How-To-Do-It

"Visualization" of Biochemical Pathways with Enzyme Markers

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Living organisms use biochemical pathways to conserve chemical energy for work and to obtain precursor molecules for macromolecular synthesis. Students usually are asked to learn a few of these energy transducing pathways and to associate these pathways with specific cellular sites. Yet sometimes it is difficult for an undergraduate student to gain an understanding of these abstract chemical concepts and to relate function to structure. This paper will present laboratory techniques that will enable a teacher to design an undergraduate laboratory investigation for the visualization of biochemical pathways at the optical microscope level within plant and animal tissues. The visualization is accomplished by the localization of a marker enzyme for the glycolytic, citric acid cycle and electron transport pathways. Marker enzymes provide, pedagogically, a way to help a student: 1) associate a biochemical pathway with specific structural organization; 2) gain an understanding of the relationship of function and structure; and 3) correlate enzyme activity and energy transduction.

The aim of this paper is to interest the undergraduate teacher in providing students with an unique opportunity for a "hands-on" approach in relating cellular function to structure. This goal necessitates that the teacher: 1) be familiar with general background about the biochemistry of the cell; 2) study the individual experimental protocol; and 3) design and write an investigative procedure that is best suited to a student's learning environment.

Protocol presented in this paper is documented by a list of references that provide background information applicable to plants and animals, and a list of suppliers. Tissue preparation and each marker enzyme assay stand alone. The teacher, therefore, can construct a learning experience that fits almost any schedule. The activities require a refrigerated cold microtome, viz., a cryostat, and some chemical reagents not usually found in the undergraduate laboratory but readily available from a chemical supplier.

Methods and Materials

General—Fresh tissue is embedded in Tissue Tek II-OCT compound, temperature equilibrated and sectioned in a cryostat. Unfixed, cryostat sections are histochemically assayed for the activity of a specific marker enzyme: glyceraldehyde-3-phosphate dehydrogenase, succinic acid dehydrogenase and cytochrome oxidase that specifically mark, respectively, the glycolytic (Embden-Meyerhof) pathway, the citric acid (Kreb's) cycle and the electron transport system. For these studies, unfixed plant shoot or root meristematic tissue, or animal liver tissue is very reactive and allows the student an opportunity to study cells preserved in a life-like condition.

Cryostat procedure—In using a cryostat, it is necessary to attach the unfixed tissue, 1-5mm in thickness, to a specimen holder of the microtome. This is achieved by: 1) placing several drops of a tissue support medium, Tissue Tek II-OCT compound (American Scientific Products), on a grooved brass specimen holder and quick-freezing the OCT until it hardens; 2) adding one drop of room temperature OCT on top of the frozen mound of OCT and rapidly inserting the tissue; 3) quick-freezing the OCT-tissue in the cryostat for approximately three minutes; and 4) temperatue equilibrating in the cryostat chamber for 25 minutes (animal) or one hour (plant) to minimize tissue damage during the sectioning process. Within six hours after embedment, frozen tissue blocks are sectioned, with the aid of an anti-roll plate, at 8-16 μm, and at a knife temperature of −18C to −25C. Jensen (1962), Thompson (1966) and Pearse (1980) contain excellent information on the use of a cryostat.

Once a section is obtained, the anti-roll plate is flipped back and the section is transferred to an uncoated, room temperature glass slide. This is accomplished by: 1) positioning the slide just above the frozen section; 2) picking up the section with a cold metal spatula; and 3) lowering it gently to the slide. Some workers use a gelatin-coated slide to adhere the section, but I found that gelatin inhibits the activity of some enzymes. To obtain a wrinkle-free section it is essential to remove OCT debris and frost from the knife edge.

Histochemical protocol—General—Protocol for the localization of marker enzyme activity within tissues and cells differs somewhat from those procedures usually found in general microtechniques, or with the localization of other cellular constituents. There are certain problems in the histochemical demonstration of enzyme activity: 1) maintenance and specificity of enzyme activity; and 2) diffusion of colored, reaction end-product (Jensen 1962). It is important to test the specificity of the enzyme catalyzed reaction through the use of two main controls: 1) incubation minus substrate; 2) addition to the reaction medium of an inhibitor that is specific to the enzyme.

All reaction media contain polyvinyl alcohol (G 04/140) (J. T. Baker Chemical) to prevent the release of enzyme from the tissue (Altman 1980) and to keep the sections firmly adhered to the glass slide (Orr, unpublished). All reacted tissue sections are mounted in polyvinyl pyrrolidone (PVP-40) (Sigma Chemical) to minimize leaching of the colored reaction product (Burstone 1957).

Stated parameter for incubation time and temperature may vary among tissue type. All incubation parameters for the reactions in this paper

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are suitable for plant tissues. A slightly higher temperature may be required for some animal tissue.

Glyceraldehyde-3-phosphate dehydrogenase (GPD)—A two-step assay system has been developed for visualizing the activity of GPD in animal sections (Henderson 1976) and recently in plant tissue (Orr, unpublished): Fructose 1,6-diphosphate is used as the primary substrate and the demonstration of GPD activity depends upon the conversion of this compound into the specific GPD substrate, glyceraldehyde 3-phosphate, by aldolase. Gahan (1984) presents the conceptual ideas for the use of tetrazolium salts (i.e., NBT), NAD^+ and mPMS in the reaction medium.

Twenty-minute, air-dried sections are incubated for 30 minutes at 30C in a fresh reaction medium: fructose 1,6-diphosphate, 3 mg/ml (Sigma); NAD^+, 2.5 mg/ml (Sigma); KH_2PO_4, 0.5 mg/ml; nitroblue tetazolium (NBT), 3 mg/ml (Kodak); methoxyphenazine methosulfate (mPMS), 0.2 mg/ml (Sigma); PVA, 0.5 mg/ml; aldolase (type X, from rabbit muscle), 80 ug/ml (Sigma); glycylglycine, 0.05 M (Sigma) at pH 8.5. The reaction should be performed in a darkened room. Fresh solutions containing NBT and mPMS should be stored in the dark. Control sections are incubated with reaction medium minus substrate, fructose 1,6-diphosphate, or with reaction medium containing sodium azide (2×10^{-3} M) (Sigma). The site of GPD activity will appear blue.

Succinic Acid dehydrogenase—To determine SDH activity, 20-minute air-dried sections are incubated for one hour at 35C in a fresh reaction medium: sodium succinate, 27 mg/ml (Sigma); nitroblue tetrazolium, 0.5 mg/ml; PVA, 0.5 mg/ml; 0.2 M phosphate buffer at pH 7.2 (Nachlas, Tsow, De Souza, Cheng & Seligman 1957). Sections are rinsed in water (30 seconds), fixed in neutral 10 percent formalin (Fisher) for five minutes, dehydrated through an alcohol series (30, 50, 70, 90, 100 percent for five minutes each), immersed in xylene (five minutes) and mounted in PVP-40 (Berlyn & Miksche 1976; Petersen & Orr 1983). Control sections are incubated in a reaction medium containing the enzyme inhibitor malonic acid (8×10^{-3} M) (Sigma), or a reaction medium minus the substrate sodium succinate. Sites of SDH activity are blue.

Cytochrome oxidase(CO)—To determine CO activity, 20-minute air-dried sections are incubated in a reaction medium: variamine blue RT base (0.3 mg/ml) (Sigma); 8-amino-1, 2, 3, 4-tetrahydroquinoline, 1 mg/ml (Sigma); .02 M trizma buffer a pH 7.4, 0.3 ml/ml; cytochrome C, 0.4 mg/ml (Sigma); catalase, 0.04 mg/ml (Sigma); PVA (0.5 mg/ml) (Burstone 1960; Person, Burston & Fine 1962; Orr 1984). Tissue sections are transferred to a colbaltous acetate (10%) (Sigma)—formalin (10%) (Fisher) buffered (pH 5.2) solution at 0.2 M for 90 minutes, rinsed in water, and mounted in PVP-40. Control sections are incubated with reaction medium containing the enzyme inhibitor, Na_2S (1×10^{-3}M) (Sigma) (Jensen 1962; Harwig 1967) or with the reaction medium minus either variamine blue RT base or 8-amino-1, 2, 3, 4-tetrahydroquinoline. The site of CO activity will appear blue.

Photography—Black and white photography of reacted tissue sections may be done with Kodak Technical Pan film (2415) and two Kodak Wratten gelatin filters (No. 15, 58). Color photographs are obtainable with Kodak Photomicrography Color film (2483).

Summary

Cytochemical methods presented in this article are used to demonstrate the site of biochemical pathways at the tissue, cellular and subcellular (mitochondria) level. Through this simple enzymic marking procedure students can gain insight into catabolic metabolic pathways, enzyme action, and can integrate structure and function. Information on an anabolic biochemical pathway, the pentose phosphate pathway, can be obtained by assaying tissue sections for glucose-6-phosphate dehydrogenase (Gahan 1984; Orr 1985).

It should be seen that these procedures also can elucidate metabolic differences between cells and tissues, enabling one to obtain information about energy transduction activities in special or abnormal material. Further, these histochemical enzyme assays can be used to mark biochemical pathway activity at different stages of growth and differentiation, e.g., flowering (Orr & Schoneman 1986). Visualization of a biochemical pathway with an enzyme marker is a useful, pedagogical tool in the undergraduate laboratory.

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