

## A VERSATILE GIEMSA PROTOCOL FOR PERMANENT NUCLEAR STAINING OF FUNGI

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A variety of cytological stains and staining procedures including Giemsa-HCl (23), acetic-orcein (11), propionic-carmin (17), iron haematoxylin (20), safranin O (1), aniline blue or trypan blue (5), toluidine blue (9), and basic fuchsin or Feulgen stain (4, 16) have been used to investigate the nuclear condition of reproductive and somatic structures of fungi. Fluorescent stains such as acriflavin (19), acridine orange (22), 2,5-bis(4-aminophenyl)-1,3,4-oxadiazole (BAO) (14), and especially 4,6-diamidino-2-phenylindole (DAPI) (3, 13, 18) have been employed more recently to differentiate and improve the resolution of nuclear DNA from other cellular constituents and to quantitate genomic DNA through microspectrophotometric analyses. Most of these staining procedures were developed to produce temporary slides. Destaining solutions often are used as mounting media for temporary slides and usually cause stained nuclei to fade or become less differentiated within several hours to several days after preparation. Consequently, slide preparations from such procedures must be viewed and photographed during the short period when they are optimally differentiated. Since many nuclear-staining procedures are useful when only temporary slides are required, there is a need for a protocol that provides stable nuclear staining on permanent slides.

Giemsa stain has been used successfully for many years on a large diversity of fungi with various procedures and modifications. This paper describes a protocol that allows preparation of slides for permanent nuclear staining of fungal structures with minimal fading. The procedure is useful for revealing numbers and positions of nuclei and providing tentative indications of ploidy as inferred from the numbers and sizes of nuclei within cells. It provides the capability of

locating and counting nuclei in large numbers of samples especially when permanent records are required. The procedure is not as useful for determining mitotic stages, DNA content, or chromosome numbers, particularly for Basidiomycetes.

Spores and hyphae of fungi may be prepared by a variety of methods for use with this procedure. For best results, however, specimens should contain actively growing young cells or at least viable cells when fixed since nuclei often migrate toward growing points (especially in fast-growing fungi) leaving older hyphae devoid of nuclei. Spore suspensions concentrated by low speed centrifugation or hyphal squashes of sporulating material may be applied directly to the slide and air-dried at room temperature with the coverslip removed. When germinated spores are desired, suspensions of spores that germinate readily in films of distilled water may be placed overnight on slides in moist chambers to induce germination prior to air-drying. Thin agar blocks containing germinating spores or sections of fungal colonies may be cut directly from cultures and inverted onto slides. If delicate structures must remain intact, e.g., conidia attached to conidiophores, the Riddell slide culture procedure (21) provides excellent results. Thin sections of paraffin-embedded material also are readily adaptable to the procedure. In each case, the slide is air-dried 5-10 min, flooded with filtered Haupt's adhesive in 4% formalin (2) to chemically fix and adhere the specimen to the slide, and heated at 40-45 C overnight. The slides with attached specimens can then be stored indefinitely prior to staining.

The staining procedure is initiated by hydration of the specimen on slides or cover slips in 60 C distilled water for 5-7 min. Paraffin sections must be hydrated by removing the paraffin in xylene, reaffixing the sections to the slide with Haupt's adhesive, and passing the specimens through a descending ethanol series followed by

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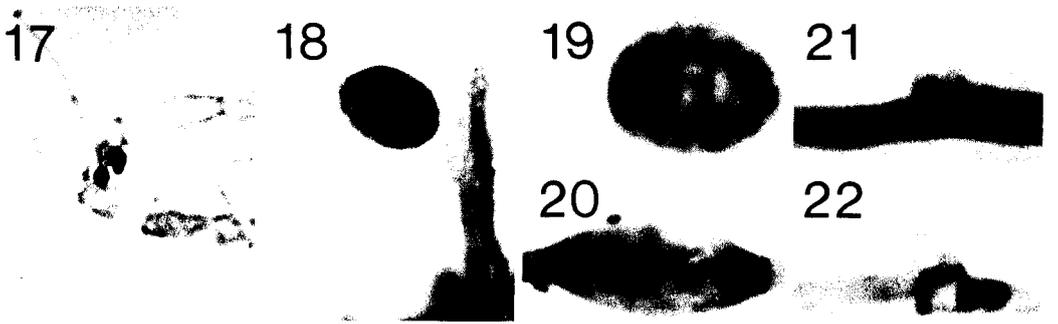
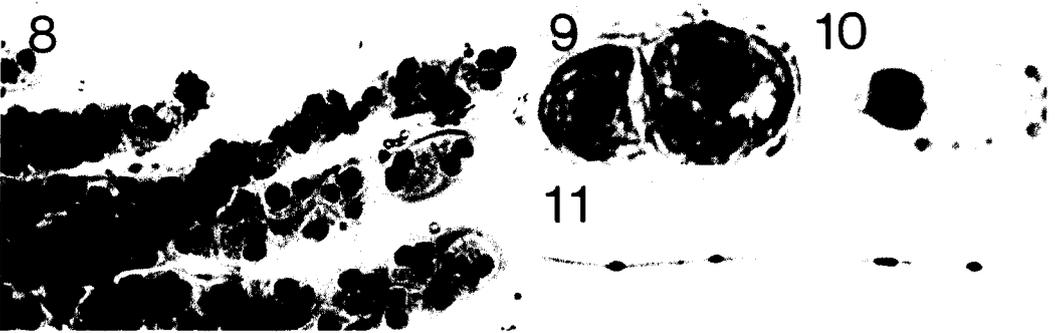
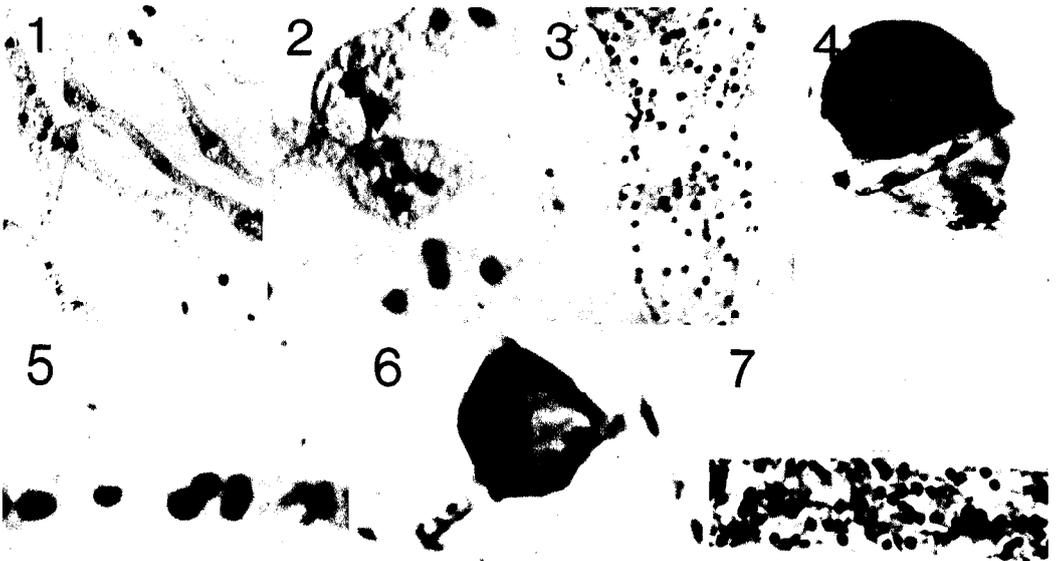
several changes of distilled water. The specimens are then hydrolyzed in 5 N HCl at 60 C for approximately 2 min, or 15-30 sec for paraffin sections. When agar blocks from cultures are used, the agar is hydrolyzed leaving the hyphae or spores attached to the slide. The acid is removed and neutralized by four 1-min rinses in distilled water and a final 1-min rinse in 0.15 M potassium phosphate buffer. For photographic purposes, a buffer pH of 6.5 yields purple-staining nuclei while pH 7.2 produces magenta-staining nuclei. The specimens are stained for 30-120 min in a 1:25 Giemsa-phosphate buffer solution (1 part Giemsa to 25 parts phosphate buffer) prepared from anhydrous methanol-Giemsa stock immediately before use. Staining times vary considerably with different fungi, although 1 hr is usually sufficient. Excess stain is removed in a brief phosphate buffer rinse. Specimens may be mounted in glycerin or buffer at this point if only temporary slides are required. The slides are then dipped in a dilute Tween-20 solution (2-5 drops per L) to facilitate the removal of water, wiped dry on the nonspecimen side, and air-dried for 5-10 min at 21 C. The specimens are differentiated and dehydrated in a 19: 1, 14:6, and 6: 14 acetone-xylene series for 20-40 sec each. Acetone differentiates nuclei by removing background stain and lipid droplets that can be confused with nuclei. The slides are passed through 2-3 changes of absolute xylene and mounted permanently in a resin-based medium such as Coverbond (Harleco, Gibbstown, New Jersey). Lead weights are placed on the cover slips and the slides are dried at 40-45 C for 1-2 days to set the resin. Excess resin may be removed from slides and coverslips using paper tissues soaked in xylene.

The entire protocol is most easily done using a slide holder and staining containers that will accommodate 20-30 slides in a batch. However, each slide should be processed individually through the acetone-xylene series until optimal times for differentiation and destaining are determined. For example, spores often destain more quickly than hyphae, thus spores often must be differentiated for only 5-10 sec at each step to avoid excessive destaining. The acetone-xylene and absolute xylene solutions should be changed after 2-3 batches of slides are processed to prevent water droplets from accumulating. The HCl, water, and buffer solutions also should be changed after 3-5 batches of slides have been processed.

The author has used this protocol on fungi from all major taxonomic groups and in cytological studies of plant pathogens (24, 25). Results from permanent, stained mounts of some representative members of the major taxa are presented in Figs. 1-22. The procedure also has been used by other workers in investigations of a variety of somatic and reproductive structures (6, 7, 8, 10, 12, 15). The versatility of the procedure lies in its adaptability to a wide range of fungi and to a variety of specimen preparation methods prior to staining. The acetone-xylene dehydration and resin-mounting steps halt the destaining process and provide the options and conveniences of permanent cytological preparations. It enables one to photograph and take data from slides even years after preparation and to avoid the urgency of examining nuclei during the brief period when they are optimally stained. The procedure also allows one to maintain permanent records of the nuclear condition of fungi at each stage in their life history for future reference. Furthermore, it offers an excellent op-

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FIGS. 1-22. Nuclear staining of somatic and reproductive structures of various taxa on permanent Giemsa slides. 1-7. Lower Fungi. 8-12. Ascomycetes. 13-17. Deuteromycetes. 18-22. Basidiomycete. 1. Hyphae and zoosporangia of *Hyphochytrium catenoides* Karling,  $\times 1100$ . 2. Zoosporangia of *H. catenoides*,  $\times 2660$ . 3. Hypha of *Achlya ambisexualis* Raper,  $\times 740$ . 4. Sporangium of *Saprolegnia* spp.,  $\times 730$ . 5. Hypha of *Pythium ultimum* Trow.,  $\times 4320$ . 6. Sporangium of *P. ultimum*,  $\times 2320$ . 7. Hypha of *Rhizopus stolonifera* (Ehrenb. : Fr.) Vuill.,  $\times 1480$ . 8. Ascospores in asci of *Mycosphaerella rabiei* Kovachevski,  $\times 2280$ . 9. Ascospore of *M. rabiei*,  $\times 4390$ . 10. Conidium of *M. rabiei*,  $\times 2160$ . 11. Ascospore of *Claviceps purpurea* (Fr. : Fr.) Tul.,  $\times 1590$ . 12. Ascogenous hyphae in a telomorph of a *Paecilomyces* spp.,  $\times 1490$ . 13. Phialides and conidia of a *Paecilomyces* spp.,  $\times 1640$ . 14. Phialide and microconidia of *Fusarium solani* (Mart.) Sacc.,  $\times 850$ . 15. Intercalary chlamydospore of *F. solani*,  $\times 4610$ . 16. Germinating macroconidia of *F. solani*,  $\times 1550$ . 17. Nuclear exchange between anastomosed hyphae of *Rhizoctonia cerealis* Van der Hoeven,  $\times 2260$ . 18. Basidium and immature basidiospore of *Echinodontium tinctorium* (Ellis & Everh.) Ellis & Everh.,  $\times 4030$ . 19. Mature basidiospore of *E. tinctorium*,  $\times 4020$ . 20. Haploid chlamydospore of *E. tinctorium*,  $\times 2120$ . 21. Nuclear pair in clamp connection of *E. tinctorium*,  $\times 2970$ . 22. Dikarotic hypha of *E. tinctorium*,  $\times 2870$ .



**portunity as a teaching tool for demonstrating the nuclear condition of various fungal taxa in the classroom or in seminars and workshops.**

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