Stripping Film Technic for Radioautographs. (17350)

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(Introduced by E. M. Landis.)
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The method of radioautography for localization of radioactive tracers in tissues is finding increasingly broad application as a new approach to histochemistry. One of the potential hazards in connection with all histochemical technics, including that of radioautography, is the leaching out or displacement of material under investigation which may occur as a result of tissue processing or staining. For example, studies at this laboratory[1] on radioautographic localization of P^{32} in brain tissue have shown that marked losses of the tagged element are incurred in the course of fixing and staining for routine paraffin sections. Perhaps the surest way to avoid artifacts of this nature is to use completely unprocessed frozen material for the radioautograph exposure, omitting all fixing and staining until after the autograph has been completed. An advantage of the method to be described is that it not only makes such post-staining possible, but also makes the staining process quite independent of the radioautograph development. As a result, the tissue may be stained by any of the well-documented histochemical technics as adjunct to or control of the isotope localization. An outline of the technic and an example of its application has been described in a preliminary report from this laboratory.[2] Similar applications of stripping film have also been reported by Pelc, Boyd and others.[3] A more general discussion of radioautographic technics may be found in the review article by Evans[4] and the book of Yagoda.[5]

Method. A. Films. In a series of experiments[6] in which nineteen commercial emulsions were investigated with respect to sensitivity, contrast and grain size, Type M stripping film (now commercially available) was selected as best suited for our radioautograph technic on the basis of the following qualities: fine grain, high contrast, low background fog and thinness of emulsion. Emulsions with finer grain and consequently greater resolving power are available (as, for example, Ansco Reprolith ortho stripping film), but these are very much less sensitive and so call for exposures which are impracticable for most work. Type M stripping film represents a satisfactory compromise between the mutually exclusive extremes of fine grain and high sensitivity in commercial emulsions.

The emulsion of Type M stripping film is about 10 microns in thickness and is coated on a 7 micron transparent cellulose acetate stripping base. Emulsion and stripping base are in turn mounted on a transparent supporting base about 0.15 mm thick, as shown in Fig. 1. At the time of use the emulsion on its 7 micron base is stripped away from the supporting material and can then be handled almost as though it were an autonomous 10 micron layer of emulsion. To facilitate later steps, it is advantageous to have on hand pieces of film just large enough to overlap the edges of a standard microscopic slide.

B. Tissue Preparation. 1. Frozen sections. Our best histology has been obtained by freez-

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1 Steinberg, D., and Selverstone, B., unpublished data.

2 MacDonald, A. M., Cobb, Jock, and Solomon, A. K., *Science*, 1948, **107**, 550.

3 Pelc, S. R., *Nature*, 1947, **160**, 749; Boyd, G. A., and Williams, A. I., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 225; Endicott, K. M., and Clark, F. A. (Ref. 5, p. 48).

4 Evans, T. C., *Nucleonics*, 1948, **2**, 52.

5 Yagoda, H., Radioactive Measurements with Nuclear Emulsions, John Wiley and Sons, Inc., New York, 1949.

6 Cobb, Jock, and Solomon, A. K., *Rev. Sci. Inst.*, 1948, **19**, 441.

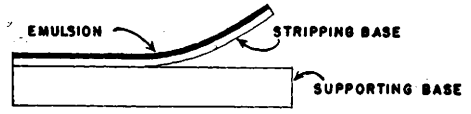

FIG 1.

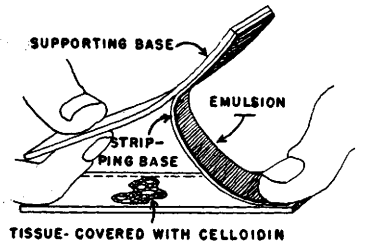

FIG 2.

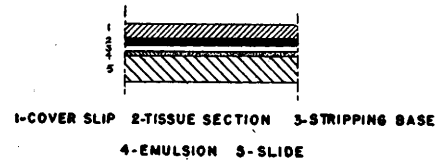

FIG 3.

ing the tissue to be studied immediately on removal in iso-pentane at liquid nitrogen temperatures, according to the technic of Linderstrøm-Lang and Mogensen.[7] It is then transferred to a refrigerated room at −10 to −15°C and cut on a microtome equilibrated at that temperature, making use of a modified Linderstrøm-Lang "window",[8] to prevent rolling of the sections. These are melted onto slides previously prepared by coating with 0.1% gelatin which is then hardened by dipping in 10% formalin. With this technic sections can be allowed to dry at room temperature without distortion. They are then ready for the application of the film in the manner described below.

2. Paraffin sections. When there is no problem of leaching out or displacement of the material under investigation, routine fixing, embedding and sectioning technics are employed. Needless to say, tissue detail obtained with this technic is decidedly superior to that of even the best frozen sections, and hence this technic is to be preferred whenever possible. The slides are stained in the usual manner up to the absolute alcohol stage. They are not carried through into xylol since the film is to be mounted with an alcohol base adhesive mixture. If the film cannot be mounted at the time staining is completed, the slides may be carried through to xylol, provided it is removed with absolute alcohol prior to mounting.

C. Application of the Film. All handling of film is carried out under Wratten Series 2 safelight. First, the emulsion and stripping base are separated from the heavy supporting base as shown in Fig. 1, leaving the last ¼ inch adhering to the base to facilitate manipulation. Stripping can usually be started at a corner of the film by stroking the emulsion away from the support with the ball of the thumb.§ Stripping should be done slowly to prevent any static discharge between stripping base and support, as such a discharge causes a considerable increase in background fog.

A 1% solution of celloidin in methyl alcohol is used to cement the film, *emulsion side up*, to the tissue. If the celloidin is dissolved in the conventional ether-alcohol mixture, irregular black splotches appear on the developed film. These artifacts have been eliminated by the use of methyl alcohol as solvent. The slides are laid tissue section up on a piece of filter paper and the alcohol from the paraffin sections is allowed to evaporate almost completely. Then a drop or two of the celloidin solution is placed directly on the tissue section and another drop on the slide to either side of the section. The partially stripped film is then applied to the section as shown in Fig. 2, and stripping of the film is completed at the same time. Next, the trans-

[7] Linderstrom-Lang, K., and Mogensen, K. R., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, 1938, **23**, 27.

[8] Coons, A. H., private communication.

§ To facilitate stripping, Dr. George A. Boyd[3] has suggested turning the film emulsion side down and slitting the supporting base with a razor blade. The backing can then be cracked along the slit. While this procedure simplifies stripping, it also increases the background fog due to the static discharge accompanying the breaking of the support. See Boyd's article for details.

parent supporting base is laid over the emulsion to serve as protection, and the preparation is smoothed with the fingers. Finally, a second microscope slide is placed on top of the supporting base and the entire "sandwich" wrapped with scotch tape. It is our practice to wrap each preparation individually in opaque paper and place it immediately under a pressure of 8 lbs/sq inch. Resolution is considerably improved by the more intimate tissue-film contact obtained with pressure. After 24 hours under weights the preparation is transferred to the refrigerating unit or dry ice box and stored at freezing temperature for the duration of the exposure time. Removal from under the weights at this time, after the celloidin has set, causes no apparent loss in resolution. Estimation of proper exposure times will be discussed below.

D. Developing and Mounting. Autographs ready for development are unwrapped in the darkroom and the scotch tape is cut along the edges of the "sandwich" with a razor blade. Unwinding the tape leads to a grossly visible blue static discharge which can fog the film quite badly. After removal of the guard slide and the supporting base, the bottom slide, bearing tissue and adherent stripping film, is placed in the developer *in toto*. It is carried as a unit through the stop bath and fixative. Each slide is developed in a separate test tube into which the developer and other solutions are successively poured.

In order to control the many variables that may affect film sensitivity we have adopted the following precautions as part of our standard procedure. (a) All films are kept in a desiccated box in the refrigerating unit. (b) Films are allowed to come to room temperature before use. (c) Exposures are all made at the same temperatures. (d) Films are developed immediately at the end of the exposure period. (e) Wratten Series 2 safelight at 3 feet is the only illumination used.

The developing process is controlled in the following ways. (a) Distilled water is used in all solutions. (b) Developer is stored in full, brown, stoppered bottles. (c) 50 cc of fresh developer is used for each preparation. (d) Each tube is inverted gently every 30 seconds during development. (e) All solutions are brought to 68° ± 0.5°C and kept at this temperature during development.

During the processing, the emulsion is directly and evenly exposed to the solutions, thus insuring complete, uniform development. The tissue is protected by the intervening stripping base and the celloidin coating, so that it is preserved from the action of the developing solution Since the stripping film gradually separates from the slide during processing, however, it is important to limit the time in the solutions. Five minutes in Kodak D-19 developer, 15 seconds in 1% acetic acid stop bath and two minutes in Kodak F-5 fixing-hardening solution constitutes a satisfactory routine procedure. After 5 to 10 minutes washing in cold water, the stripping film has become partially detached from the slide and a gentle, steady pull on one end suffices to complete the separation. The tissue section adheres to and comes away with the stripping film which can be left in a beaker of distilled water until it is mounted.

At this point we have the tissue section adherent to one side of the stripping base and on the opposite side the developed grains of the tissue's own radioautograph. We now simply take this preparation up through alcohols to xylol, and, after trimming away the excess film around the tissue, mount with balsam, or a substitute, and a cover slip. Usually, it does not matter whether the tissue is above or below the autograph, but when the image is very dense, the tissue detail is better examined with the tissue uppermost. The orientation of the final preparation is shown in Fig. 3.

At low magnification, the close juxtaposition of tissue with its autograph makes it possible to survey the preparation with a single fixed plane of focus. At high magnification, a slight change of microscope focus correlates the grain density pattern in the autograph with the histologic pattern.

E. Post-staining. When unstained sections have been used, the steps are exactly the same up to the point at which the preparation is removed from the wash water. In order to stain the tissue and at the same time to

protect the emulsion from the stain the stripping film is now cemented *emulsion side down* onto a clean slide with Kodalith Stripping Film Cement or with 1% celloidin in ether-alcohol. This leaves the tissue section exposed and the slide can be handled through the staining process in the routine manner. After staining, the excess film peripheral to the tissue section itself is trimmed away and the preparation covered with balsam, or a substitute, and a cover slip. In order to obtain a uniformly flat field it is necessary to weight the cover slip rather heavily until the balsam has dried.

F. Time of Exposure. Data have been presented elsewhere[9] that permit the calculation of exposure time for Type M stripping film for 5 important isotopes, C^{14}, Ca^{45}, I^{131}, P^{32}, and Zn^{65}, provided the activity of the preparation in terms of disintegration per minute per unit area is known. For problems in which such assay is inconvenient, a satisfactory empirical procedure has been developed, based on the use of Eastman No-Screen x-ray film, a film many times more sensitive than the Type M. Using this film, "trial" radioautograph preparations are made using tissue sections from the same block. The film is merely laid over the tissue section without adhesive, covered with a guard slide and "sandwiched" with scotch tape as above. At the time of development the "sandwich" is opened and the film removed and processed. Several such preparations are put up and developed at intervals. From the ratio of sensitivities of the No-Screen x-ray and Type M stripping films the exposure time needed to get an equivalent density with the latter can be calculated. Ratios of initial activity requirements from Table I indicate the relative sensitivities of the two films.

Table I (taken from reference 9) gives the activities required at the surface of the tissue to produce radioautographs of image density 0.1 and 0.6 above background with 15 day exposures to uniform sources of C^{14}, Ca^{45}, P^{32}, I^{131} or Zn^{65}. It must be kept in mind that generally one is interested in some par-

9 Steinberg, D., and Solomon, A. K., *Rev. Sci. Inst.*, in press.

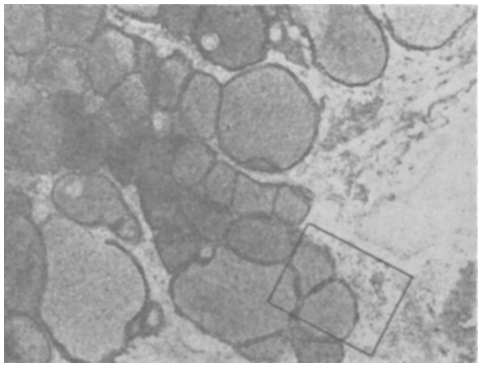

FIG. 4.

Radioautograph of rabbit thyroid. Animal was sacrificed 16 hours after intraperitoneal tracer dose of 270 microcuries I^{131}. Tissue was formalin fixed and stained with hematoxylin-eosin. Type M stripping film, exposed 16 hours, developed 5 minutes in Kodak D-19. Magnification 200×.

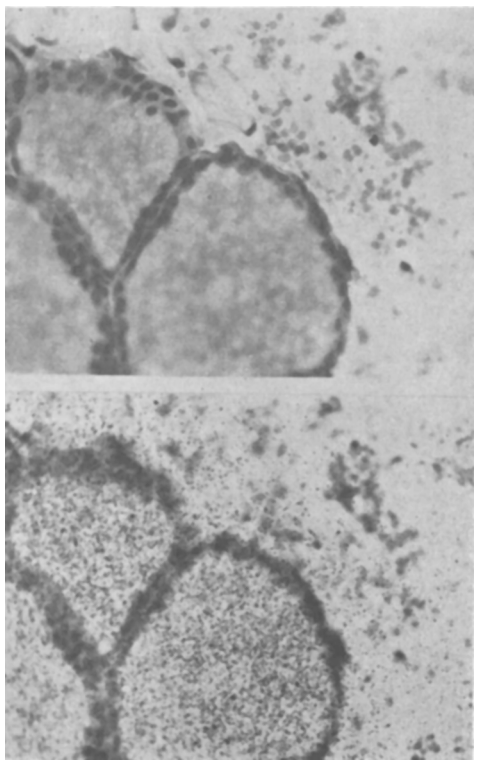

FIG. 5.

Enlargement of area blocked out in Fig. 4.

Upper: Focused in plane of tissue to bring out histologic detail.

Lower: Same area focused in plane of photographic grains for study of distribution and contrast. Magnification 800×.

TABLE I.
Initial Activity Required in Disintegrations per Minute per cm² of Tissue.

	Eastman no-screen x-ray film		Eastman type M stripping film	
	0.1> Bkgd.	0.6> Bkgd.	0.1> Bkgd.	0.6> Bkgd.
C^{14}	124	806	2,280	11,400
Ca^{45}	124	1,030	3,260	27,000
I^{131}	414	2,180	18,500	75,200
P^{32}	843	4,610	36,700	160,000
Zn^{65}	4,250	27,400	138,000	741,000

Values given assume a 15-day exposure and 5 minutes development in Kodak D-19. Data are for a uniform source, and so apply only to mean activity of tissue section.

ticular structure and so the average density of the autograph may be a very unreliable guide to optimum exposure. Both when counting the initial activity of tissue sections and when examining the images of preliminary No-Screen x-ray autographs, these areas of interest should where possible be the basis of estimation. It is absolutely essential that the precautions in handling of films and development described above be observed in order to obtain reproducible results.

Discussion. The advantages of the present technics are several:

1. The staining and developing processes are independent of one another.

2. The thickness of the emulsion is equal over the entire preparation so that differences in grain density at different points can be used as an index of the activity of the underlying point in the tissue.

3. Tissue and emulsion are held in a fixed relationship from the very beginning of the procedure through to the time of examination. Since one can look through both the autograph and the tissue simultaneously under the microscope, correlation and interpretation are made simple.

One limitation of the technic lies in the 7 micron separation of tissue and emulsion. While in many studies this very small separation may not be of importance, it does limit the theoretical resolution possible with the technic. However, our results with Type M stripping film indicate that resolution to 10-15 microns can be obtained, as illustrated by Fig. 4 and 5.

These autographs show the very different rates of uptake of I^{131} by different thyroid follicles. The fine grain of the emulsion can be noted and the order of magnitude of the background can be estimated from the areas of emulsion not overlying tissue. At 16 hours after an injection of iodide the material is known to be widely disseminated throughout the gland and this is evidenced in the autographs. Of particular interest is the rather sharp demarcation of the high activity colloid material from the low activity thyroid stroma.

The data of Table I for Type M stripping film should make clear a limitation of the radioautographic technic that is too seldom emphasized, the need for high tissue activity. Unless the material under investigation is highly concentrated by the organism as in the case, say of radioiodine, the specific activity requirements are high and in some cases forbiddingly high. With the aid of data on exposure, such as that referred to above, it is often possible to determine beforehand the feasibility of a study when the specific activity of the material and the probable biologic dilution are known.

Summary. A technic for radioautography is described which makes the developing and staining processes independent and, through the use of stripping film, provides an emulsion of uniform thickness. The method of mounting permits uniform development of the entire emulsion and maintains a fixed tissue-autograph relationship.

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