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Chapter 33

An Improved Method for Assessing Antigen Presentation on the Surface of *Plasmodium falciparum*-Infected Erythrocytes by Immuno-Electron Microscopy

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Abstract

Immuno-electron microscopy can detect and localize antigens in cells or tissues at a resolution of several nanometers. In the case of *P. falciparum*-infected erythrocytes, immuno-EM studies are frequently hampered by the electron-dense nature of the hemoglobin and access of antibodies to antigenic sites, particularly if the targeted protein is presented on the host cell surface or lies in proximity to the host cell cytoskeleton. Here, we describe an improved immuno-EM protocol that overcomes these problems. The improved signal to noise ratio and the enhanced access to antigenic sites now allows one to obtain information regarding target density and distribution and, hence, additional insights into the architecture and function of parasite-induced, or -affected, structures.

Key words PfEMP1, Knobs, Antigen accessibility, Hemoglobin, Immuno-EM, Stereology

1 Introduction

To view cells and tissues under the electron microscope, the sample must be thin. This is typically achieved by sectioning an embedded specimen into thin slices using a diamond knife. Each cut exposes a new layer of the cell interior. The antigens that are located directly on the newly exposed surface are accessible to antibodies, unlike antigens located deeper in the section, which are not accessible because of restricted penetration by antibodies. For instance, antibodies can penetrate merely a few nanometers into samples embedded in epoxy resins [1]. The penetration can be extended to 20–25 nm in unembedded tissue slices, such as those prepared by the Tokuyasu method [2]. Nevertheless, the accessibility to antigenic sites diminishes with the distance from the surface. As a result, it is difficult if not impossible to obtain semi-quantitative

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information regarding the number and density of a given target protein in a certain subcellular structure or compartment.

One of the hallmarks of erythrocytes infected by *P. falciparum* is the induction of a system of thousands of adhesive knobs on the surface of the host cell. Due to their small size, the organization of the knobs cannot be investigated with optical microscopy, but necessitates the use of electron microscopy. However, the limitations described above have so far hampered a detailed and comprehensive description of knobs and the factors that form, and associate with, knobs, in spite of knobs being readily discernable in EM images as electron dense protrusions of the plasma membrane of *P. falciparum*-infected erythrocytes. Given that knobs have a three-dimensional structure with a diameter of ~80 nm and a height of ~6–8 nm [3, 4], they extend into the non-penetrable part of the EM section. As a consequence, antigenic sites of the knobs close to the surface of the EM section are labeled more intensely with a specific antibody than sites deeper in the section. Even improved visualization, as can be achieved by 3D tomography, cannot avoid artificial label distribution. Thus, an antibody directed against the PfEMP1 adhesins that are presented on the surface of knobs would only detect a limited number of molecules owing to the penetration problem. It is even more difficult to antibody-label a target protein that is a constituent or an associated factor of knobs. In the latter case, the antibody penetration problem would be compounded by the high density of the surrounding hemoglobin and an unfavorable background to noise ratio.

Here, we describe improved immuno-EM methods that assure equal accessibility of primary antibodies to antigenic sites located i) on the cell surface and ii) at the cytoplasmic side of the plasma membrane. The first protocol relies on antibody labeling of intact, unfixed parasitized erythrocytes in suspension. The second approach requires hemolysis of the infected erythrocyte and exposure of the cytoplasmic face of the plasma membrane and, where necessary, fenestration or complete removal of the plasma membrane with a detergent. We provide here a detailed description of our modifications; however, for the details on classical EM procedure, we refer the interested reader to the relevant literature [5].

2 Materials

2.1 P. falciparum-Infected Erythrocytes

1. Purified *P. falciparum*-infected erythrocytes. A convenient way to enrich for infected erythrocytes is to use a strong magnet (*see* Chapter 34). For most experiments described in this chapter, sufficient amounts of purified infected erythrocytes at the trophozoite stage can be obtained from a 10-cm culture dish containing 0.5 ml packed cells in 10–15 ml supplementary

RPMI 1640 culture medium at a parasitemia of 2–5% (*see* **Notes 1** and **2**).

2.2 Reagents and Solutions

Prepare all solutions using deionized water and store the solutions at 4 °C (unless stated otherwise). Diligently follow all waste disposal regulations. Pay particular attention to properly disposing aldehyde fixatives and heavy metal salts (uranyl acetate, osmium tetroxide, lead citrate, etc.). The amounts of fixatives and contrasting medium should match the experimental needs; avoid preparing excess amounts and, thus, unnecessary disposal.

1. Fixation solution: 4% paraformaldehyde (PFA) and 0.016% glutaraldehyde (GA) in phosphate buffer saline (PBS) (unless indicated otherwise) (*see* **Note 3**).
2. Fixative quenching solution: 0.15% glycine in PBS.
3. Blocking solution: 0.8% bovine serum albumin (BSA) and 0.1% fish skin gelatin (FSG) in PBS (*see* **Note 4**).
4. Final fixative (after immunolabeling procedure): 1% GA in PBS.
5. EM grids with appropriate mesh size (e.g., Cu 200 mesh, coated with plastic film and reinforced with a 2-nm carbon coat).
6. Lysis buffer: 10 mM sodium phosphate, pH 8.0, 10 mM NaCl.
7. Detergent containing lysis buffer: 5 mM sodium phosphate, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DDT, 0.2% NP40-S.
8. Distilled water.
9. Embedding medium: Mix 0.5 ml of 2% uranyl acetate aqueous solution and 0.5 ml of 2% methyl cellulose aqueous solution. 1 ml of embedding medium is sufficient for five grids.
10. Filter paper with coarser fibers.
11. Loops made of thin metal wire, with a loop diameter of 4–5 mm—to fit the grid for drying.
12. Primary antibodies: Use home-made or commercially available polyclonal or monoclonal antibodies specific to surface antigens, such as PfEMP1 (*see* Chapters 28–30), or components of the knobs, such as KAHRP, or the membrane skeleton, such as actin, spectrin or ankyrin.
13. Secondary antibodies: colloidal gold (optimum 10 nm) coupled secondary antibodies specific for the primary antibodies, e.g., a goat-anti human antisera if the primary antibody is of human origin. If a gold-conjugated secondary antibody is not available, use a rabbit anti-primary antibody in the second step followed by colloidal gold (protein A with 10 nm gold) in the third step.

14. Glow discharge apparatus.
15. The python scripts described in this chapter can be downloaded from: https://github.com/usschwarz/PfEMP1-Distribution.
16. Transparent paper.
17. Pencil or felt pen.
18. 0.1% poly-L-lysine in PBS.
19. Blotting paper.
20. Forceps.
21. Parafilm.

3 Methods

All procedures presented in this chapter derive essentially from classical and well-established immuno-EM labeling protocols [5]. For protocols on preparation of samples for transmission electron microscopy (TEM), troubleshooting, and general questions regarding optimization of immuno-EM images, we refer the reader to the relevant literature [5] (*see* **Note 5**). Carry out all procedures at room temperature (RT) unless specified otherwise.

3.1 Immunolabeling of Surface Markers on Knobs

3.1.1 Sample Preparation

1. Transfer the purified suspension of parasitized erythrocytes to a 1.5- or 2-ml Eppendorf tube.
2. Centrifuge cells for 2 min at 700 × *g* in an Eppendorf centrifuge.
3. Rinse cells with PBS (or fixative quenching solution, when fixed cells are used) and spin two times at 700 × *g* for 2 min each (*see* **Note 6**).
4. Discard the supernatant.
5. Add blocking solution to cells for 30 min. Spin for 2 min. Keep approximately 20 μl suspension on top of pellet (do not dry the sample).
6. Add primary antibody (e.g., human anti-PfEMP1) diluted in blocking buffer at desired concentration (or use series of dilutions, *see* **Note 7**). Pay attention to the fact that the volume of the cell pellet dilutes the antibody concentration. Mix well and incubate for 60 min (30–90 min, depending on labeling efficiency), while gently agitating (*see* **Note 7**). Double (multiple) labeling might also be possible; although it is not considered in this protocol.
7. Rinse in blocking buffer and spin as above (**step 2**) two times for 2 min.

8. Add secondary antibody (e.g., goat anti-human, coupled to 10 nm protein A-gold (PA-gold)) (*see* **Note 8**) diluted in blocking buffer as specified by the manufacturer (e.g., 1:20) for 30–60 min (*see* **Note 9**).
9. Rinse in blocking buffer and spin at 700 × *g* for 2 min.
10. Prepare sections of, e.g., 200 nm thickness, using standard methodology for preparation of tissue sections [5]. Thicker sections increase the chance of capturing complete knobs in side views and also include more markers in the field of view. Alternatively to classical TEM preparation, fix sample in final fixative, put a drop of suspension on EM grid and preserve in embedding medium (*see* Subheading 3.2.3, **steps 10–15**). Some areas of slack plasma membranes should be thin enough for viewing in TEM. Alternatively, break the plasma membrane using a hypotonic solution to release hemoglobin (*see* Subheading 3.2.1).

3.1.2 Data Analysis and Spatial Distribution of Marker Protein

In this section, we describe a protocol to determine the spatial distribution of PfEMP1 molecules on knobs [3]. However, the procedure can be transferred to other markers or, for that matter, to other defined surface structures. It is important to note that as many as 200–300 knobs need to be imaged and analyzed in order to obtain statistically reliable data. One further needs to take into account that only a fraction of available antigenic sites is effectively labeled in immuno-EM and also that the physical distance of the electron dense marker from the actual epitope can be as much as 30 nm when primary and secondary antibodies were used for labeling.

1. From 2D EM images (Fig. 1a), select those knobs for further analysis where the sectional plane includes both the base and the tip of the knob, such that the full contour of the knob is visible.
2. Rotate extracted images to view the knobs at the same orientation, e.g., horizontal or vertical.
3. Measure knob dimensions, e.g., the size of the knob base and the knob height (or any other characteristic relevant to the study). If all knobs are very regular in size and shape, proceed straight to **step 4**; if knobs are heterogeneous, group them into classes.
4. For each class, create a template on transparent paper by drawing a series of parallel lines that vertically divide the knob into sections of 5 nm line-to-line distance each and which extend to each side of the knob base but not beyond. Other distances between lines can also be chosen, depending on the accuracy of the experiment. Number each section from the center of the knobs outward, such that the two sectors in the center of the

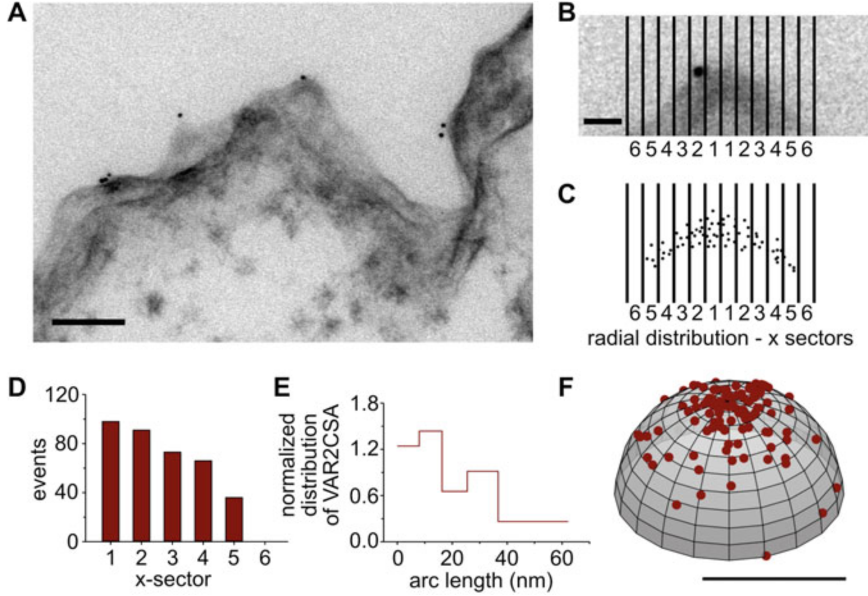

Fig. 1 Immunolabeling on cell surface: Spatial arrangement of VAR2CSA molecules on knobs. (**a**) TEM image showing a thin EM sections through the cell margin with some knobs decorated with gold on the surface. The section was labeled with a VAR2CSA monoclonal antibody as a primary antibody and, as a secondary antibody, with a goat anti-human antibody coupled to 10 nm colloidal gold. Scale bar, 100 nm. (**b**) Extracted knob decorated with gold markers. The image is overlaid with a line grid, dividing the image into sectors of equal size. The sections were numbered from the center of the knobs outward. Bar, 30 nm. (**c**) Template indicating the position of gold markers, as observed by examining 100 or more knobs. (**d**) Number of gold markers per section. (**e**) Distribution of VAR2CSA molecules on knobs as a function of the arc length (from center to periphery), assuming knobs have a hemispherical shape [6]. (**f**) A spatial probability distribution model showing the predicted location of 100 VAR2CSA molecules on an idealized hemispherical knob. Bar, 50 nm

knob have the number 1, the sectors flanking the central sectors the number 2, and so on.

5. Properly place the template over the knob image and copy with a felt pen or pencil the position of the gold label (Fig. 1b).
6. Repeat the procedure on a large number of knobs to reach a statistically significant label distribution (Fig. 1c). This may require analyzing 200–300 knobs.
7. Create a bar chart by plotting the number of gold labels as a function of the sector, yielding a probability distribution that reveals sections of high and low label encounter (Fig. 1d). (In the case of the PfEMP1 variant VAR2CSA, the probability to encounter a gold-labeled VAR2CSA was the highest at the tip of the knob and declined toward the knob base [3]).
8. Convert the 2D data into a discrete radial distribution model and, hence, a spatial projection model, by taking into account the thickness of the EM slice and assuming, in a first approximation, that knobs have a hemispherical shape (Fig. 1e). To

convert the measured density in x-sectors to a density in circular rings along the arc length of a half-sphere, use the python script “density-in-sectors.py” [6].

9. Use the python script “distribution-on-half-sphere.py” to generate a 3D distribution model that localizes the targeted protein on an idealized hemi-spherical knob (Fig. 1f) [6].
10. Calculate the nearest neighbor distance between targeted molecules on knobs, using the python script “average_distances.py” [6].

3.2 Immunolabeling at the Cytoplasmic Side of the Plasma Membrane

There are different ways to improve access to antigenic site close to the plasma membrane of the infected erythrocyte, such as components of knobs or the membrane skeleton. Possible strategies include cell lysis, treatment with detergent, or a combination of both.

3.2.1 Hypotonic Cell Lysis on EM Grid

1. Use grids of the desired mesh size (*see* **Note 10**).
2. Treat the grid in a glow discharge apparatus to make the surface slightly hydrophilic.
3. Treat grid surface with 0.1% poly-L-lysine for 20 min immediately before experiment.
4. Wash grid extensively with PBS.
5. Add 3–5 μl (1×10^6 cells) of purified parasitized erythrocytes on EM grid, incubate for 5 min at room temperature to let cells sediment and adhere to the grid surface.
6. Lyse the cells, using lysis buffer. To do so, place the grid on blotting paper. Lift up one edge of the grid with forceps and hold it, while applying with a pipette a steady flow of 50–80 μl double distilled water for 30 s—from one edge of the grid down to the edge of the grid that is still placed on the blotting paper. Wash with distilled water, as described above. Do not let the grid dry; always keep the grid moist (*see* **Note 11**). With this procedure, the infected erythrocytes are lysed, while parts of the erythrocyte plasma membranes remain attached to the grid surface in an exposed fashion. Hemoglobin and the majority of parasites are washed away.
7. Proceed to Subheading 3.2.3 for immunolabeling.

3.2.2 Detergent-Induced Cell Lysis on EM Grid

1. Prepare grid as described in Subheading 3.2.1, points 1–4.
2. Place the grid on a drop of detergent containing lysis buffer for 10 min. Avoid wetting the back side and sinking of the grid, which often happens when incubating the grids in solutions containing strong surfactants, such as common detergents. To prevent grid sinking, either invert the drop (thereby creating a kind of a “hanging drop” with grid floating below) or use a

detergent concentration close to, or only slightly above, the critical micellar concentration value.

3. Wash grid five times for 1 min each, by placing it on drops of PBS (~30 μl on a parafilm).
4. Proceed to Subheading 3.2.3 for immunolabeling.

3.2.3 Immunolabeling with Specific Antibodies

Follow a classical immunolabeling protocol established for immunolabeling of Tokuyasu and plastic sections [5]. The following steps, i.e., labeling, washing, and embedding, are done by floating the grid on drops of 30–50 μl of the respective solution (*see* **Note 12**). The specimen containing side of the grid is always facing the liquid. Avoid drying the specimen between steps until final embedding.

1. Prepare grids, parasitized erythrocytes, conduct cell lysis, and apply the material on an EM grid as described above.
2. Float the grid on a drop of blocking solution for 10 min.
3. Incubate grid on a 10–20 μl drop of primary (e.g., anti-PfEMP1) antibody diluted in the blocking solution at the desired concentration for 30–90 min.
4. Wash the grid on drops of blocking solution three times for 5 min each.
5. Incubate the grid on a drop of secondary antibody-PA gold conjugate, diluted in blocking buffer at the concentration specified by the manufacturer (e.g., 1:30) for 30 min.
6. Wash the grid on a drop of blocking solution once for 5 min.
7. Wash the grid on a drop of PBS once for 5 min.
8. Fix the grid on a drop of final fixative (*see* Subheading 2.2) for 10 min.
9. Rinse the grid on two drops of distilled water twice for 1 min each.
10. In addition, rinse the grid on two drops of ice-cold distilled water twice for 1 min each on ice.
11. Embed in embedding medium on ice (fast wash on two drops and incubate for 8 min on the third drop).
12. Collect the grid from the last drop of embedding medium with a wire loop.
13. Blot excess of embedding medium by smearing the edge of the loop on filter paper with a larger fiber size and a high soaking speed.
14. Air-dry the grid in the loop overnight and collect it carefully with forceps.

15. View the specimen under the electron microscope. Focus on patches of membrane where the cytoplasmic side of the erythrocyte plasma membrane is exposed. Avoid folds in membranes and not completely opened erythrocytes. This assures that the images represent areas of unrestricted access of antibodies and electron dense probes to antigenic sites. Collect EM-projection images or tomographic tilt series.

4 Notes

1. It is advisable to prepare an excess amount of purified infected erythrocytes to compensate for cell loss during the preparation, especially when labeling epitopes on the cell surface.
2. Preferentially use infected erythrocytes immediately after purification. If it cannot be avoided, cells can be kept for less than 2 h at RT. If cells need to be stored for longer periods, then it is recommended to fix the cells mildly in PBS buffer containing 4% paraformaldehyde and 0.016% glutaraldehyde. Fixed cells can be kept for several days at 4 °C before immunolabeling. However, fixation can reduce the labeling efficiency, as we observed when labeling surface proteins.
3. Fixation with paraformaldehyde (PFA) is reversible and, after removing the fixative, the protein reverses to its natural conformation, enabling binding of specific antibodies. However, the fixation with PFA is believed to preserve biological structures and cell morphology only partially. In comparison, fixation with glutaraldehyde preserves biological material very well, but generally leads to irreversible changes in the structure of molecules, which, in turn, can affect antibody binding. Nevertheless, some molecules maintain their affinity to a specific antibody even after GA fixation at higher concentrations. How susceptible a given epitope/antibody reaction is should be tested experimentally. In case antibody binding is lost after GA fixation, one may consider using only PFA as fixative. Conversely, if the labeling efficiency is not affected, one might consider increasing the GA concentration in the fixative solution gradually up to 1%.
4. The efficiency of labeling and the specific signal to background ratio partially depend on the blocking solution used in the washing steps and for diluting antibodies and gold markers (among other factors that are listed in **Note 5**). It is, therefore, advisable to optimize the blocking solution for each experiment to achieve optimal results. The blocking solution may contain only BSA (1–3% in PBS), only FSG (0.8–3% in PBS), or a mixture of BSA and FSG in PBS at a ratio of, e.g., 0.1–1.5%, 0.5–1%, or other combinations.

5. The selectivity and specificity of labeling depend on a number of factors, including specimen handling, quality of primary and secondary antibodies, affinity of antibodies to corresponding epitopes, time and temperature of labeling, and blocking conditions (*see* **Note 4**). Adjust these factors to achieve best results.
6. If starting sample is small, or if substantial amounts of cells are lost in each spinning step, consider spinning fewer times than indicated. However, this likely decreases the fidelity of the labeling and may result in lower and less specific labeling and increased background.
7. Use antibody dilutions of 1:5, 1:20, 1:100, or similar. As a rule of thumb, use a 100-fold higher concentration of primary antibodies than recommended for, e.g., Western analysis. This rule applies to labeling of surface and intracellular antigens. To improve labeling efficiency and the signal to noise ratio, adjust antibody concentration, temperature (ice, RT, 37 °C), agitation, and blocking conditions.
8. If a secondary antibody coupled to protein A-gold is unavailable apply three-step labeling procedure: Use a rabbit secondary antibody against the primary antibody in the second step, then rinse in blocking buffer, and then, in a third step, add protein A-gold in blocking buffer at the concentration as specified by the manufacturer.
9. To improve the signal to noise ratio, one can vary the secondary antibody dilution (such as 1:20; 1:30; 1:50, etc.), the time of incubation (20–60 min) and the temperature (ice; RT; 37 °C). One may further consider using an antibody from a different supplier as the quality of an antibody may vary between manufacturers and sometimes even between different batches from the same manufacturer. Other factors that may affect the labeling efficiency and the signal to noise ratio are the age of the antibody and the storing conditions. Use the desired size of PA-gold, such as 5 nm, 10 nm, 15 nm; although according to our experience, 10 nm gold most often gives the best results. Consider negative control experiments, using secondary antibodies only.
10. Use EM grids with continuous plastic film (either home-made or purchased from a commercial supplier), stabilized with a 2–5 nm carbon coat. Use grids of the desired mesh that allows for film stability, e.g., square or hexagonal 200 or 300 mesh copper grids. However, for tomographic data collection use slot grids, or grids with a larger mesh (e.g., 100), containing a continuous formvar film coated with 2–5 nm carbon.
11. If hemolysis is incomplete or if the exposed membranes are covered by contaminating hemoglobin, it is advisable to repeat the cell lysing with fresh purified erythrocytes under conditions

of increased water flow and/or longer washing times. Contaminating hemoglobin will unnecessarily increase the mass thickness of the specimen, obstruct access of antibodies to antigenic sites, reduce the contrast in EM images and tomograms, and hinder the detection of fine structural details. In cases in which parasites still remain attached to, or trapped in, host membranes, continue with sample preparation and data collection, provided there are large enough areas of free and exposed plasma membrane.

12. If the antibody is precious, reduce the drop size to 10 μl or even 5 μl. When floating grids on very small drops of liquid, assure it is done in humid conditions, e.g., in a small petri dish with wetted tissue inside.

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