Reflectance enzyme histochemistry (REH): visualization of cerium-based and DAB primary reaction products of phosphatases and oxidases in cryostat sections by confocal laser scanning microscopy

Accepted: 2 October 1995

Abstract In the present study the reflectance mode of confocal laser scanning microscopy was adapted to detect and to assess semiquantitatively cerium-based primary reaction products of oxidases (Ce(IV) perhydroxide) and phosphatases (Ce(III) hydroxyphosphate converted into Ce(IV) perhydroxyphosphate) as well as of the 3,3'-diaminobenzidine (DAB)-based primary reaction product of cytochrome c oxidase in cryostat sections. Confocal laser scanning microscopy offers a unique way of making visible histochemical reaction products which are weakly absorbant but sufficiently reflective. It was easily possible to record simultaneously the reflectance signals at the wavelength of the exciting laser (preferentially 488 nm) and the autofluorescence signals (>580 nm in our set-up) of glutaraldehyde-fixed tissue. The results of an imbibition study of cerium-containing model precipitates indicate that the cerium, generally, should be oxidized prior to observation because the index of refraction of Ce(IV) compounds is considerably higher than that of the corresponding Ce(III) compounds. An attempt at comparative numerical assessment of reflection intensities from reflectant parts in morphologically similar sections is presented. The proposed technique may open new possibilities in enzyme- and immunohistochemistry.

Introduction

Catalytic histochemistry as a highly specific method in morphological research provides qualitative and quantitative information about histochemical enzyme localization and has become indispensable to histochemical investigations. Today, enzyme histochemistry represents an interdisciplinary link between biochemistry and morphology and even a bridge to molecular biology (Meijer 1975; Hardonk et al. 1977; Lojda et al. 1979; Wohlrab et al. 1979; Wohlrab and Gossrau 1992). Enzymes become more and more important as histochemical tools for identification and localization of characteristic structural elements and macromolecules of cells (Bendayan 1981; Coulombe et al. 1988; Seno et al. 1989), which are of special relevance for the improvement of diagnostic pathology as well as for the biological understanding of cellular pathological processes (Rath 1981; Hulstaert et al. 1989). Last, but not least, enzymes are effectively used as tracers in immunoassays enzyme-linked immunosorbent assays, radioimmunoassays, in protein blotting, in immunocytochemistry (Cuello 1983; Polak and van Noorden 1983; Polak and Varndell 1984; Luppa et al. 1986), and in hybridization histochemistry (Lande-gent 1987). From this wide range of applications of enzyme histochemistry it is seen that further developments in the field of catalytic enzyme demonstration techniques are of considerable interest.

Confocal laser scanning microscopy (CLSM; for reviews see Cheng and Summers 1988; Pluta 1989; Gu and Sheppard 1993; Rigaut et al. 1993; Shotton 1995) offers a unique way of making visible histochemical reaction products which are weakly absorbant but sufficiently reflective. An example of such reaction products is the insoluble precipitates of the primary cerium reaction product occurring in the histochemistry of phosphatases as well as oxidases (for reviews see Hulstaert et al. 1989; van Noorden and Hulstaert 1991; van Noorden and Frederiks 1993; Halbhuber et al. 1994). Without any additional visualization procedures, precipitates of that kind appear under microscopical dark-field observation as well-localized, faint but frequently very bright, light-scattering patterns (Sebastian and Bock 1987). Dark-field microscopy, however, suffers from the drawback that portions of the tissue which are free from light-scattering material are poorly visible. CLSM, on the other hand, by using two detection channels, permits the simultaneous imaging of light-scattering structures together with their non-luminous surroundings. Of course, it...
Reflected confocal laser scanning microscopy has been used in histochemistry under various aspects. Robinson and Batten (1989b, 1990) employed reflectance laser scanning microscopy to localize sites of hydrogen peroxide release from stimulated glass-adherent neutrophils by means of cerium-based reaction products as well as lysosomal acid phosphatase in various cultured cells without additional visualization steps. Robinson and Batten (1989a, c) as well as the group of Turner et al. (1993) also described the detection of 3'3'-diaminobenzidine (DAB) polymers after treatment with the exogenous tracer horseradish peroxidase in lysosomes of neutrophils and in mouse oocytes as well as in the CA3 region of the rat hippocampus. Lewis et al. (1990), Paddock et al. (1991), and Watanabe et al. (1995) utilized confocal reflectance images from developed silver grains in autoradiographic specimens to localize 35S-labeled human immunodeficiency virus RNA and 3H-fucose as well as 125I-insulin or 125I-epidermal growth factor in blood smears and paraffin sections, respectively. Deitch et al. (1990a, b, 1991) studied the reflectance of biocytin-filled dendrites visualized with DAB-Ni. Arnold et al. (1992) utilized the reflective properties of DAB polymers for in situ hybridization experiments using digoxigenin-labeled DNA probes. Whallon et al. (1994) investigated the intracellular distribution of hexokinase in PC12 cells by confocal imaging of the reflection of DAB polymer formed by immuno-bound horseradish peroxidase. In recent studies, Rawlins and Shaw (1990), Rigaut et al. (1993), and Linares-Cruz et al. (1994) applied the method using in situ hybridization for the detection of riboprobes labeled by 1- to 5-nm (also silver enhanced) colloidal gold particles. Kazama et al. (1994) described an immunohistochemical and enzyme-histochemical double staining utilizing the reflective properties of colloidal gold particles as well as lead phosphate precipitates in the laser scanning confocal microscope. Ploton et al. (1994) described the visualization of silver dots on proteins of the nucleolar organizer regions (Ag-NOR proteins) after a one-step silver staining technique with the confocal laser scanning reflectance mode. Recently, Duschneser (1995) proposed the use of confocal laser reflectance microscopy to estimate changes in the dental enamel of humans.

The aim of our study was to adapt the CLSM reflectance mode developed by Robinson's group to enzyme- and immunohistochemical studies with cryostat sections. We demonstrated the feasibility of reflectance studies for a broad variety of enzymes employing cerium-based and DAB-based primary reaction products (PRPs). Furthermore, we studied the reflective properties of various precipitates of cerium compounds. Also, an attempt was made at a comparative assessment of the amount of primary cerium reaction products.

## Materials and methods

### Chemicals

Unless otherwise stated biochemicals were obtained from Serva (Heidelberg, Germany) and Sigma Chemical (Deisenhofen, Germany). Buffer components and inorganic chemicals from Merck (Darmstadt, Germany) were of the purest available grade.

### Animals

All enzyme demonstrations have been carried out on tissues of adult Wistar rats of both genders weighing 200-300 g. The animals were kept under standardized conditions (21°C±1°C; 12 h light/dark cycle). They were fed a standard diet with free access to food and water. They were killed under light ether anesthesia.

### Tissue sampling and cryostat sections

Tissues were removed and immediately immersed in Freon 12, cooled by liquid nitrogen. Sections, 10-μm thick, were cut on a Reichert-Jung or a Sileo motor-driven cryostat at −25°C (native cryostat sections). In order to minimize the non-specific reflectance arising from the submicroscopic inhomogeneities of the tissues, which may diminish the contrast between the specific enzymatic reaction product and non-specifically reflecting background under CLSM observation in the reflection mode, air-drying of the sections after mounting onto glass slides was avoided. Instead, the slides with the wet native cryostat sections were immediately transferred into the fixative. To prevent loosening of sections, the slides were precoated with chromealum gelatin. After fixation, the sections were rinsed successively in double-distilled water (ddH2O) (twice, 5 min at 1°C), cold 50 mM glycine in 50 mM TRIS-HCL buffer at pH 7.0, ddH2O (twice, 5 min at 1°C) and, lastly, in ddH2O at room temperature (20°C).

### Enzyme histochemistry

**d-Amino acid oxidase (AAOX), α-hydroxy acid oxidase (HAOX), urate oxidase (UOX), xanthine oxidase (XOX), and monoamine oxidase (MAOX)**

Activities of these H2O2-generating oxidases were detected according to the methods of Angermüller and Fahimi (1988a, b), Angermüller (1989), Frederiks and Marx (1993), as well as that of Nakos and Gossrau (1993) in a modified form using fixed cryostat sections with incubation in an aqueous HEPE- or HEPPSO-buffered and dextrane-stabilized incubation medium (Halbhuber et al. 1991). To remove non-specifically bound cerium from tissues, the sections were treated with EGTA.

Fixation: 2-5 min at 1°C (ice bath) 1% glutaraldehyde (GA) in 0.1 M cacodylate buffer (pH 7.2-7.4). Rinsing: twice ddH2O at 1°C, 50 mM glycine (pH 7.0) 5 min at 1°C, twice ddH2O at 1°C, ddH2O at 20°C 1 min. Incubation: 30-120 min at 37°C under constant stirring in 0.1 M HEPE or HEPPSO (pH 7.4), 1% dextrane T70, 50 mM aminotriazolo, 3 mM levamisole, 10 mM CeCl3, 5 mM substrate (AAOX: d-proline; HAOX: α-hydroxybutyric acid; UOX: 0.5 mM sodium urate; XOX: 0.5 mM hypoxanthine; MAOX: 5 mM tyramine). Rinsing: twice in 0.025 M glycine-NaOH buffer (pH 10.0). EGTA treatment: 2 mM EGTA in 0.1 M cacodylate buffer (pH 6.5) 15-45 min at 20°C. Reoxidation: 5% H2O2 in 0.025 M glycine-NaOH buffer (pH 10.0), 100 mM NaN3, 15 min at 20°C. Rinsing: twice in 0.025 M glycine-NaOH buffer (pH 10.0).

The specimens were dehydrated over graded alcohol and covered with xylol in Canada balsam. It is important to use freshly prepared alcohol series without any contaminations to avoid deposition of possibly reflectant products of non-specific origin.