Some Fluorescent Counterstains for Neuroanatomical Studies

L.C. SCHMUEDE, L.W. SWANSON, and P.E. SAWCHENKO

The Salk Institute, La Jolla, California 92037
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Methods for counterstaining neural tissue that contains fluorescent markers have been developed. Acridine orange is useful for localizing cells that are retrogradely labeled with the fluorescent tracers true blue, bisbenzimide, and nuclear yellow because at low concentrations it yields a green Nissl stain when excited with blue, but not with ultraviolet, light; since the tracers fluoresce only when exposed to ultraviolet light, they are not masked by the counterstain. In addition, counterstaining at pH 2 increases bisbenzimide fluorescence considerably. Ethidium bromide is useful for immunohistochemistry (IHC) because it yields a bright red Nissl counterstain when excited by green light, and is only faintly visible when the fluorescent marker is excited with blue light, or when ultraviolet excitation is used. Ethidium bromide is therefore a good counterstain for fluorescent retrograde tracer and for combined IHC-retrograde tracer studies as well. Certain dyes are also useful for studies of the normal morphology of neural tissue. For example, bisbenzimide and nuclear yellow at low concentrations produce a brilliant Nissl stain at pH 2, and stain only nuclei at pH 7.2. The latter procedure may be particularly useful for cell counts. Finally, neutral red, astrazone red, and safranin-O differentially stain cells and myelinated fibers, producing fluorescence analogs of the Klüver-Barrera stain.

KEY WORDS: Fluorescent counterstains; Retrograde tracers; Immunohistochemistry; Acridine orange; Ethidium bromide; Bisbenzimide; Nuclear yellow.

Materials and Methods

Tissue processing. Most of the studies were carried out on adult albino rat brains that were processed by a method previously described in detail (Sawchenko and Swanson, 1981) for the simultaneous demonstration of the retrogradely transported fluorescent tracer true blue, and a specific antigen localized with an indirect immunofluorescence technique. In brief, true blue was injected stereotaxically into any one of a number of sites in the brain or spinal cord, and 3 to 7 days later the animal was anesthetized and perfused through the ascending aorta with 10% neutral buffered formalin (following a brief rinse with normal saline). The brains were removed, immersed overnight in fixative containing 15% sucrose, and sectioned at 30 µm on a freezing microtome. Free-floating sections were then incubated in a primary antiserum (raised in rabbits) that was then localized with an indirect immunofluorescence technique. In brief, true blue was injected stereotaxically into any one of a number of sites in the brain or spinal cord, and 3 to 7 days later the animal was anesthetized and perfused through the ascending aorta with 10% neutral buffered formalin (following a brief rinse with normal saline). The brains were removed, immersed overnight in fixative containing 15% sucrose, and sectioned at 30 µm on a freezing microtome. Free-floating sections were then incubated in a primary antiserum (raised in rabbits) that was then localized with fluorescein isothiocyanate-conjugated (affinity purified) anti-rabbit immunoglobulin G (FITC-ARG). The sections were then mounted on gelatin-coated slides, dried, and counterstained. Finally, the sections were mounted with phosphate buffered glycerol (1:1; pH 8.6), and observed with a Leitz Dialux 20 fluorescence microscope equipped with a Ploem illumination system and a 100 W Hg light source. The following Leitz filter systems were used: system "A" (wide-band ultraviolet excitation for retrograde tracers); system "L" (blue excitation for FITC); and system "N" (green excitation for certain counterstains). The characteristics of these filter systems are given in Table 1.

In other experiments, two fluorescent retrograde tracers that label different parts of the neuron, i.e., true blue for cytoplasm, and bisbenzimide or nuclear yellow for nuclei (Kuypers et al., 1980), were
Table 1. Properties of most useful fluorescent dyes for staining neural tissue

<table>
<thead>
<tr>
<th>Dye</th>
<th>Typical concentration (%)</th>
<th>pH</th>
<th>uv (A)</th>
<th>Excitation blue (N)</th>
<th>green (N)</th>
<th>Color of fluorescence</th>
<th>Cytochemical selectivity</th>
<th>Major use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisbenzimide</td>
<td>0.0001</td>
<td>2.0</td>
<td>+ + +</td>
<td>yellow</td>
<td>blue</td>
<td>cytoplasm + nucleus</td>
<td>normal cytoarchitectonics</td>
<td>cell counts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.2</td>
<td>+ + +</td>
<td>blue</td>
<td>cytoplasm + nucleus</td>
<td>nucleus</td>
<td>normal cytoarchitectonics</td>
<td>cell counts</td>
</tr>
<tr>
<td>Nuclear yellow</td>
<td>0.0001</td>
<td>2.0</td>
<td>+ + +</td>
<td>blue</td>
<td>cytoplasm + nucleus</td>
<td>nucleus</td>
<td>yellow</td>
<td>cytoplasm + nucleus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.2</td>
<td>+ + +</td>
<td>yellow</td>
<td>cytoplasm + nucleus</td>
<td>nucleus</td>
<td>yellow</td>
<td>cytoplasm + nucleus</td>
</tr>
<tr>
<td>Neutral red</td>
<td>0.1</td>
<td>4.5</td>
<td>+ +</td>
<td>red</td>
<td>orange</td>
<td>myelin</td>
<td>normal morphology</td>
<td>cytoplasm + nucleus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>blue</td>
<td>neuropil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acridine orange</td>
<td>0.0001</td>
<td>2.0</td>
<td>+ +</td>
<td>green</td>
<td>cytoplasm + nucleus</td>
<td>nucleus</td>
<td>red+b</td>
<td>retrograde tracer studies (with true blue, bisbenzimide, nuclear yellow)</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>0.00002</td>
<td>7.2</td>
<td>(+) (+)</td>
<td>red</td>
<td>cytoplasm + nucleus</td>
<td>nucleus</td>
<td>IHC, tracers</td>
<td>retrograde tracers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>(+) (+)</td>
<td>red</td>
<td>cytoplasm + nucleus</td>
<td>nucleus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


1Ethidium bromide fluorescence appears orange when viewed with filter systems "A" and "L."

Results

Normal Material

During the course of this study it became clear that several dyes might be useful for cytoarchitectonic studies and for cell counts in normal material. Two dyes commonly used as retrograde tracers, bisbenzimide and nuclear yellow, were found to be particularly effective as counterstains at concentrations between 0.0001 and 0.001% (Table 1). Interestingly, at pH 2.0 both dyes produced a brilliant yellow Nissl stain, while at pH 7.2 only the nucleus, and a ring around the nucleolus, were fluorescent when excited with ultraviolet light (Figure 1). At pH 7.2 bisbenzimide fluorescence was light blue and nuclear yellow fluorescence was yellow; at pH 2 the color of the fluorescence for the two dyes was reversed. The sections were stained for 2 min, rinsed in buffer at pH 7.2 or 2.0, washed briefly in distilled water, dehydrated, and mounted in DPX. This staining procedure can also be applied to mounted paraffin sections that have been deparaffinized and rehydrated, and to thin (about 1 μm) plastic (embedded in Spurr’s medium) sections.

Neutral red differentially stains Nissl substance, neuropil, and myelinated fiber tracts when viewed in either the light or the fluorescence (ultraviolet or blue excitation) microscope, and is thus somewhat analogous to a Klüver-Barrera (Klüver...
and Barrera, 1953) stain (Figure 2A, Table 1). The staining was optimal when rehydrated sections were pretreated for 1 min in a mixture of 70% ethanol:2% glacial acetic acid (50:1), rinsed for 1 min in distilled water, stained for 2 min in 1% neutral red (in phosphate buffer at pH 4.5), and differentiated in acid alcohol. We also found that safranin-O gives a similar staining pattern, and that astrazone red, which has been used to stain plant tissues (Maćz and Vágás, 1962), differentially stains cells (brilliant pink fluorescence) and myelinated fiber tracts (orange fluorescence).

We found that sections could be stained successfully with a great number of dye combinations, which differentially stain particular cytological features. One of the more useful and esthetically pleasing of these is shown in Figure 2B; when the sections were stained with nuclear yellow and ethidium bromide (see below) at pH 7.2, the nuclei of neurons (and other cells) fluoresce bright yellow and the cytoplasm (presumably the Nissl substance or RNA) fluoresces red when excited with ultraviolet light.

Immunohistochemistry

There are several requirements for adequate counterstaining of immunofluorescence material. The counterstain should be water-soluble and should not mask the FITC labeling; that is, it should fluoresce a different color and it should be at best only weakly visible when using filter system "I." In addition, it must be effective at neutral or basic pHs, since FITC labeling deteriorates seriously in acidic solutions. The counterstain that meets these criteria best is ethidium bromide (Table 1). At concentrations between 0.0001 and 0.00001%, this dye produces a moderately bright red Nissl stain (neuronal cytoplasm and nucleioli, and glial nuclei) when excited by green light, but is only weakly fluorescent when excited by blue or ultraviolet light. Thus, when exciting FITC-labeled cells or fibers (greenish-yellow fluorescence) with blue light, faint red to orange cell staining is also seen (especially at higher magnifications). However, the weak ethidium bromide staining is helpful in localizing the FITC-stained cells, and masking is minimal because of the contrast in colors. We have used ethidium bromide counterstaining successfully in conjunction with a variety of antisera. For example, Figure 2E shows a group of dopamine-b-hydroxylase (DBH)-stained cells in the A2 catecholamine group of the dorsal vagal complex. The antiserum, which was raised against rat adrenal DBH (kindly supplied by Dr. K.B. Helle, University of Bergen, Norway; see Helle et al. (1979)), was used at a dilution of 1:750. The air-dried sections were rehydrated in distilled water (2 min), counterstained for 30 sec to 2 min (in phosphate buffer at pH 7.2), rinsed in phosphate buffer (pH 7.2) for 1 min, washed briefly in distilled water, and mounted in glycerol (pH 8.6).

Fluorescent Retrograde Tracers

We have found that counterstains suitable for localizing cells that are labeled with retrogradely transported fluorescent markers (which are excited by ultraviolet light) must be excited primarily by other than ultraviolet light, since the tracers are easily masked. It is also preferable that the dye used for counterstaining be water-soluble, because several of the tracers (e.g., bisbenzimide and nuclear yellow) fade when placed in alcohols (Sawchenko and Swanson, 1981). And finally, the counterstain should be easy to differentiate, because the tracers appear to diffuse readily from retrogradely labeled cells in a variety of liquid media (Bentivogio, et al., 1980; and unpublished observations). Ethidium bromide (above) and acridine orange proved to be the best counterstains for localizing these tracers. At a concentration of 0.0001%, acridine orange provided a green cell (nucleus and cytoplasm) stain when excited with blue light and was not visible with ultraviolet light (Figure 2C,D; Table 1), hence it can be used in conjunction with true blue, bisbenzimide, and nuclear yellow. Interestingly, when counterstaining is carried out at pH 2 (citrate-HCl...
gradely labeled pyramidal cells in field CA, of the hippocampus are magnification \( x \times 650 \). (C and D) Two photomicrographs of the same section, taken with different filter systems. The tracer true blue was injected into the lateral septal nucleus, and the appearance of retrogradely labeled pyramidal cells in field CA of the hippocampus are shown in C, as viewed with filter system "A". With filter system "I" (D), the acridine orange counterstain is visible. Original magnification \( x \times 150 \). (E) A low-power photomicrograph of a section through the caudal medulla (note the central canal at the bottom). The section was stained with an indirect immunofluorescence method for DBH, and shows bilaterally symmetrical groups of noradrenergic cells (greenish-yellow fluorescence at the top) centered in the nucleus of the solitary tract. Cytoarchitectonic features in the section (such as the hypoglossal and dorsal motor nuclei) are revealed by an ethidium bromide counterstain. Blue excitation (system "I"). Original magnification \( x \times 60 \). (F--H) Three photomicrographs of the same section to show the location of noradrenergic cells in and near the locus coeruleus that project to the spinal cord. (F) The region of the locus coeruleus as shown by an ethidium bromide counterstain; green excitation (system "N"). (G) Noradrenergic cells as shown by an indirect immunofluorescence stain for DBH, using FITC as a marker; blue excitation (system "I"). (H) Cells retrogradely labeled by true blue, after an injection of the tracer in the spinal cord; ultraviolet excitation (system "A"). Three DBH-labeled cells in the subcoeruleus area that project to the spinal cord are indicated by arrows. Dorsal: right; lateral: bottom. Original magnification \( x \times 150 \).

buffer), the bisbenzimide fluorescence is considerably brighter and the true blue fluorescence is not significantly altered. Although we do not know the reason for this enhanced fluorescence, we did note (see above) that when bisbenzimide is used as a counterstain, the fluorescence was brighter, and was selective for nuclei and cytoplasm at acidic pHs. Staining with acridine orange is quite simple: air-dried sections are dipped in distilled water, stained for 30 sec to 2 min, washed in citrate-HCl buffer at pH 2 (or in phosphate buffer at pH 7.2), rinsed briefly in distilled water, dried, dehydrated in xylene, and mounted in DPX. The related acridine derivative, quinacrine, is also a useful counterstain. It fades less rapidly than acridine orange, but does not stain cells as crisply. The protocol for counterstaining with quinacrine is similar to that for acridine orange.

It was also found that sections containing cells that are brightly labeled with true blue can be counterstained with neutral red or astrazone red, which differentially stain cells and myelinated fiber tracts (see above).

**IHC with Retrograde Tracers**

Immunofluorescence and retrograde transport methods can be used concurrently to characterize biochemically the cells that project to a particular terminal field. For this purpose, a counterstain is needed that does not mask either FITC or true blue fluorescence, and that can be used in neutral or somewhat basic solutions. As indicated above, ethidium bromide (Table 1) meets these requirements and is the best counterstain we have found for this purpose. The use of this counterstain is illustrated in figure 2F--H. True blue was injected into the spinal cord and sections that contained retrogradely labeled cells in the locus coeruleus were incubated in the antiserum to DBH described earlier. The sections were then counterstained with ethidium bromide as described above.

**Other Applications**

It is worth noting that ethidium bromide may be a useful counterstain with a variety of methods. For example, we have found (unpublished observations in collaboration with R. Peterfreund and W. Vale, the Salk Institute) that dissociated hypothalamic cells, which have been plated to a culture dish and incubated in anti-somatostatin (and detected with FITC-ARG), are nicely counterstained with ethidium bromide, following the method described above. This approach may be useful for determining the portion of FITC-labeled cells in a heterogeneous population of cells. In addition, cell cultures that are obtained by placing a glass slide in contact with fresh tissue slices of the hippocampal formation are similarly counterstained with ethidium bromide (unpublished observations in collaboration with J. Davidenas, the Salk Institute).

We have also found (in collaboration with R. Loy, University of California, San Diego) that histofluorescence material, prepared according to the Bloom and Battenberg (1976) method, is adequately counterstained with ethidium bromide using the protocol described above. And, considering their binding properties to neural tissue, there is reason to believe that tissue containing procion yellow—or lucifer yellow—stained cells could also be successfully counterstained with ethidium bromide.

**Discussion**

We have developed a number of counterstains that can be used successfully in conjunction with a variety of neuroanatomical methods based on the detection of fluorescent markers. Direct visualization of cytoarchitectonic boundaries is preferable when tracing pathways in the central nervous system, but the traditional basic aniline Nissl stains are unsatisfactory when applied to IHC or fluorescent tracer material because the fluorescent label is greatly masked, or fades rapidly (Sawchenko and Swanson, 1981). In the past, three general approaches have been used to circumvent this problem. First, adjacent series of sections have been stained with conventional Nissl techniques. Projection drawings are then made and used to plot the distribution of fluorescently labeled cells in the adjacent section. This approach has the obvious disadvantage that the transfer of information is only as accurate as one's ability to detect appropriate landmarks in the experimental material. Second, fluorescent material can be photographed, counterstained, and rephotographed. This approach is tedious and difficult to apply to sections (particularly for IHC) that have been cover slipped. And third, X-Y plotters have been used to map the distribution of fluorescent cells. The same
section can then be counterstained, and cytoarchitectonic boundaries can be added to the original plot. This method is relatively accurate (see Swanson and Kuypers (1980)), but requires elaborate equipment. The use of fluorescent counterstaining as described here avoids many of these problems when an appropriate dye, at an appropriate concentration and pH, is chosen.

We are not aware of previous systematic attempts to counterstain neural tissue for the purposes described here, although the idea of developing counterstains for more general use in conjunction with immunofluorescence methods is by no means novel (see e.g., Schenk and Cherukian (1975); Pearse (1980); Franklin and Locker (1981)). Furthermore, little can be said with certainty about the chemistry of the staining reactions we have found useful, since the interaction of the compounds used with formalin-fixed tissue has not been determined. Acridine derivatives are essentially cationic dyes that become intercalated within or between nucleic acids (Pritchard et al., 1966; Pearse, 1980) and, at the concentration of acridine orange used here on formalin-fixed tissue, both the nucleus and the cytoplasm display green fluorescence, suggesting that monomeric binding to nucleic acids has taken place (Rigler, 1966). On the other hand, quinacrine appears to bind selectively to guanine-cytosine base pairs of DNA (Caspersson et al., 1968), and bisbenzimide is said to have a high affinity for adenine-thymine base pairs (Weisblum and Haenssler, 1974). That labeling is restricted to the nucleus when bisbenzimide is used as a counterstain at pH 7.2 is consistent with this view. It was surprising to find, however, that bisbenzimide (and nuclear yellow) stained both the nucleus and the cytoplasm at pH 2, suggesting that their preferential affinity for DNA is pH dependent. Ethidium bromide, which is known to bind to nucleic acids (LePeceq, 1971), yielded a pattern that resembled conventional Nissi staining most closely, since only neuronal cytoplasm and nucleioli, and glial nuclei were brightly fluorescent. In conclusion, the counterstains we have described here may be used to localize, in neural tissue, a variety of fluorescent markers that are excited by ultraviolet and blue light.

**Literature Cited**


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