CC/NUMBER 29
JULY 18, 1988

This Week's Citation Classic®

Mesulam M-M. Tetramethyl benzidine for horseradish peroxidase neurohistochemistry: a non-carcinogenic blue reaction-product with superior sensitivity for visualizing neural afferents and efferents. *J. Histochem. Cytochem.* 26:106-17, 1978.
[Bullard and Denny-Brown Labs., Harvard Neurological Unit, Beth Israel Hosp., Boston, MA]

This paper described a method for the histochemical visualization of horseradish peroxidase (HRP) in neuroanatomical tracing experiments. The high sensitivity for the detection of transported HRP allowed a more complete and consistent demonstration of anterograde and retrograde neural connections. [The *SCI*® indicates that this paper has been cited in over 2,100 publications.]

M.-Marsel Mesulam
Division of Neuroscience and
Behavioral Neurology
Bullard and Denny-Brown Laboratories
Dana Research Institute
Beth Israel Hospital and
Harvard Medical School
Boston, MA 02215

January 19, 1988

The introduction of amino acid autoradiography and horseradish peroxidase (HRP) histochemistry in the early 1970s was met with great enthusiasm[1-3] and led to a striking invigoration (and democratization) in neuroanatomy. The tracing of neural pathways ceased to be the province of the initiated few and came within the grasp of any neuroscientist who wished to ask questions about connectivity.

At that time I was working with Deepak Pandya, Gary Van Hoesen, and Douglas L. Rosene, first at Boston City Hospital and then at Beth Israel Hospital. The laboratory was deeply immersed in tracing cortical connectivity in the brain of the rhesus monkey. We welcomed the introduction of HRP as a novel tool for locating the cell bodies giving rise to neural projections. However, our HRP experiments (with the recommended diaminobenzidine procedures) did not demonstrate all the connectivity that we had come to expect on the basis of prior experience with silver impregnation methods. Was our interpretation of the silver material in error or was it possible, as some had come to suggest, that HRP was not being transported along all neural pathways?

It became clear that the neuroanatomical applications had not paid sufficient attention to the underlying enzymatic reaction. The demonstration of HRP transport was indirect and required the generation of a reaction product. The detection threshold for HRP could vary substantially according to the choice of histochemical parameters.

I first turned to benzidine dihydrochloride as an alternative chromogen.[4] This had been used by W. Straus to trace endocytosis into renal tubular cells. The deep blue reaction product and its contrast with the neutral red counterstain was quite pleasing to the eye and provided additional motivation for pursuing these experiments. Considerable manipulation of histochemical variables eventually led to a method that was more sensitive than the diaminobenzidine procedure and helped us demonstrate the existence of limbic projections from the cingulate gyrus into the parietal association cortex in the monkey brain. These observations proved that the visibility and sensitivity of a reaction product can determine whether a neural connection gets to be demonstrated with HRP. Additional experiments by Rosene showed the importance of fixation for overall sensitivity.[5]

High carcinogenicity made the handling of benzidine dihydrochloride quite cumbersome. I then came across tetramethylbenzidine (TMB), which had been introduced as a noncarcinogenic alternative to benzidine dihydrochloride for the detection of occult blood. A major problem was its hydrophobic properties. One strategy was to make the incubation solution ethanolic, but this diminished sensitivity. The answer came to me in, of all places, the dentist's office. A great deal of TMB would dissolve in warmed absolute ethanol and small aliquots could introduce precise amounts of TMB without changing the ethanol content of the final solution by much. I asked my dentist if I could use his telephone, and I relayed a new set of instructions to my laboratory assistant.

Encouraging results allowed a parametric study of histochemical variables, leading to the method that is described in this paper. Sensitivity could be enhanced without sacrificing much specificity, and the effect of altering each of the many variables could be predicted reliably. This made it possible to exploit the potential of HRP transport more extensively. The first full test was done in tissue from a rhesus monkey with an HRP injection in the frontal eye fields. We were able to visualize all known anterograde and retrograde connections of the frontal eye fields. The TMB method also proved that the uptake and transport of macromolecules occurs not just in the retrograde direction but also anterogradely, from dendrites and perikarya into axonal terminals.[6]

This method (with minor modifications)[7] was applied to demonstrate a large number of afferent and efferent pathways where the minute quantities of transported HRP had remained beyond the resolution of previously available procedures. Laboratories throughout the world reported the use of HRP and TMB for tracing the connectivity of visceral organs, skin, muscle, peripheral nerves, ganglia, and just about every structure in the central nervous system. This broad range of applications is probably responsible for the frequency of citation.

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2. **Kristensson K & Olsson Y.** Retrograde axonal transport of protein. *Brain Res.* 29:363-5, 1971. (Cited 310 times.)
3. **LaVail J H & LaVail M M.** Retrograde axonal transport in the central nervous system. *Science* 176:1416-7, 1972. (Cited 595 times.)
4. **Mesulam M-M.** The blue reaction product in horseradish peroxidase neurohistochemistry: incubation parameters and visibility. *J. Histochem. Cytochem.* 24:1273-80, 1976. (Cited 480 times.)
5. **Rosene D L & Mesulam M-M.** Fixation variables in horseradish peroxidase neurohistochemistry. I. The effects of fixation time and perfusion procedures upon enzyme activity. *J. Histochem. Cytochem.* 26:28-39, 1978. (Cited 265 times.)
6. **Mesulam M-M & Mufson E J.** The rapid anterograde transport of horseradish peroxidase. *Neuroscience* 5:1277-86, 1980. (Cited 85 times.)
7. **Mesulam M-M, Hegarty E, Barbas H, Carson K A, Gower E C, Knapp A G, Moss M B & Mufson E J.** Additional factors influencing sensitivity in the tetramethyl benzidine method for horseradish peroxidase neurohistochemistry. *J. Histochem. Cytochem.* 28:1255-9, 1980. (Cited 150 times.)

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