Developed Scanning Electron Microscope Technique for Red Blood Cell Morphological Analysis

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ABSTRACT

Abnormal morphology of periperal red blood cells (RBCs) is commonly used for diagnosis and moritoring various clinical diseases including anemia, infection, thrombosis and some cancer. The blood film analysis is routine laboratory work. However, some poikilocytes such as crenated cells are appearred as artifct that occur during specimen processing for light microscope (LM). In fact, the small number of true crenated cells can be seen in patients with hypothyroidism, heart disease, uremia, cancer of stomach and bleeding peptic ulcer. That is useful for screening test. In this study, RBC morphology from healthy subjects were analyzed using LM as conventional method compared to the scanning electron microscope (SEM). Developed SEM technique with fresh EDTA blood fixed with 2.5%glutaldehyde solution before dehydration at 50%, 70%, 95% and absolute ethanol was performed. This technique is suitable for routine electron microscopic laboratory analysis.

Keywords : Scanning electron microscope, Red blood cell morphology

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Introduction

Red blood cell (RBC) morphology and deformability play an important role as cellular marker for various clinical diseases including anemia such as hereditary spherocytosis, hereditary elliptocytosis, sickle cell anemia, thalassemia, etc. Several evidences indicated that the shape of RBCs is associated with pathogenesis and pathophysiology. For example, spherocytes are abnormal cell membrane protein that may be caused by hereditary or acquired disease. Spherocytes have excessive intracellular sodium than in plasma when they reach the microenvironment of spleen, the active-passive transport system is unbalanced with increased sodium and decreased glucose resulting in cell swelling and hemolysis (Perrotta, Gallagher, & Mohandas, 2008). Although the peripheral blood film is a rountine laboratory for cytology of blood cells on a slide for light microscope (LM), the artifacts are usually appear as results of spreading, fixing, drying, staining and observation in unsuitable areas. Crenated cells (echinocytes in SEM) are most common observed as an artifact. The indication of the true crenated cells, irregularly sized with unevenly spaced spicules, is useful for mornitoring and diagnosis such as the small number of crenated cells can be seen in immediately following an injection of heparin, patients with hypothyroidism, heart disease, uremia, canner of stomach and bleeding peptic ulcer (Jones, 2009). Scanning electron microscope (SEM) is used for the assessment of morphologic details such as blood cells that can excluded the undersirable artifacts caused by the process of blood smear preparation for LM. The aim of this study was optimized and developed SEM technique for RBC morphological analysis that could be advantaged to study on defect of RBC or some RBC disorders.

Objective of the study

To developed scanning electron microscpe for red blood cell morphological analysis.

Methodology

Subjects

One mililiter of venous blood samples from 10 health subjects who had hemoglobin typing A_2A and no anemia were collected into EDTA anticoagulant tube for SEM and LM analysis or directly mixed with fixative solution for SEM analysis. The study protocol was approved by the Mahidol University Institutional Review Board (approval number 2015/076.1906). Written informed consent was obtained from all individual participants included in the study. Complet blood count analysis of subjects in this cohort was 4.3-4.8 $\times 10^6$ /μL of RBC count, 12.0-14.2 g/dL of hemoglobin concentration, 35.6-41.7% of hematocrit, 12.5-15.1% of red cell distribution width with 82.8-86.9 femtoliters of mean corpuscular volume, 27.9-29.8 of mean corpuscular hemoglobin and 33.7-34.7 of mean corpuscular hemoglobin concentration.

Light microscope (LM)

EDTA blood samples were smear on slides and allowed air dry. Blood smear specimen were fixed with absolute methanol. After air dry, specimens were stained by Wright's-Giemsa's solution (BecTech, Thailand) according to manufacturer's recommended. One thousand RBCs were count and classified to normocyte, ovalocyte,

spherocyte, tear drop cell, sickle cell, target cell, stomatocyte, crenated cell, spur cell, keratocyte, schistocyte and bite cell (Jones, 2009). The images were illustrated with E-330 digital camera, CX-31 light microscope (Olympus) and analyzed with Cell^A software (Olympus).

Scanning electron microscope (SEM)

Fixed blood sample preparation for SEM was performed by two different processing; 1) fresh blood without anticoagulant (from syringe) and 2) EDTA blood. One milliliter of blood samples was fixed with 9 ml of fixative solution (0.5% or 2.5%glutaldehyde) in 0.1 M sodium phosphate buffer (mixture of 19 ml 0.2 M $NaH_2PO_4.H_2O$ and 81 ml 0.2 M $Na_2HPO_4.7H_2O$), pH 7.4 at 4°C for overnight (16-18 h). The fresh blood samples from syringe were immediately fixed with either 0.5% or 2.5% glutaldehyde solution within seconds of being withdrawn. On the other hand, EDTA blood samples were separated into three different time points after blood collection; 1) within seconds, 2) 6 h stored at 4°C and 3) 24 h stored at 4°C. Then, blood samples were fixed with 2.5% glutaldehyde solution. After fixation, the fixed samples were wash twice with 0.1 M sodium phosphate buffer, pH 7.4 at 3,000 rpm for 5 min at room temperature (RT). RBC pellets were performed dehydation step.

The dehydration was separated into two sets; 1) The dehydation Set “A”, RBCs was dehydrated with two washes in each solution of 50%, 70%, 95% and absolute ethanol. 2) The dehydation Set “B”, RBCs was dehydrated with two washes in each solution of 50%, 60%, 70%, 80%, 95% and absolute ethanol. After that, cells were wash twice in acetone. The pellet RBC was resuspended into 2 ml acetone. Five microliters of dehydrated RBC were applied to a coverslip and allowed to dry. Coverslips were mounted onto carbon tabs which were already placed onto SEM mount pin type. The coverslips were stored in a desiccator until electron microscopy was performed. Before electron microscopy, the specimen was coated with a thin layer of palladium. One hundred RBCs were count and classified to discocyte, elliptocyte, spherocyte, dacryocyte, drepanocyte, codocyte, stomatocyte, echinocyte (stage 1-3), acanthocyte, keratocyte, schistocyte and dangocyte (Bunyaratvej, Sahaphong, Bhamarapravati, & Wasi, 1985; Jones, 2009). The illustration of SEM analysis was performed by SNE-3200N/SNE scanning electron microscope (Extrad Instruments, Korea).

Statistical analysis

Data were analyzed using SPSS Version 18.0 (IBM, Chicago, USA). Comparisons between parameters were evaluated with a T-Test. Pearson’s correlation test was used to correlate RBC morphology from different techniques. The threshold for statistical significance for all comparisons was $P < 0.05$.

Results

Fixative optimization for SEM

RBCs are pink in color when stained with Rowmanosky dye such as Wright’s-Geimsa’s solution because the hemoglobin content of the RBC picks up eosin, the acidophilic components of the dye. Under LM, a normal sized

RBC is comparable to the size of a nucleus of small lymphocyte. The normal RBC (called discocyte by SEM analysis or normocyte by LM analysis) is biconcave disc-shaped, central pallor (approximately a third of the red cell diameter) and lacks intra-cytoplasmic inclusions, about 7-8 μm in diameter. Representative examples of scanning electron micrographs of RBCs from fresh blood sample immediately fixed in syringe shown RBC clumping with some plagues in 0.5% glutaldehyde fixative solution as fixative (Fig. 1A) compared with those in 2.5% glutaldehyde fixative solution (Fig. 1B). The 2.5% glutaldehyde fixative solution is the fixative that can preserve the normal discocyte shape of RBCs. However, the peripheral blood cells without anticoagulant are not rountine perform and required a special preparation before sample collection. It might be error from partial blood clot or hemolysis. Therefore, the EDTA blood samples at either within seconds or 6 h or 24 h after venous puncture were fixed with 2.5% glutaldehyde solution and performed dehydration and SEM analysis comparing to fresh blood samples immediately fixed in syringe. The results shown that the EDTA blood samples at those three different time point can used for RBC morphological analysis by SEM (Fig. 1D-E). However, 24 h-EDTA blood samples were partial hemolysis. Here, we recommended that specimen for RBC morphological analysis by SEM could be fresh EDTA blood samples and fix with 2.5% glutaldehyde solution within 6 h after withdraw.

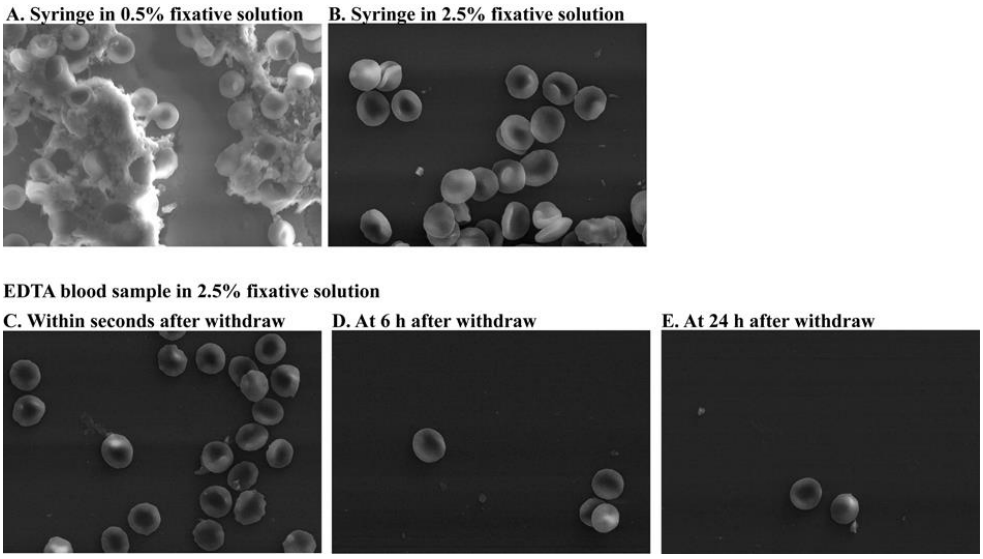

Figure 1 Red blood cell morphology identified from a scanning electron microscope. Fresh blood samples from syringe were fixed with (A) 0.5% glutaldehyde fixative solution or (B) 2.5% glutaldehyde fixative solution within seconds after withdraw. The EDTA blood samples from the same subjects were also fixed with 2.5% glutaldehyde fixative solution within (C) seconds, (D) 6 h and (E) 24 h after withdraw. After fixation, the dehydration was performed by following the dehydration Set "A" (See in Methodology). (Experiment repreated three different subjects) (magnification ×2,000)

Dehydrated optimization for SEM

The dehydration is another common trouble for RBC morphology. No significant difference of the percentages of discocytosis from peripheral blood samples that were dehydrated by following the dehydration Set "A" (50%, 70%, 95%, absolute ethanol and acetone) (mean±SD 90±19%) (SEM in Fig. 1C and Fig. 3A-B, LM in Fig. 2) and the dehydration Set "B" (50%, 60%, 70%, 80%, 95%, absolute ethanol and acetone) (94±15%) (Fig. 3C-D). The RBC morphological changes of each step of dehydation from 50% ethanol to 70% to 95% was analyzed by LM (Fig. 2). It is resulting in RBCs were no hemolysis and no poikilocyte that was caused by dehydration. Only the echinocyte stage 1, regularly placed projections on RBC surface, was mainly found as artifact (10±19% and 6±15%of total cells, respectively).

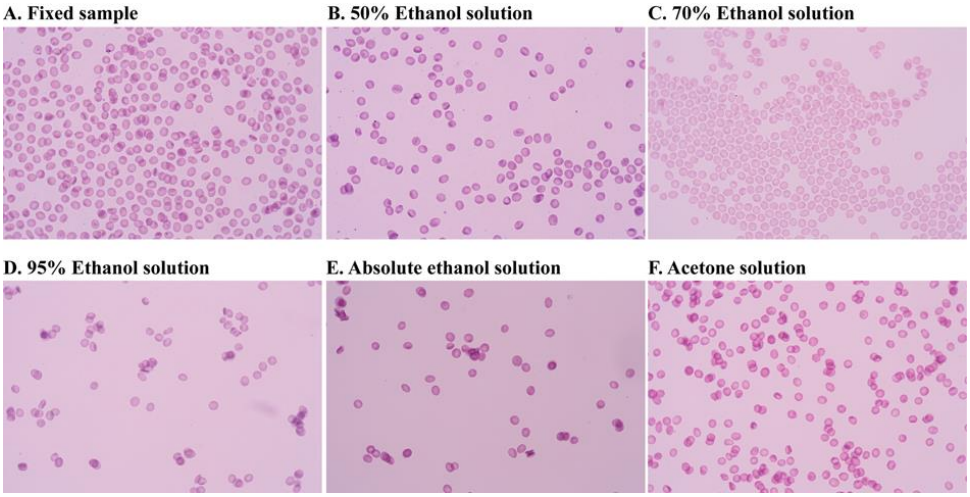

Figure 2 Wright's-Giemsa's stained red blood cell samples during each step of dehydration. The EDTA blood samples from healthy donors in 2.5% glutaldehyde solution within seconds after withdraw were performed washes twice with (A) buffer, then continuous washes twice with (B) 50% ethanol, (C) 70% ethanol, (D) 95% ethanol, (E) absolute ethanol and (F) acetone. (Experiment repreated three different subjects) (magnification ×1,000)

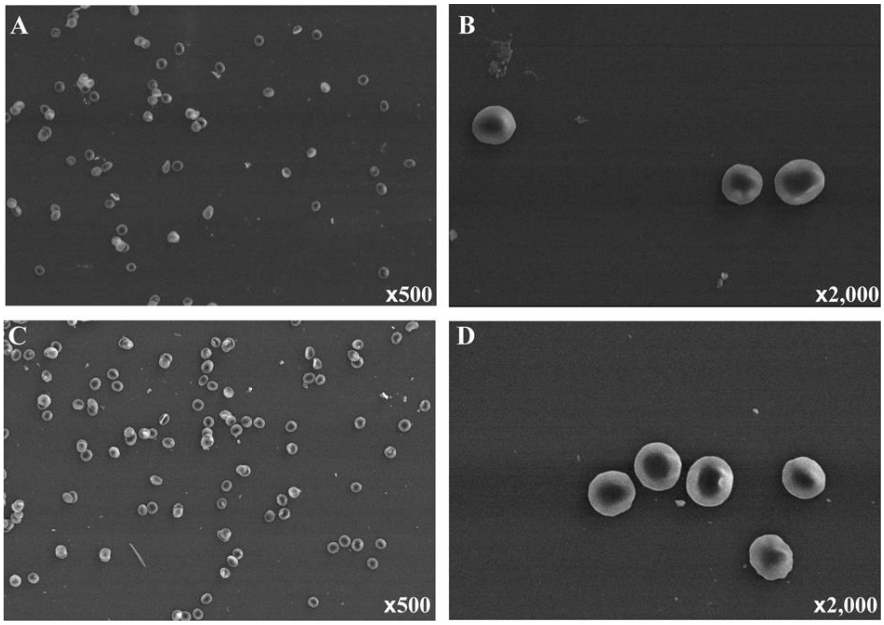

Figure 3 Red blood cell morphology identified from a scanning electron microscope. The EDTA blood samples from healthy donors in 2.5% glutaldehyde solution within seconds after withdraw were performed washes twice with buffer, 50%, 60%, 70%, 80%, 95% ethanol, absolute ethanol and acetone. (Experiment repreated three different subjects)

RBC morphology from peripheral blood donor (n = 10) were examined by 2 different slides for LM analysis and 2 different coverslips for SEM analysis. RBC morphological analysis by LM and SEM was correlated (Fig. 4). The echinocyte stage 1-3 and acanthocytes were clearly analysis by SEM. The echinocyte stage 1 was not count and identified as artifact. While echinocyte stage 2 (varying numbers of spines occurring at irregular intervals on the cell membrane) and 3 (spiculated red cells with equally spaced, short spines, over the entire surface of RBC) and acanthocytes (several fine, spine-like projections spaced unevenly over RBC surface) were count.

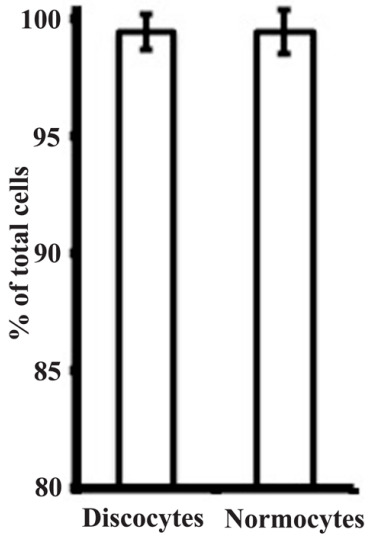

Figure 4 RBC morphological analysis by SEM (discocytes) and LM (normocytes).

Discussion and Conclusions

The high resolution and high manification with clearly identification of RBC shape were necessary for clinical features of some diseases such as anemia. SEM was choosen as the standard investigating tool that can observed blood cell morphological details in the 3-dimensions and excluded artifact. Our technique for RBC morphological analysis by SEM was highly specific, simple, practical for routine laboratory, quick report in a day. The fresh EDTA blood tube as common specimen can be used. The recommended procedure for SEM analysis was as follows. Blood sample in EDTA can be fixed by 2.5% glutaraldehyde solution either immediately or within 6 hours after withdraw. Dehydration steps of twice washing in 50%, 70%, 95%, absolute ethanol and acetone were recommended before apply on coverslip and air dry then mount onto carbon tap. Just before SEM analysis, the specimen was coated with a thin layer of palladium. Finally, the SEM analysis can be performed in any routine electron microscopic laboratory.

Acknowledgements

This work was supported by Faculty of Science, Mahidol University; Faculty of Science and Faculty of Medicine Ramathibodi Hospital, Mahidol University; Mahidol University Research Grants; Office of the Higher Education Commission and Mahidol University under the National Research University Initiative; Thailand Research Fund (TRF) (IRG5780009); and Research Chair Grant, National Science and Technology Development Agency, Thailand.

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