2/2 Cat# S173-7 or R3.5/1 Cat#S177-7 from PLANO, GmbH or R2/2 from Ted Pella, USA), or gold lacey carbon grids (Cat# LC300Au25, van Loenen Instruments, the Netherlands). For correlative approaches carbon-coated gold finder grids can also be used (Ted Pella, USA) (*see* **Note 4**).
2. Glow discharging unit (e.g., Edwards auto 306 HT).
3. Method of holding the grids for glow discharging: e.g., clamping in sheets with flexible sluts (Leica AC20 sheets are preferred), or alternatively they can be glued via their edges to double-sided tape.
4. Fibrinogen: 100 μg/mL.
5. Hepes Tyrode buffer (129 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3).
6. Blocking buffer: 1% BSA in Hepes Tyrode buffer with 1 mg/mL glucose, pH 7.3.
7. Humidifier apparatus such as a glass beaker or petri dish placed upside-down on wetted filter paper.
8. Whatman filter paper.
9. For vWF surface labeling: primary anti-vWF antibody and 10 nm protein A gold. (In the case of monoclonal anti-vWF, an intermediate bridging antibody should be used).

2.7 Correlated Light and Electron Microscopy (CLEM)

1. CLEM Microscope system such as the iCorr™ (FEI, Eindhoven, The Netherlands).

   The basic goal in CLEM studies is that regions of interest, identified by their fluorescent signal at low magnification by fluorescence microscopy (FM), can be subsequently analyzed at the ultrastructural level with TEM. There are several ways to perform CLEM. The approach that we describe here is based on the use of the iCorr™ (Fig. 3a–c, Supplementary Movie 1) [27, 28]. The iCorr™ (FEI Company, Eindhoven, The Netherlands) is a prototype that involves a Tecnai 12 Twin transmission electron microscope, equipped with a fully

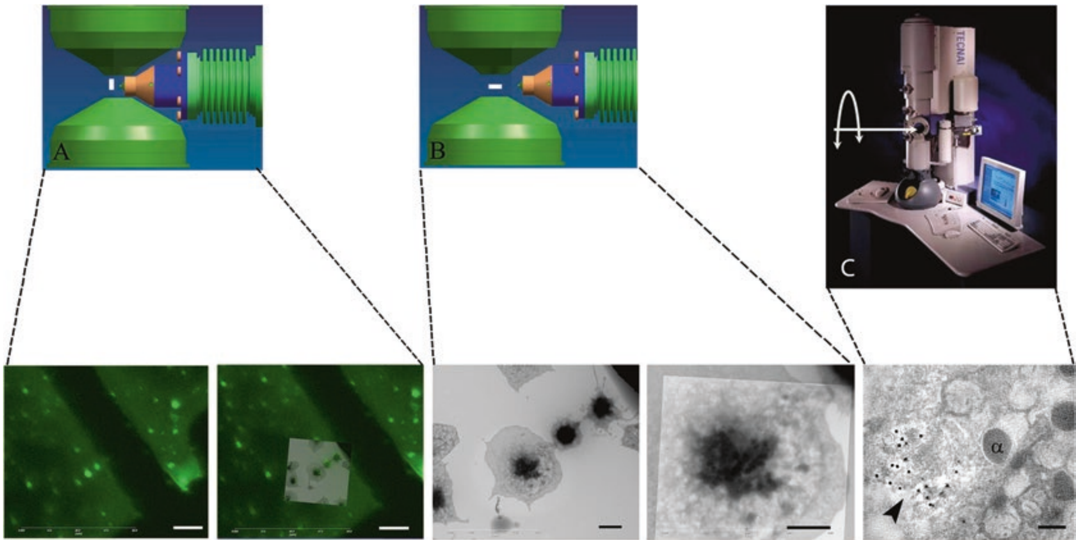

Fig. 3 RT-CLEM of platelet whole mounts. Platelets spread on fibrinogen-coated EM supports are sequentially immunolabeled with Alexa 488-conjugated anti-vWF and 10 nm protein A gold. (**a**) Adherent platelets undergoing vWF secretion (highlighted region with fluorescent dots) are imaged at ambient temperature using a Tecnai 20 with integrated IF microscope (iCorr^{tm} FEI Company). (**b**) The IF objective is withdrawn from the EM column and the grid is switched 90° and EM overlays of ROIs representing vWF release are stored on the computer using the iCorr software package mode. (**c**) Dual axis tilt series are recorded, aligned, and reconstructed using the IMOD software package. Arrowhead in the highlighted right panel shows immunogold labeled vWF on the surface of the adherent platelet. Bars from left to right **a**, 12.5 μm; **b**, 2.5 μm and 1.25 μm; **c**, 200 nm. *See* Supplementary Movie 1

integrated LED-based wide field fluorescence microscope (excitation light ranging from 460 to 500 nm) located at the normal sample position in the TEM column (Fig. 3a). This setup allows for consecutive acquisition of fluorescence and TEM data (Fig. 3b) on the same grid within the same microscope and enables the direct correlation of the fluorescent signal with ultrastructural features. The iCorr™ microscope is equipped with an Eagle 4x4k CCD camera. Correlative imaging is performed using the iCorr work flow [27, 28], which creates a shared coordinate system for IF labeled ROIs and TEM data recordings. Using the common EM specimen stage, the sample is tilted 90° followed by insertion of the objective lens of the optical unit close to the specimen. Light microscopic images are then recorded in the fluorescence mode and stored on the computer. After fluorescence imaging (Fig. 3a), the iCorr™ is switched to TEM mode by which the optical element is retracted and the specimen holder is rotated back to its 0° position (Fig. 3b). The iCorr™ system is suited for RT and frozen-hydrated samples. For cryo sampling the specimen is inserted in a cryo specimen holder (Gatan 626, Gatan, Abingdon, UK), ensuring that the speci-

men remains vitrified. The iCorr™ is also equipped with the tomography data acquisition software package Xplore 3D (FEI Company, Eindhoven, the Netherlands). This allows us to immediately record tomographic data sets from the fluorescently identified regions of interest.

In our studies platelets are allowed to spread on Au-carbon-coated grids that have been functionalized with a physiological substrate (fibrinogen, vWF). The adherent platelets are simultaneously labeled with antibody-conjugated fluorescent dyes and gold particles, and sequentially imaged in cryo FM and TEM mode in the iCorr electron microscope (Figs. 3 and 4). Correlative approaches can be performed at room temperature (Fig. 3) or under cryo conditions (Fig. 4), provided that the samples are thin enough to be visualized in the TEM. CLEM can also be applied on semi-thin Tokuyasu sections (Fig. 1, lane B). This can be very useful in whole bone marrow for example, to identify specific target cells and/or MK maturation stages in the crowded environment of the bone marrow, or to identify MKs that have been transfected to express specific GFP-tagged proteins. In all these cases specific ROIs are first selected in IF mode and next analyzed by electron tomography for ultrastructure analysis. We here describe the protocol for two CLEM approaches for whole adherent platelets using iCorr, but other approaches that make use of finder grids and specific navigation software (MAPS and CorrSight, FEI Company) can also be used [29].

2. Materials as described in Subheading 2.6, **items 1–9**.
3. Parafilm.
4. Alexa 488-conjugated anti vWF or anti-fibrinogen antibodies.
5. 10 nm protein A gold.

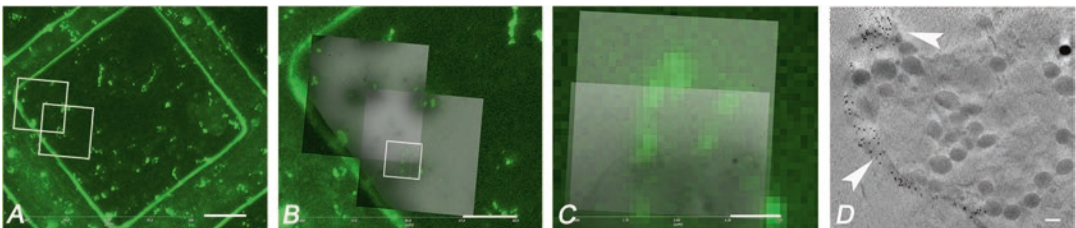

Fig. 4 Cryo-CLEM of platelets adhering to a fibrinogen substrate. The platelets are immunolabeled with Alexa 488-conjugated anti-vWF and 10 nm protein A gold. The grids are immediately plunge-frozen in liquid ethane, transferred to a Gatan cryo-holder, and imaged in the cryo-stage of a Tecnai 20 with integrated IF microscope (iCorr™ FEI Company). Regions of interest are recorded in both IF and EM mode using the iCorr software package. (**a**) Low magnification overview recorded in IF mode. (**b**) Highlighted areas representing spread platelets with released vWF (IF dots). (**c**) High magnification with selected EM overlay of a single platelet suitable for cryo electron tomographic recording. (**d**) Tomographic slice of area outlined in C taken after the tilt series recording. The black dots represent immunolabeled vWF on the surface of the spread platelets and are used as fiducial gold markers to aid alignment of the tilt series. Bars: **a**, 25 μm; **b**, 12.5 μm; **c**, 1.75 μm; **d**, 200 nm

2.7.1 RT-CLEM (Fig. 2, Lane A)

1. 2% PFA and 0.2% GA in 0.1 M phosphate buffer (*see* Subheading 2.4, **item 2**).
2. 2% uranyl acetate (pH 7) or uranyl oxalate (pH 7), both dissolved in AD, (Aqua Dest distilled water by Medicalcorner24®), pH adjusted with 1 M NaOH.
3. Mixture of 1.8% methylcellulose and 0.3% uranyl acetate in Milli-Q® water.
4. Whatman filter paper.

2.7.2 Platelet Vitrification and Cryo CLEM (Fig. 2, Lane B)

Since resting platelets in suspension are too thick for whole cell vitrification we use platelets spread on a fibrinogen substrate (*see* Subheadings 3.3.4 and 3.3.5; Materials as described in Subheading 2.6, **items 1–9**. Gold holey carbon quantifoil R2/2 (Ted Pella, USA) or lacey carbon grids (van Loenen, the Netherlands) (*see* **Note 5**)).

1. Apparatus for vitrification such as the Vitrobot Mark IV (FEI, Eindhoven, The Netherlands).
2. Whatman grade 4 qualitative filter paper, pore size 20–25 μm.
3. Liquid ethane and liquid nitrogen.
4. Cryo boxes suitable for storage of grids.

2.8 Equipment for Cryo Electron Tomography and Analysis

The specialized equipment required for cryo ET is discussed in Subheading 3.4. For acquisition this includes a high-tilt cryo tomography holder (e.g., Gatan 914 from Gatan Inc., USA) and a TEM (e.g., Tecnai 12 Twin TEM or Tecnai 20 STEM, equipped with a 4x4K CCD Eagle camera; FEI Company, USA). In addition, specialized software is required for automated data acquisition and data analysis such as the Xplorer 3D package (FEI, Eindhoven, the Netherlands) and the IMOD package [30] from Colorado University (Boulder, USA).

3 Methods

3.1 Preparation of Washed Human Platelets

First step in studying platelet adhesion and activation processes is the isolation of platelets from whole blood (Fig. 1).

1. Whole blood is drawn by venipuncture from healthy volunteers into 0.105 M sodium citrate anticoagulant (1 part acid citrate to 9 parts blood) (*see* **Note 6**).
2. Centrifuge the whole blood at 160 × *g* for 15 min and remove the upper platelet-rich plasma (PRP).
3. Determine the mean platelet volume (MPV) in the PRP using a cell analyzer.
4. Add 1/10 volume of ACD buffer (containing 85 mM sodium citrate, 71 mM citric acid and 111 mM D-glucose) and mix gently.

5. The platelets are isolated from the PRP by centrifugation at 360 × *g* for 15 min.
6. The pellet is resuspended in modified Tyrode buffer, pH 6.5.
7. Thaw a 25 μL aliquot of 10 μg/mL prostacyclin and add 225 μL 0.1 M TRIS-buffered saline (TBS, pH 9.0) and keep on ice.
8. Add prostacyclin 0.1 μg/mL (final concentration) to the suspension.
9. The platelets are washed once via centrifugation (360 × *g* for 15 min) and suspended in modified Tyrode buffer, pH 7.3.
10. Remeasure the MPV, count the number of platelets and adjust to the desired concentration (*see* **Note** 7).
11. Usually the platelet concentrations required for HPF of resting cells should be in the range of 600–2000 × 10^9/L. For the spreading assays onto grids much lower densities are used (≈100 × 10^9/L).

3.2 Preparation of Bone Marrow Megakaryocytes

Follow local ethical guidelines and regulated procedures for perfusion fixation of 8–10 week old BALB/c mice (*see* Chapter 14, this volume for further details of a perfusion fixation protocol).

Dissect out the femurs, cut the epiphyses and flush with 0.1 M sodium cacodylate buffer into a petri dish using a syringe (*see* Chapters 12 and 13 for further guidance on flushing marrow).

Immediately process small pieces of the flushed marrow for classical resin embedding (Subheading 3.3.1), the Tokuyasu method (Subheading 3.3.2), or the HPF-FS method (Subheading 3.3.3).

3.3 Platelet Preparation Procedures

Platelets have been analyzed at the ultrastructural level for many years with what we now call "conventional electron microscopical methods" (Fig. 1, lane A). The conventional approach is based on chemical fixation, followed by resin embedding, sectioning of thin sections, contrasting with heavy metals and then transmission electron microscopy. This is still a widely used approach but in the last decade several alternative approaches and data recording and analysis methods have been developed and applied by us and others in platelet research (see Fig. 3 for comparison of the different fixation protocols).

3.3.1 Protocol for Resin Embedding (Fig. 1, Lane A)

1. Centrifuge the platelets 1–2 min @ 8 K RCF in warm (approximately 40 °C) low melting point 2% agarose in 0.1 M Phosphate buffer. Typically a 1 mL suspension of ≈600 × 10^9/L is sufficient to obtain a pellet for resin embedding.
2. Solidify by placing the vial with pelleted platelets on ice.
3. Isolate the platelet pellet and dehydrate at room temperature in a graded series of ethanol (70%, 90%, 96% for 15 min and 3 × 30 min in 100%, respectively).

4. Transfer the pellets into 1,2-propylene oxide (RT, 2 × 10 min).
5. Infiltrate the pellets with a series of resin-propylene oxide mixtures (1:3, 1:1, and 3:1, respectively) for 1 h each. We generally use Epon-812 as the embedding resin.
6. Finally, transfer the platelet pellets into pure resin for overnight infiltration.
7. The next day the resin is refreshed and the platelet pellets are incubated for another 2 h before resin polymerization in flat embedding molds is performed at 60 °C.
8. After polymerization, section the resin blocks on an ultramicrotome using a diamond knife.
9. Collect serial gray sections floating in a water trough on formvar and carbon-coated 200 mesh copper grids.
10. Stain with heavy metals to provide contrast to the membranes, usually a combination of uranyl acetate followed by lead citrate (*see* **Note 8**). Conditions: 35 min at 45 °C with 0.5% uranyl acetate, followed by thorough rinsing with Milli-Q®, 20 min at 25 °C, with 3% lead citrate, followed by rinsing with Milli-Q® water and air drying.

3.3.2 Tokuyasu Method (Fig. 1, Lane B)

The Tokuyasu technique is named after his inventor Kiyoteru Tokuyasu and is further optimized in the lab of Slot and Geuze. Since then the method has become the method of choice for high-resolution immunogold localization studies of frozen specimens (see for details of the procedure [18]).

1. Platelets are mildly fixed with a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer for 60 min followed by centrifugation into a pellet.
2. After washing with 0.1 M phosphate buffer the pellet is immersed for 10 min at 37 °C in 12% gelatin in PHEM buffer.
3. After gelation at 4 °C, small blocks (1 mm^3) are trimmed and infiltrated with a sucrose-based cryoprotectant solution at 4 °C.
4. Individual blocks are placed on small pins and excessive sucrose solution is removed with a Whatman filter paper.
5. The samples are frozen in liquid nitrogen and stored until cryo sectioning.
6. A cryo-ultramicrotome is used for sectioning 60–70 nm thin sections at temperatures between minus 80 °C and minus 140 °C.
7. The sections are collected on 200 mesh formvar-coated grids using a wire loop filled with a drop of 1% (w/v) methylcellulose and 1.15 M sucrose in PHEM buffer.
8. Simultaneous immunolabeling (Fig. 1, lane B) is performed by floating the grids successively on drops containing antibodies and detection probes (fluorophores and/or protein A gold).

9. Sections are post-stained with 2% uranyl acetate (pH 7) or uranyl oxalate (pH 7), rinsed on successive drops of AD and floated for 10 min at 4 °C on drops containing a mixture of 1.8% methylcellulose and 0.3% uranyl acetate (*see* **Note 9**).
10. The grids are lifted from the methylcellulose drops using a small loop and excess methylcellulose is removed by touching the edge of the loop to well-absorbing filter paper and dragging the grid carefully along the filter paper edge until no more uranyl acetate/methylcellulose comes off. In this way a thin film of uranyl acetate/methylcellulose is left on the grid.
11. After air drying, the grids are ready for analysis in the electron microscope.

3.3.3 High Pressure Freezing (HPF) and Freeze Substitution (FS) (Fig. 1, Lane C)

Membranes display the most obvious distortions when chemical fixation protocols are used (Fig. 5a, d and e). Vesiculation or “blebbing” of cellular and organelle membranes are frequently observed in aldehyde-fixed cells as a consequence of fast membrane flow and local breakdown of membrane–cytoskeleton coupling [13]. In terms of optimal preservation the best choice is imaging platelets in the frozen-hydrated state (Fig. 5c, g). The freezing conditions must be sufficiently rapid to prevent serious damage of the membranes by ice crystals. The most widely used procedure at the moment is high-pressure freezing (HPF) followed by freeze substitution.

During HPF, platelets are pressurized to about 2000 bar and then cooled by liquid nitrogen within milliseconds. The goal is to extract the heat from a sample before cell water can rearrange into ice crystals. At this level of pressure, the freezing point of water is lowered down to about −20 °C, and the nucleation of ice crystals as well as their growth is slowed down. For more details about the theory behind this method, see the articles by Riehle and Höchli [19], Studer et al. [20], and Vanhecke et al. [21]. HPF is capable of freezing cells and tissue samples up to 200 μm (*see* **Note 10**).

1. Platelet preparation: Since PRP contains insufficient platelets for HPF-FS, enriched samples of washed platelets are prepared by centrifuging for 15 min at 300 × *g*, and resuspension at densities >600 × 10^9/L in 20% human albumin serum.
2. Marrow preparation: For the study of megakaryocytes or other bone marrow cells by HPF, fresh mouse bone marrow is harvested from the femurs of 8- to 10-week-old BALB/c mice by flushing with 0.1 M sodium cacodylate buffer into a petri dish using a syringe. Small pieces of the flushed marrow are then immediately transferred into the carrier of the HPF. An example of a mouse bone marrow sample prepared according the HPF-FS protocol is shown in Fig. 6.

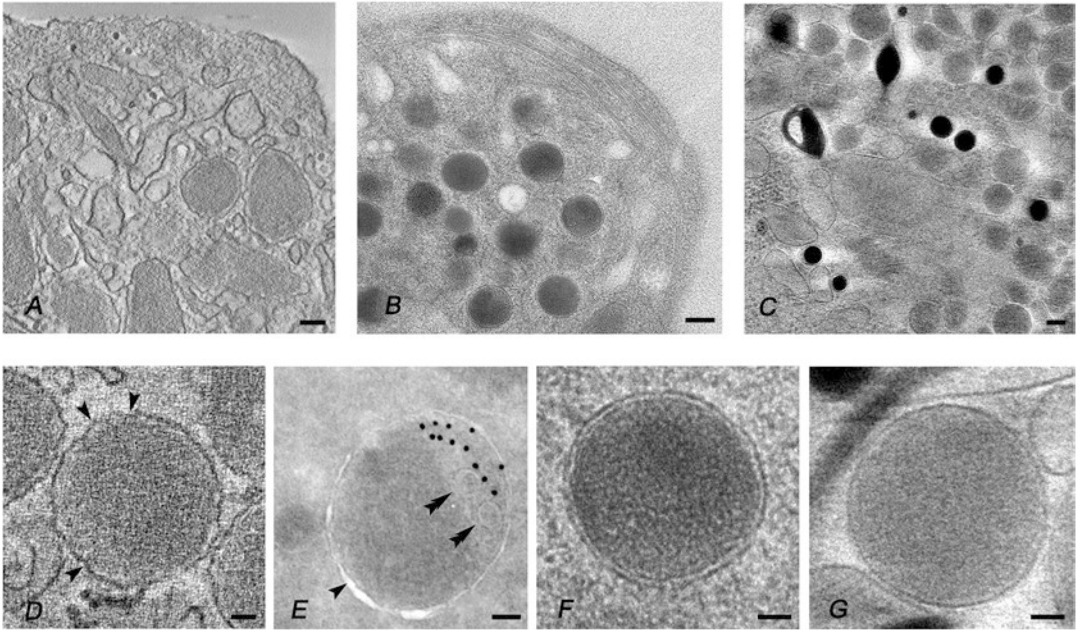

Fig. 5 Effect of different fixation and preparation protocols on membrane morphology. (**a**–**c**) Electron tomography generated by three different fixation protocols: (**a**) chemical fixation, dehydration, and embedding in Epon; (**b**) high-pressure freezing (HPF) followed by low-temperature freeze substitution and plastic embedding in Epon; (**c**) whole vitrified adherent platelet. (**d**–**g**) Alpha granule substructure following different fixation and preparation protocols; (**d**, **f**, **g**) are thin slices (≈5 nm) extracted from tomograms, while (**e**) is a 60 nm cryosection: (**d**) conventional chemical fixation and plastic embedding; (**e**) Tokuyasu method, immunogold labeling of vWF (double arrowheads indicate luminal vesicles); (**f**) HPF/FS and plastic embedding; (**g**) vitrified platelet. Note that alpha granules show membrane distortions and shrinkage in (**d**, **e**) compared to the more regular limiting membranes after HPF and vitrification (**f**, **g**). The membrane preservation in chemical fixed thin-frozen sections is close to the conventional resin-embedded sections. The membrane preservation in HPF/FS method is much closer to the native vitreous state than the chemical-fixed sections. Bars: **a**, 100 nm; **b** and **c**, 200 nm; **d**–**g**, 50 nm

3. Precoat the flat HPF specimen carrier (0.2 mm deep and 1.2 mm diameter carrier) of the HPF apparatus with 20% HAS solution.
4. Platelets in 20% human albumin solution (HAS), or fresh bone marrow samples are transferred into the flat specimen carrier (e.g., 0.8 μL platelet suspension in 0.2 mm deep and 1.2 mm diameter carrier) of the HPF apparatus.
5. Cryo immobilization is performed at a pressure of 2000 bar (2×10^8 Pa) according to the manufacturer's manual within 1 min after transfer of the platelets.
6. The cryo-fixed carrier samples are then transferred to the acetone-based substitution medium in 1.5 mL micro tubes placed at minus 90 °C in a cryo substitution apparatus. Here, frozen water in the sample is replaced by the precooled substitution fluid. Chemical fixatives (uranyl acetate, osmium

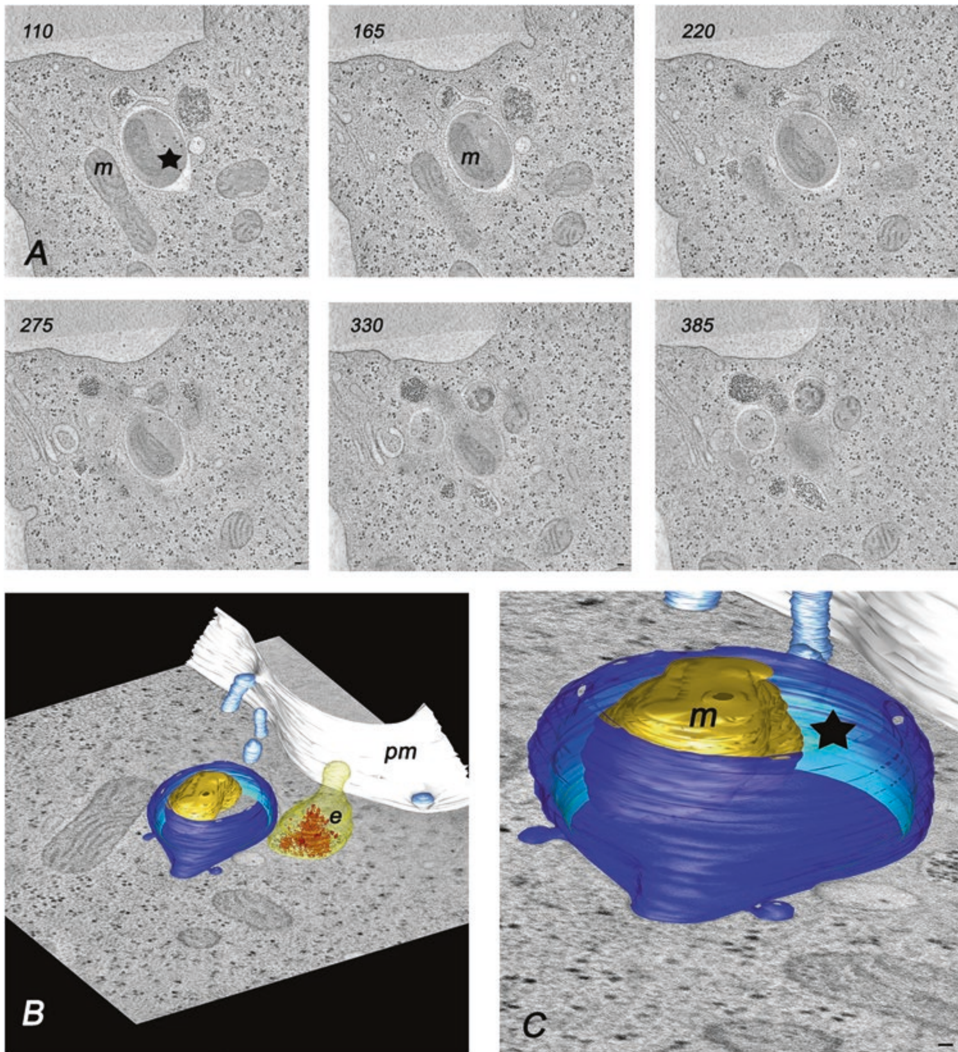

Fig. 6 Electron tomography of erythroblast from mouse bone marrow, prepared according to the HPF-FS protocol (see Fig. 1, lane C). (**a**) Series of 6 tomographic slices through a 300 nm thick section taken at different *z*-axes. Numbers in the top left indicate different *z*-positions in nm. The images show an autophagosome (star) containing a mitochondrion. (**b**, **c**) 3D reconstruction and modeling. *pm* plasma membrane, *m* mitochondrion, *e* endosome (transparent yellow) containing ferritin particles (red). Bars: A, 50 nm; C, 20 nm

tetroxide, glutaraldehyde) in different combinations can be added [14, 31]. We prefer a mixture of 0.5% glutaraldehyde (GA), 0.25% uranyl acetate (UA), 1% OsO_4 (osmium), and 3% H_2O in anhydrous acetone. The platelets are then dehydrated and fixed in this solution as follows.

7. Keep at −90 °C for 48 h.
8. The temperature is raised to −60 °C (2 °C/h).
9. Samples are kept at −60 °C for 8 h.
10. Next the temperature is raised to −30 °C (2 °C/h).

11. Keep at −30 °C for 8 h. When the substitution solution contains UA it is washed off by rinsing four times with the same substitution medium but without UA.
12. The samples are removed from the substitution apparatus and placed on ice for 1 h.
13. After dehydration and fixation during cryo substitution, the platelets are transferred to an Epon-acetone mixture for plastic embedding (as described in Subheading 3.3.1).
14. After **step 11** in the HPF-FS procedure the fixed samples can also be rehydrated on ice in six steps of 10 min each in subsequent series of 95, 90, 80, and 70% (v/v) acetone in distilled water, then 50% (v/v) acetone in PHEM buffer and finally in 30% acetone in PHEM buffer (*see* **Note 11** and ref. 31.
15. Platelets are washed four times for 10 min in PHEM buffer.
16. Finally, the platelets are immersed for 10 min at 37 °C in 12% gelatin in PHEM buffer.
17. After gelation at 4 °C small blocks are trimmed for 2.3% sucrose infusion and cryo sectioning (*see* **Note 12**) as described for the Tokuyasu procedure (*see* Subheading 3.3.2). The only difference is that in the section fixation procedure, phosphate-containing buffers are avoided at all stages.

3.3.4 Whole Mount Procedure (Fig. 2, Lane A)

The limited thickness of spread platelets allows the application of a technique that we have named "whole mount electron tomography." To this end, platelets are allowed to adhere to EM supports. Platelets spread equally well on formvar, quantifoil, or lacey carbon grids, provided that they have been functionalized with a physiological substrate (fibrinogen or vWF, Fig. 7). For tomography purposes fiducial markers (i.e., 10 nm colloidal gold particles coupled to protein A) can be applied to the grids. The fiducial gold markers are extremely convenient for aligning the recorded data set of projection images to make a tomogram. Adherent platelets can also be quickly

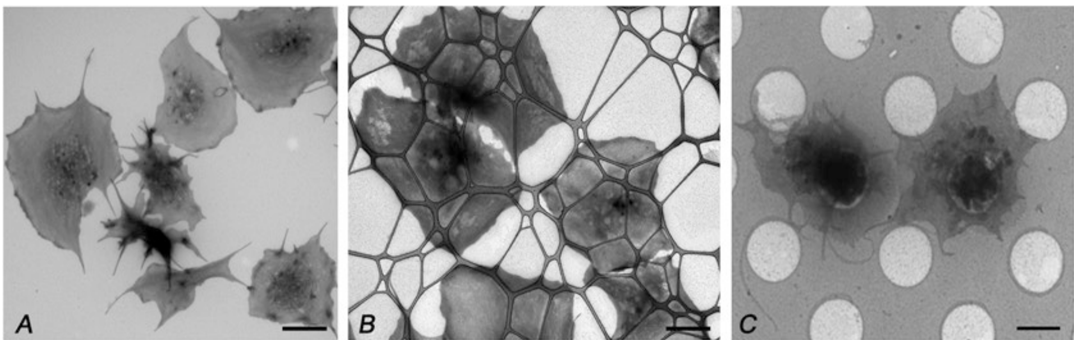

Fig. 7 Platelets spread equally over formvar carbon-coated grids (**a**), gold lacey-carbon (**b**), and gold quantifoil grids (**c**), provided that they are glow-discharged and precoated with a physiological substrate (i.e., fibrinogen or vWF). For correlative purposes carbon-coated Au-finder grids can also be used. Bars: A, 3 μm; B and C, 2 μm

immuno-gold labeled (after **step 6**) using antibodies that identify a protein of interest. These specific gold particles can then be simultaneously used as fiducial markers.

1. The grids are freshly glow discharged. For this process, the grids can be clamped in sheets with flexible sluts (Leica AC20 sheets are preferred), or alternatively glued with their edges to double-sided tape (*see* **Note 13**).
2. The grids are coated for 30 min at room temperature (RT) with fibrinogen (100 μg/mL) in a humidified environment.
3. Excess fibrinogen is removed using a piece of fast absorbing Whatman paper.
4. The grids are then exposed to blocking buffer for 30 min at RT, still in a humidified environment.
5. Excess blocking buffer is removed with Whatman paper.
6. Small drops of the washed platelet suspension (100–150 × 10^6/L) are added to the grids and platelets allowed to settle for ~7 min at RT on the fibrinogen substrate.
7. The grids with attached platelets are removed from the flexible sluts and immediately put (with platelets facing down) on successive drops of Hepes Tyrode buffer with glucose. The drops are placed on Parafilm and covered with petri dishes to prevent dust contamination. Unbound platelets are washed away over several drops of buffer.
8. To allow platelets to spread further, the grids are left for an additional 10–12 min at RT on Hepes Tyrode buffer with glucose.
9. vWF surface labeling can be performed by transferring the grid to successive drops containing anti-vWF antibody (~2 min is sufficient) in Hepes Tyrode with glucose and 10 nm protein A gold (3 min) (*see* **Note 14**).

3.3.5 Correlated Light and Electron Microscopy CLEM (Fig. 2)

1. Carbon-coated formvar, gold lacey carbon, or quantifoil grids are glow-discharged and coated with 100 μg/mL fibrinogen or 100 μg/mL vWF as described in Subheading 3.3.4, **steps 1–4** (*see* **Note 15**).
2. After a BSA block, washed platelets (~100 × 10^9/L) are allowed to spread on the grids (*see* Subheading 3.3.4, **steps 5–6**).
3. After spreading for 20 min on the physiological substrate, the intact platelets are immunolabeled (2 min) by floating them on small drops on Parafilm containing Alexa 488-conjugated anti-vWF or anti-fibrinogen antibodies diluted in Hepes Tyrode buffer with glucose.
4. Rinse several times on successive drops of Hepes Tyrode buffer with glucose.
5. Incubate with protein A gold (3 min).
6. Quickly rinse 3× on drops in Hepes Tyrode with glucose.

From here the sample preparation methods for RT-CLEM and cryo CLEM go separate ways. RT-CLEM requires chemical fixation whereas for cryo CLEM the adherent platelets are vitrified.

RT-CLEM (Fig. 2, Lane A, Supplementary Movie 1)

1. For RT-CLEM, platelets are chemically fixed with 2% PFA and 0.2% GA in 0.1 M phosphate buffer.
2. The adherent platelets are rinsed 5 × 1 min on successive drops of PBS, followed by 5× rinsing on Milli-Q® water drops.
3. Analogous to the Tokuyasu sections (*see* Subheading 3.3), the whole mount platelets are stained for 5 min by floating the grids on 2% uranyl acetate (pH 7) or uranyl oxalate (pH 7).
4. Rinse 3 × 1 min with Milli-Q® water.
5. Transfer the grid to a drop containing a mixture of 1.8% methylcellulose (MC) and 0.3% uranyl acetate (UA) on ice and leave for 10 min at 4 °C.
6. Pick up the grid with a loop and touch the edge of the loop to well-absorbing filter paper and drag the grid carefully along the filter paper edge until no more UA/MC comes off into the filter paper. In this way a thin even film of UA/MC is left on the grid.
7. The grid remains adhered to the loop until it is air-dried.
8. Grids can now be inspected in the i-Corr electron microscope successively in IF and TEM mode using the iCorr work package (Fig. 3) [27] (*see* **Note 16**).

Cryo CLEM (Fig. 2, Lane B)

1. For cryo CLEM grids are immediately transferred to the Vitrobot Mark IV and plunged into liquid ethane for vitrification (*see* Subheading 3.3.6).
2. Grids are stored under liquid nitrogen until transfer to the cryo-stage of the iCorr electron microscope.
3. In the iCorr platelets are inspected successively in IF and EM mode using the iCorr work package and tomographic datasets can be recorded of regions of interest (Fig. 4).

3.3.6 Platelet Vitrification (Fig. 1, Lane C; Fig. 2, Lane B)

Accurate ultrastructural analysis of platelet organelles and simultaneous visualization of macromolecular complexes in their native state requires rapid freezing procedures in combination with high resolution cryo tomography of the whole vitrified platelet. Vitrification immobilizes the membrane dynamics in milliseconds, while preserving the native molecular structure in the hydrated state.

Two strategies can be applied to vitrify tissue samples or platelets. Whereas HPF is capable of freezing cells and tissue samples up to 200 μm, plunge freezing is suitable for cells up to 10 μm in diameter. HPF vitrified samples are generally too thick for tomography and must be thinned. This can be achieved by cryo electron microscopy of vitreous sections (CEMOVIS) or by cryo Focused Ion Beam (FIB) milling. Both CEMOVIS and cryo FIB milling are beyond the scope of this chapter. For technical details and procedures of these techniques we refer to the excellent review papers on these topics [32, 33].

The relative small thickness of blood platelets, particularly when spread on a physiological substrate like fibrinogen or vWF,

allows for whole cell vitrification and offers possibilities for whole cell cryo tomography. Cryo TEM is a demanding "expert" technique that requires expertise and advanced equipment and addresses special demands on platelet preparation methods. We here describe our methods to vitrify resting and substrate-adherent human blood platelets.

1. For plunge vitrification we use a Vitrobot Mark IV operating at 37 °C and 100% humidity.
2. For preparation of adherent cell specimens, *see* Subheading 3.3.4.
3. After the final rinsing step (Subheading 3.3.4, **step 9**) the grids are immediately transferred to the Vitrobot.
4. Excess liquid is removed by blotting either manually or automated (*see* **Note 17**) from both sides with Whatman paper. The fluid absorbing speed of Whatman paper, blotting force and duration are crucial to obtain a thin layer of ice and need to be adjusted experimentally.
5. Immediately after blotting the grid is plunged into liquid ethane to create the vitrified sample. The ethane should be partly solidified. Retract the vitrified grid slowly out of the ethane and blot away remaining ethane with a piece of Whatman paper precooled with liquid nitrogen.
6. Plunge-frozen grids are transferred to cryo boxes and stored in a liquid nitrogen container until transfer to the cryo-stage of the electron microscope.

3.3.7 Labeling of Platelets

Immunogold labeling on thin Tokuyasu cryo sections of chemically fixed cells is one of the most favorable protocols available, and has been used in many MK and platelet studies [34, 35]. With this IEM or so-called Tokuyasu method, sensitive immunoreactions are achieved in non-resin-embedded thin cryo sections of room temperature fixed cells. Fixation is usually with a mixture of formaldehyde and/or low concentrations of glutaraldehyde. The advantage of this method is that the cells do not go through a series of dehydration processes and embedding in resins, and thus maintain epitope access for labeling. Furthermore, the use of different sizes of gold particles guarantee an optimal resolution, and make double labeling and colocalization studies a simple option. Many EM labs can perform the basic steps of fixing (either chemical or the more advanced high-pressure freezing followed by freeze-substitution), resin embedding, and sectioning and contrasting of sections. These approaches usually provide good morphological quality for tomography analysis, but are limited in immune localization options because penetration of antibodies and gold probes into the plastic-embedded section is not possible. Electron tomography can also be applied on semi-thin (~150 nm) immunolabeled cryo sections. Although the labeling is restricted to the section surface, *z*-axis information is still obtained in the thin subsequent slices of the tomogram (Fig. 8). This method can also be applied on HPF-frozen and rehydrated cryo sections [31].

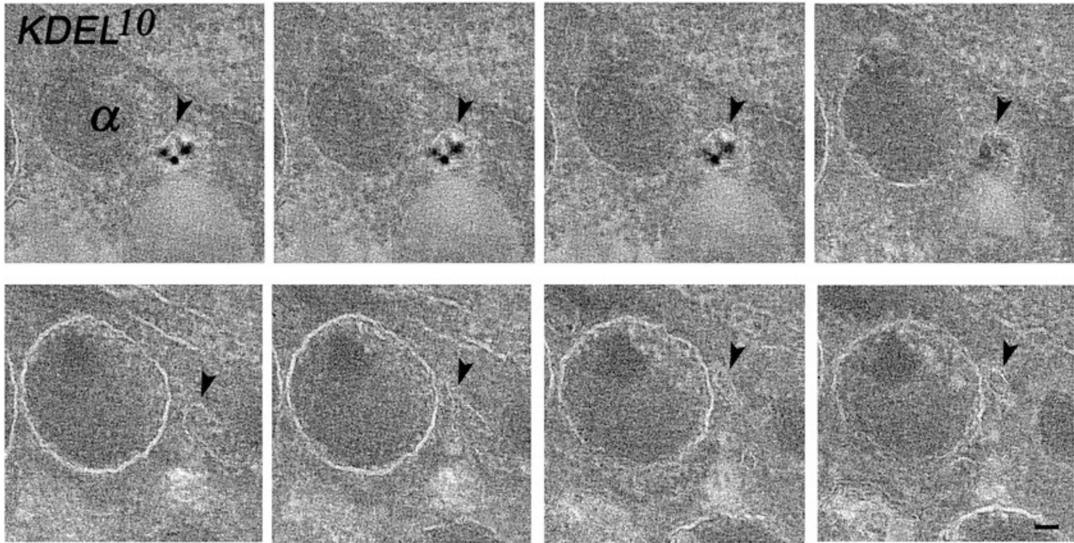

Fig. 8 Electron tomography of 150-nm semi-thin cryo-section, prepared according to the classical Tokuyasu method. Platelets were stimulated for 30 s with CRP. Series of sequential tomographic slices show immunogold labeling of KDEL in reticular tubule-vesicular structures in close position to alpha granules representing the DTS (arrowheads). Note that the gold label is restricted to the top of the section (upper slices). Bar 50 nm

3.4 Automated Data Acquisition Methods for Tomography

3.4.1 Tomography

Electron tomography (ET) is a general approach that we apply to obtain three-dimensional (3D) information regardless of the platelet preparation pathway used. It is based on an old concept, in which projection images of thin specimens (sections or whole mount platelets in the range of 200 to 400 nm) are acquired with an electron microscope by tilting the specimen through a range of tilt angles (typically −60° to +60°) at a predefined interval [36, 37]. However, due to increased sample thickness at higher tilt angles and mechanical limitations by the holders, the tilting range is limited. This results in loss of information (the so-called missing wedge) in the final tomogram. From HPF and chemically fixed thick sections we can generate a dual axis tilt series. This means that after the first tilt series, the specimen is rotated 90°, and a second tilt series is recorded of the same area. The two tilt series are then combined into one tomogram.

Imaging vitreous platelets for tomography is an additional challenging aspect of the technique. It is more complicated due to two factors—the low inherent contrast of the sample and the high sensitivity to electron radiation damage. The sensitivity for electron damage places limitations on the cryo-ET data acquisition. These include the magnification (image pixel size), the tilting scheme (single axis vs dual axis), and the amount of signal to noise in the images [38]. The grids with adherent vitrified platelets are mounted in a Gatan 914 high-tilt cryo-tomography holder (Gatan Inc., USA), and transferred to the stage of the Tecnai 12 Twin TEM or Tecnai

20, equipped with a 4x4K CCD Eagle camera (FEI Company, USA). Due to radiation sensitivity only single-axis tilt series are collected (electron dose <20 e/Å2), using a goniometer tilting range from −60 to +60, with angular increment of 2°. Total electron dose for the entire tilt series should not exceed 100 e−/Å^2. To increase contrast we use a defocus range from −8 to −12 μm.

3.4.2 Software

Besides hardware, specialized software is required for automated data acquisition and data analysis. A broad range of software packages is available for these purposes. Some are commercially available (often expensive) others are academic (much cheaper or even free available). The main goal of the automated data acquisition software is to accurately collect a tilt series of digital images from a fixed location on the specimen. Irregularities in stage movement (x, y) and focusing (z) during the recording of the tilt series must be corrected for. We use the Xplorer 3D package (FEI, Eindhoven, The Netherlands) for data acquisition at our electron microscopes. The 3D reconstruction software performs two tasks: alignment and reconstruction. Currently, we use the IMOD package [30] from Colorado University (Boulder, USA) to align the recorded projection images to create our platelet tomograms, preferably by using fiducial markers. The platelet volume can then be analyzed by visual inspection and segmentation of specific elements of interest. For example, the autophagosome and mitochondrion as depicted in Fig. 6b, c.

The resolution-weighted back projection algorithm [39], which is implemented in the IMOD package is a widely used method for computing the tomogram. For a more detailed description of this package we refer to Kremer et al. [30]. In cases of very low contrast images (i.e., in low-dose cryo-tomograms), we use the SIRT (Simultaneous Iterative Reconstruction Technique) a modules that is also available in the IMOD package. Depending on your computer the weighted back projection algorithm is relatively fast. The SIRT reconstruction algorithm has the disadvantage that it is computationally more intensive.

3.5 Future Perspectives

Several of the methods described above, in particular the cellular cryo-electron tomography (cryo ET) are time consuming and require high technical skills. Yet the future looks bright for cryo ET. The so-called resolution revolution in structural biology [40], where cryo EM is slowly replacing the more traditional X-Ray crystallography opens up great opportunities for cellular cryo ET. It is therefore to be expected that cellular cryo ET will become a dominant technique for studying the structure of dynamic interacting macromolecules at sub-nanometer resolution in the native context of the cell. The increasing interest in cryo EM and the combined forces of many cryo EM laboratories and companies will result in rapid improvements in the entire workflow of cryo EM. Improvements are currently being made in automated cryo

specimen preparation, such as cryo focused ion-beam (cryo FIB) milling of snap-frozen whole cells, automated approaches for correlative cryo LM and TEM, and TEM instrument developments (phase plates, direct detection cameras). Undoubtedly these improvements will enter the platelet and MK research field and result in exciting new discoveries.

4 Notes

1. To study resting platelets we add 1/10 volume of ACD buffer (containing: 85 mM sodium citrate, 71 mM citric acid, and 111 mM D-glucose) and 1 μg/mL prostacyclin (final concentration).
2. All animal work must follow local and national ethical guidelines and be carried out by licensed, competent staff.
3. All buffer and other aqueous solutions are prepared with Ultrapure Water obtained from a Milli-Q® Integral Water Purification System.
4. In the case of vitrification of adherent platelets, carbon-coated formvar grids and copper lacey carbon or quantifoil grids should be omitted because of thick ice formation and the induction of cytotoxic effects during adhesion, respectively.
5. When platelets are allowed to spread on a physiological substrate it is necessary to use golden quantifoil grids because platelets are very sensitive to copper ions and become activated.
6. Extraction of blood must only be taken by a trained and competent phlebotomist after obtaining informed consent from the donor and authorisation by the relevant local ethical committee.
7. Normal MPV (mean platelet volume) for resting platelets is 6.0–8 fL, and should not increase by more than 1.5 fL during the platelet washing procedure.
8. The heavy metal staining can be performed manually, as described here, or automatically with the Leica EM AC20 automated stainer.
9. To prevent uranyl phosphate precipitation, phosphate-containing buffers should be avoided in the final rinsing steps before staining with uranyl acetate and embedding in methyl-cellulose.
10. HPF equipment is available from several providers (Leica Microsystems, Vienna, Austria; Baltzers, Liechtenstein). We have used the Leica EMPACT2 high pressure freezer and the Leica EM AFS2 freeze substitution apparatus.
11. Recently, a novel IEM method has been developed by which cells are fixed by HPF and then rehydrated and processed for

cryosectioning and immunolabeling according to the Tokuyasu method (Fig. 1, lane C) [31].

12. Alternatively, direct cryo sectioning of HPF frozen platelets can also be performed (CEMOVIS) [32]. The frozen platelet samples are transferred to a cryo microtome specimen holder and glued with a drop of a viscous mixture of 2-propanol/ethanol (3/2), at −145 °C that hardens when the temperature is again lowered to −160 °C. Trimming and sectioning can then be performed at −160 °C using a dry diamond knife (Element Six B.V., Cuijk, the Netherlands or Diatome AG, Biel, Switzerland). Sections can be pick-up directly on sandwiched grids for direct transfer to the cryo-stage of the TEM or picked-up with a fixative-containing solution for fixation during thawing). This latter so-called SFM method has only been used in two studies so far, see for additional details reference [31, 41].
13. Clamping the quantifoil grids into the flexible sheet is a critical point. Care should be taken that the fragile carbon layer is not touched. This is best achieved using a binocular microscope or a well-illuminated loupe.
14. The gold particles are conjugated to Protein A, which is used here as the secondary labeling step. To improve efficiency of labeling or in cases that certain moAbs do not recognize Protein A, an intermediate Ab is used.
15. To facilitate correlation also finder grids (Ted Pella, USA) can be used.
16. Since the electron beam bleaches the fluorescence signal very rapidly, particularly at room temperature, make sure that all IF imaging and recording are done prior to the analysis in TEM mode. Tomographic data sets can be recorded from regions of interest. For room temp iCorr imaging and tomography, grids with spread platelets can also be first fixed after spreading, followed by the same immunolabeling procedure but then for 30 min.
17. Blotting is a delicate and critical step in cryo sample preparation, especially when adherent whole cells are used. It determines the ice film thickness and the final quality of the preparation. Most devices, e.g., the Vitrobot (FEI), offer automated blotting and plunging functions. Manual blotting, however, may sometimes be useful to prevent cell damage. Excess solution is removed by blotting with a piece of Whatman filter paper, producing a thin liquid film spanning the holes of the grid. The exact amount of blotting time and force used, in both manual and automated devices must be adjusted to the nature of the sample. For both adherent and resting platelets we have used blotting times between 5 and 10 s and blotting forces up to −5.

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