ANDREW W. ROGERS

TECHNIQUES OF AUTORADIOGRAPHY

Second revised and enlarged edition

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by

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ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM/LONDON/NEW YORK/1973

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
335 Jan van Galenstraat
P.O. Box 1270, Amsterdam, The Netherlands

AMERICAN ELSEVIER PUBLISHING COMPANY, INC.
53 Vanderbilt Avenue
New York, New York 10017

SECOND EDITION 1973

Library of Congress Card Number: 72–87964

ISBN 0-444–41057–0

With 92 illustrations

Printed in The Netherlands

Preface to the Second Edition

Autoradiography has progressed considerably since the first manuscript of this book was written. In fact I have been embarrassed to find the first edition deficient or misleading in many places and downright wrong in several. I have therefore largely rewritten the book, encouraged by those who felt the first edition was useful, and trying to benefit from the criticisms of those who did not.

The book is now in three sections. The first deals with the principles underlying the method; the second with the collection and interpretation of data from autoradiographic experiments; the third with the techniques of preparing autoradiographs. The major changes will be found in the second section, since the questions now being asked of autoradiography have become considerably more sophisticated in recent years.

Each chapter is intended to stand on its own. This has inevitably meant a certain amount of repetition, but I feel this is perhaps better than assuming that every reader will read the whole book from beginning to end.

It is a great pleasure to acknowledge the collaboration of Dr. **J.M. England** in producing a new section on the statistical analysis of autoradiographic data. In the section on electron microscope autoradiography, I have relied heavily on the advice of Dr. Miriam Salpeter, in whose laboratory at Cornell University this manuscript was started, while I was a guest on sabbatical leave there, and of Dr. M.A. Williams, of Sheffield. The section on diffusible materials has benefited greatly from discussions with Dr. W.E. Stumpf and Dr. W.B. Kinter. To them and to many others who have made available to me their data for building up this account of the present state of the art of autoradiography, I wish to record my thanks.

I am grateful to the following authors for permission to reproduce material which was used in the first edition: to Dr. H. Levi for Figs. 10, 11, 12, 13 and 85 from work carried out together; to Dr. M.M. Salpeter for kindly providing Figs. 2, 86, and 91, and for permission to reproduce Figs. 23, 25, 87, 88 and 90; to Dr. L.G. Caro for Figs. 3 and 22; to Dr. Z. Darzynkiewicz for Fig. 37; to Dr. R. Ross for Fig. 39; to Dr. L. Lajtha for Fig. 81; to Dr. S. Ullberg for Fig.

77; to Dr. L. Schwartz for Fig. 78. The range-energy data in the Appendix is based on material published by Dr. P. Demers. The table of data on radioisotopes is taken from data published by the United Kingdom Atomic Energy Authority.

My thanks are due to the Editors of the following journals for permission to reproduce material already published by them, which had been used in the first edition: *Proceedings of the Royal Danish Academy of Sciences*,, Figs. 10, 11, 12, 13 and 85; the *Journal of Cell Biology*, Figs. 3, 22 and 88; *Laboratory Investigation*, Figs. 23, 25, 87 and 90; the *Journal of Anatomy*, Fig. 52; *Experimental Cell Research*, Fig. 56; *Radiation Research*, Fig. 29; the *Journal of Histochemistry and Cytochemistry*, Fig. 39: and *Leitz Mitteilungen*, Figs. 47, 49 and 51.

Of the new material in this second edition, I wish to express my thanks to Dr. M.M. Salpeter for permission to use Figs. 17, 18, 19, 20, 24, 63, 64, 67, 74, 74 and 75; to Dr. S. Bleecken for Fig. 21; to Dr. W.B. Kinter for Figs. 40 and 41; to Dr. J.M. England for Fig. 70 and new tables in the Appendix; and to the Research Laboratory of Ilford, Ltd., for kindly providing Fig. 1.

My thanks are due to the editors of the following journals for permission to use material already published by them: *Philosophical Transactions of the Royal Society*, Figs. 17, 18, 19 and 68; *Journal of Microscopy*, Figs. 54, 55, 57, 58 and 59; *Journal of Cell Biology*, Figs. 24, 73 and 74; *Journal of Clinicial Investigation*, Figs. 40 and 41; *Zeitschrift für Allgemeine Mikrobiologie*, Fig. 21; *Journal of Anatomy*, Fig. 43; *Journal of Endocrinology*, Fig. 44, and *Academic Press*, Fig. 30.

I am most grateful to Mrs. Betty Hammond for the care with which she has typed the mauscript. Miss Christine Court has once again provided several excellent diagrams, while Miss Tania Williams and Miss Barbara Liddiard have helped in many ways with the preparation of material for this edition. My wife has not only shown great tolerance during the gestation of the book, but has carried out an extensive literature search.

Finally, my thanks are due to the Medical Research Council, to the late Prof. G.W. Harris, F.R.S., and to Dr. A.G.M. Weddell for their interest and support while working here in Oxford.

Oxford, April 1972 ANDREW W. ROGERS

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CONTENTS

PART 1: THE THEORETICAL BASES OF AUTORADIOGRAPHY

CHAPTER 1

The Uses of Autoradiography

HISTORICAL INTRODUCTION

The first undoubted autoradiograph was obtained almost exactly 100 years ago. In 1867, Niepce de St. Victor[1] published an account of the blackening produced on emulsions of silver chloride and iodide by uranium nitrate and tartrate. It is curious that the blackening of photographic emulsions by radioactive material should have been observed in this way so long before the realisation that radioactive phenomena even existed. Niepce found this blackening to occur, even when the uranium salt was separated from the emulsion by sheets of glass of different colours. He interpreted his results in terms of luminescence.

In 1896, Henri Becquerel[2] repeated and extended Niepce's observations, again in the belief that he was investigating mechanisms of fluorescence. He used crystals of uranyl sulphate, and showed that, after exposing them to sunlight, they were able to blacken a photographic plate through two layers of black paper. On one occasion, it seems that the sun did not shine for several days, and the uranyl sulphate remained in a closed drawer together with the photographic plate. This plate was also found to be blackened. Through this experiment, and the work of the Curies in 1898, radioactivity was first demonstrated. So autoradiography is in fact older than the knowledge of radioactivity itself, and contributed directly to its discovery.

After these first, almost accidental, autoradiographs of crystals of uranium salts, autoradiography remained a curious observation rather than a scientific technique for a quarter of a century. Not until 1924 did Lacassagne and his collaborators begin to use this response of photographic emulsions to ionising radiations in order to study the distribution of polonium in biological specimens[3,4]. Their work, which followed sporadic experiments by other investigators, was the first systematic and successful attempt to exploit the phenomenon observed by Becquerel as a means of observing the sites of localisation of radioactivity within biological specimens.

References p. 8

The development of autoradiography as a biological technique progressed very little from Lacassagne's work until after the 1939-45 war. Physicists were using photographic methods of recording and studying radioactive phenomena, but the application of similar techniques to biological material was limited by two factors. The first, and most important, was that the few naturally occurring radioactive substances were of very little biological interest. In the second place, autoradiography was dependent on emulsions prepared for photographic purposes: the few autoradiographs that were made involved pressing the specimen against a photographic plate. The first fifty years of autoradiography saw very little accomplished, apart from the study, on a macroscopic scale, of the distribution of various salts of radium, thorium, or uranium in a few plants and animals[5].

The revolutionary advances in physics during and after the Second World War brought a new impetus to autoradiography. The study of cosmic rays and of the particles which could increasingly be generated in the laboratory created the demand which led to the production of nuclear emulsions – photographic emulsions with specialised characteristics, which recorded the tracks of charged particles with greater precision and sensitivity. From the work of such men as C.F. Powell[6,7], a wealth of new information became available, both on the techniques of handling this new recording medium, and on the interpretation and analysis of the observed particle tracks. Several fundamental particles were first described on the basis of their tracks in nuclear emulsions.

Controlled nuclear fission brought a further impetus to autoradiography. The advent of the atomic bomb made it vitally important to know the distribution in plants and animals of the fission products of radioactive fallout. At the same time, new radioactive isotopes became available, opening up new possibilities in the investigation of biological systems. It is not surprising that the physicists and biologists working in these new fields should have adopted the techniques and emulsions of the particle physicists.

In 1940, Hamilton, Soley and Eichorn[8] demonstrated the uptake of radioactive iodine by the thyroid gland, and Leblond[9] soon afterwards prepared autoradiographs showing its distribution in the gland. These were still made with the old technique of placing the sectioned specimen in direct contact with a lantern plate. By 1946, Bélanger and Leblond[10] had evolved a technique with liquid emulsion that gave considerably better resolution. The molten emulsion was removed from the lantern slides, and painted on the specimens with a fine paintbrush. It was not long before Arnold[11], who was studying the retention of long-lived isotopes in the body, adapted this technique for use with nuclear emulsions. The following year (1955), Joftes and Warren[12] described dipping

slides in molten nuclear emulsion, a technique which has been widely used, and is the basis for present-day liquid emulsion methods.

During the same decade, a parallel group of techniques was emerging. The lantern slide provided the starting point once again, and several authors [13, 14] attempted to improve the contact between emulsion and specimen by stripping the emulsion off its glass support and applying it directly to the specimen. As was the case with liquid emulsions, a new technique employing a nuclear emulsion soon made its appearance. At the suggestion of S.R. Pelc, Kodak Ltd. began the manufacture of a special autoradiographic stripping film[15, 16]. Since the publication of these two papers, the stripping film technique, which brought great advances in resolution and in reproducibility over any of the techniques that had been tried up to that time, has probably yielded more autoradiographic information than any other single method.

Both the liquid emulsion and the stripping film techniques produce emulsion layers a few microns thick over the surface of the specimen. Charged particles coming from the specimen only leave one or two silver grains to show their passage in this type of preparation. A few autoradiographers, however, saw possibilities in the more direct application to biology of the physicists' techniques of recording particle tracks. This approach is direct and simple in the case of α particles, which leave a very characteristic track that is easy to record and recognise. β Particles are not so amenable to track methods, but, thanks to the pioneering work of physicists such as Hilde Levi[17–19] and C. Levinthal[20], β-track autoradiography has developed into a technique of great quantitative precision.

The last decade has seen a rapid transformation of cellular biology due to the development of the electron microscope. It was inevitable that attempts should be made to link the techniques of autoradiography to this new method of observing biological material. The first, and rather unpromising, autoradiographs viewed in the electron microscope were published in 1956 by Liquier-Milward[21]. Since then, new techniques have been proposed, and new nuclear emulsions produced, to meet the requirements of this approach for extremely high resolution. It is now possible to resolve the site of incorporation of radioactive material to within 500–700 Å in favourable circumstances, and further improvements are certain to come.

Radioactivity is no longer the property of a few rare elements of only minor biological interest. An increasingly wide range of compounds is now available labelled with a radioactive isotope, opening up new possibilities in the study of living systems. In consequence, the blackening of an emulsion of silver halides by uranium salts observed by Niepce a century ago has evolved into a wide

References p. 8

spectrum of techniques for recording and measuring radioactivity in biological material.

RADIOACTIVE ISOTOPES

What place do radioactive isotopes have in the study of living systems?

The majority of techniques available to the biologist are basically analytical. In other words, by their application a mixture of individuals (which may be molecules or cells or animals) can be separated into groups on the basis of some common similarity between the members of each group. The techniques of biochemical analysis, such as chromatography, for instance, can give detailed and quantitative information on the molecules out of which cells and cell products are made. The techniques of histology and histochemistry provide an analysis of the cells and tissues of the body on the basis of their appearance and chemical constitution.

In living systems molecules and cells, and even whole organisms, undergo rapid and often surprising transformations. An aminoacid may be synthesised into a protein, which is subsequently degraded, yielding the original aminoacid again. The large, multinucleate megakaryocyte forms the small blood platelets. By their very nature, analytical procedures are cumbersome and unreliable for the study of these transformations. The relative sizes of the aminoacid and protein compartments of a cell are a poor measure of the rate of transformation of the one into the other.

If, however, aminoacid molecules labelled with a radioactive isotope can be introduced into such a system, and their recognition combined with subsequent analysis, the synthetic pathways by which they are incorporated into specific proteins may be studied, and the rates of these transformations measured with considerable precision.

This is the basic pattern of the tracer experiment. Whatever the material under examination, the pattern is the same. A population that is heterogeneous is separated into homogeneous groups by an analytical technique after the addition to it of labelled members of one group. The possible transformations that may occur between that group and the others are then determined by looking for the distribution of radioactivity in the analysed population.

The chief value of radioactive isotopes in biological research has been to provide precisely this dynamic information to supplement the analytical techniques as they have been applied at every level from the molecular upwards. In every field of biology, the combination of radioisotope techniques with the analytical methods available has added another dimension to the observations

that can be made. It is difficult to see how the work of the past 20 years on oxidative respiration, photosynthesis, or the control of protein synthesis by the nucleic acids, to quote only these examples, could have been carried out without the advances in nuclear physics that made radioactive isotopes so freely available.

In addition to this use of isotopes in the tracer experiment, there have evolved a number of techniques in which radioactivity has been exploited in a purely analytical way. The precision with which relatively small numbers of labelled atoms may be detected and measured has led to methods of analysis more sensitive than those otherwise available. In radioactivation analysis, for example, a method has developed for measuring the yield of certain elements in biological specimens at a sensitivity which is often far higher than that available with any other existing technique. The principle involved is neutron irradiation of the specimen in order to induce radioactivity in the element under study. The characteristic radiation from this activity is then detected and measured[22] .

Another example of the use of radioactive isotopes as the basis for an analytical technique comes from histochemistry. In 1961, Ostrowski and Barnard[23] suggested the use of isotopically labelled enzyme inhibitors as histochemical reagents. Following their application to the tissue under study, the distribution of radioactivity could be observed by autoradiography. From this pattern, the distribution of the enzyme to which the inhibitor was bound could be inferred, and measurements of the radioactivity present in a particular cell or structure could be used to estimate the number of molecules of enzyme present there. Reference will be made to this interesting approach later, in the chapters dealing with quantitative measurements by means of nuclear emulsions, and to some of the results that have been obtained through its application.

Apart from tracer experiments and the analytical techniques based on radioactive isotopes, the third main group of experiments that involve the use of isotopes comes under the heading of radiobiology. Studies on the distribution and retention within the body of ingested radioisotopes and on the effects of radiation on the surrounding cells and tissues combine elements of the tracer experiment with the analytical approach.

These are the three principal ways in which radioactive isotopes are used in studying living systems. The techniques available for recording and measuring radioisotopes will next be considered, to try and pinpoint the characteristics of nuclear emulsions which make them suitable for particular experiments, and to relate these features to the other methods of detecting radiation.

References p. 8

AUTORADIOGRAPHY IN RELATION TO OTHER TECHNIQUES OF DETECTING RADIOISOTOPES

The methods available for the detection and measurement of radioactivity can be classified under three headings.

The first of these is the group of electrical methods that depends on the production of ion pairs by the emitted radiation. The geiger tube, the ionisation chamber, and the gas-flow counter are all examples of this approach, in which the ionisation caused by the passage of a particle or γ ray through the sensitive volume of the counter is recorded as an electrical pulse, which can be then amplified and registered.

The second group relies on the property, possessed by a number of materials, of absorbing energy from the incident radiation, and re-emitting this in the form of visible light. In a scintillation counter, these minute flashes of light are detected and converted into electrical pulses by a photomultiplier tube, and may then be amplified and registered in the same way as in the ionisation detectors.

These two groups of techniques have much in common. A β particle entering the sensitive volume of the counter produces a transient effect which is converted into an electrical pulse. These pulses can be handled by data processing systems rapidly and reliably. The pulse counting techniques, whether based on ionisation or scintillation, can provide accurate measurements of the radioactivity in a source, but each measurement is a sum of the radiation entering the sensitive volume of the counter. Variations in radioactivity from one part of the sample to another are not detected.

Autoradiography differs from the pulse counting techniques in several important respects. Each crystal of silver halide in the photographic emulsion is an independent detector, insulated from the rest of the emulsion by its capsule of gelatin. Each crystal can respond to the passage through it of a charged particle, with the formation of a latent image that persists throughout the counting or exposure period, and is made permanent by the process of development. The record provided by the nuclear emulsion is cumulative, and spatially accurate.

By responding in this strictly localised fashion to incident charged particles, a nuclear emulsion is ideally suited to studies of the distribution of radioactivity within a sample, a function that the pulse counters cannot perform. But while the emulsion can and does respond in a quantitative fashion to radiation, it is often a slow and difficult process measuring the overall activity of a sample in this way, by comparison to the speed and simplicity of the pulse counters.

There is thus little point in autoradiographing a specimen that is homogeneous. But where the specimen is made up of different components the measurement of the radioactivity present in bulk samples by pulse counting techniques only gives a mean value for the whole specimen. An extreme case of heterogeneity within the specimen is provided by animal or plant tissues. Pulse counting from a gram of homogenised liver gives a rapid and accurate assessment of the total radioactivity present, but no evidence on whether it is intra- or extracellular, in parenchymal cells or other cell types, nuclear or cytoplasmic, and so on. The earliest experiments in autoradiography were concerned solely with the localisation of radioactivity within a specimen, and this probably remains the most frequent goal of biologists using nuclear emulsions.

The strict localisation of the response of a nuclear emulsion to those grains through which an incident particle passes, means that it is possible to study sources of very small size within a larger specimen. It is possible to observe the nucleus of a single cell, and determine whether or not it is labelled, or an individual chromosome in a squash preparation of a dividing cell.

It may be impossible to isolate sources as small as these from the tissue to present them to a pulse counting system. Even if microdissection is possible, the levels of radioactivity in such minute specimens are usually too low for detection against the background of the pulse counter. In such cases, there is no alternative to using the nuclear emulsion itself as a measuring instrument.

Nuclear emulsions have a very high efficiency for β particles, particularly those with low energies. Fortunately, many of the elements of interest to the biologist have suitable isotopes – tritium, carbon-14, sulphur-35 and iodine-125 for example. If the volume of emulsion to be examined is restricted to the immediate vicinity of the source, the effective volume of detector may be as little as 100 cubic microns. Reducing the detector volume also reduces the probability of observing a background event, due to cosmic rays, for instance. It may be weeks or months before background in such small volumes of emulsion builds up to restrictive levels. It is possible, therefore, to combine a high efficiency for low energy β particles with very long counting times. With suitable techniques, sources the size of a single cell or smaller can be accurately measured at decay rates as low as 1 disintegration per day. By contrast, most commercially available pulse counters have backgrounds of 10–20 counts per minute.

In summary, then, autoradiography supplements the data provided by pulse counting techniques when the specimen is relatively large, indicating the distribution of radioactivity between the various parts of the specimen. With sources of cellular dimensions, pulse counting is often impossible, and measurements of radioactivity may have to be made by autoradiography.

References p. 8