How to stain and count nuclei.
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Background

Conventional, nonfluorescent dyes can be used, but they give variable results. The Feulgen reaction is generally not satisfactory with fungal nuclei. Fluorescent staining is now the method of choice.

Fluorescent staining. The following is copied from Raju (1982), with minor changes:

"This note is intended for Neurospora workers who have access to a fluorescence microscope but do not have easy access to advice on nuclear staining. The section on equipment provides basic information for equipping and using a fluorescence microscope.

Fluorescent staining of fungal nuclei is quick, easy and much simpler than conventional methods (See e.g. Lemke *et al.* 1978 J. Cell Sci. 29: 77; Williamson and Fennel1 1975 Methods in Cell Biol. 12: 335; Slater l977 Methods in Cell. Biol. 20: 135; Martegani and Trezzi 1979 Neurospora Newsl. 26: 20). Several articles in "Flow Cytometry and Sorting" (eds. Melamed *et al*., John Wiley & Sons, New York, 1979) give useful information on the use of numerous nucleus-staining fluorochromes.

I have been exploring possible applications of fluorescence microscopy to routine Neurospora cytology. I have used five fluorochromes that have specificity for DNA: DAPI (diamidino phenylindole), Hoechst 33258, olivomycin, auramin-O and acriflavin. DAPI, Hoechst 33258 and olivomycin are simplest and quickest to use. Acriflavin is very good for observations on ascus nuclear cytology (see Raju 1986).. DAPI and Hoechst 33258 require a mercury light source and fluorite objectives. With auramin-O, the staining solution must be prepared fresh every time, and requires filtering. (See Lemke *et al.* 1975 Exptl. Cell Res. 96: 367.) Acriflavin and auramin-O are very inexpensive and easily available. The other three are more expensive, but their cost is not prohibitive because of the very small quantities needed. Acriflavin- and auramin-O staining procedures are based on Feulgen, and use of acriflavin for quantitative DNA measurements has been reported 9Tanke and van Ingen 1980 J. Histochem. Cytochem. 28: 1007). Some fluorochromes (DAPI, olivomycin, Hoechst 3342) are also useful as vital nuclear stains.

Based on my experience using an Olympus microscope fitted with epifluorescence accessories, the fluorescent methods are indeed very simple, yet highly reliable for staining Neurospora nuclei in conidia, mycelia and asci. Nuclear counts are reliable and painless. In addition, because of the specificity of several fluorochromes for DNA, they can serve a diagnostic function that is especially needed in fungi where Feulgen staining is difficult or unsuccessful."

Procedure

A. *DAPI and Hoechst 33258* (available from Calbiochem and Sigma, respectively) are specific to AT-rich regions. DAPI (and perhaps Hoechst 33258) also interacts with polyphosphate and S-adenosylmethionine, resulting sometimes in lower contrast between nucleus and cytoplasm. Stock solution may be prepared at 1 mg/ml in distilled water and can be stored in the refrigerator for several months. 1) Place cells or mycelium in 3:1 ethanol:acetic acid for at least 10 minutes. (Fixation in 1% glutaraldehyde, 4% formalin or 70% ethanol is also satisfactory.) Store in freezer if they are not to be used immediately. 2) Wash cells in water once. 3) Dilute the fluorochrome to 0.1 to 0.5 µg/ml (final concentration) and suspend cells in it for ten minutes at room temperature. 4) Wash cells in water once or twice. 5) Suspend cells in 25% glycerol solution. 6) Place a drop of cell suspension on a clean glass slide, squash under a cover glass and seal as necessary. The slides keep well for a week or more if stored in a refrigerator.

B. *Olivomycin* (available from Calbiochem) is structurally related to mithramycin, with specificity for G-C- rich regions. Stock solution is prepared as 1 mg/ml 0.067 M Sorenson's phosphate buffer, pH 7.0, and can be stored in the refrigerator for a year or more. Place cells in ethanol:acetic acid (3:l) for at least ten minutes and store in the refrigerator if they are not to be used immediately. (Glutaraldehyde, ethanol or formalin may also be used for fixation.) Rinse cells in the phosphate buffer. Stain cells for five to ten minutes in 100 µg olivomycin per ml buffer to which magnesium chloride is added (25 mM). Follow other steps as given for DAPI above. The slides may be stored in the refrigerator for several months without loss of fluorescence intensity. According to the literature, mithramycin (available from Sigma) can be handled in a similar manner (Slater 1977 Methods in Cell Biol. 20: 135).

C. *Acriflavin* (from Sigma) and auramin-O (from Kodak) staining protocols resemble the conventional Feulgen procedure (Cirssman *et al.* 1975 Methods in Cell Biol. 9: 179; Tanke and Van Ingen 1980 J. Histochem. Cytochem. 28: 1007, Lemke *et al.* 1975 Exptl. Cell Res. 96: 367). A simplified acriflavin method involves acid hydrolysis of unfixed cells in 4N HCl for 15 to 30 min at 30°C and staining in a solution containing acriflavin (100-200 µg/ml) and K_2S_2O (5 mg/ml) in 0.1N HCl for 20 to 30 min at 30ºC. (The shorter hydrolysis and staining times are for conidia and mycelia, whereas the longer times are for asci inside intact perithecia.) The stained cells are washed at least three times (5 min each) in an HCl-70% ethanol mixture (2:98 v/v) at 30°C and twice in distilled water, to remove the noncovalently bound stain from cells. Cells are squashed under a coverglass in a drop of 25% glycerol. Raju (1986) recommends the use of acriflavin for detailed meiotic chromosome analysis. See also "*How to stain meiotic chromosomes using acriflavin*".

Equipment

For most observations, I recommend an epifluorescence microscope (also called reflected- or incident-light fluorescence microscope) employing a super-pressure mercury lamp. A mercury light source is very versatile because of its multiple emission peaks, including near-UV. The near-UV is essential when employing DAPI or Hoechst 33258. Olivomycin, acriflavin or auramin-O do not require the near-UV emission, and can be used with either a mercury or a quartz-halogen light source. In addition to the light source, one or more sets of specifically matched excitation filter/dichroic mirror/barrier filter combinations are needed to deliver the radiation of desired wavelength to the specimen and to transmit the resulting fluorescence to the observer.

In an epifluorescence microscope, the light source is located at shoulder level. Radiation from the lamp travels a horizontal path through a 'narrow pass' excitation filter. A dichroic mirror placed at a 45º angle to the horizontal path reflects the radiation downwards so that it goes vertically through an objective to the specimen. The fluorochrome-DNA complex in the specimen absorbs this radiation and in turn, emits light of longer wavelength, which travels up through the same objective and through the dichroic mirror directly to the observer. (The dichroic mirror is opaque to shorter wavelengths but transparent to longer wavelengths.)

The transmission peak of the excitation filter should match as closely as possible the absorption maximum of the fluorochrome-DNA complex (365 nm with DAPI or Hoechst 33258, and about 425 nm for olivomycin). The dichroic mirror selected in each case should be opaque for excitation wavelength but transparent to the fluorescence emitted by the specimen (470 nm with DAPI or Hoechst 33258, and about 540 nm with olivomycin). Acriflavin or auramin-O fluorescence may be examined with the same filter/mirror combination as for olivomycin. A barrier filter placed between the dichroic mirror and the eyepiece improves contrast by absorbing shorter wavelengths. It is chosen to have a cut-off point somewhere above that of the dichroic mirror but just below the emission maximum of the fluorochrome-DNA complex.

Since the epifluorescence method uses the microscope objective as its own condenser, the objective must be capable of transmitting the desired wavelengths to the specimen. Fluorite objectives such as Zeiss Neofluors, Olympus UV-FL, Nikon UV-F, and Leitz FL, among others, are most useful for DAPI or Hoechst 33258. These objectives are also better than achromats for bright-field microscopy. The most expensive planapochromats (with their many lens elements) may not transmit the near-UV wavelengths necessary for DAPI or Hoechst 33258. Excitation of olivomycin, acriflavin and auramin-0, when complexed with DNA, is in the blue range requiring a different filter/mirror combination than that used for DAPI. In this mode, normal bright-field as well as fluorite objectives can be used.

Most existing research microscopes can be adapted for work with various DNA-specific fluorochromes by fitting them with UV, violet, blue or green excitation accessories. Cost varies from $3,000 to over $10,000 for accessories alone, depending on microscope make. New Olympus or Nikon research microscopes equipped for epifluorescence work appear to be as good for our purposes as are the much more expensive Leitz, Zeiss or Reichert units.

Observation

I routinely observe cells at 400× or higher magnification. High magnification objectives have the advantage of producing a brighter image because of their larger numerical aperture. After staining with DAPI or Hoechst 33258, nuclei as well as DNA-containing cytoplasmic organelles fluoresce bluish whereas olivomycin-, acriflavin-, or auramin-O-stained nuclei fluoresce golden yellow against a generally dark background. Both auramin-O and acriflavin are very good for staining Neurospora for nuclear counts as well as for quantitative DNA measurements. I use acriflavin because of its brighter fluorescence and because the staining solution need not be prepared fresh every time.

Fluorescent images fade as a result of intense excitation radiation: Fading is more rapid when high magnification objectives are used. DAPI and Hoechst 33258 are relatively stable and allow observations to be made for 5 to 10 minutes on each microscope field. Olivomycin (or mithramycin) fluorescence fades dramatically within a few seconds, especially in fresh preparations. Despite the initial rapid fading in these fresh specimens, residual fluorescence is adequate to allow reliable counting of nuclei and observation of major nuclear features. After storage for a week or more at 4°C, the olivomycin-stained specimens show remarkable resistance to fading and observations may be made for up to ten minutes on each microscope field. Acriflavin- or auramin-O-stained specimens fade less rapidly, allowing for observations to be made for five minutes or longer, even on fresh specimens.

Fluorescent fungal nuclei are especially difficult to record photographically because of their small genome size and the consequent problem of rapid fluorescence fading. Standard fine-grain films require unusually long exposures (4 minutes or longer) and fast films are very grainy at the desired magnifications. I find the recently introduced Ilford XP2 (ASA 400) black and white film exceptionally good for photographing fluorescent Neurospora nuclei and chromosomes. This film combines the fine grain of slow films and the high speed of fast films. See also "*How to do film and digital imaging"*.

Nonfluorescent staining

Conventional dyes may be used for nuclear counts as well as for general cytology (Barratt and Garnjobst 1949, Barry 1966; Duran and Gray 1989, Raju 1984, 1998, Raju and Newmeyer 1977, Somers *et al.* 1960). See "*How to prepare aceto-orcein squashes, especially for pachytene chromosomes", "How to use hematoxylin for cytological studies",* and *"How to obtain naked nuclei extruded from the cell"*.

References

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