Simultaneous Staining of Feulgen with Ki-67 and Phosphohistone H3 Antibodies

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Introduction

Digital image analysis of cell nuclei is useful for grading morphometric parameters, in order to obtain quantitative information for the diagnosis and prognosis of cancer. Feulgen and Ki-67 staining have been used for quantification of the essential characteristics of malignant tumors, including cell proliferation, nuclear size, chromatin patterns and ploidy. 1,2 Recently, studies have shown that phosphohistone H3 (pHH3) antibody is a specific marker for mitosis and when used in combination with Ki-67 can provide prognostic and predictive values. 3,4 In this abstract, we will present both single and double stains for Feulgen plus Ki-67 and pHH3 in a single tissue section for evaluation of the labeling and mitotic index.

Materials and Methods

Formalin-fixed paraffin-embedded tissues (FFPE) were retrieved in a high pH solution using a Decloaking Chamber (pressure cooker). Immunohistochemical staining was performed using antibodies to Ki-67 (M) and pHH3 (P). For single stains, a universal polymer detection system was employed; for double stains, a double stain polymer was used for detection, followed by dual chromogens. Sections were rinsed in deionized water, followed by a modified Feulgen staining procedure.

Tissues

Colon cancer and melanoma

Pretreatment of sections

Tissue sections were retrieved with a high pH Tris buffer (Borg Decloaker, Biocare Medical) under 125 °C for 30 seconds (Biocare's Decloaking Chamber). Tissues were allowed to cool for 10 minutes then washed in distilled water.

IHC Stain Procedure

1. Primary Antibody

When used as single stains, Ki-67 (clone MM1) and pHH3 (polyclonal) were incubated for 30 minutes followed by MACH 2 mouse and rabbit polymer detections (Biocare Medical) for 30 minutes at room temperature (RT).

2. Ki-67 + pHH3 Cocktail

The Ki67 + pHH3 antibody cocktail was incubated for 30 minutes at RT followed by MACH 2 Double Stain kit # 1 (Biocare Medical)

3. Chromogens

a. Ferangi Blue Chromogen (Cat# FB813) was applied for 7 minutes.
b. Deep Space Black Chromogen (Cat# BRI4015) was applied for 5 minutes.
c. Wash in distilled water to prepare for the Feulgen stain.

Feulgen Stain Procedure

(Feulgen Stain Kit, American Mastertech, Cat #KTFEU)

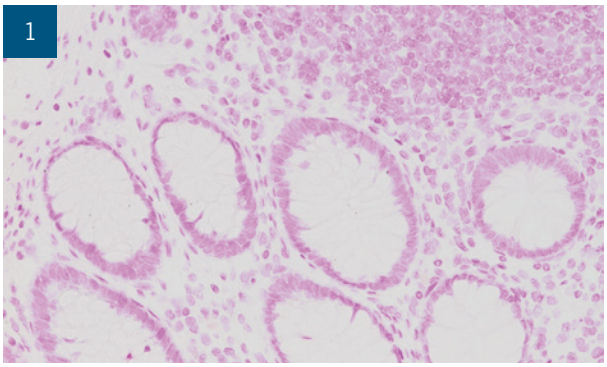
1. Place slide in 1N HCl, preheated to 60°C in a water bath for 15 min.
2. Without rinsing, Place slides directly into 3 changes SCHIFF'S REAGENT for 15 minutes each at RT.
3. Without rinsing, place slides in 2 changes of SULFUROUS RINSE for 3 minutes each.
4. Wash in distilled water 3 times.

Results

Feulgen staining identified DNA in cell nuclei on normal colon (figure 1). Double stains including Feulgen + Ki-67 (figure 2), Feulgen + pHH3 (figure 3) and Feulgen + Ki-67 + pHH3 (figure 4) were successfully stained in single sections of FFPE tissue.

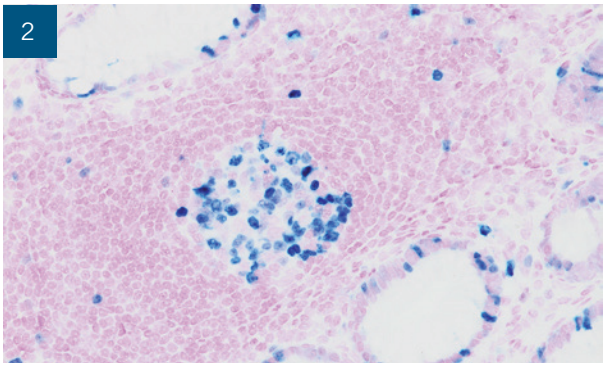
Figures

1



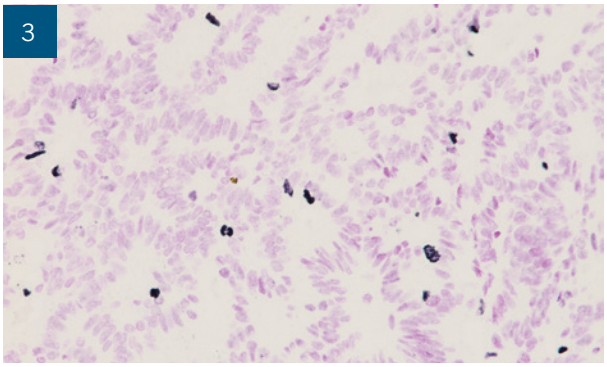
Feulgen on normal colon

2



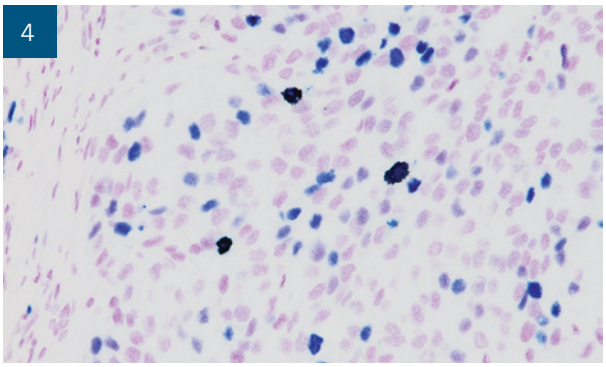
Feulgen + Ki-67 on normal colon & lymphatic germinal center

3



Feulgen + pHH3 on colon cancer

4



Feulgen + Ki-67 + pHH3 on melanoma

Discussion

In a study by Kolles1, a Feulgen stain + Ki-67 double stain protocol was successfully reported. Following their protocol, in our tissurs we found the Feulgen stain in nuclei to be much lighter and unsatisfactory. Evaluating a variety of temperatures for antigen retrieval and different retrieval solutions found that a high pH Tris retrieval solution provided increased Feulgen staining in nuclei and was superior to citrate buffer formulations; however, Feulgen staining of nuclei was still too light compared to the standard Feulgen stain without antigen retrieval. By using 3 changes of Schiff's reagent for 15 minutes each, staining was restored to the intensity observed with the standard Feulgen stain protocol. Feulgen histochemical stain must be performed after antigen retrieval, and primary antibodies and detection must be applied before the Feulgen stain.

Conclusion

Ki-67 + pHH3 antibodies were combined with Feulgen staining in a single section, and may be suitable for quantitative evaluation of morphometric parameters and DNA ploidy. In the future, other proliferative and quantitative markers might be adapted using this procedure.

References

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2. Nielsen B, *et.al.* Automatic segmentation of cell nuclei in Feulgen-stained histological sections of prostate cancer and quantitative evaluation of segmentation results. J of Cytometry A, 2012 Jul;81(7):588-601

3. Nasr MR, El-Zammar O: Comparison of pHH3, Ki-67, and survivin immunoreactivity in benign and malignant melanocytic lesions. J Dermatopathol 2008, 30(2):117-122

4. Kim YJ, Ketter R, Steudel WI, Feiden W: Prognostic significance of the mitotic index using the mitosis marker anti-phosphohistone H3 in meningiomas. Am J Clin Pathol 2007, 128(1):118-125.

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