Elimination of Autofluorescence in Archival Formaldehyde-Fixed, Paraffin-Embedded Bone Marrow Biopsies

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Context.—High levels of autofluorescence in bone marrow tissue constitute a major obstacle to immunofluorescence analysis of bone marrow biopsies.

Objective.—To present a simple, efficient method to eliminate autofluorescence in bone marrow biopsies.

Design.—Autofluorescence of paraffin bone marrow tissues was examined in different hematologic disorders with confocal laser scanning microscopy. Strong autofluorescence was observed in primary myelofibrosis and acute leukemia with reticulin myelofibrosis in 488-nm and 561-nm channels. To eliminate autofluorescence, AutoFluo Quencher was used on bone marrow sections with different incubation times. The effects of AutoFluo Quencher on immunofluorescence analysis of bone marrow biopsies was tested using antibodies tagged with different fluorophores.

Results.—AutoFluo Quencher thoroughly eliminated the strong autofluorescence of bone marrow but did not decrease the intensity of fluorophores, leaving the specific signals of target proteins clearly visible.

Conclusions.—This study presents a simple, efficient method to eliminate autofluorescence in bone marrow paraffin tissue, and it opens the way to better results in the immunofluorescence analysis of bone marrow biopsies.

MATERIALS AND METHODS

Tissue Sections and Equipment

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks of primary myelofibrosis (PMF), acute monocytic leukemia (AML) with reticulin myelofibrosis, multiple myeloma, chronic lymphocytic leukemia, and idiopathic thrombocytopenic purpura (ITP) were obtained from the Blood Diseases Hospital, Chinese Academy of Medical Sciences, Tianjin, China.

The main equipment used was the UltraVIEW Vox confocal laser scanning microscope (PerkinElmer, Waltham, Massachusetts), equipped with laser diodes (405, 488, 561, and 633 nm) and Volocity 4.0 software (PerkinElmer; Table).

Observation of AF on Bone Marrow Sections

The FFPE sections were cut at 4 μm, and then oven dried for 2 hours at 65°C. For removal of paraffin, sections were immersed in xylene (twice for 15 minutes) and graded ethanol (twice for 10 minutes in 100%, twice for 10 minutes in 95%, 10 minutes in 80%).

After they were stained with 4',6-diamidino-2-phenylindole
Figure 1. Autofluorescence in bone marrow paraffin tissue of different hematologic disorders. Bone marrow paraffin sections of primary myelofibrosis (A through D), acute monocytic leukemia with reticulin myelofibrosis (E through H), multiple myeloma (I through L), chronic lymphocytic leukemia (M through P), and idiopathic thrombocytopenic purpura (Q through T) with 4′,6-diamidino-2-phenylindole (DAPI) staining were exposed to 405-, 488-, 561-, and 633-nm channels in confocal laser scanning microscopy (original magnification ×200).
(DAPI, 0.5 μg/mL; Sigma, St Louis, Missouri) for 15 minutes and mounted, sections were observed using confocal laser scanning microscopy (CLSM) within 24 hours. The selected area of each section was sequentially exposed to 405-, 488-, 561-, and 633-nm laser channels. Fluorescence intensity of myelofibrosis sections without DAPI staining in the 4 channels was measured by Volocity 4.0 software. Relative fluorescence intensity in 488-, 561-, and 633-nm channels was obtained with that in the 405-nm channel used as a control.

### IF Staining

The epitopes of deparaffinized, rehydrated tissues were retrieved in 0.01 M sodium citrate buffer (pH 6.0) for 15 minutes at 95°C by microwave heating. Sections were incubated for 12 hours with primary antibody in blocking buffer in a humidified chamber at 4°C and secondary antibodies for 1 hour at room temperature. Primary antibodies were as follows: rabbit polyclonal antibody to CD45 (Abcam, Cambridge, Massachusetts), rabbit monoclonal antibody to hemoglobin α (Boster, Wuhan, China), mouse monoclonal antibody to CD68 (Abcam, Cambridge, Massachusetts), and mouse monoclonal antibody to PM-2K (Abcam). Secondary antibodies were as follows: Alexa Fluor 647–conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, Pennsylvania), Alexa Fluor 488–conjugated rabbit anti-mouse (ThermoFisher, Waltham, Massachusetts), and Alexa Fluor 594–conjugated rabbit anti-mouse (ThermoFisher). Rabbit immunoglobulin G (IgG) monoclonal (Abcam) and mouse IgG monoclonal (Abcam) were used for isotype controls. Sections were washed in phosphate-buffered saline–Tween and counterstained with DAPI. Antifade mounting medium (Beyotime, Haimen, China) was added. Sections were mounted with cove slips and observed using CLSM within 24 hours.

### Sections Treated With Ammonia–Ethanol

The protocol was referred to by Baschong et al. While rehydrating the deparaffinized sections in graded alcohol, sections were immersed for 1 hour in 70% ethanol supplemented with 0.25% ammonia (NH₃ · H₂O), and rehydration was resumed by immersion in 50% ethanol for 10 minutes, after which the sections were washed in distilled water.

### Sections Treated With AutoFluo Quencher

After immunofluorescence and DAPI staining, sections were washed in phosphate-buffered saline–Tween 3 times for 5 minutes and in distilled water twice for 3 minutes. Then AutoFluo Quencher (C1212, Beijing APPLYGEN Technologies Inc, Beijing, China) was applied. Different incubation times (5, 30, 60, and 90 minutes) were applied. Sections were washed in distilled water 3 times for 3 minutes and were covered with antifade mounting medium.

### Statistical Analysis

Data were analyzed by SPSS 22.0 software (IBM, Durham, North Carolina). Relative fluorescence intensities are shown as mean ± SD.

### RESULTS

#### Features of AF in FFPE Bone Marrow Tissue

Formalin-fixed, paraffin-embedded bone marrow sections of PMF, AML with reticulin myelofibrosis, multiple myeloma, chronic lymphocytic leukemia, and idiopathic thrombocytopenic purpura with DAPI staining were exposed to 405-, 488-, 561-, and 633-nm channels in CLSM. There was strong AF in the 488- and 561-nm channels on sections of PMF and AML with reticulin myelofibrosis, but not on sections of multiple myeloma, chronic lymphocytic leukemia, and idiopathic thrombocytopenic purpura, which suggested myelofibrosis may play an important role in the production of AF (Figure 1). Relative fluorescence intensity of myelofibrosis sections (PMF and AML with reticulin myelofibrosis) without DAPI staining in 488-, 561-, and 633-nm channels were 5.1 ± 1.2–fold, 3.9 ± 0.8–fold, and 0.6 ± 0.2–fold, respectively, of that in the 405-nm channel (Figure 2).

### Sources of AF

To analyze the sources of AF in bone marrow tissue of myelofibrosis, PMF sections stained with hemoglobin α primary antibody, Alexa Fluor 647–conjugated secondary antibody, and DAPI were exposed to 405-, 488-, and 633-nm channels in CLSM. Not only nucleus but also autofluorescent cells were labeled by DAPI (Figure 3, A). Strong AF was clearly visible in 488-nm channels, emitted from small cells with abnormal morphology which showed the expression of hemoglobin α (Figure 3, B through D). The fact that certain hemoglobin α–positive cells exhibited strong AF illustrated that some erythrocytes accounted for the strong AF.

| Channel | Fluorophore Used in This Study | Excitation Laser, nm | Filtered Emission, nm | Image Display Color |
|---------|--------------------------------|----------------------|-----------------------|--------------------|
| 405-nm  | DAPI                           | 405                  | 455–515               | Blue               |
| 488-nm  | Alexa Fluor 488                | 488                  | 500–550               | Green              |
| 561-nm  | Alexa Fluor 594                | 561                  | 580–650               | Red                |
| 633-nm  | Alexa Fluor 647                | 633                  | 660–750               | Red                |

Abbreviation: DAPI, 4′, 6-diamidino-2-phenylindole.
To compare the efficiency of ammonia–ethanol and AutoFluo Quencher in eliminating AF in FFPE bone marrow tissue, serial sections from the same patient were treated with ammonia–ethanol or AutoFluo Quencher. Untreated sections were used as a control (Figure 4, A through D). DAPI staining was performed to ensure the presence of bone marrow cells, and images were acquired in 405-, 488-, and 561-nm channels.

Treatment with ammonia–ethanol on bone marrow sections led to the reduction of background signals in both 488- and 561-nm channels. However, it failed to reduce the bright AF (Figure 4, E through H). On the other hand, AutoFluo Quencher eliminated the strong AF in 488- and 561-nm channels at 5 minutes after incubation, leaving weak background signals (Figure 4, I through L). AutoFluo Quencher also reduced the background signals of bone marrow sections in a time-dependent manner and performed best at 60 minutes after incubation (Figure 4, I through X).
Figure 4. Elimination of autofluorescence in bone marrow paraffin tissue. Serial sections from the same patient were treated with ammonia–ethanol (E through H) or AutoFluo Quencher for 10 minutes (I through L), 30 minutes (M through P), 60 minutes (Q through T), 90 minutes (U through X). Sections with no treatment were used as a control (A through D). Staining with 4',6-diamidino-2-phenylindole (DAPI) was performed to ensure the presence of bone marrow cells, and images were acquired in 405-, 488-, and 561-nm channels. Merging (D, H, L, P, T, and X) of these 3 channels was performed (original magnification ×200).
Effects of AutoFluo Quencher on FFPE Bone Marrow Sections

To test the effects of AutoFluo Quencher on IF staining of FFPE bone marrow sections, markers of monocytes (coexpression of CD68/PM-2K and CD45 antigens) were detected by IF staining in AML with reticulin myelofibrosis. Strong AF concealed the specific signals of CD68 antigen labeled by Alexa Fluor 488 in the 488-nm channel (Figure 5, I through L) or signals of PM-2K antigen labeled by Alexa Fluor 594 in the 561-nm channel (Figure 6, I through L) on unblocked sections. Although specific signals of CD45 antigen labeled by Alexa Fluor 647 were detectable in the 633-nm channel, coexpression of CD68/PM-2K and CD45 antigens still could not be achieved. On AutoFluo Quencher blocked sections, AF was eliminated thoroughly; thus, specific signals of CD68/PM-2K antigen labeled by Alexa Fluor 488/594 were clear. As a result, markers of monocytes on FFPE bone sections were detected by IF staining (Figures 5 and 6).

COMMENT

As a common laboratory technique, IF staining shows great value in the diagnosis of hematologic disorders. The AF of tissues arising from cell endogenous fluorophores serves as a major obstacle to IF analysis. Our results showed that archival FFPE bone marrow biopsies of myelofibrosis have strong AF in the commonly observed channels (488 and 561 nm) in CLSM, which illustrates the importance of eliminating AF.

In this study, AF in FFPE bone marrow tissue of different hematologic disorders was examined with CLSM. To our surprise, sections of myelofibrosis displayed strong AF, whereas sections from other entities did not, demonstrating the correlation between AF and myelofibrosis. The AF of erythrocytes has been observed in human placenta paraffin tissue\textsuperscript{12} and murine renal paraffin tissue.\textsuperscript{13} In our study we found some erythrocytes displayed AF in human myelofibrosis paraffin tissue. It has been reported that the production of AF in erythrocytes is the result of the peroxidation reaction. The interaction between malondialdehyde and the amino groups of phospholipids and proteins

Figure 5. Effects of AutoFluo Quencher on immunofluorescence staining of CD68 and CD45. Serial bone marrow sections of acute monocytic leukemia with reticulin myelofibrosis were treated (A through H) or not treated (I through L) with AutoFluo Quencher. CD68 was labeled by Alexa Fluor 488 (F and J), and CD45 was labeled by Alexa Fluor 647 (G and K). Isotype controls of CD68 (B) and CD45 (C) were used for negative controls. Images were acquired in 405-, 488-, and 633-nm channels. Merging (D, H, and L) of 488- and 633-nm channels was performed (original magnification $\times$200).
during lipid peroxidation results in the formation of fluorescent chromolipids. Lipid peroxidation accounts for the AF of erythrocytes in thalassemic and uremic patients. Oxidative damage induced by humic acid results in the AF of erythrocytes. Senescent erythrocytes display enhanced green AF as oxidative damage accumulates. Ultraviolet irradiation induces AF enhancement via production of reactive oxygen species and photodecomposition in erythrocytes. Therefore, we speculate there is oxidative damage to some erythrocytes in myelofibrosis, which leads to AF.

Bone marrow sections of myelofibrosis displayed AF of different intensity in 405-, 488-, 561-, and 633-nm laser channels. Strong AF was mainly observed in 488- and 561-nm channels, thus interfering with the analysis of fluorescence emitted by the commonly used fluorophores, such as fluorescein isothiocyanate, tetramethylrhodamine, phycoerythrin, Alexa Fluor 488, and Alexa Fluor 594. In our studies, specific signals of CD68 antigen labeled by Alexa Fluor 488 and signals of PM-2K antigen labeled by Alexa Fluor 594 could not be detected because strong AF confounded specific fluorescent signals or hid weak specific signals of the markers in 488- and 561-nm channels. Therefore, it was impossible to detect fluorescent markers in bone marrow paraffin sections in the 488- and 561-nm channels using CLSM or fluorescence microscopy.

Removal of AF was crucial for the successful visualization of fluorescent markers. The commonly used fluorescence quenching agents, such as ammonia–ethanol, Sudan Black B, and borohydride, were examined in bone marrow, myocardium, and cartilage paraffin tissues by Baschong et al. They found the agents’ effects depended on the type of tissue and technique of tissue processing. Treatment with borohydride in bone marrow paraffin tissue induced bright AF of erythrocytes. Both ammonia–ethanol and Sudan Black B reduced the AF of myeloid cells and trabecular cells, but ammonia–ethanol was superior to Sudan Black B. In our studies, ammonia–ethanol and AutoFluo Quencher were each used respectively to remove AF in the bone marrow paraffin tissue of myelofibrosis. We found ammonia–ethanol could not reduce AF completely, whereas AutoFluo Quencher eliminated AF signals thoroughly.

AutoFluo Quencher is a fluorescence quenching agent made in China. It contains ions that are able to capture electrons emitted by the autofluorescent particles by way of collision, inhibiting unwanted back-hopping of electrons.

Figure 6. Effects of AutoFluo Quencher on immunofluorescence staining of PM-2K and CD45. Serial bone marrow sections of acute monocytic leukemia with reticulin myelofibrosis were treated (A through H) or untreated (I through L) with AutoFluo Quencher. PM-2K was labeled by Alexa Fluor 594 (F and J), and CD45 was labeled by Alexa Fluor 647 (G and K). Isotype controls of PM-2K (B) and CD45 (C) were used for negative controls. Images were acquired in 405-, 561-, and 633-nm channels. Merging (D, H, and L) of 561- and 633-nm channels was performed (original magnification ×200).
from the excited state to the ground state, which leads to the quenching of AF (information from product manual). The quenching efficiency of this agent varies in different tissues. It failed to remove AF in human sweat gland paraffin tissue but was effective in the removal of AF in murine spinal cord paraffin tissue. In our studies, it proved to be very effective in eliminating AF in bone marrow paraffin tissue. Besides, it did not decrease the fluorescence intensity of exogenous fluorophores, such as DAPI and Alexa Fluor 488/594/647. Therefore, fluorescent markers of monocytes can be detected easily on bone marrow paraffin sections of AML with reticulin myelofibrosis.

In conclusion, our studies provide a simple, efficient method to eliminate AF in FFPE bone marrow tissue, and they open the way to better results in IF analysis of bone marrow biopsies.

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