TECHNIQUE OF PREPARATION, STUDY AND PHOTOGRAPHY OF BENZYL-BENZOATE CLEARED MATERIAL FOR EMBRYOLOGICAL STUDIES

MARGARET WARD ORSINI

Department of Anatomy, University of Wisconsin, Madison, Wisconsin, U.S.A.

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Summary. The entire reproductive tract with its mesenteries is removed intact and fixed in AFA (30 cc 95% alcohol, 10 cc commercial formalin, 10 cc glacial acetic acid, 50 cc water). The tract is dehydrated through ascending alcohols, with two changes of each. A few drops of peroxide are added to each of the 70 and 80% alcohols for bleaching. From 100% alcohol, the tracts are passed through 100% and benzol, to two changes of benzol, and to benzyl benzoate where the tract is stored and observed.

Cleared material may subsequently be returned to benzol, infiltrated with paraffin and sectioned for histological study.

The construction of a box providing oblique light for gross observations and photography, and the technique for study of fine detail with a dissecting microscope and photography with a Micro Ibso is discussed.

Implantation sites prior to gestational swelling, placental scars, stages of development, abnormalities of development, body form, skeletal structure, vascular pattern of the uterus and details of gross anatomy may be observed and studied in material prepared by the above technique.

INTRODUCTION

Material cleared in benzyl benzoate and studied under varying conditions of illumination shows implantation sites prior to gestational swelling, placental scars and abnormalities in foetus and placenta. Details of internal structure and anatomy of the reproductive tract are clearly shown. Such cleared material may be stored indefinitely or used for histological studies. The method of clearing, modifications required for different sized tracts, the type and construction of apparatus required for study and photography will be discussed.

MATERIALS AND METHODS

TECHNIQUE USED FOR FIXATION AND CLEARING

Fixation

The entire uterus with cervix, ovaries and attached mesenteries is removed as a single unit, pinned by the mesenteries through aluminium foil to a supporting card and fixed in AFA (30 cc 95% alcohol, 10 cc commercial formalin, 10 cc commercial formalin, 50 cc water). The tract is dehydrated through ascending alcohols, with two changes of each. A few drops of peroxide are added to each of the 70 and 80% alcohols for bleaching. From 100% alcohol, the tracts are passed through 100% and benzol, to two changes of benzol, and to benzyl benzoate where the tract is stored and observed.

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10 cc glacial acetic acid, 50 cc water). Use of aluminium foil prevents adhesion of the paper sizing to the tract. It is extremely important that the tract be as clean as possible, any excess blood should be wiped off. The tract should be pinned as flat as possible, for ease in future study, but stretching should be avoided lest distortion ensue. Fixation and clearing are best carried out in a dish large enough to contain the entire tract without bending. Finger bowls that can be stacked have proved invaluable for this purpose. Fixation can be carried out in formalin, Bouin’s or Zenker’s fluid, but the coloured fixatives are difficult to bleach and the AFA provides better histological detail than the formalin. Pins should be removed immediately after fixation in order to prevent metallic staining of the adjacent tissue.

Dehydration and bleaching

From the AFA, the tract is labelled and carried through two changes each of ascending alcohols, i.e., 50, 70, 80, 87, 95 and 100 % alcohol. A few drops of hydrogen peroxide are added to the 50, 70 and 80 % alcohols for bleaching. The tract, removed from the supporting card, is labelled by a tag attached by a fine thread through the cervix. All labels should be made with very dark lead and on one side only, they should be attached by threads long enough to allow the label to be pulled to one side out of the field of vision during photography. Material stored in fixatives or alcohols for long periods of time prior to dehydration and clearing is difficult to bleach satisfactorily, requiring long periods in the peroxide alcohols.

Clearing

From 100 % alcohol, the tracts are passed into 100 % alcohol and benzol (to save solutions an approximately equal volume of benzol is simply added to the second 100 % alcohol) then through two changes of benzol to benzyl benzoate, where they may be stored indefinitely and studied. Tracts have been stored for over 2 years without change. Anethole may be substituted for the benzyl benzoate, but methyl salicylate (oil of wintergreen) cannot, as the clearing is greatly inferior.

Timing of fixation, dehydration, bleaching and clearing

The timing of the clearing technique, as outlined above, can be varied according to the size of the tract involved. It is possible to fix, dehydrate, bleach and clear a mouse tract in early gestation (up to 5 days and 12 hr gestation) within 24 hr. A guinea-pig tract can be carried through the entire process within 10 days, provided that it is no older than 15 days gestation. In general, one must allow ample time for adequate fixation, then sufficient time in the 70 and 80 % alcohols plus peroxide to bleach out the blood and muscle pigments. This can be judged accurately by the naked eye. When bleached, the tract appears almost white; repeated changes in the peroxide-bearing alcohols will expedite this and peroxide may also be added to the 87 % alcohol in instances where speed is desirable. The tracts begin to turn yellow when in the 100 % alcohol plus benzol. Tracts may be rebleached if bleaching has been inadequate, by retracing the process.
Preparation of cleared uteri

The amount and concentration of peroxide used should also be varied with the size of the specimen involved; excess peroxide can ruin specimens, causing distortion of the lumen of the uterus and of the foetal membranes, or causing a fine white flaking of the surface, similar to the white peroxide burn on the investigator’s fingers. Approximately four to five drops of 8% peroxide/80 cc alcohol should be used for small tracts, and the material carried through within a few days; in a few instances a mouse tract has been found to crumble after a prolonged clearing process. Superoxal (30% hydrogen peroxide) five to eight drops to 250 cc alcohol is used for larger tracts. Since peroxide loses its activity with time it should be purchased in as small amounts as possible, and dated when opened; this serves as a simple control of its activity.

Histological preparations

For histological study the desired area is cut out and run back to benzol, or to toluene. Several changes of either fluid are desirable to remove the dense benzyl benzoate prior to infiltration with paraffin.

EQUIPMENT AND TECHNIQUE USED FOR STUDY AND PHOTOGRAPHY

For both routine study and photography, the number of layers of glass should be reduced to a minimum and all glass used should be free of scratches and all optical aberration. Special glass dishes may be constructed inexpensively by fusing glass cylinders of the desired height to ordinary window glass by use of the resin Epox 346. Dishes should be shallow enough to allow the investigator to manipulate the specimen with forceps in order to study all angles, but deep enough so that the specimen is completely covered. In order to eliminate dust from the specimen and fluid, dishes should be covered when not in use and the benzyl benzoate constantly refiltered to remove extraneous material.

For gross observations and photography, a light box such as is shown in Pl. 1, Fig. 1 should be constructed. The black baffle in the box is coated beneath with aluminium foil, and the interior of the box is painted white or lined with aluminium foil. The 22-watt fluorescent circular tube in the base of the box provides the source of light, which is deflected by the baffle, thus providing oblique illumination for the specimen which rests on a removable top. These tops, with central apertures of varying sizes to accommodate various sized dishes, rest on supporting brackets. Photographs can be taken with a single-lens reflex camera equipped with extension tubes or supplementary portrait lenses.

For careful study and detailed observations, a dissecting microscope with fully adjustable substage mirror and a source of strong light is required. Unfortunately many of the newest dissecting microscopes have substage mirrors which are limited in scope of movement; it must be possible to swing the mirror to one side and then pivot it to provide oblique or direct illumination.

Where only dissecting microscopes lacking stages are available, it is possible to construct a small table with removable glass stage, and to use a car mirror mounted on a pivot with a suction base as a reflecting mirror. When this is necessary, it is best to make the aperture for the glass stage equivalent to a 5” x 7” photographic glass plate, since whenever benzyl benzoate penetrates
between the glass support and the specimen dish both must be changed for visibility. Such photographic plates are optically clear and readily available.

The most convenient light source is a simple goose-neck lamp with a 75-watt spot or flood bulb. Reflected or transmitted oblique light can be used. A Micro Ibso MIKAS micro-attachment with a Leica back can be inserted in either ocular of the microscope for photography. This particular attachment gives excellent optical detail and is easy to use (Pl. 1, Fig. 2). Adox 17 or Anscochrome (tungsten) was used for all the Figs. except Pl. 1, Figs. 1 and 2. These two films have identical speeds; thus, once one has determined the correct timing for the ocular and objective used, it is possible to transpose between black and white and colour film with no difficulty; with the use of two camera backs, one is able to maintain routine records in both film types. Filters may be inserted beneath the specimen, in front of the light, or in the objective of the microscope.

**OBSERVATIONS**

With ordinary transmitted light, the cleared tracts appear yellow and no fine detail is visible, Pl. 2, Fig. 6. With oblique light, the same tract, Pl. 2, Fig. 7, is slightly bluish, the muscularis appears as a white refractile line and early implantation sites appear as slightly opalescent, white, opaque dots. The value of this technique for the visualization of implantation sites prior to the appearance of visible swelling in various species has been described in other papers (Orsini, 1962; Orsini & Mossman, 1961). This technique should be of value in screening experimental procedures aimed at prevention of implantation.

Placental scars are clearly visible in the cleared tracts of common laboratory rodents. Pl. 2, Fig. 8 shows a portion of a hamster tract removed 13 days after the birth of the first litter, and 4 days and 22 hr pregnant with a second. The hamster has no post-partum ovulation. In this case, the young of the first litter were removed immediately after birth, the animal checked daily to determine the recurrence of oestrus and mated on the second oestrus after parturition. Three intensely orange placental scars appear in the mesometrium at the uterine-mesometrial junction; opaque white areas in the uterus proper mark the sites of the current conceptuses. Note that the intergestational areas in the uterus are still filled with haemosiderin from the first litter; the placental scars overlie these involuting areas; implantation of the second litter occurs between the scars of the first. Thus the relation of one litter to the position of the previous litter may be studied.

The extreme clarity of material prepared by the above technique reveals minute details overlooked at autopsy or by the wintergreen clearing technique. Pl. 1, Fig. 4 shows a portion of a uterus from a hamster spayed the 2nd day after mating and provided with minimal exogenous progesterone. At autopsy, two approximately normal sites and one resorbing were observed; after clearing, an additional tiny opaque dot was visible (at left in Pl. 1, Fig. 4) which on sectioning revealed decidua and a few foetal cells, suggesting that implantation began but could not be maintained.

This technique can also be used to observe normal and abnormal development in later stages of pregnancy. With experience one can distinguish actual stages of development, i.e. between 5th, 6th, 7th, 8th and 9th days of develop-
Fig. 1. Light box used for gross photography. The door of the box is open, showing the 22-watt fluorescent bulb mounted in the base of the box and the baffle above, which provides for oblique light. The Pentacon camera is shown in place on the photographic stand, above the specimen. The apparatus was used for photographing Figs. 4 and 5.

Fig. 2. The dissecting microscope used for detailed study with the Micro Ibso MIKAS with Leica back. Figs. 6, 7 and 8 were taken with this. Note the spot bulb used as light source. Oblique reflected light may also be used as well as oblique transmitted light.

Fig. 3. Photomicrograph of implanting hamster blastocyst. This uterus was removed at 4 days and 8.5 hr developmental age. The entire tract was fixed in AFA and cleared by the technique described in this paper. This area was selected by the visible opacity, infiltrated with paraffin and sectioned at 8 µ. Note that the histological detail has not been adversely affected. ×150.

Fig. 4. A cleared uterus from a hamster that had been spayed 2 days subsequent to mating and given a minimal dose of progesterone. At autopsy, three degenerating sites were visible, two large and one small; after clearing, the minute degenerating site opacity at the left of the figure was observed. ×2.25.

Fig. 5. The cleared uterus of a hamster removed during parturition. Eight young had been born at the time the animal was killed; opaque areas at the uterine-mesometrial junction in the empty horn at the left mark the recent separation sites. Three young are still in utero. Note the skeletal detail visible. ×0.75.
Fig. 6. Cleared hamster tract pregnant for 4 days and 13 hr as seen by direct transmitted light. ×8.

Fig. 7. Same tract as that shown in Fig. 6, but photographed with oblique transmitted light. Note that two conceptual areas are shown as white opaque regions in the endometrium. ×8.

Fig. 8. Photograph of a portion of a hamster uterus viewed by oblique transmitted light. This animal was killed when 13 days post partum and 4 days and 22 hr pregnant with its second litter. The hamster has no postpartum ovulations. To obtain such a specimen, the young of the first litter were removed immediately after birth, the animal checked daily for the recurrence of oestrus, and mated on the second oestrus after parturition. Three intensely orange placental scars can be seen in the mesometrium at the utero-mesometrial junction. Three opaque white regions within the endometrium mark the sites of the current conceptuses. The intergestational areas of the endometrium are still filled with haemosiderin from the first litter. Note that the placental scars overlie these involuting regions, and that implantation of the second litter is definitely between the scars of the first litter. ×8.
ment in the hamster, between the 13th, 14th, 15th and 16th day of development in the guinea-pig, and between the 6th, 7th and 8th days in the rat. In such instances, the pattern of the opacity, the development of foetal body form, the shape and position of the heart and allantois serve to distinguish between these early days of development. Skeletal outline and development may be observed in terminal stages of development in small rodents. Pl. 1, Fig. 5 shows a hamster uterus fixed in mid-parturition. All the young had been born from one horn, three foetuses are still in situ in the other. Complete detail of the skeleton is visible in these foetuses.

Such material provides a three-dimensional view of the gestational area. Although in large specimens the mass of the cleared specimen limits the photography to optical sections, the use of dissection overcomes this difficulty, but with experience the observer can make out details in varying optical levels. Areas of special interest may be cut out, examined from different angles, or partially dissected for further detail. Moreover, cleared specimens are ideal for localization of desired areas, and for orientation for subsequent histological studies. Pl. 1, Fig. 3 shows the cytological detail after fixation in AFA clearing by this method, and subsequent sectioning at 8μ.

The bleaching is always relative, the arteries and veins are clearly distinguishable in the mesometrium and with care may be followed through the uterus; thus vascular patterns are visible by this technique (see Orsini, 1957).

DISCUSSION

The above technique was developed over a number of years and is derived from the commonly known classic clearing techniques. It is fundamentally the same as that presented by H. Lundvall (in Romeis, 1948), who, in studies of the skeletal system, after fixation in formalin, bleached foetuses with peroxide and cleared in benzyl benzoate. However, the application of this technique to study of the differential changes in the uterus during implantation, the value of oblique light, the detail as to procedures for study and photography, and the fact that such material is suitable after AFA fixation for subsequent histological studies have not been previously pointed out.

This technique is of value for studies of development, reproductive history, teratology and pathology, and for study of the gross anatomy of the reproductive tract.

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