Preface

In our first protocols book, *Free Radical and Antioxidant Protocols* ***(1)***, reference to in vivo, ex vivo, or *in situ* techniques were few compared to classical biochemical assays and only 6 of the 40 chapters were concerned with these applications. In our second book, *Oxidative Stress Biomarkers and Antioxidant Protocols* ***(2),*** which is being published concurrently with this third volume, *Oxidants and Antioxidants: Ultrastructure and Molecular Biology Protocols*, the number of such chapters has increased. The literature dealing with histochemical/cytochemical and immunohistochemical techniques and staining to identify cellular/subcellular sites of oxidative stress has expanded rapidly, as has the molecular biology methodology used to analyze free radical and antioxidant (AOX) reactions, as well as the monitoring of living tissue.

A two-way search was performed for each technique listed in **Table 1**, coupled with "oxidative stress" using the PUBMED search engine from the National Library of Medicine at NIH. Most of the techniques involved in measuring oxidative stress employ molecular biology or ultrastructural approaches. Of these techniques, histology, polymerase chain reaction, and Western blotting are the most widely used. Several forms of therapy are now available for patients with increased oxidative stress. In addition to standard antioxidant therapy supplementation in vivo and in vitro, photodynamic therapy (PDT) employs excitation of a photon-emitting compound delivered systemically for free radical-mediated necrosis of affected tissues, and stem cells are also being used to induce signaling events or replace antioxidant enzymes.

From **Table 1**, one can appreciate how these various techniques have currently become valuable options for pathophysiological studies, especially in ultrastructure. Treatment is another important category: in most instances these are non-invasive. Consequently, this third volume in the series contains additional chapters written by authors who collectively provide 109 methods of oxidative stress measurement across the three books.

Some of the procedures listed within this volume require sophisticated instrumentation not generally found in the routine laboratory. However, some devices are usually available somewhere in a given institution and collaboration is often possible. Techniques introduced here alert the reader to the high level that oxidative stress measurements have reached. Imaging techniques employing photon biotechnology, confocal microscopy, and photodynamic therapy have been included in the present book chapters, a development that illustrates the degree of sophistication available for study at the molecular level.

Table 1
Recent Citations of Oxidative Stress Biomarkers

		1997	1998	1999	2000
Molecular Biology	Western blot	17	31	26	47
	Northern blot	21	23	29	21
	Southern blot	5	4	4	4
	PCR	33	22	55	61
	Differential display	5	9	7	6
	Mobility shift assay	8	14	16	14
	In situ hybridization	21	12	18	26
Ultrastructure	Histology	87	91	150	160
	Intracellular	94	122	155	204
	Cytology	431	523	641	820
	Immunohistochemistry	56	91	119	118
Treatment	AOX therapy	121	158	209	288
	PDT	4	3	7	12
	Stem cells	24	44	50	66

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Donald Armstrong

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2

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 lipoxygenase. 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 amoung 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 many 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* Na_2HPO_4.
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* Na_2HPO_4/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* Na_2HPO_4 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% OsO_4 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**).

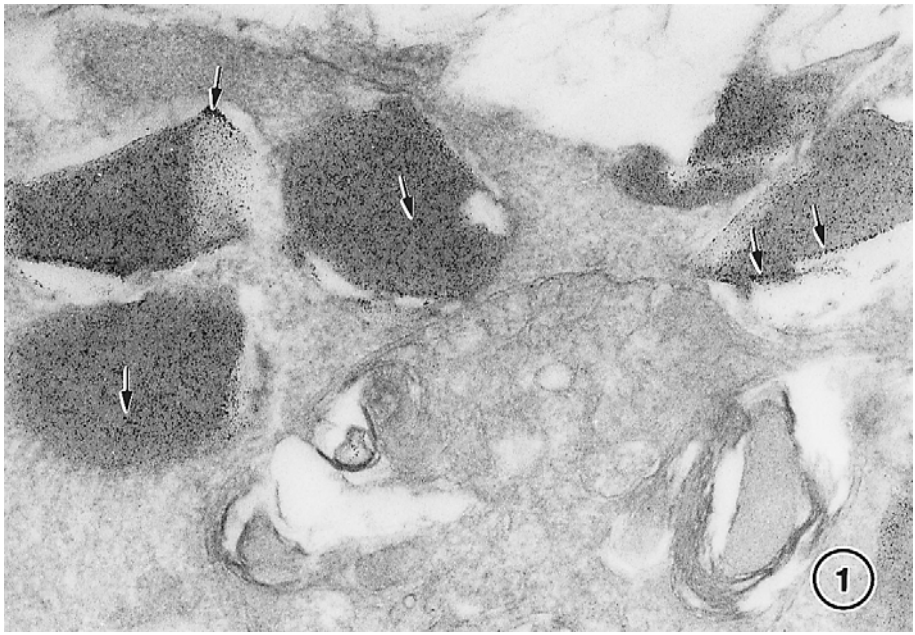

Fig. 1. Localization of LHP by the TMB reaction (arrows) in a retinal pigment epithelial cell macrophage. The retina was injected 2 wk earlier with 50 µg of authentic 18:2 linoleic LHP. ×50,000.

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. Osmiun 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

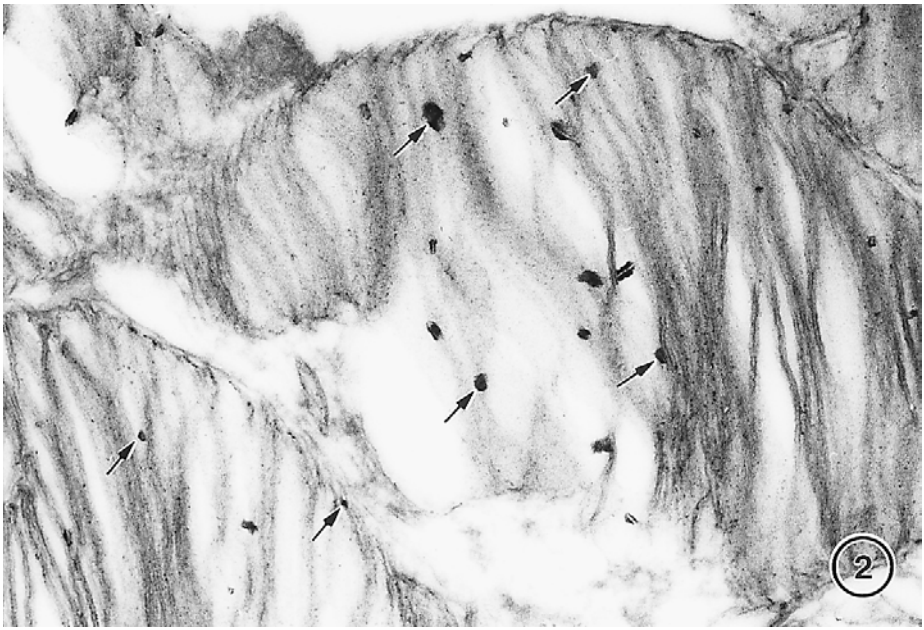

Fig. 2. Localization of LHP by the TMB reaction (arrows) in the outer segments of a diabetic rat with uncontrolled hyperglycemia for 6 mo. ×50,000.

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 ferrucyanide 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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