Practical autoradiography

Review 20

The Radiochemical Centre
Amersham England

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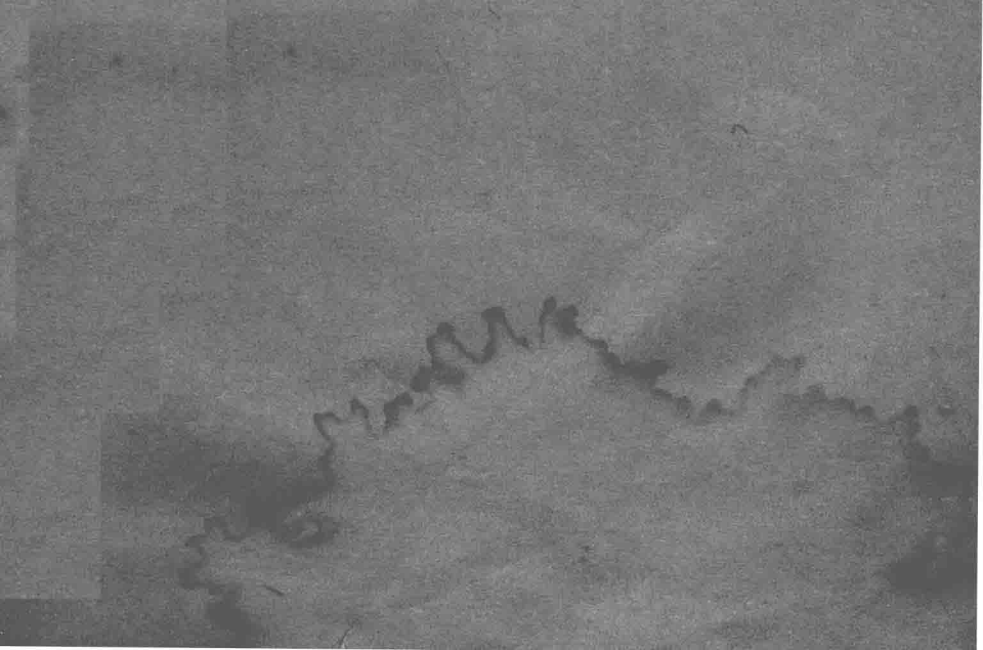

Practical autoradiography

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Introduction

A radiograph is a picture or pattern produced by radiation other than visible light. As in a photograph, a source of radiation, an object to be imaged and a recording medium are required.
In an autoradiograph, by contrast, the specimen is itself the source of the radiation.
Photographic emulsion is by far the most commonly employed recording medium in autoradiography. It is possible to use thin metal foils and layers of plastics as detectors for alpha particles and for even heavier fission products[1], and the patterns produced in these detectors are just as much autoradiographs as those recorded in photographic emulsions. In this review we shall only consider photographic detectors, which remain the most effective means of imaging the beta particles and extra-nuclear electrons which are given off by the radioactive isotopes of primary interest to the biologist.
Autoradiography is an old technique, preceding and contributing to the discovery of radioactivity. In 1867, Niepce de St. Victor published the observation that uranium salts could blacken emulsions of silver chloride and iodide. But it was Henri Becquerel, in 1896, who found that this effect of uranium salts on photographic emulsions could take place in the dark, without prior exposure to sunlight. His work, together with that of the Curies published in 1898, led to the recognition of radioactivity.
Naturally occurring radionuclides are, in general, of high atomic number, and of little interest to the biologist. Autoradiography remained a curiosity rather than a technique until the great strides in nuclear physics of the 1940's resulted in the increasing availability of artificially produced radioisotopes of elements such as hydrogen, sulphur, carbon and phosphorus. Autoradiography as we know it really dates from Leblond's demonstration of the distribution of radioactive iodine in sections of the thyroid gland in the early 1940's[2]. Since then, it has grown and diversified into a spectrum of techniques of wide application.
Why has this rather simple idea of putting a radioactive specimen in contact with a photographic emulsion developed into such a widely used group of techniques? The answer lies in the properties of the photographic emulsion, and these make it different in many important respects from the other methods available for detecting and measuring radioactvity. The emulsion consists of a large number of silver halide crystals, suspended in a solid phase which is usually gelatin. Each crystal within the emulsion is a separate detector for nuclear particles, isolated from its neighbours by the surrounding gelatin. Geiger counters and scintillation counters, which measure with great accuracy the number of nuclear particles entering the sensitive volume of the

counter, give no information on the distribution of these particles in space. The photographic emulsion, on the other hand, gives a permanent record of the tracks of these particles, allowing the patterns made by them in the emulsion to be related to the structural patterns within the specimen.

Autoradiography is useful not only to biologists. Any source which is heterogeneous in structure, and is also radioactive, can be studied. The distribution of radioactivity in rocks, in fibres and metals, even in biscuits and cakes, has been studied using autoradiography. But it is in the study of living systems, with their elaborate and beautiful microstructure, that autoradiography has made its greatest contribution. It puts biochemistry in its place, allowing one to observe reactive sites and processes against the background of the tissues, cells and organelles in which they are taking place.

How to use autoradiography

Is autoradiography the technique to use?

Genius has been defined as an infinite capacity for taking pains, a totally unacceptable definition, since it ignores the most crucial decisions of all—which pains are the ones most worth taking? There are no prizes for exhaustive and meticulous work if it could have been largely avoided by selecting a more appropriate technique. Unfortunately, we are all unduly influenced by the skills of those with whom we are in contact. It often seems easier to learn a method that is established in a nearby laboratory than to examine the range of methods available, and to select deliberately the one most closely matched to the problem being investigated. Taking over a method designed for a different problem frequently involves more work and less clear results in the long run.

The first choice in an experiment involving radionuclides is whether to use autoradiography at all. Pulse counters, in particular scintillation counters of various sorts, have reached such a degree of sophistication, are so easy and rapid to use and are so quantitatively reliable, that, if possible, they should always be the method of data collection. If, by some separation technique, the tissue element you are interested in can be isolated for pulse counting, that should be the method of choice, rather than autoradiography. An experiment into the time course of the uptake of a radioactive drug into one cell type may be performed and the results analysed in a day, given a method of collecting relatively pure samples of the cells concerned for scintillation counting. It might well take several weeks to get the same result by autoradiography and grain counting.

In many cases, of course, it will not be possible to isolate from the tissues the structures of interest, whether they be the cell nuclei in metaphase, or the nucleoli of the epithelial cells only, and it will be clear from the start that autoradiography is necessary. But pulse counting and autoradiography are seldom alternative techniques; they are complementary, and in almost any experiment with a complex tissue or organ, it is worth counting the radioactivity in samples of the whole tissue in a pulse counter before analysing the distribution of this radioactivity within its component structures.

The unique advantage of autoradiography is that it is spatially accurate. But this is true only up to a given point. The passage of nuclear particles through the emulsion is recorded, but there remains the problem of relating their pattern in the emulsion to the structure of the biological specimen. For any autoradiograph, there is a distribution of developed silver grains around a source of radioactivity in a specimen, and thus an uncertainty in identifying that source from the observed pattern of developed grains. This uncertainty becomes greater the higher the magnification used to

view the preparation. A scatter of grains of up to several microns from the source presents no problem in whole-body autoradiographs viewed directly: a scatter of up to 0·2μm creates great difficulties in the analysis of autoradiographs at the electron microscope level.
In general, the lower the magnification at which the autoradiograph is to be viewed, the simpler the techniques of preparation and analysis. For example, it makes no sense to prepare light microscope autoradiographs of the brains of rats and to count the silver grains under the oil immersion lens, if one wishes to compare the radioactivity in the supra-optic nucleus with that in the visual cortex: measurements of the blackening produced on X-ray film by structures of that size would be far quicker and just as reliable.
In many instances, the identification of which cell is radioactive, and whether the radioactivity is nuclear or cytoplasmic, can be carried out more quickly and with less effort using light microscope techniques than at the electron microscope level. There is no point in choosing a technique that gives better resolution than the project demands, unless you have time and energy to waste.
A summary of the autoradiographic techniques in use is given in Table 1. Details of the techniques and their quantitation will be given in the appropriate chapters.
There are sometimes choices to be made between autoradiography and other non-radioactive techniques, such as histochemistry or fluorescence microscopy. For instance, should one produce a coloured product at the site of a particular tissue component by histochemical means, or a radioactive one, to be visualized by autoradiography? Should one use a fluorescent label on the antibody, or a radioactive one? If localization is the aim of the experiment, autoradiography should in general be avoided. Histochemical and fluorescence techniques are usually simpler and more rapid, and have much more visual impact. Just occasionally, however, the quantitative possibilities of autoradiography make it preferable. One can, for instance, estimate the number of binding sites for a radioactive antibody on the surface of a cell autoradiographically, whereas this would be virtually impossible by fluorescence microscopy. With high specific activity substrates available the level of detection of reactive sites using autoradiography is usually considerably lower than is possible by labelling with coloured or fluorescent molecules.

Autoradiography and the preparation of the specimen

The main value of autoradiography is the correlation between the distribution of radioactivity and the structure within a specimen. It follows, therefore, that adequate histological demonstration of the structural patterns is essential. The first autoradiographs of biological specimens were produced by pressing the cut surface of a block of tissue embedded in paraffin wax against a photographic plate

Table I.
Summary of autoradiographic techniques

Level of magnification	*Technique*	*Emulsions*	*Quantitation*	*Diffusible material*
macroscopic	contact	X-ray film	densitometry or scintillation counting	possible with standard technique
light microscopic	grain density (i) dipping	liquid emulsions	^{3}H and ^{125}I: unreliable at higher energies	possible with modified technique
	(ii) stripping film	AR-10	possible for all nuclides	
	track techniques	liquid emulsions	^{14}C or higher energy emitting nuclides	
electron microscopic	dipping or loop	L4 or 129-01	possible, but time-consuming	not possible

For manufacturers of these emulsions see text

to demonstrate the distribution of injected polonium[3]. However, the routine histological techniques that have evolved for light and electron microscopy may not necessarily be directly applicable to autoradiography. Two main questions should be considered when designing an experiment:

i What effect do the histological methods have on the distribution of radioactivity in the specimen?

ii Do the histological methods affect the emulsion in any way?

The effect of histological methods on the distribution of radioactivity in the specimen

The standard histological techniques involve rendering the structural protein of the specimen insoluble by fixation, dehydrating the tissue in increasing concentrations of alcohol, and impregnating the tissue, either with paraffin wax or with plastic, to give a block from which thin sections can be cut successfully.

In some cases, this routine, which has evolved without any regard for the fate of any radioactive material that may be present in the specimen, gives a perfectly acceptable result. For example, if tritiated thymidine is injected into an animal to label newly synthesized DNA, the radioactivity in the tissue block may be in many forms, including tritiated water, labelled precursors of DNA and DNA itself. It so happens that almost any histological fixative will precipitate the DNA satisfactorily, and the subsequent washing and dehydration steps will remove the other labelled compounds. This leaves only the newly synthesized DNA as a source of radioactivity in the section. But it is clear that this good fortune is likely to be limited to relatively few experimental situations.

For example RNA is likely to be moderately well preserved by Carnoy's fixative, but by few others, and proteins only by formaldehyde or by Bouin's fixative[4]. In all these cases, the routine processing for light microscopy results in retention of the end product of biosynthesis with the removal of soluble precursors. At the electron microscope level, glutaraldehyde fixation and post-fixation with osmium tetroxide give good retention of DNA and protein, but there is evidence to suggest that considerable loss of RNA from the tissue results during washing, dehydration and embedding. (This is discussed in greater detail in "Techniques in Autoradiography" by A. W. Rogers, see General References, p.74).

In experiments where specific sites in tissue are labelled by reacting them *in vivo* with a radioactive inhibitor, drug or hormone, it may be necessary to carry out separate experiments to demonstrate that the radioactivity remains quantitatively in the tissue during histological processing. One instance where this is not the case is the binding of labelled steroids to their receptor proteins in target tissues, where the radioactivity does not survive routine processing[5]. Since dehydration in organic solvents is one of the steps in histological

processing, it is not surprising that the preservation of lipids for autoradiography has proved very difficult. A number of possible alternatives have been investigated.

Alternatively, you may wish to investigate the distribution of some highly diffusible drug or small molecule, or even of ions such as sodium or iodide. There is clearly no hope of finding these in tissue that has been through routine histological procedures. You will almost inevitably be forced to use one of the methods available based on frozen tissue.

It is possible to freeze small tissue fragments very rapidly, and to cut sections from the frozen block, placing them in contact with an emulsion layer at low temperature, and keeping the section firmly frozen until the end of autoradiographic exposure. Theoretically, there is then no chance for diffusible materials to move from their position *in vivo*. There are, however, difficulties with this approach. Firstly, ice crystals form within the tissue on freezing. Secondly, the optimum temperature of sectioning becomes lower the thinner the section required: thus at 15μm the optimum temperature is −15°C, at 3μm −30°C, at 1μm −50°C, and so on. And the thinner the section the more difficult it is to be certain that transient thawing does not take place during cutting. Finally, freeze-drying of the tissue and section inevitably occurs with the resulting concentration of the remaining solutes either on to adjacent membranes or perhaps into small aggregates, thus introducing uncertainty into the interpretation of the autoradiograph.

These difficulties are not significant in the autoradiography of diffusible substances at the macroscopic level. This is a relatively easy technique. At the light microscope level, it is a difficult and demanding technique, but quite possible. But at the electron microscope level, it is not yet possible, and represents a challenging and unclimbed Everest.

A number of acceptable techniques exist for autoradiography at the light microscope level, and these will be discussed in greater detail in the appropriate chapter. It is sufficient to note here that if the radioactive compound under study is not likely to survive routine histological processing, the onus will be on the experimenter to demonstrate that the technique selected retains the radioactivity quantitatively in the section. Whether you modify the fixing and embedding in some way or choose a completely "dry" frozen technique, the experiment will be more difficult and time consuming.

So far we have discussed the possibility of removal of radioactivity from the specimen by histological processing. Curiously enough, the reverse can also happen. Many histological fixatives act by cross-linking protein molecules: formaldehyde and glutaraldehyde are good examples of this. It is possible in some circumstances for labelled amino-acids to be attached to the denatured and precipitated protein by the fixative, so that they cannot be washed out from the tissue in

the later processing[6]. This gives spurious labelling, mimicking the synthesis of protein. In fact, this effect is only likely to be significant if concentrations of labelled amino-acid are rather high, such as in cells cultured in the presence of amino-acid or in animal tissues a very short time after injection of radioactivity. A similar retention of precursors of RNA to give spurious labelling can occur with osmium tetroxide[7].

2 *The effect of histological methods on the photographic emulsion*

Turning now to possible interactions between the histological and photographic processes: some fixatives, particularly those containing metals, and many stains will affect the emulsion directly. They may either cause the appearance of silver grains unrelated to radioactivity, or render the emulsion insensitive and unable to record the passage of beta particles. Lists of such interactions exist, but should not be regarded as absolute, since local variations in the composition of reagents are common. It is advisable to check that staining before contact with the emulsion does not fog or desensitize the emulsion. Should this happen, it may be necessary to change the routine and stain after autoradiography. Alternatively, it may be possible to apply a hydrophobic and inert layer between the stained section and the emulsion[8]. A layer of carbon evaporated on to the section can serve the same purpose in electron microscope work. Some stains when applied before the emulsion, are removed or altered by photographic development and fixing.

If sections are stained through the emulsion layer after photographic processing, there is the possibility, particularly with acid solutions, of removing the silver grains. A few stains are heavily taken up by the gelatin of the emulsion, while some produce a granular precipitate which may resemble silver grains. In general, however, staining after autoradiography is less likely to cause trouble than staining beforehand.

Autoradiography requires a high standard of specimen preparation. Cleanliness is essential if the emulsion is to be given a reasonable chance of functioning, and dirt and stain precipitates make the recognition of silver grains more difficult. Such things as variations in section thickness may matter critically if the radioactivity per unit is being measured. An autoradiograph is only as good as the section from which it is made.

The facilities and equipment needed

The darkroom

One seldom has the privilege of designing a darkroom for autoradiography from the drawing-board. If this opportunity occurs, Kopriwa's recommendations will be most useful[9]. In nearly every case, however, one is faced with adapting existing accommodation on a tiny or non-existent budget. The important points to work for are the following:

a *Size*

Unlike most photographic procedures, autoradiography requires considerable periods of working time to be spent in the darkroom. A blackened broom-cupboard will be so unpleasant that the quality of work will suffer. Space is needed for a refrigerator—preferably used only for autoradiography—a sink and bench space measuring at least $3m^2$.

b *Light trap*

Emulsions require periods of up to several hours to dry after preparation. It is intolerable to have to remain in the dark, doing nothing, for this period of time. The provision of double doors, with a space between them big enough for two people to stand in reasonable comfort, will enable you to come and go, carrying equipment or slides, without introducing light.

c *Real darkness*

Although nuclear emulsions are not very sensitive to light, they are often exposed on the bench in the darkroom for hours. Check that no stray light is entering when you are fully dark-adapted: this takes at least 20 minutes. Electrical switches and contacts can flash. Some fluorescent light tubes continue to emit a significant amount of light for minutes after switching off: these should be replaced. Signal lights on apparatus should be removed or covered. It must be possible to lock the door, and to indicate that the darkroom is in use. Light switches must be positioned well apart so that there is no possibility of confusing safelight with main light.

d *Cleanliness*

Autoradiographs usually demonstrate the tracks of single electrons through the emulsion layer in a quantitative manner. Observations of the developed silver grains using a light or electron microscope demand more care in preparation of the autoradiograph than in the production of routine photographs. In addition, as already mentioned, autoradiographic emulsions are exposed in the darkroom for long periods of time. The average photographic darkroom contains dried developer and fixer in the dust, and it is almost impossible to persuade photographers to adopt standards of cleanliness which are unnecessary in their own work. It is therefore much more satisfactory to have a darkroom specially for autoradiography than to prepare

autoradiographs in a photographic darkroom.

Having said all this, I should make it clear that if autoradiography is limited to placing macroscopic specimens, such as chromatograms or whole-body sections, against sheets of X-ray film, the requirements will be much less demanding, since this will not involve preparing and drying emulsion layers but simply handling a factory-made product.

Equipment and reagents

In general, equipment will be dealt with under the appropriate technique in later chapters.

It is useful to have a wall-mounted thermometer and hygrometer, since the conditions for drying emulsion layers are important, and temperature and humidity should always be recorded when preparing a batch of autoradiographs. Emulsions should never be in contact with anything except glass, some plastics or high grade stainless steel: metal ions, in particular copper, can produce remarkable artefacts at surprisingly low concentrations. Similarly, water used to dilute or wash emulsions at any stage before photographic fixing should always be distilled.

For light microscopy, any reasonable research microscope with transmitted light optics is adequate for viewing autoradiographs. It is worth noting the value of incident light darkfield methods: suitable apparatus is available from all microscope manufacturers. Vertical incident illumination can be made the basis of a semi-automatic system of grain counting which is neither very expensive nor difficult to operate[10], whereas the visual counting of silver grains is slow and not very accurate.

The choice of an emulsion of suitable sensitivity and crystal size will be dealt with in the next chapter. However there are probably several manufacturers who make a suitable product. If an emulsion has to be flown halfway round the world with all the delays that that can involve, there is a very variable period when the emulsion is out of your control, and a real probability of the batch being spoilt. Other things being equal, it is preferable to choose a manufacturer in the same country or continent.

Emulsions should be stored before use at between 0°C and +5°C. They should never be frozen. Obviously, emulsions should be protected from external radiation at all times. Check the position of any X-ray machines and whether there are any laboratories using gamma-emitting nuclides in the building.

The shelf-life of a nuclear emulsion is usually longer than that quoted by the manufacturer, particularly if the container remains unopened.

Safe lighting in the darkroom should be used as specified for the emulsion, remembering always that the recommended filter only determines the wavelength of the light. Intensity is a function both of the wattage of the bulb, and the inverse square law. Try to keep

work at least 1·5m from a 15 watt bulb.

The requirements for the histological preparation of the specimen are more exacting than for normal histology. The purity and cleanliness of the reagents used is important. High-grade reagents, stored in glass and used fresh each time are not a luxury but an insurance. One source of artefact that I had great difficulty in tracing was due to the bulk purchase by the histology laboratory of xylene in metal drums: changing to high-grade xylene in glass containers for the preparation of the sections removed the artefact completely.

Finally, I strongly recommend the serial numbering of all batches of autoradiographs, and recording the details of their preparation, drying, exposure and development in a book kept specially for this purpose. If difficulties arise from one batch of emulsion, or from a spell of weather that reduces the humidity in the darkroom to 15%, the record gives you a chance of identifying the factor concerned. Apart from its short-term value, the record will remind you of the details of preparation of the autoradiograph years later. After all, an autoradiograph is a permanent record of the distribution of radioactivity in the specimen, and it can well be re-analysed later to test a different hypothesis. In a sense, a good series of autoradiographs is a valuable heirloom, to be kept carefully and handed on to future generations of research workers.