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Volume II

John E. Johnson, Jr.

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Current Trends in Morphological Techniques

Volume II

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1. Histological techniques

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METHODS IN AGING RESEARCH

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PREFACE

Man has always been interested in the structure of the world around him, because he is a visually oriented animal. Science had its roots in philosophy, and the philosophers examined structure and inferred function. With the invention of the light microscope we realized that living things are made of cells, and we attempted to infer the function of structures within those cells. However, the limits of light microscopic optics to resolve detail were reached in the twentieth century with computerized lens designs. The smallest detail that could be discerned with the best of these glass optics is about 0.2 μ, much larger than many cellular components, and scientists had to, again, become philosophical as to what some of the subcellular organelles really were.

With the discovery that an electron beam could be used to image the fine structure of cells and other substances, the world of biology has been turned upside down and inside out, illuminating to a fascinated population the fact that a microcosm exists within us all.

Although we have reached the limits of resolution in electron microscopes, the applications of this elegant instrument have only begun to unfold. During the next decade, the use of *analytical* electron microscopy will provide information not just about the structure of small cell and material components, but what types of atoms are there, and, with a little luck, how many. The electron microscope will be a tool not for anatomists alone, but for scientists of all disciplines; will not be simply a microscope, but a microlaboratory.

Light microscopy is a powerful tool in biology, and electron microscopy may be the most powerful. Techniques in specimen preparation and handling are probably more important than technical skill on the instrument itself. Techniques abound across hundreds of laboratories, yet, because journal space is so limited and costly, rarely do we see an extensive materials and methods section in a published article. Most books published on methodology simply review what has been done in everyone's laboratory. The purpose of the present series of volumes (three to begin with in 1981; more to follow) is to describe, where possible, how a particular scientist — or group — does things in their own laboratory. I did not limit the authors to a certain length of text or a specific number of photographic illustrations. I asked for as much detail as possible including all the little "tricks" for success that usually get passed around locally but never get published. I also asked for descriptions of artifacts, something present in a sampling of material from each of our laboratories but difficult to get a scientist to show, because, in microscopy, except for freeze fracture, they are usually not very attractive.

Most of the material presented in the first three volumes is related to the Neurosciences. This is due in part to the fact that I am a neuroscientist by training, but also, to the explosion of brain research as a field.

Future volumes in this series will spread out into other techniques that span the entire field of light and electron microscopy. I am extremely pleased with the contents of the first three volumes and am proud to offer these published technical methods representing many years of concentrated effort by microscopists.

John E. Johnson, Jr., PhD.
Baltimore, 1981

EDITORS-IN-CHIEF

Dr. Richard C. Adelman is currently Executive Director of the Temple University Institute on Aging, Philadelphia, Pa., as well as Professor of Biochemistry in the Fels Research Institute of the Temple University College of Medicine. An active gerontologist for more than 10 years, he has achieved international prominence as a researcher, educator, and administrator. These accomplishments span a broad spectrum of activities ranging from the traditional disciplinary interests of the research biologist to the advocacy, implementation, and administration of multidisciplinary issues of public policy of concern to elderly people.

Dr. Adelman pursued his pre- and postdoctoral research training under the guidance of two prominent biochemists, each of whom is a member of the National Academy of Sciences: Dr. Sidney Weinhouse as Director of the Fels Research Institute, Temple University, and Dr. Bernard L. Horecker as Chairman of the Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, N.Y. His accomplishments as a researcher can be expressed in at least the following ways. He is the author and/or editor of more than 70 publications, including original research papers in referred journals, review chapters, and books. His research efforts have been supported by grants from the National Institutes of Health for the past 10 years, at a current annual level of approximately $300,000. He continues to serve as an invited speaker at seminar programs, symposiums, and workshops all over the world. He is the recipient of the IntraScience Research Foundation Medalist Award, an annual research prize awarded by peer evaluation for major advances in newly emerging areas of the life sciences. He is the recipient of an Established Investigatorship of the American Heart Association.

As an educator, Dr. Adelman is also involved in a broad variety of activities. His role in research training consists of responsibility for pre- and postdoctoral students who are assigned specific projects in his laboratory. He teaches an Advanced Graduate Course on the Biology of Aging, lectures on biomedical aspects of aging to medical students, and is responsible for the biological component of the basic course in aging sponsored by the School of Social Administration. Training activities outside the University include membership in the Faculty of the National Institute on Aging summer course on the Biology of Aging; programs on the biology of aging for AAA's throughout Pennsylvania and Ohio; and the implementation and teaching of Biology of Aging for the Nonbiologist locally, for the Gerontology Society of America and other national organizations, as well as for the International Association of Gerontology.

Dr. Adelman has achieved leadership positions across equally broad areas. Responsibilities of this position include the integration of multidisciplinary programs in research, consultation and education, and health service, as well as advocacy for the University on all matters dealing with aging. He coordinates a city-wide consortium of researchers from Temple University, the Wistar Institute, the Medical College of Pennsylvania, Drexel University, and the Philadelphia Geriatric Center, conducting collaborative research projects, training programs, and symposiums. He was a past President of the Philadelphia Biochemists Club. He serves on the editorial boards of the *Journal of Gerontology, Mechanisms of Ageing and Development, Experimental Aging Research*, and *Gerontological Abstracts*. He was a member of the Biomedical Research Panel of the National Advisory Council of the National Institute on Aging. He chairs a subcommittee of the National Academy of Sciences Committee on Animal Models for Aging Research. As an active Fellow of the Gerontological Society of America, he is a past Chairman of the Biological Sciences section; a past Chairman of the Society Public Policy Committee for which he prepared Congressional testimony and represented the Society on the Leadership Council of the Coalition of National Aging Organizations; and is Secretary-Treasurer of the North American Executive

Committee of the International Association of Gerontology. Finally, as the highest testimony of his leadership capabilities, he continues to serve on National Advisory Committee which impact on diverse key issues dealing with the elderly. These include a 4-year appointment as a member of the NIH Study Section on Pathobiological Chemistry; the Executive Committee of the Health Resources Administration Project on publication of the recent edition of *Working with Older People — A Guide to Practice;* a recent appointment as reviewer of AOA applications fo Career Preparation Programs in Gerontology; and a 4-year appointment on the Veterans Administration Long-Term Care Advisor Council responsible for evaluating their program on Geriatric Research, Education, and Clinical Centers (GRECC).

Dr. George S. Roth is a Research Chemist at the Gerontology Research Center of the National Institute on Aging, Baltimore, Md. Dr. Roth received his B.S. in Biology from Villanova University in 1968 and his Ph.D. in Microbiology from Temple University School of Medicine in 1971. He received postdoctoral training in Biochemistry at the Fels Research Institute in Philadelphia, Pa. Since coming to NIA in 1972, Dr. Roth has also been affiliated with the Graduate Schools of Georgetown University and George Washington University, Washington, D.C.

He is an officer of the Gerontological Society of America, a co-editor of the CRC series, *Methods of Aging Research*, an associate editor of *Neurobiology of Aging*, and a referee for numerous other journals. Dr. Roth has published extensively in the area of hormone action and aging and has lectured throughout the world on this subject.

THE EDITOR

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Dr. Johnson received a B.S. in psychology and a B.S. in zoology from the University of Washington-Seattle in 1968. He attended Tulane University, New Orleans, where he received his M.S. in psychophysiology in 1970 and a Ph.D. in neuroscience (anatomy and electron microscopy) in 1973. His Ph.D. thesis dealt with the effects of brain trauma at the electronmicroscopic level; specifically, how the nerve tissue responded and repaired itself.

Dr. Johnson continued his studies (1973-1977) on a postdoctoral fellowship at NASA Ames Research Center researching the effects of altered gravitational force and radiation on the brain. During this time, he received training in scanning electron microscopy in Dr. Thomas Hayes' laboratory at the University of California, Berkeley, freeze fracture electron microscopy in the Anatomy Department at the same university, and pathology at Stanford University Medical School, Palo Alto. In 1977 he went to the National Institute on Aging, National Institutes of Health, Baltimore, where he redesigned the electronmicroscopy laboratory and researched the effects of nutrition and hormone therapy on aging. For two summers he was a visiting faculty member at Berkeley where he taught neuroanatomy to students in the School of Medicine.

Dr. Johnson has published over 50 works in his field and, in addition to *Current Trends in Morphological Techniques*, has another book in press, *Aging and Cell Structure*, Plenum Press, New York. He is on the editorial board of *Experimental Aging Research*, *AGE*, and *Neurobiology of Aging*.

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Dedicated to JEJ, MEJ, SEJ, CBJ, HMS, ROE, JFE, JM, RB.

CURRENT TRENDS IN MORPHOLOGICAL TECHNIQUES

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Silver Degeneration Methods
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Chapter 1

THE TECHNIQUE OF WHOLE BODY AUTORADIOGRAPHY

Irwin Fand and William P. McNally

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I. INTRODUCTION

A. Characteristics and Advantages of Whole Body Autoradiography

The pioneer of whole body autoradiography (WBAR) is Dr. Sven Ullberg, Department of Toxicology, University of Uppsala, Sweden, and his co-workers who perfected the methodology and delineated the useful applications of these procedures for use in biologic research. Historically, WBAR was employed first by Ullberg in 1954[1] for localization studies of antibiotics in mice. The method has been subsequently refined by Ullberg et al.[2-8] as an effective tool for examining the distribution of many radioactively labeled drugs, vitamins, and hormones. These carefully worked out methods, reviewed by others,[8-10] have served as the standard for the majority of ensuing studies.

The growing interest in WBAR as a fundamental tool useful for toxicology and drug screening has created a need for a comprehensive description of the basic technique. Accordingly, the primary goal of this article is to provide a working technical guide for the preparation and analysis of whole body autoradiograms. Additional detailed descriptions of the WBAR method may be consulted.[8-10]

When comparing WBAR with alternative methods such as pulse counting from fragments of dissected tissue the following special advantages may be cited:

1. In WBAR, the speed of freezing small experimental animals such as mice permits the nearly instantaneous whole body autoradiographic localization of radionuclides, with an interval as brief as 1 min post i.v. injection.
2. Frozen sections prepared by WBAR procedures help reduce artifacts in the completed autoradiograms by avoiding all contact between tissues and customary histological fluids such as fixatives, dehydrating agents or decalcifying chemicals.
3. WBAR allows direct comparisons between localization of radioisotopes and underlying structures examined simultaneously over the entire animal.
4. WBAR is particularly valuable when tracing the precise disposition of substances in the body whose metabolic fate cannot be accurately predicted.
5. Using tracer amounts of radionuclides, WBAR makes available both qualitative and quantitative information on rates of compound absorption, metabolic transformation and patterns of excretion as a function of time, dose, and route(s) of administration.
6. Unthawed WBAR tissue sections cut from solid, frozen experimental animals minimize compound diffusion from sites of localization, eliminates post-mortem alterations and lessens the risk of tissue contamination.
7. WBAR is a valuable tool when it is desirable to investigate distribution patterns of radioactivity in pregnant animals, comparing maternal tissues to fetal structures such as placenta, yolk sac, and amniotic fluid.
8. Radionuclide distribution can be determined from whole body autoradiograms in tissues and organs not easily accessible by routine dissection, such as ear tissues, ganglia, pituitary, and adrenal medulla.
9. Employing WBAR, radioactivity content may be determined in body cavities, organ ducts, blood, lymphatics, gastrointestinal lumina, and tissue fluids for direct comparisons with radioactivity in adjacent organs and tissues.

II. DETAILS OF THE WHOLE BODY AUTORADIOGRAPHIC METHOD

A. Isotopes and Labeled Compounds

The principle isotopes used in WBAR are ^{14}C, ^{3}H, ^{125}I, and ^{35}S which exploit the property of β particle emission from the source to make visible silver grains in devel-

Table 1
PHYSICAL CHARACTERISTICS OF SOME RADIOISOTOPES COMMONLY USED IN WBAR[11,33]

Radioisotope	Half-life	Maximum β-particle energy (keV)	Average β-particle energy (MeV)
^{3}H	12.35 years	18.6	0.005
^{14}C	57.30 years	156	0.05
^{35}S	87.4 days	167	0.055
^{45}Ca	180 days	254	0.10
^{59}Fe	47 days	460	0.12
^{131}I	8 days	690	0.205
^{32}P	145 days	1710	0.695

oped photographic emulsions. Additional radionuclides finding use include: ^{131}I, ^{45}Ca, ^{32}P, ^{22}Na, ^{59}Fe, ^{57}Co, ^{58}Co, ^{60}Co, ^{203}Hg, ^{109}Cd, ^{18}F, ^{82}Br, ^{80m}Br, ^{63}Ni, ^{203}Pb, ^{137}Cs, ^{75}Se, ^{65}Zn, $^{51}Cr_2$, ^{204}Tl, ^{198}Au, ^{48}V, ^{89}Sr, ^{90}Sr, ^{133}Ba, and ^{238}Pu.[8] In making a choice of radioisotope, attention should be given to the characteristics of radiation energy. An advantage with ^{3}H compounds is their high resolving power based on the short track range of beta particles in the section and photographic emulsion, lessening the scatter of silver grains around the source in the completed autoradiogram. Moreover, ^{3}H-labeled organic compounds of biologic interest are readily available with high specific activities and are relatively economical. A major disadvantage, however, arises in using ^{3}H with respect to doses 50 to 100 times larger than ^{14}C compounds which are required to produce satisfactory autoradiograms with sections of 20 μm dimension applied to standard industrial type X-ray films. This condition is explained by the β emission energy property of ^{3}H determined at 18.6 keV (mean particle track length about 2.5 μm) compared to 156 keV for ^{14}C (mean particle track length about 100 μm).[11] The extremely low efficiency for tritium can be accounted for by the relatively short range of βemission, possible air gaps developing between section and emulsion, and the obstruction offered by the 0.5 to 1.0 μm thick gelatin antiabrasion coating deposited on X-ray films. A newly developed and relatively costly ^{3}H-sensitive film has been made commercially available which lacks an antiabrasion coat and increases the efficiency of ^{3}H registration, mainly overcoming the disadvantages of using ^{3}H for macroautoradiography.[12] For general WBAR applications, however, the high efficiency and low dose requirements weigh in favor of use of ^{14}C-labeled substances. A suitable replacement for ^{14}C is ^{35}S whose E_{max} of β emission = 167 keV is nearly identical to the ^{14}C value (see Table 1). The short half-life of ^{35}S (87.4 days) is a factor, however, which may limit its use under certain conditions. In general terms, factors to consider in establishing the optimal dose of labeled substance under investigation include: chemical nature of the nuclide, specific activity, relative compound toxicity, absorption efficiency, route of administration, and properties of the film emulsion. There is a distinct advantage to include, where possible, two differently labeled nuclides of the same substance, enabling the evaluation of position in the molecule of the radioactive atom(s) as it may be affected by metabolic cleavage of the molecule in the organism. A useful working reference standard in our laboratory is the administration of 0.20 to 0.25 μCi/g of ^{14}C-compounds when routine 20 to 30 μm sections are exposed against industrial-type X-ray films for a period of 2 to 3 weeks. Under similar experimental conditions the required dose of ^{35}S is 0.20 to 0.50 μCi/g body weight.

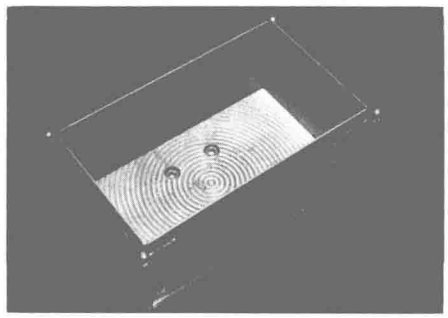

FIGURE 1. Freeze mounting frame assembled.

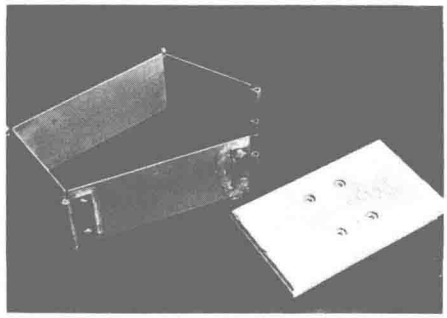

FIGURE 2. Components of mounting frame.

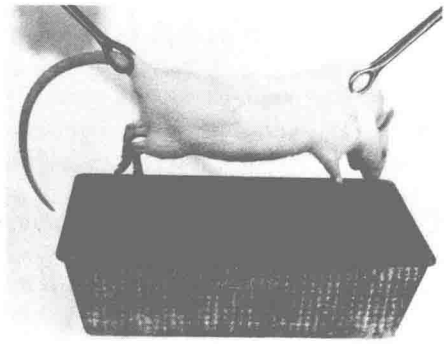

FIGURE 3. Lightly anesthetized rat prior to immersion in freezing mixture (liquid N).

B. Routes of Administration for Labeled Compounds

Depending on the metabolism and position of the label within the compound being studied radioisotopes may be introduced orally, subcutaneously, topical cutaneously, intramuscularly, or by intraperitoneal or intravenous injection.

Administration of label by the intravenous route leads to rapid body localization of radioisotopes and makes possible instantaneous uptake studies. Concentration gradients from depot sites are not present with i.v. injection, a condition which increases the accuracy of quantitation of radioactivity in autoradiograms.

A detailed procedure for i.v. injection of rodents has been presented by Ullberg.[8] The basic steps may be summarized as follows:

1. Place mouse or rat in a clear plastic cage with a hole provided for the tail, allowing visualization of the animal.
2. Cage dimensions are made sufficiently large to permit the animal some degree of body motion during injection, thereby avoiding undue stress associated with body restraint.
3. An injection needle, mounted on a disposable tuberculin syringe, is divided in two segments near the cone and the resulting parts of the needle are then connected by a length of clear polyethylene tubing.
4. The needle tip is inserted in the tail vein and a volume of about 0.2 mℓ fluid, for an adult mouse, is injected under observation in the polyethylene tubing.
5. Nonwater-soluble compounds may be injected as a suspension using phospholipid as emulsifier[13] or in solution with small amounts of dimethyl sulfoxide (0.02 mℓ for an adult mouse).

For administration of labeled substances by the oral route a small flexible tube attached to syringe may be employed with animals rendered quiescent by light anesthesia to reduce compound loss by sudden body movements. In long term experiments it is advisable to maintain animals in screen-bottomed cages which allow feces and urine to drop through, preventing coprophagy. Reingestion of radioactive materials in waste may lead to inaccurate interpretations of completed autoradiograms.

C. Freezing and Mounting of Specimens

The routine procedures are as follows:

1. Animals are customarily anesthetized with chloroform or ether, although our experience has shown that ethrane (Enflurane®) may be superior to both of these agents for the reasons that animals appear to be less traumatized with Enflurane® and explosive urination does not occur with this agent.
2. To freeze animals, a suitable microtome stage (4 cm × 12 cm for mice; 7.5 cm × 15 cm for rats) is surrounded by a rustproof metal frame with removable sides; the surface of the mounting stage is provided with elevated ridges or prongs in order to firmly grip the bottom surface of the frozen blocked animal (see Figures 1 and 2).
3. To allow for some flexibility with large or oddly shaped animals, cardboard walls taped to the bottom of object holders form convenient boats for carboxymethyl cellulose (CMC) gel mounting.
4. A stock solution of 50 g CMC per liter of cold water is prepared, kept at 4°C overnight to facilitate solution, and is stirred gently before use to insure an homogeneous solution.
5. The lower portion of the metal or cardboard enclosed stage is filled with the cold viscous CMC mixture, the animal is positioned as desired and the preparation is lowered into the freezing mixture long enough to permit substantial solidification to occur (see Figures 3 to 6).
6. Additional CMC gel is poured in until the animal is covered and a glass rod is used to dislodge bubbles (see Figure 7).
7. The entire unit comprising animal in CMC gel enclosed by retaining frame is then slowly lowered with tongs (to prevent cracking open of specimens) into a choice of several freezing mixtures: aromatic hydrocarbon-free hexane or acetone and dry ice, isopentane cooled with liquid nitrogen or liquid nitrogen alone.

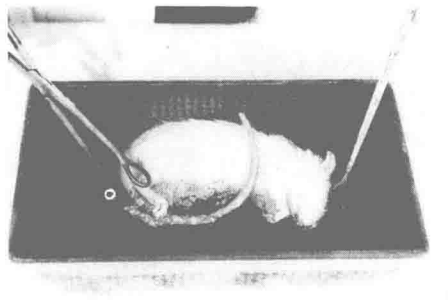

FIGURE 4. The animal remains in the freezing mixture until completely frozen, usually 3 to 5 min for a 100 g rat.

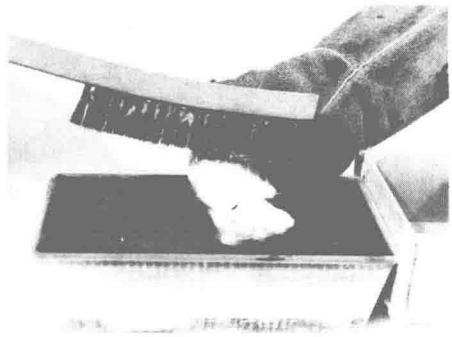

FIGURE 5. To facilitate the firmness of mounting in CMC gel, animals may have hair removed at liquid N temperature with a stiff wire brush.

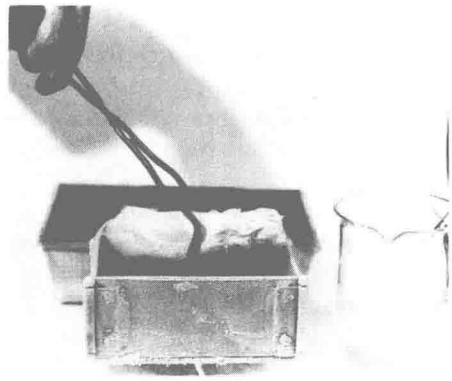

FIGURE 6. In the initial mounting step, a shallow layer of CMC gel is poured into the precooled frame and the frozen animal is held in the desired position until the gel freezes.