Easy methods for fluorescent staining of Neurospora nuclei.

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Abstract
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Nemeti, F. M., M. Schablik and G. Szabo

Experiences with Metzenberg’s method for preparing Neurospora DNA.

Metzenberg and Baisch (1981 Neurospora Newsl. 28: 20) reported an easy method for preparing high molecular weight DNA from Neurospora crassa RL3-8 A. We confirmed their results: however, in our hands the nucleic acid prepared by Metzenberg’s method contained large amounts of RNA and polysaccharides. To reduce contamination in our DNA preparation the original procedure was modified as follows: (a) digestion with RNase was extended to 3 hours; (b) final precipitation of DNA was performed with 0.5% volume of iso-propanol.

Since the product obtained in this way still contained equal amounts of DNA and RNA, as determined by the di-phenylamine (Richards 1974 Anal. Biochem. 57: 369) and phloroglucine (Dische and Barenfreund 1957 Biochim. Biophys. Acta 23: 639) methods, respectively, an additional treatment with RNase was performed. The RNA content of the final product was less than 10%.

The DNA prepared either by the method of Metzenberg or according to the above modification contained about equal amounts of DNA and polysaccharides (w/w): the latter was determined with the anthron reagent (Roe 1955 J. Biol. Chem. 212: 335).

The molecular weight of DNA was determined by agarose gel electrophoresis (Aaij and Borst 1972 Biochim. Biophys. Acta 269: 192) with λ-phage DNA used as standard, and was found to be about 49 kilobase pairs (kb). About 5% of the product were fragments of lower molecular weight (between 23 and 49 kb).

In conclusion, although Metzenberg’s method provides an easy way for obtaining high molecular weight DNA, the DNA prepared in that way is contaminated to a significant degree with RNA and polysaccharides.

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Raju, N. B.

Easy methods for fluorescent staining of Neurospora nuclei.

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.


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-0 and acriflavin. DAPI, Hoechst 33258 and olivomycin are simplest and quickest to use. Acriflavin is very good for observations on ascus nuclear cytology. DAPI and Hoechst 33258 require a mercury light source and fluorite objectives. With auramin-0, the staining solution must be prepared fresh every time, and requires filtering. (See Lenke et al. 1975 Exptl. Cell Res. 96: 367.) Acriflavin and auramin-0 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-0 staining procedures are based on Feulgen, and use of acriflavin for quantitative DNA measurements has been reported. 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.

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:
These objectives are also better than achromats for bright-field microscopy. The most expensive planapochro-
rics require a separate filter/mirror combination as compared to DAPI. In this mode, normal bright-field as well
in the blue range requirings a separate filter/dichroic mirror/barrier filter combination are needed to deliver the
radianc of desired wavelength to the specimen and to transmit the resulting fluorescence to the observer.

A cronymc (available from Calbiochem) is structurally related to mithramycin with specificity for G-C. Stoc
slation is prepared as 1mg/ml 0.067 M Sorenson's phosphate buffer, pH 7.0, and stored in the refriger-
ator for a year or more. Place cells in ethanol: acetic acid (3:1) for at least ten minutes and store in the re-
imer 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 stained 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 (Grissman et al. 1975 Methods in Cell Biol. 9: 179; Tanke and Van Ingen 1980 J. Histochem Cytochem
sis 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 K2S2O4 (5 mg/ml) in 0.1N HCl for 20 to 30 min at 30°C. (The shorter hydrolysis and
staining times are for conidia and mycelia, whereas the longer times are for asci insides intact perithecia.)
The stained cells are washed at least three times (5 min each) in HCl-70% ethanol mixture (2:98 v/v, at 30°C)
coverglass in a drop of 25% glycerol.

Since the epifluorescence method used the microscope objective as its own condenser, the objective must
be capable of transmitting the desired wavelength 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 planapochro-

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coverglass in a drop of 25% glycerol.

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, 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 'narrowpass' excitation filter. A dichroic mirror placed at a 45° 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.)

Most existing research microscopes can be adapted for work with various DNA-specific fluorochromes by
flying 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 out' purposes as are the much more expensive Leitz, Zeiss or
Reichert units.

Observation

I routinely observe cells at 400x 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 for five to ten minutes on each microscope field. DAPI and Hoechst 33258 are relatively stable and allow observations for five to ten minutes on each microscope field. DAPI and Hoechst 33258 are relatively stable and allow observations for five to ten minutes on each microscope field. 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. Acriflavine- or auramin-O-stained specimens fade less rapidly; observations may 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 min. or longer), and fast films are very grainy at the desired magnifications. I find the recently introduced Ilford XP1 (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. (Supported by U.S. Public Health Service Grant AI-01462.) - - Department of Biological Sciences, Stanford University, Stanford, California 94305.

Rigby, D. L., A. J. Baron, and A. Radford

New assays for carbamyl phosphate synthetase applicable in the presence of exogenous carbamyl phosphate.

While investigating the effect of exogenous carbamyl phosphate (CP) on pyrimidine-specific carbamyl phosphate synthetase (CPS) activity, it became necessary to develop an assay which did not depend on the estimation, directly or indirectly, of CP present at the end of the reaction. One method was devised which instead estimated the synthesis of another product of CPS, the glutamate derived from glutamine. This glutamate was used as a substrate for the glutamate dehydrogenase assay. The reaction mixture contains the sample in 100 mMolar tris acetate buffer at pH7.0, 6 mMolar glutamate, 12 mMolar ATP, 30 mMolar potassium bicarbonate, and 0.4 mMolar NADP+. Synthesis of NADPH is followed by absorbance at 340 nm over a period of 120 minutes at 37°C. Using a Unicam SP-800 dual beam spectrophotometer. As a control, ATP, a substrate of CPS, was omitted from the reaction. This still retained some nonspecific reaction involving NADP and glutamine in both the experimental and control mixtures, and the reaction trace produced is shown in the figure. To find that part of the trace corresponding to the CPS activity, a further control was introduced by the use of an extract of the CPS- strain pyr-3 (KS20), and this trace is also shown in the figure. It can be seen that the positive slope after the initial nonspecific peak corresponds to CPS activity, the initial peak being nonspecific. Comparison of this method with that of Prescott and Jones, assaying in the absence of exogenous carbamyl phosphate, gave identical results.

The second method is based upon the conversion of 14C bicarbonate, to carbamyl phosphate and beyond. The reaction mixture used is basically as for the Prescott and Jones assay, with a 3 ml reaction mixture containing approx. 2 mg/ml of crude Neurospora extract. Duplicate 0.1 ml samples are taken with time. To one sample is added 0.2 ml 50% sulfuric acid, and the mixture is then heated at 90°C to liberate any bicarbonate and CP as CO2, so that only ureidosuccinate (US) remains. To the second is added 0.1 ml 1M ammonium chloride, followed by 90°C for 10 minutes, then 0.1 ml 50% sulfuric acid and again 90°C for 10 minutes. This liberates bicarbonate but retains both CP (fixed as urea) and US. Thus the 14C incorporated into CP and US can be estimated by scintillation counting. - - - Department of Genetics, Leeds University, Leeds LS2 9JT, U.K.