A reliable and sensitive method for fluorescent photoconversion

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Abstract

The objective of this study was to develop a reliable and sensitive method of diaminobenzidine (DAB) photoconversion through systematic variation of the objectives and the method of DAB enhancement employed. Results showed that reliable photoconversion of Dl-labeled macaque monkey cortical cells and processes can be obtained consistently by using fresh filtered DAB solutions, clean slides, and a bright fluorescent light source. Using a 20 x objective and enhancing the DAB reaction product with Giemsa stain provided the most sensitivity and best resolution in the photoconverted area. Objective size was found to correlate directly with sensitivity and indirectly with both the size of the area photoconverted and the time to complete photoconversion. Nitro blue tetrazolium (NBT) did not produce as much contrast in the photoconverted cells as Giemsa stain, but allowed for the best visualization of finer processes because it somewhat enlarged the fiber diameter. Nickel and cobalt used together, but not separately, were found to provide adequate enhancement. These results are likely to apply to photoconversion and enhancement of all fluorescent material, but may require slight modifications in protocol.

Keywords: Diaminobenzidine; Dl; Intensification; Giemsa stain; Nitro blue tetrazolium; Macaque monkey; Visual cortex

1. Introduction

Fluorescent markers are used to trace pathways and examine morphological details in both living and fixed (including postmortem) tissue and are compatible with several immunocytochemical techniques (Bartheld et al., 1990; Casagrande and Hutchins, 1990; Papadopoulos and Dori, 1993; Supprian et al., 1993). Most fluorescent dyes, however, have the disadvantage of fading rapidly upon visualization. This disadvantage can be overcome by converting the fluorescent dye into a permanent label. Maranto (1982) was the first to use 3,3'-diaminobenzidine (DAB) and fluorescent illumination to photoconvert the intracellularly injected fluorescent dye, Lucifer Yellow, into a stable, ultrastructurally useful label. Others subsequently discovered that photoconversion could be used with a variety of fluorescent markers (e.g., Propidium Iodide, Fluoro-Gold, Fluoro-Ruby, and Fast Blue; see Sandell and Masland, 1988; Bentivoglio and Su, 1990; Lubke, 1993a, b; Schmued and Snavely, 1993) in various tissue types.

A frequent criticism of DAB photoconversion is that it produces variable and inconsistent results with loss of the fluorescent detail. The objective of this study was to develop a reliable and sensitive method of DAB photoconversion by systematically varying basic parameters of the method. Given that it is possible to convert a variety of fluorescent labels using the same basic DAB procedure (Lubke, 1993b; Bentivoglio and Su, 1990), it seems likely that our findings using Dl generalize to other fluorescent markers. Moreover, it is likely that the protocol we suggest would also enhance the quality of any material taken to the ultrastructural level, although we did not perform specific experiments to test this possibility.

2. Materials and methods

2.1. Subjects and fluorescent dye labeling

Blocks of fetal day 135 Macaca nemestrina cortex fixed in 4% paraformaldehyde in 0.1 M phosphate buffer were incubated in the intermediate zone (below the layers of the cortical plate) with 1,1-diiododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) using standard procedures (see Casagrande and Hutchins, 1990). After a 12-month incubation period at room temperature, each
piece was embedded in agarose gel and cut at 75 μm on a vibratome into 0.1 M phosphate buffer. Well-labeled sections from striate cortex were selected for photoconversion.

2.2. General procedure for photoconversion

We followed the standard procedure described by Maranto (1982) and Sandell and Masland (1988) with some modifications. The Dil-labeled sections were first rinsed briefly (3–4 times) in cold (4°C), filtered 0.1 M Tris buffer, pH 8.2. To avoid fading, sections were photoconverted within 1–2 days of cutting. Next, sections were pre-incubated for 45–60 min at 4°C in the dark in DAB (1.5 mg DAB/ml of Tris, pH 8.2) filtered with an acrodisc (0.45 μm sterile syringe filter; Gelman Sciences, Ann Arbor, MI). Following incubation, the sections were rinsed again with Tris and flattened onto a depression slide (which can be made using a ‘Pap-Pen’ (Electron Microscopy Services, Ft. Washington, PA) or nail polish) without a cover slip. Excess buffer on the flattened section was removed using small pieces of filter paper and replaced with 1–3 drops (or enough to cover the area of interest) of fresh DAB solution. The area of interest was then located and irradiated using an Olympus Inverted Research Microscope model IMT-2 equipped with epifluorescence and a 100 W mercury bulb using a rhodamine filter cube under the desired objective. The DAB solution was changed after 10 min of irradiation and then replaced with fresh, cold, filtered DAB every 20–30 min during the remainder of the photoconversion. The progress of the reaction was monitored by switching periodically between fluorescent and white light until a dense, brown DAB product was visualized. After photoconversion, the sections were rinsed briefly in Tris and the DAB rinses were disposed of in a Clorox-filled waste container.

2.3. Comparisons: objective lenses and DAB enhancement

The results obtained by photoconverting with different objective lenses and methods of DAB enhancement were compared on sets of adjacent sections. Three different objectives (SPlan 4PL 4 × Olympus objective with numerical aperture (NA) of 0.13; EA10 10 × Olympus objective with NA of 0.25; CDPPlan 20PL 20 × Olympus objective with NA of 0.40) were used to determine: (1) the diameter of the region photoconverted, (2) the amount of detail recovered from the fluorescent tissue after photoconversion, and (3) the length of time required to complete photoconversion. In order to enhance the DAB reaction product, we compared: (1) a Giemsa staining method (adapted from Inguz et al., 1985; personal correspondence with Dale Hogan, University of Tennessee in Memphis, and Harvey Karten’s laboratory), (2) enhancement with Nitro Blue Tetrazolium (NBT) salt (see Vaney, 1992), and (3) heavy metal intensification with nickel (Ni), cobalt (Co), or both (see Adams, 1981). The details of the enhancement procedures we used are as follows.

2.3.1. Giemsa stain

1. Preparation: 0.4% Giemsa stain, pH 6.9 (Sigma #GS-500, St. Louis, MO) 1:1 in phosphate-buffered saline (PBS, pH 7.4).
2. Mount sections on gelatinized slides and dry overnight.
3. De-fat tissue in 95% ethyl alcohol/chloroform (1:1) for 1 h.
4. Rehydrate in 90% and 70% ethyl alcohol and deionized (d) H₂O for 3 min each.
5. Wash in dH₂O for 10 min.
6. Counterstain tissue in warm Giemsa mixture (50°C) for 2–5 min (or more if necessary; check periodically).
7. Dip in PBS, then differentiate in 95% ethyl alcohol for 5–20 s.
8. Leave in PBS and check staining (if staining too dark, may place back into 95% ethyl alcohol for a few more seconds to quickly remove some of the stain; watch closely to prevent stain from leaching out).
9. Fix stain in 1% ammonium molybdate for 5 min followed by a running dH₂O rinse for 30–45 s.
10. Dehydrate in 70%, 25% and 2 rinses of 100% ethyl alcohol for 3 min each. Leave in xylene at least 15 min.
11. DAB reacted cells turn black and the remaining tissue looks like a blue nissl stain.

2.3.2. NBT (photochromic reaction product)

1. Preparation: 0.02% NBT (Sigma, St. Louis, MO) in 0.1 M Tris buffer, pH 8.0–8.2. Keep dark-stored at 4°C. Use in well-ventilated room.
2. Pre-incubate sections for 5 min in NBT solution.
3. Coverslip section on non-gelatinized slide using NBT as a mounting medium. Illuminate with a green filter for most rapid enhancement (rhodamine optics: 530–585 nm) until desired intensification is obtained (generally 2–5 min). Can move section around or vary objectives to focus on areas that looked weakly photoconverted.
4. Rinse section briefly in Tris buffer to remove residual NBT. Discard NBT waste in waste container (do not mix with other chemicals; NBT cannot be neutralized and is extremely toxic).
5. DAB cells are dark-brown to black and the background is brownish-purple (does not obscure cell structure).

2.3.3. Ni or Co or both (heavy metal intensification)

1. Preparation: 0.04% Ni or Co in Tris-buffered saline (TBS, pH 7.4–7.6). For Ni and Co, make 0.02% Ni and 0.02% Co in TBS.
2. Flatten section on slide and cover with metal solution for 30 s, rinse well with Tris.
3. Repeat until desired product is visualized. Note that leaving sections in too long will simply bring up the background. These metals turn the cells a medium to dark brown.
and Masland (1988) suggest replacing the DAB every 10 min, but we did not find this absolutely necessary unless the stock DAB solution was not fresh (i.e., over 2 h old). To limit the amount of debris forming on the section, we used non-gelatinized slides and did not apply mounting media to the section. When locating an area to photoconvert, we simply mounted the section on an acid-cleaned slide and used 0.1 M filtered phosphate buffer as the coverslipping medium. Third, it was important to use a well-focused, bright light source. Our efforts to photoconvert tissue using a dim light source (because the bulb was less than 100 W or the light source was out of alignment)

After photoconversion and intensification, the sections were nissl stained (except for the Giemsa-stained sections) and coverslipped using Permount.

3. Results

3.1. General observations

We found three factors to be important to ensure optimal photoconversion. First, it was essential to pick the area of the tissue which had the most clearly and brightly labeled elements of interest (see Fig. 1A). Our attempts to convert dimly labeled cells and processes were generally not very successful since the prolonged time required to react the tissue resulted in high background (see also Sandell and Masland, 1988). Second, it was important to keep all solutions filtered, fresh, and cold. DAB forms clumps in solution as it oxidizes. These clumps appeared to be seeded by dirt in the solution and precipitated out more readily onto dirt on the sections or slides. Sandell

Fig. 1. Comparison of the fluorescent image (A) and opaque DAB product (B) after photoconversion, but without enhancement. A 10× objective lens was used to photoconvert the section. Note that the detail obtained from the photoconversion is very similar to that of the original fluorescent material. Scale bar: 100 μm.

Fig. 2. Comparison of the size of the area obtained from photoconverting using 4× (A), 10× (B), and 20× (C) objective lenses. The diameters of these photoconverted areas ranged from 2.5 to 4.0 mm with the 4× objective, 1.0–1.5 mm with the 10× objective, and 0.5–0.75 mm with the 20× objective. The figure shows adjacent sections in the same region intensified with the Giemsa stain. Scale bar: 500 μm.
resulted either in poor photoconversions or required excessive periods of irradiation or both.

While irradiating the tissue, the fluorescent material was gradually replaced with an opaque, brown DAB product, with a short intermediate period where there was neither any fluorescent material visible under fluorescent light nor traces of a DAB product visible under white light. After this period, a brown product was seen under white light. The final DAB product closely resembled the fluorescent image (compare Fig. 1A and B).

3.2. Objective lens considerations

Our results suggest that as the size of the photoconverted area is decreased (e.g., with increasing lens power), photoconversion sensitivity (i.e., the amount of detail that

Fig. 3. Higher-magnification comparison of the photoconversion sensitivity obtained through photoconversion with the 4× (A and B), 10× (C and D), and 20× (E and F) objectives. A, C, and E: general features of cells and processes in the upper portion of layer III of the striate cortex. B, D, and F: details of the apical dendrites of these cells. Arrowheads indicate corresponding features in the adjacent photomicrographs (A, B, C, D, and E, F). More morphological details are evident following photoconversion using a higher-power objective (compare B with F). A, B, C, D, and E, F are higher magnifications of the sections shown in Fig. 2A, B, and C, respectively. Scale bar (A, C, E): 50 μm. Scale bar (B, D, F): 10 μm.
becomes photoconverted) increases (see Figs. 2 and 3). An area of 2.5–4.0 mm in diameter was successfully photoconverted using the 4× objective (Fig. 2A). The labeled neurons, however, did not appear as detailed as they had originally under fluorescent light. The neurons photoconverted with the 4× objective were not well resolved at higher power nor were their processes well-filled (see Fig. 3A, B). With the 10× and 20× objectives, 1.0–1.5 mm and 0.5–0.75 mm diameter areas, respectively, were photoconverted (Fig. 2B, C). The labeling of the neurons in the area of photoconversion was comparable to that seen in the fluorescent image using both lenses (Fig. 3C–F). How-

Fig. 4. Comparison of the effects of the intensifying agents on the final photoconverted product (using the 10× objective for photoconversion). A: Giemsa stain; B: NBT, C: Ni and Co; D: Ni; E: Co. Figures show layer III cell basal dendrites in striate cortex. Dendritic spines show a similar morphology using the Giemsa stain to that demonstrated by the Golgi stain method (e.g., see Lund et al., 1977). The Giemsa stain consistently stains spines more discretely and more darkly than the other methods used (see arrows). Scale bar: 10 μm.
ever, details of neuronal spines and growth cones were better preserved under the 20 × lens (compare Fig. 3D with 3F).

We also found that when we used a higher power lens, the time required for photoconversion was reduced. The first signs of a DAB product under white light were visible with the 4 × objective within 1 h. The reaction was completed after 1.5–3 h depending upon the original intensity of the fluorescent label. When using the 10 × and 20 × objectives, the time necessary to see an initial DAB product under white light was 25 min and 10–15 min, respectively. The complete reaction took between 30 and 85 min with the 10 × lens and between 20 and 30 min with the 20 × lens.

Another consideration was how to prevent contamination of the objective lenses by the solutions. The risk of lens contamination was decreased by using an inverted microscope; the use of this microscope permitted both easier manipulation of the specimen and the maximum intensity of light to be focused on the specimen. With enhanced light intensity, photoconversion time was decreased, which is important for minimizing background label. The tissue can generally be used for only one irradiation since the DAB appears to make the non-illuminated areas of the tissue less reactive by causing the fluorescence to fade.

3.3. Comparison of intensifying agents

DAB enhancement mainly increased the contrast between the DAB label and the background. The quality of the enhancement, however, appeared to depend not only on the degree of increased contrast, but also on a possible increase in the resolution of fine axonal processes through deposition of additional material. Figs. 4 and 5 compare the enhancement procedures that we employed.

The Giemsa method provided consistently better enhancement of all the photoconverted material and higher contrast of the cells and processes against a blue Nissl background. The higher contrast allowed for better visualization of fine morphological details. The Giemsa stain seemed to enhance spines exceptionally well (similar to that seen in a Golgi stain; see Lund et al., 1977; see arrows in Fig. 4A), possibly because it enhances the spine necks enough to bring the entire spine to the level of resolution visible with low-power objectives. This method also has the added advantages of being easy to use, less hazardous, and allows for a simultaneous nissl counterstain.

Similarly, the NBT method was very effective in revealing the fine details of neuronal processes, especially axons (see arrows Figs. 4B and 5B). This method also allowed for adjustment and microscopic monitoring of the intensification parameters (e.g., size and location of an area or intensity of enhancement). The other enhancement procedures are ‘all-or-none’ techniques. Even though the NBT method produced slightly less contrast between neurons and the background than Giemsa stain, resolution of many processes was better using lower power objectives with NBT than with Giemsa stain. This finding may be due to the observation that NBT increases neurite size. Vaney (1992) speculates that when NBT is excited by light, it may be reduced by the DAB reaction product to form an insoluble formazan compound. This compound could enlarge the processes and therefore allow for better visualization of finer processes. In fact, Fig. 5B shows that the axons and growth cones of an NBT-intensified section are larger than axons and growth cones revealed by the Giemsa method (see Fig. 5A). The increase in process size can be especially useful in tracing axons over long distances, even at the periphery of the photoconverted region. Unfortunately, deposition of additional material has the disadvantage that it slightly obscures dendritic spines (Fig. 4B, arrows). This might be a problem if one wished to take the material to the ultrastructural level.

Finally, Ni and Co were more effectively used together than used separately. The heavy metal intensification techniques, however, did not increase the contrast of the photoconverted cells and processes much more than the
original DAB product (see Fig. 4C–E compared to Fig. 1B).

Successful enhancement critically depends on a good initial photoconversion. If the section photoconverts well, then the type of intensification method selected will depend upon the particular needs of the investigator. No intensification method can compensate for a poor photoconversion.

4. Discussion

Our results suggest that it is possible to obtain reliable and sensitive photoconversion of Dil-labeled tissue if one is careful to: (1) restrict photoconversion to brightly fluorescent tissue, (2) keep all material that comes in contact with the tissue clean, (3) use a bright, well-focused light source, (4) use at least a 10 × objective, and (5) enhance the final product with either the Giemsa or the NBT method. Our best results for fine detail and contrast were obtained with a 20 × lens and Giemsa stain.

The investigator is advised to keep in mind both the strengths and the limitations of this technique before photoconverting and intensifying fluorescent material.

4.1. Strengths

Photoconversion allows the detailed morphology of fluorescently labeled tissue to be preserved for repeated viewing and quantification. Photoconversion also can improve the visibility of details because of increased contrast under white light and the limited resolution of fluorescent images with standard microscopes (i.e., higher background and lower optical quality of a fluorescent versus light microscope; Sandell and Masland, 1988). In addition, photoconversion is compatible with many other methods including immunocytochemistry and electron microscopy. Even though we did not attempt to take our material to the ultrastructural level, others have shown that photoconverted tissue can be examined with an electron microscope albeit with some loss of tissue preservation, presumably due to extended fixation periods (see Bartheled et al., 1990; Linke and Frotscher, 1993; Lubke, 1993b; Papadopoulos and Dori, 1993).

4.2. Limitations

Photoconversion is limited to a small region of tissue. The tissue around this region generally cannot be photoconverted due to fading. Photoconversion is also very time consuming, requiring hours of work for just a few photoconverted regions. Photoconversion is not a forgiving method and requires careful monitoring and good technique. In addition, most of the reagents involved in photoconversion are very toxic and require appropriate precautions. Moreover, modifications of our protocol are required when other fluorochromes are used. In particular, we found that fluorochromes excited by shorter wavelengths (e.g., 4-(4-dihexadecylyaminostyril)-N-methylpyridinium iodide (DiA), a yellow fluorochrome) require longer wavelengths (550 nm) and much longer time periods (3–5 h with a 10 × objective) to be successfully photoconverted (results not shown; see Bentivoglio and Su, 1990). The same procedures, however, for enhancement can be used and the end product is similar to that we have described for Dil, suggesting that these photoconversion and intensification methods can be used reliably with other fluorochromes.

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