NEUROLOGY AND NEUROBIOLOGY VOLUME 1

Alan R. Liss, Inc., New York

Cytochemical Methods in Neuroanatomy

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Alan R. Liss, Inc. • New York

Address all Inquiries to the Publisher
Alan R. Liss, Inc., 150 Fifth Avenue, New York, NY 10011

Printed in the United States of America.

Library of Congress Cataloging in Publication Data
Main entry under title:

Cytochemical methods in neuroanatomy.

(Neurology and Neurobiology; v. 1)
Papers presented at a conference held at the National Institutes of Health in Bethesda, Md., June 10-12, 1981.
Includes bibliographies and index.
1. Neuroanatomy. 2. Cytochemistry. I. Chan-Palay, Victoria. II. Palay, Sanford L. III. Series. [DNLM: 1. Histocytochemistry — Methods — Congresses. 2. Nervous system — Analysis — Congresses. 3. Neuroanatomy — Methods — Congresses. W1 NE337B v.1/WL 101 C997 1981]

QM575.C97 611'.8'028 82-7826
ISBN 0-8451-2700-4 AACR2

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Preface

The ancients believed that nerves were hollow conduits designed to convey animal spirits from the brain to the muscles and sense organs. Similarly, the substance of the brain was thought to be merely the receptacle for the ventricles in which such important functions as sensation, thought, and memory occurred. These ideas were remarkably tenacious, persisting, with some modification, into the nineteenth century. Even though Vesalius protested that he could not see any hollows in the nerves that he dissected, he declared that he could hardly deny the teachings of his predecessors on this subject. The hollows could be too small to see, like the fluid channels in the pith of plant stems. Even more than a century later, Thomas Willis, who appreciated that the ventricles merely contained fluid and concluded that the fabric of the brain was the important part of this organ – the same Thomas Willis of the arterial circle at the base of the brain – claimed that since there were no visible hollows in nerves, the marvelous animal spirits must flow through them in the manner of sunlight through glass. At the beginning of the eighteenth century, Leeuwenhoek also failed to perceive the hollows in nerves with his little microscope; instead he discovered that a nerve consists of myriad parallel hollow vessels too small to be discerned with the naked eye. Clearly these were the channels through which the animal spirits flowed!

Towards the end of the eighteenth century, animal spirits lost their appeal as an explanation of nervous activity and were replaced by electricity, an idea that lasted until the middle of the twentieth century, with the modification that the current was carried by ions. Well into the 1840's nerve fibers were still considered to be fluid-filled tubes, as the German term Nervenröhrchen – little nerve tubes – signified. With the discovery of the axis cylinder in the late 1830's and the gradual acceptance of its continuity with the nerve cell body in the late 1840's, the central issue in the understanding of the nervous system became the morphological and functional organization of its constituent cells. That central issue has remained to this day the essential task of neuroscience.

Today we no longer think that animal spirits flow through our nerves, but once more we regard the processes of nerve cells – axons and dendrites – as channels for transport, not of animal spirits, but of specific definable chemicals, proteins, enzymes, peptides, and various complex but definable particulates. They are transported in a still mysterious fashion from the nerve cell body to the synaptic terminals, not only in that orthograde direction but also in the opposite direction, retrogradely, and we conceive of this transport as proceeding independently in both directions simultaneously. This is a most remarkable and startling idea, which would have been just as astounding to the neuroscientists of even forty years ago as it is reasonable and convincing to us. It comes from almost two centuries of building upon the original understanding that the axis cylinder is continuous with the nerve cell body, from the discovery of the synapse and the true ends of nerve fibers, from the biophysical and neurochemical dissection of the nerve impulse, from the discovery of chemical synaptic transmission, from the discovery of neurosecretion, and from studies on degeneration and regeneration, culminating in the

discovery of axoplasmic flow. It has been a slow and halting progress, but it has brought us to this one grand idea, which is the bedrock on which all our methods for unraveling the organization of the nervous system are based. It has proved to be the essential phenomenon of the nerve cell and it is a dynamic clue to the organization of the nervous system. Our tests are all derived from this one property of the nerve cell – the retrograde or chromatolytic reaction, Wallerian degeneration, regeneration, collateral sprouting, plasticity, tracing pathways with isotropically labeled amino acids, horseradish peroxidase and other foreign proteins, transmitters, and even various dyes. The papers in this book attest to the fruitfulness of this fundamentally cytological insight for analyzing the most complicated biological system that we know of. They constitute the report of a conference held at the National Institutes of Health in Bethesda, Maryland from June 10 to 12, 1981. About thirty neuroscientists of various persuasions – neuroanatomists, neurophysiologists, neurochemists, and neuropharmacologists – met together for two and a half days in order to discuss cytochemical methods in the analysis of the nervous system – the advantages and drawbacks of these methods, their promises and pitfalls – and to exchange information that is accumulating as a result of their use. The participants included scientists from the United States, England, Europe, and Australia, and the meeting was attended by a large number of auditors from the Baltimore-Washington research community, as well as a few from more distant points. The purpose of this book is to make the conference available to the larger community of neuroscientists and students who were unable to attend.

We are grateful to the Fogarty International Center of the National Institutes of Health and the National Institute of Mental Health for their support of this conference. We wish to acknowledge especially our debt to Dr. Earl C. Chamberlayne and Mrs. Nancy E. Shapiro of the Fogarty International Center, Conference and Seminar Program Branch, for their generous help in organizing the Conference.

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December 17, 1981

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The Orthograde Axoplasmic Transport Autoradiographic Tracing Technique and Its Implications for Additional Neuroanatomical Analysis of the Striate Cortex

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When I was a graduate student in the early 1960s, my anatomical conception of the brain was usually that of a jumbled collection of isolated Golgi-impregnated neurons set against a cresyl violet background. I was delighted to hear from our postdoctoral teaching assistant about a new silver technique that would specifically stain degenerating pathways, because it seemed that finally this would provide an experimental method to unravel the wiring patterns between all of those many brain nuclei that we were struggling to memorize. In fact, about this time, the Nauta silver degeneration stains did begin the entire reawakening of neuroanatomy and revealed a wealth of brain pathways that were previously unknown. I was quite disappointed to learn, however, that although my teaching assistant could give me the recipe for the Nauta technique, he really couldn't explain how or why the technique worked on a biochemical basis. I would just have to apply it to the brain of my particular animal and obtain success by trial and error. Now I have nothing against recipes, as my local fame as a cook will confirm, but as an anatomist who aspired to correlate cell function with structure it struck me as all wrong that the available neuroanatomical methods at that time contained more art than biochemistry.

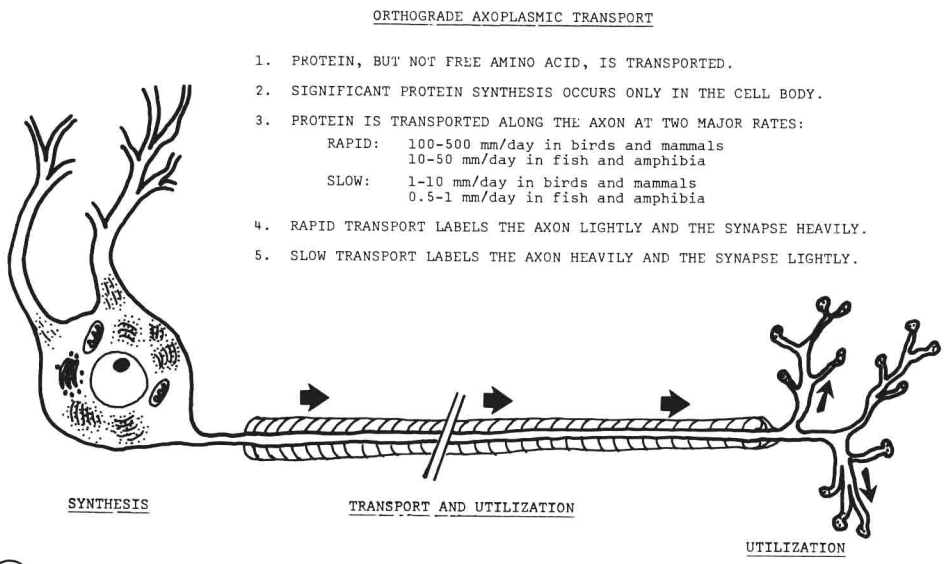
When I was a beginning instructor in the late 1960s, the tide finally began to turn. Weiss and his collaborators [5, 30, 31] reported the presence of a regular movement of protein in the brain which was synthesized in the neuronal cell body and then actively transported to its axon and synapses. We were studying the anatomy of monkey retinal input to thalamus, and were having great difficulty obtaining reliable results with silver degeneration stains in primate brain. It occurred to me, why not use this normal function of the neuron and mark the protein by making it radioactive? It seemed an excellent, sensitive, and reliable way to label the primate visual pathway for anatomy, and I was skilled in both light and EM autoradiography so I didn't have to learn a new technique. Carl Kupfer and I first injected ^{3}H-leucine into the vitreous of a monkey eye which resulted in the retinal ganglion cell protein becoming radioactive, and then I did autoradiography on the brain to localize this protein and mark the distribution of the retinal ganglion cell axons. It worked beautifully, the first time and every time, for both the light and electron microscope [10, 17]. The other appealing aspect of this approach was that the abundant biochemical data that were rapidly accumulating

about axoplasmic transport made it a method whose rationale could be explained, controlled, and expanded on the basis of cell biology (compare Lasek [22] with Wilson and Stone [32], Schwartz [27], or Grafstein and Forman [7]). That is the way that our group began to contribute to the development of the orthograde axoplasmic transport autoradiographic tracing method [4].

Now I am a professor. In less than 20 years, over the span of my academic career, I have seen neuroanatomy revitalize, snowballing into cell biology with orthograde and retrograde transport tracing techniques, radioactive transmitter uptake and transport, the metabolic autoradiographic method based on deoxyglucose, immunocytochemistry, receptor binding identified autoradiographically on sections, HRP filling of physiologically characterized neurons, and much more, now commonplace in the laboratory and literature. Virtually all of the methodology that will be discussed in these 3 days has developed during my rather short scientific career, so I guess that makes me an appropriate lead-off author for this volume on cytochemical neuroanatomy.

I will briefly discuss the now almost classic (i.e., 10 years old) method of orthograde axoplasmic transport autoradiographic tracing (OAAT). I will then show how information derived from OAAT about the neuroanatomical organization of monkey striate cortex has led to the application of new and different cytochemical techniques to understand better the anatomy and possibly the physiology of this tissue. I have chosen the monkey striate cortex because this tissue is an excellent example of how the provocative findings from one technique can stimulate the application of other techniques to answer still unresolved questions.

OAAT is based on a normal and necessary biochemical process in neurons, axoplasmic transport (Fig. 1). This literature has been recently reviewed [7, 27, 32]. Neurons as cells are characterized by the spatial separation of component parts, very long processes, and compartmentalization within specialized cell regions of single functions. Neurons are metabolically very active cells, which require a constant synthesis of



(1)

Fig. 1. A schematic neuron and a list of the essential facts about axoplasmic transport which form the basis of the orthograde axoplasmic transport tracing technique.

complex molecules including proteins. The cell body and to a lesser degree the dendrites contain all of the rough endoplasmic reticulum and ribosomes required for extramitochondrial protein synthesis, so these are the only regions of the neuron in which radioactive precursors like tritiated (^{3}H)-amino acids can be synthesized into proteins. The ^{3}H-labeled protein is then loaded onto the intracellular transport system of smooth endoplasmic reticulum and microtubules and actively transported away from the cell body along all of the cell branches, especially to the axon and its synaptic terminals. Two general rates of movement occur: A rapid phase moves at 100–500 mm/day and a slow phase moves at 1–20 mm/day in mammals and birds, whereas in reptiles and amphibians these phases are each an order of magnitude slower [6, 22]. Different precursors end up in the two different phases in different proportions; for instance, ^{3}H-proline or ^{3}H-fucose heavily label the rapid phase whereas ^{3}H-leucine heavily labels the slow phase. The different phases also distribute to different regions of the neuron. The rapid phase contains organelles such as mitochondria and smooth endoplasmic reticulum, proteins, polypeptides, carbohydrates, lipids, and transmitters [see 7, 24, 27, 32 for details]. It distributes heavily to the synaptic terminals and lightly to the axon [11, 24, 26]. The slow phase contains relatively few proteins including tubulin, actin, and myosin (discussed by Black and Lasek [2] and seems to renew the axon, which results in heavy label of axon pathways but only light labeling of the synaptic terminal [11].

It must be stressed that OAAT involves the second order detection system of autoradiography (Fig. 2). The radioactive protein is in the neuron contained within the tissue section, but the detection label, the silver grain, is in the overlying photographic emulsion. The distance that separates the radioactive decay in the tissue from its resulting silver grain is called "resolution." This distance depends on the energy of the isotope and the thickness of the section and emulsion. For the final analysis of the results obtained by OAAT (Fig. 3) you are faced with two steps. First, you must visualize the neuron and its processes by special CNS stains, which can be a problem because many of the components of the neuron are difficult to differentiate and resolve in the light microscope. Then you must relate the silver grains to their origin in the tissue, which can be another problem in that a silver grain contains no inherent information about its source; it always looks the same whether it is caused by the radioactive neuronal protein, cosmic rays, or a stray light leak in the darkroom. Despite these limitations, as witnessed by its rapid adoption by the neuroscience community and by its widespread use, OAAT has obvious advantages as a neuroanatomical technique. It answers the question, "Where do the cell bodies in a specific region of the brain send their axons to form synapses?" The advantages of OAAT are:

1. All known neurons have an axoplasmic transport system, so the method is universally applicable regardless of species or localization within the central or peripheral nervous system.

2. Ninety-nine percent of neuronal protein is made in the cell body, so the exact origin of labeled protein is known to be the neuronal cell bodies lying within the injection site.

3. Axons cannot transport free amino acids for any distance nor synthesize protein, so the axons passing through the injection site do not influence the final autoradiographic pattern. This is a great advantage over both the Nauta silver degeneration stains and horseradish peroxidase retrograde tracing techniques; in both of these, axons that lie within the lesion or injection site can subsequently stain for degeneration or transport horseradish peroxidase in both orthograde and retrograde directions. This transport specificity is confined only to amino acids and not to some other precursors such as ^{3}H-fucose, which are transported for long distances by axons [9].

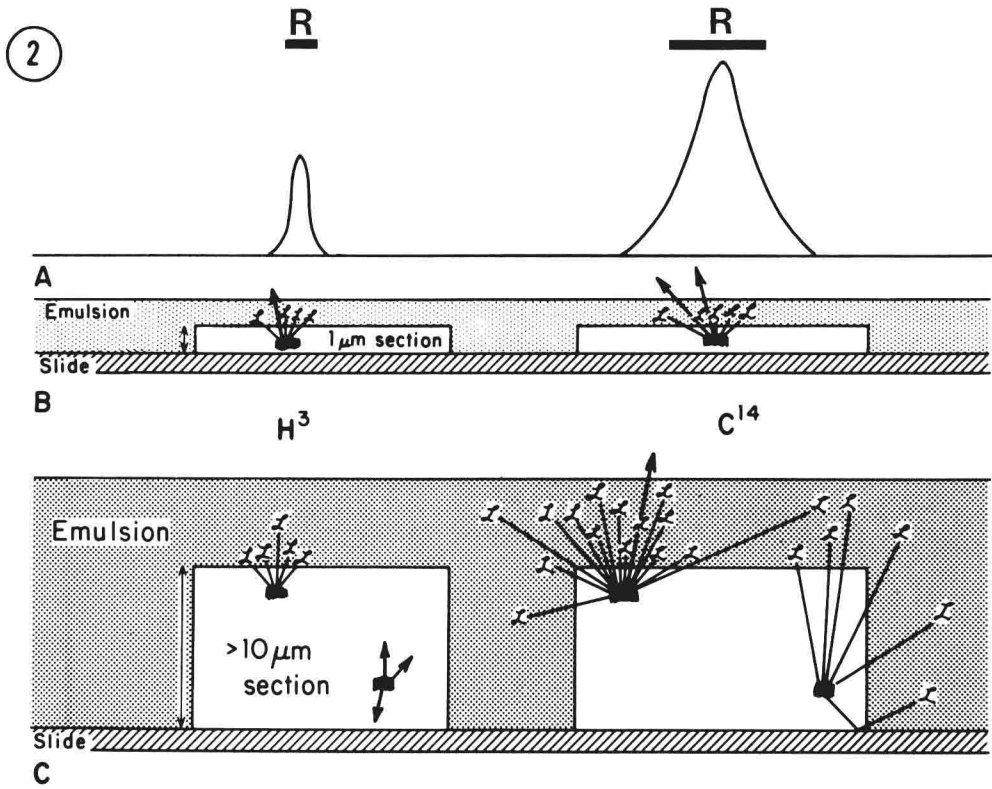


Fig. 2. A schematic representation of autoradiography. A) shows the average resolution (R) when a low energy β particle like tritium (^{3}H) or a higher energy β particle like carbon 14 (^{14}C) is used. B) When both the section and emulsion coat are thin, resolution is maintained for both isotopes, but some ^{14}C disintegrations pass through the thin emulsion without causing a latent image. C) When both section and emulsion are thick, ^{3}H forms no more latent images, but ^{14}C does, although with some loss in resolution.

4. Axon terminals do not synthesize protein, so there is no significant retrograde transport of label away from the injection site. Some precursors such as ^{3}H-proline [21] and ^{3}H-adenosine [33] have been reported to move in a retrograde direction, but the subsequent radioactivity is found over the neuronal cell body, not the neuropile, and so it is easily distinguished from orthograde label.

5. Because a significant amount of the rapidly transported protein is deposited in the synaptic terminal, the use of short survival times after injection gives a sensitive and reliable method of marking synaptic terminals. This has been confirmed by EM autoradiography, which has shown that 3 days after an eye injection 53% of the silver grains are over the retinal ganglion cell terminals and 27% are over the axons, whereas 30 days after an eye injection, when the slow phase is reaching the thalamus, the terminal labeling has decreased to 20% and the axonal label has risen to 66% [11].

6. Autoradiographic methods are easy, well documented, and reliable, and can be applied at both light and EM levels.

7. Because the emulsion will form latent images until it is saturated, exposure times can be extended to label even sparse pathways, which gives OAAT a built-in amplification factor.