

Environment-friendly Grocott stain without using chromic acid

We established a new “ecological” Grocott stain for demonstrating fungi, based upon the key word “4R’s: refusal, reduction, reuse and recycle” for waste management. Conventional Grocott stain employs environmentally harsh 5% chromic acid for oxidization. At first, we succeeded to reduce the concentration of chromic acid from 5% to 1% by incubating the solution at 60°C, and the 5-times diluted chromic acid solution was reusable. Eventually, we reached the “refusal” level: 1% periodic acid oxidization was efficient enough, when combined with preheating of sections in the electric jar, microwave oven or pressure pan. Because of the simplicity and convenience, we recommend pressure pan heating in tap water for 10 minutes. The stainability of fungi in candidiasis and aspergillosis was comparable with the conventional Grocott stain. *Mucor* hyphae showed enhanced staining. The modified sequence was further applicable to detecting a variety of mycotic pathogens in paraffin sections. Our environment-friendly Grocott stain can also avoid human risk of hexavalent chromium solution in the histopathology laboratory. The stain sequence is very simple and easily applicable to the practice worldwide.

Ref.: Shiogama K, et al. New Grocott stain without using chromic acid. *Acta Histochem Cytochem* 2015; 48(1): 9–14. doi: 10.1267/ahc.14045

Principle of environmentally-friendly stains

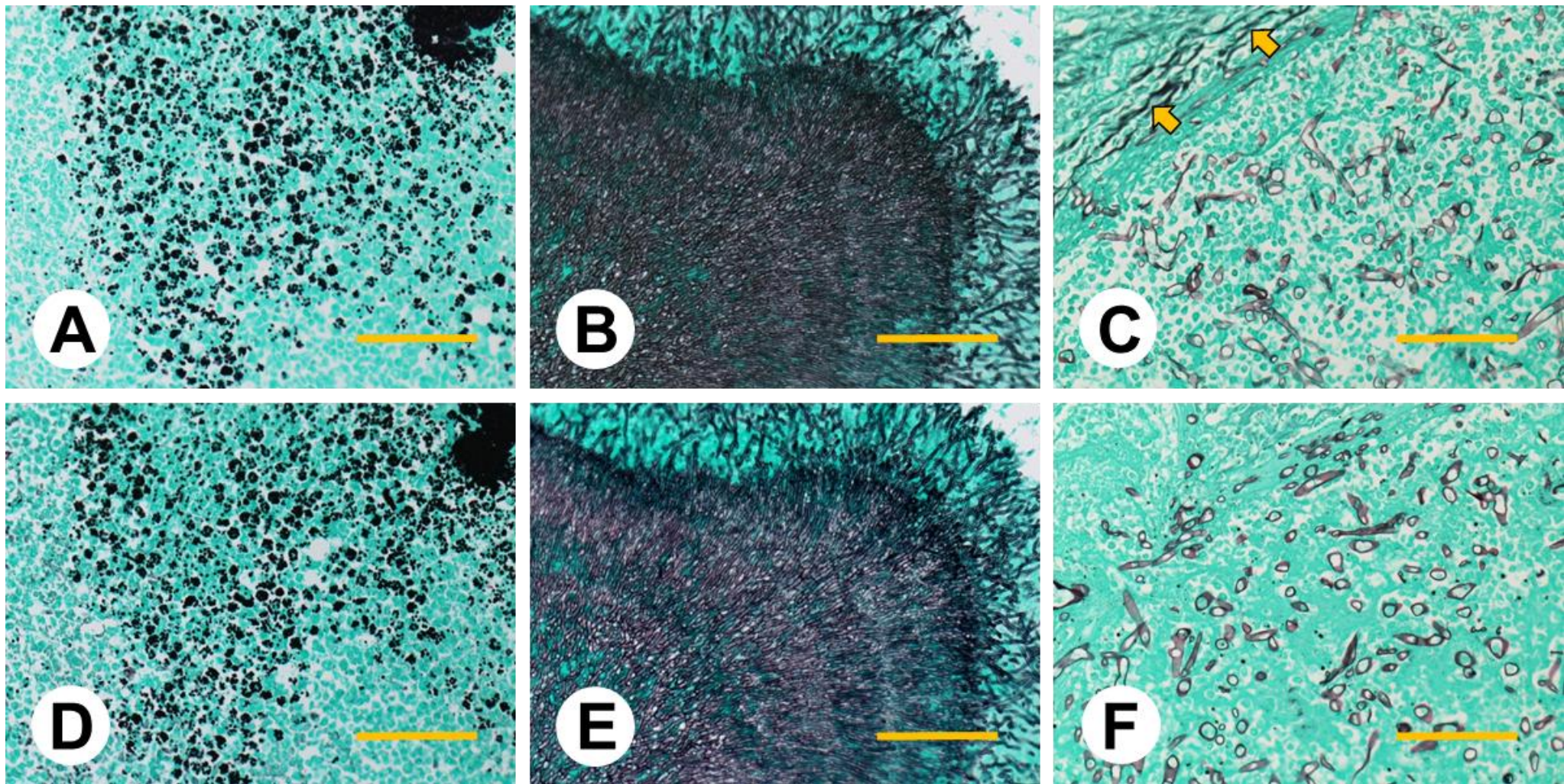
A strategy for curbing the environmental impact of waste is simple, and represented by the 4R principle of refusal, reduction, reuse, and recycle [17]. In order to develop an environmentally-friendly or “ecological” modification of Grocott stain, we firstly tried to reduce and reuse/recycle and finally to refuse chromic acid solution.

Preparation of the methenamine silver solution

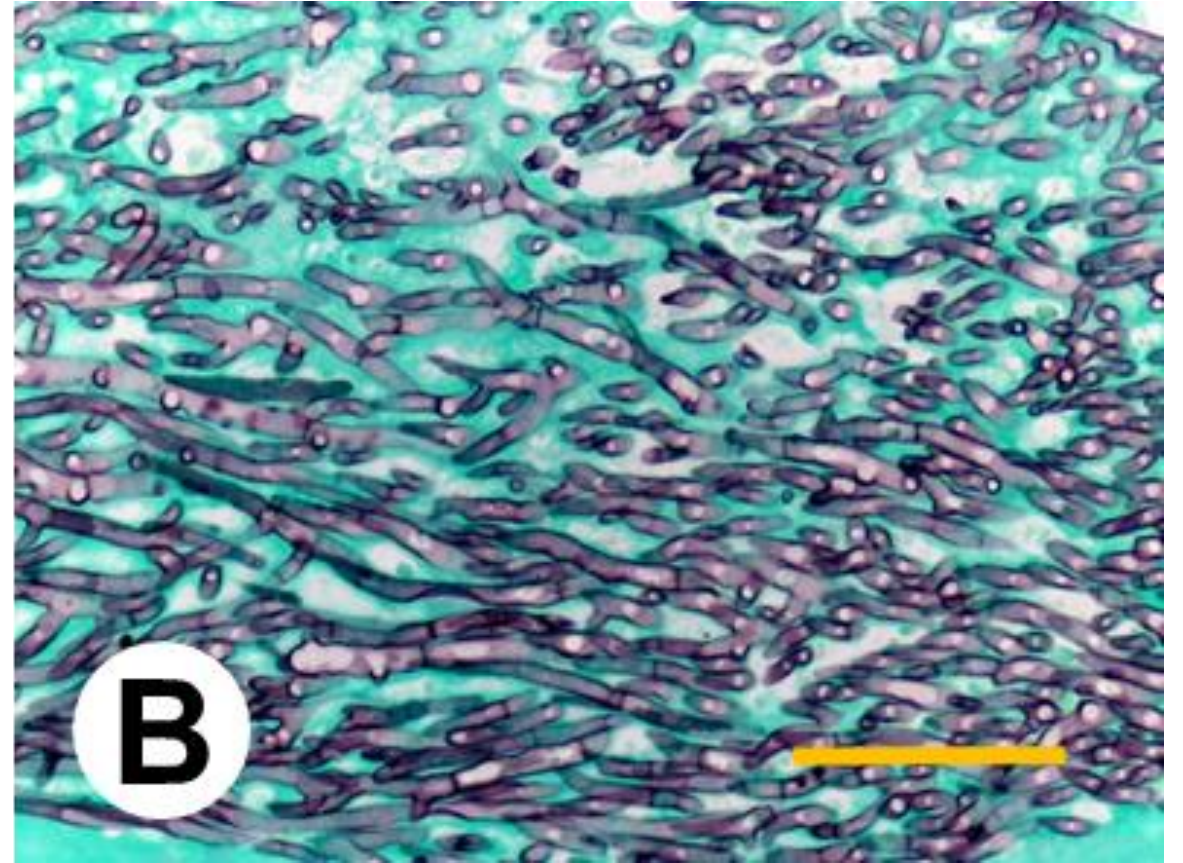
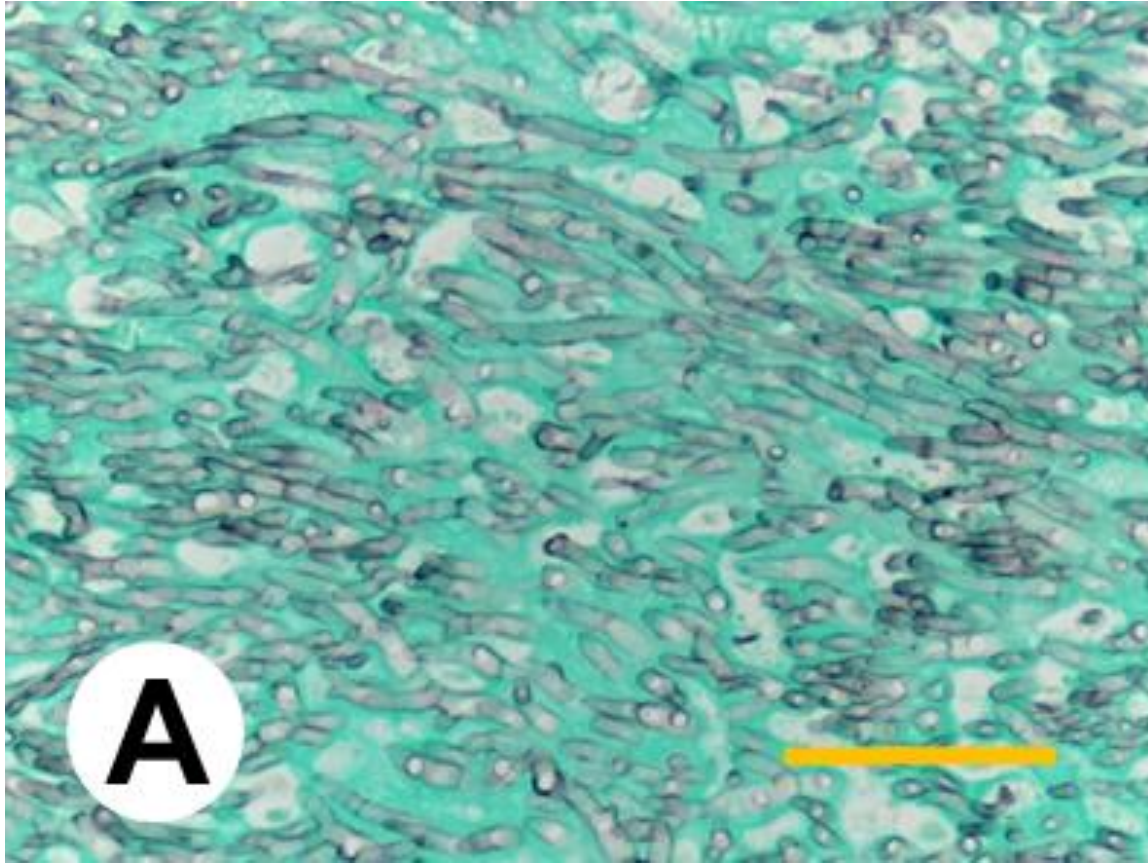
- Prepare the methenamine-silver nitrate solution just before use.
 - 1) Add 5 ml of 5% silver nitrate stock solution (AgNO_3) to 100 ml of 3% methenamine (hexamethylenetetramine) stock solution $[(\text{CH}_2)_6\text{N}_4]$.
 - 2) Then, add 25 ml of distilled water and 4 ml of 5% sodium borate stock solution ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) to the 25 ml of the mixture solution.
 - 3) Finally, mix 2-3 drops of 5% gelatin to avoid the silver mirror reaction.

Procedures for the new Grocott stain

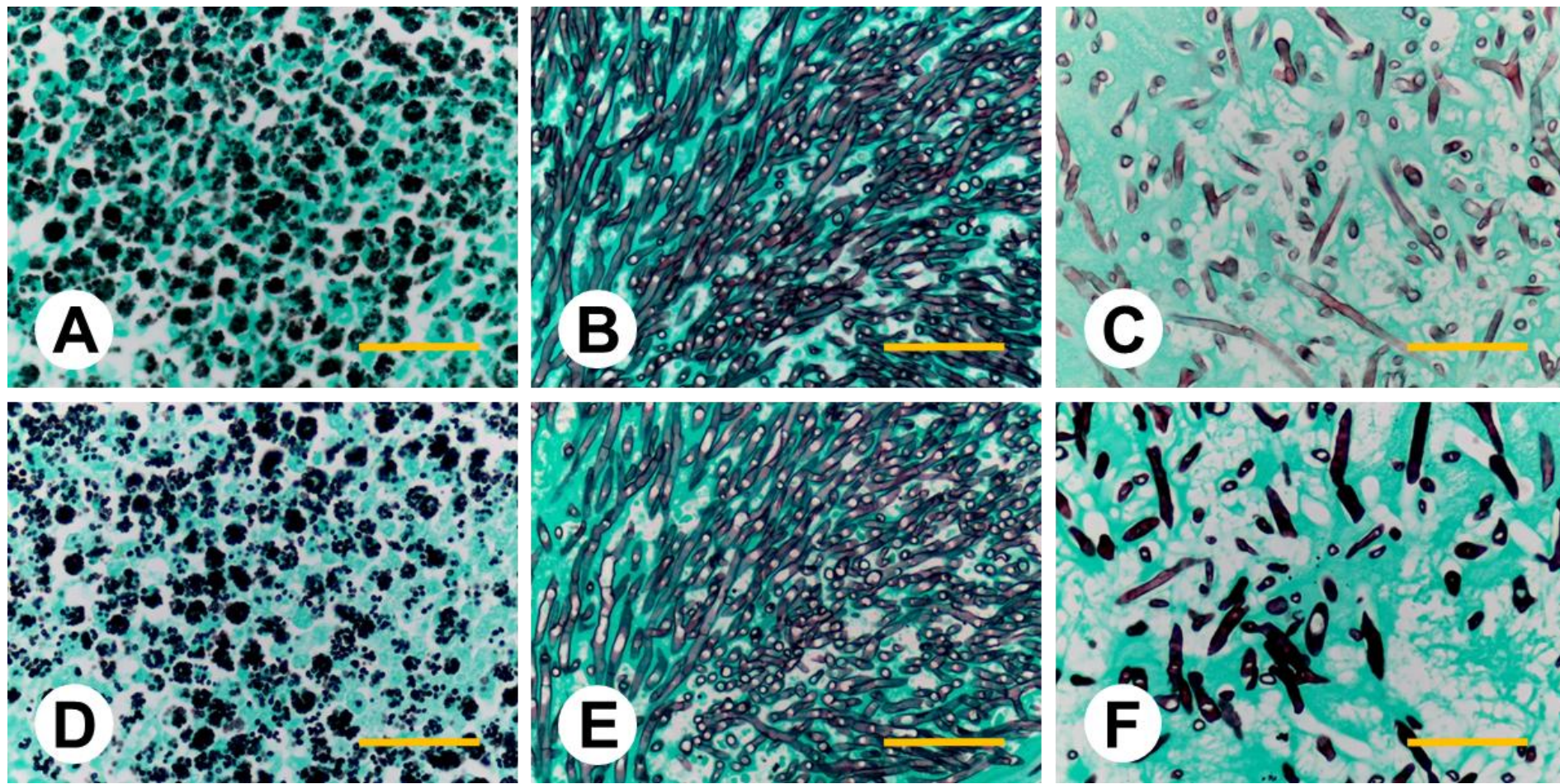
- 1) Deparaffinize sections in xylene and soak them in alcohol.
- 2) Rise sections in running tap water.
- 3) Heat sections in tap water using either electric jar at 95°C for 30 minutes, 500 watt microwave for 10 minutes or pressure pan for 10 minutes.
- 4) Cool down sections for 15 minutes on a table and then rinse them in running tap water.
- 5) Keep methenamine-silver nitrate solution in a 60°C oven.
- 6) Incubate sections in 1% periodic acid solution at room temperature for 10 minutes.
- 7) Rinse sections in running tap water and then in distilled water (3 changes).
- 8) Soak sections in the methenamine-silver nitrate solution pre-heated at 60°C for 40 to 60 minutes.
- 9) Rinse sections in distilled water (3 changes).
- 10) Replace silver with 0.1% gold chloride acid tetrahydrate ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$) at room temperature for 3 minutes.
- 11) Rinse sections briefly in distilled water.
- 12) Fix sections in 2% sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) at room temperature for 3 minutes.
- 13) Rise sections in running tap water.
- 14) Counterstain sections in 0.04% light green solution at room temperature for 5 minutes.
- 15) Rise sections in running tap water.
- 16) Dehydrate sections in ethanol, change in xylene and mount with a cover glass.



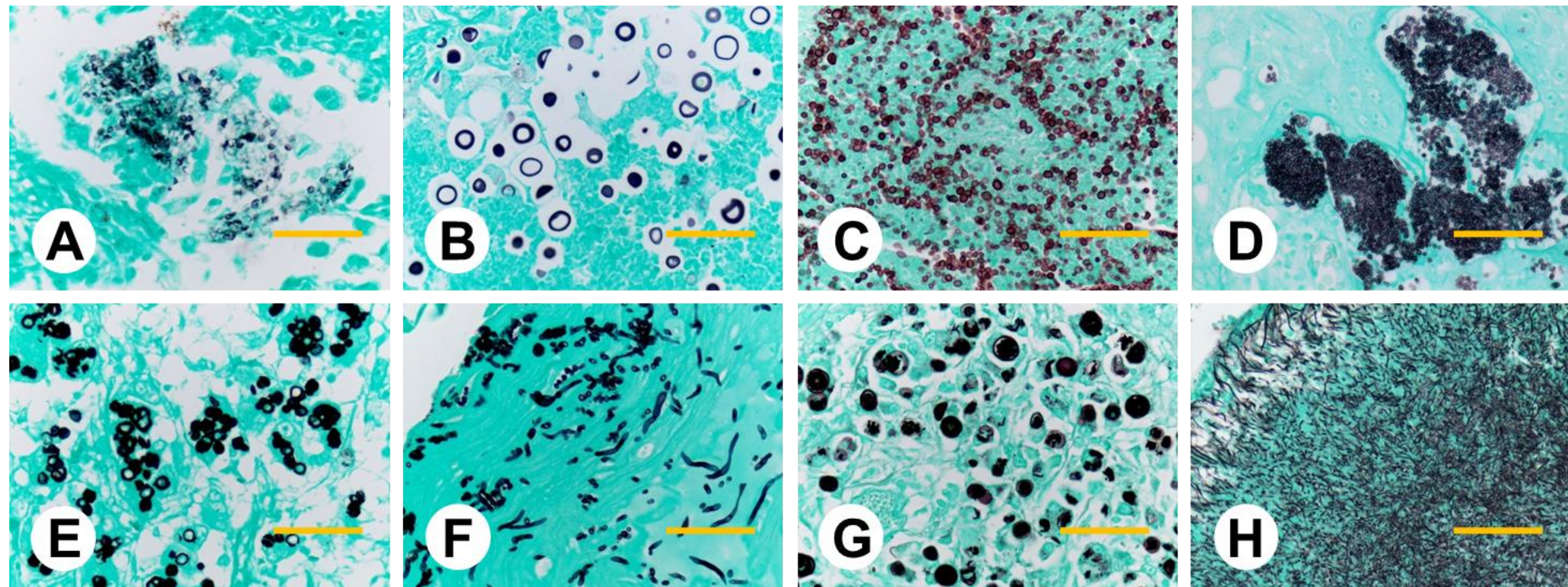
Grocott stain with a reduced concentration of chromic acid. (A&D) candidiasis, (B&E) aspergillosis, and (C&F) mucormycosis. (A, B, C) the conventional Grocott stain with 5% chromic acid oxidation at room temperature for 1 hour, (D, E, F) modified Grocott stain with 1% chromic acid oxidation at 60°C for 1 hour. The modified Grocott stain with 1% chromic acid solution gave satisfactory staining comparable with the conventional method. The co-staining in the vascular connective tissue component seen in the conventional method in panel C (arrows) disappeared in the modified sequence (panel F). Bars= 50 μ m.



The effect of preheating in demonstrating *Aspergillus fumigatus* in the lung. Periodic acid oxidation was performed before (A) and after (B) heating in tap water in an electric jar. Effect of heat pretreatment is evident. Bars= 50 μ m.



The effect of preheating in demonstrating fungi in the lung with the modified Grocott stain employing 1% periodic acid oxidation at room temperature for 10 minutes. (A&D) candidiasis, (B&E) aspergillosis, and (C&F) mucormycosis. (A-C) conventional Grocott stain using 5% chromic acid solution, (D-F) pressure pan cooking in tap water for 10 minutes. Both conditions gave distinct staining for *Candida albicans* and *Aspergillus fumigatus*. Mucor hyphae showed enhanced staining in our modification (panel F), when compared with the conventional Grocott stain (panel C). Bars= 50 μ m.



Demonstration of mycotic pathogens in biopsy specimens with our modified Grocott stain avoiding the use of chromic acid. (A) pulmonary pneumocystosis in AIDS, (B) pulmonary cryptococcosis in AIDS, (C) pulmonary histoplasmosis, (D) cutaneous penicilliosis marneffeii in AIDS, (E) cutaneous chromomycosis in hemodialysis, (F) dermatophytosis, (G) cutaneous chlorella infection in bharal (Himalayan blue sheep), and (H) pharyngeal actinomycosis. Both yeast type and hypha-forming fungi (A-F), as well as chlorella (G) and filamentous bacteria (H), are stained black with our ecological sequence. Bars= 50 μ m.

Conclusion

Our new “ecological” Grocott stain is definitely friendly to both environment and human body. Total staining time is on a par with conventional Grocott stain. Since our modified technique requires no special equipment or additional reagents, we believe our simple and practical method is easily applicable worldwide.