

Localization of Intracellular Lipid Hydroperoxides Using the Tetramethylbenzidine Reaction for Transmission Electron Microscopy

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1. Introduction

Histochemical reactions for lipid hydroperoxides (LHP) using indophenol, benzidine, or phenylendiamine as the electron donor have been described previously for auto-oxidized adipose (1) and neuronal (2) tissue. More recently, tetramethylbenzidine (TMB) has been proposed as another chromagen (3). The usefulness of the TMB reaction for ultrastructural studies of lipid peroxidation was demonstrated in retina where LPH was generated by incubation with exogenous lipooxygenase. The glutaraldehyde fixed tissue, which was reacted with TMB and then postfixed in osmium tetroxide, showed an electron-dense product (4). This technique allows intra- and extracellular localization, as well as a comparison of relative intensity among various cell types and subcellular organelles (5). In light-induced lipid peroxidation, discs of the outer segments, which are rich in oxidizable long-chain polyunsaturated fatty acids, stain strongly and appear as bubble-like structures (6). These are however, quite similar to fingerprint profiles seen acutely in outer segments and chronically in neurons, which are visualized without TMB following exogenous exposure to LHP (7,8). A possible caveat to the reported method is that peroxidized protein and carbohydrates may also react and so the TMB method has not been proven to be specific for LHP only.

The present method uses in vivo exposure of tissue to pure 18:2 linoleic acid LHP and tissue from obese, diabetic rats with known elevation of endogenous LHP as a definitive marker of lipid peroxidative processes occurring in vivo.

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2. Materials

2.1. Equipment

This protocol is for ultrastructural demonstration of LHP and is done best by technical staff who are experienced in processing tissue for transmission electron microscopy.

1. Fume hood for osmication and embedding tissue.
2. Shaking water bath for TMB reaction and osmication.
3. Ultramicrotome (Reichert Ultracut S).
4. Transmission electron microscope (Hitachi H-7000).

2.2. Reagents

1. 0.1 M citric acid.
2. 0.2 M Na₂HPO₄.
3. Osmium tetroxide (Ted Pella, Inc., Redding, CA) (*see Note 1*).
4. Sodium cacodylate (Ted Pella, Inc.) (*see Note 2*).
5. 3, 3', 5, 5'-tetramethylbenzidine dichloride (Sigma Chemical Co., St. Louis, MO) (*see Note 3*).

3. Methods

3.1. Tissue Fixation

1. Fix tissue in a cold, freshly prepared, buffered aldehyde fixative for 1 h. Any standard aldehyde fixative for electron microscopy such as 2–3% glutaraldehyde, 4% paraformaldehyde, or 2.5–5% acrolein can be used (*see Note 4*).
2. Wash the tissue in several changes (4 × 15 min) of cold, buffer wash to removed unreacted fixative.

3.2. Reaction with TMB and Post Fixation with Osmium Tetroxide

1. TMB reaction: 0.5 mg/mL TMB dichloride in 0.1 M Na₂HPO₄/citric acid buffer, pH 3.0. Dissolve 0.5 mg/mL of TMB in 4 parts of 0.1 M citric acid first and then add 1 part 0.2 M Na₂HPO₄ to adjust pH to 3.0. It is not necessary to check the pH with a pH meter.
2. Incubate tissue at 4°C overnight in TMB solution. Cover the vial that contains the tissue with aluminum foil and place this in a an insulated container with cold packs to keep the temperature at approx 4°C. Place the insulated container on the shaker, which is set at a low speed, and **agitate over night**. Rinse in cold citrate/phosphate buffer. Rinse in 0.1 M sodium cacodylate buffer, pH 7.0.
3. Osmicate in 1% OsO₄ in 0.1 M sodium cacodylate buffer, pH 7.2 in shaking water bath at 37°C for 1 h. Rinse one time in 0.1 M cacodylate buffer, pH 7.2 (*see Note 5*).
4. Dehydrate in 80, 90, 95, 100% × 2 ETOH for 15 min at each step. 2 × 10 min in acetone to propylene oxide (*see Note 6*).

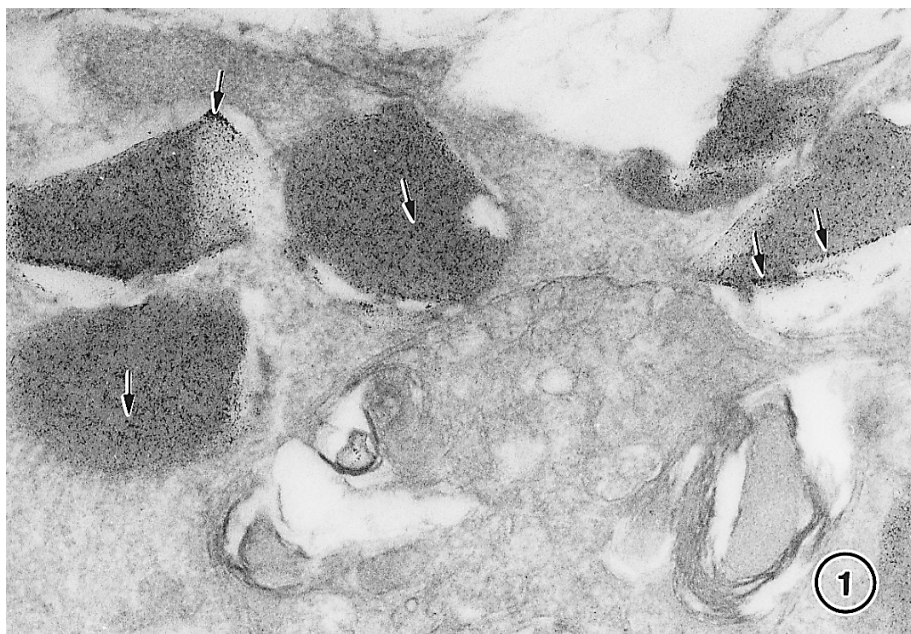


Fig. 1. Localization of LHP by the TMB reaction (arrows) in a retinal pigment epithelial cell macrophage. The retina was injected 2 wk earlier with 50 μ g of authentic 18:2 linoleic LHP. $\times 50,000$.

5. Infiltrate and embed in epoxy resin. Cut gold sections (90–100 nm) and examine in the TEM without poststaining (*see Note 7*).

3.3. Results

Figure 1 shows LHP localized with TMB in the retina of a New Zealand albino rabbit, which was injected with authentic 18:2 linoleic acid LHP. There are areas of electron dense TMB reaction product in the outer segments of the retina of a diabetic rat (**Fig. 2**).

4. Notes

1. Osmium tetroxide is extremely reactive and should be handled only in a properly functioning hood (flow rate of 100 ft/min). Osmium is also an expensive reagent and can be purchased from electron microscopy vendors as crystals or as 4% aqueous solution under an inert gas. Glassware and utensils should be cleaned in ethanol and then acetone before use with osmium tetroxide solutions. Plastic containers should not be used with osmium.
2. Sodium cacodylate contains arsenic and should be handled in an appropriate manner. Gloves should be worn when working with this buffer. If one chooses to



Fig. 2. Localization of LHP by the TMB reaction (arrows) in the outer segments of a diabetic rat with uncontrolled hyperglycemia for 6 mo. $\times 50,000$.

substitute another buffer, HEPES or PIPES are good choices. Phosphate buffers should be **avoided** since these buffers often result in nonspecific precipitates. Cacodylate can be purchased from any chemical supply company; however, it is cheaper to buy this compound from electron microscopy vendors.

3. Tetramethylbenzidine is available in several forms. Do not substitute the free base for the dichloride form recommended in this protocol. The free base is not soluble in aqueous solution without lowering the pH. The dichloride form is soluble in the buffers used in this protocol.
4. Paraformaldehyde and acrolein are extreme irritants and must be worked with in a properly functioning fume hood.
5. Osmication at room temperature or higher at neutral pH is necessary for preservation of the TMB reaction product through dehydration and embedding in epoxy resins. Optimal conditions for conversion of the TMB reaction product into the osmicated insoluble product occur at 37–45°C and pH 7.2. Use of osmium tetroxide with 1.5% potassium ferricyanide should **not** be done since this results in complete loss of the reaction product (9).
6. The TMB reaction product is soluble in lower concentrations of alcohol. Do **not** start dehydration below 80% ethanol. Do **not en bloc** stain with uranyl acetate.
7. Do **not** poststain sections with uranyl acetate and lead stains. Weak reactions can be overshadowed by uranyl acetate or removed. Staining with lead citrate

alone for 3 min can be used if necessary to improve the visibility of weak areas of TMB reaction product (10).

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