# THE ATLAS OF MOUSE DEVELOPMENT

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# Preface

The aim of this book is to provide an histological account of the development of the mouse spanning the period from fertilization to term, though the main emphasis relates to the postimplantation period. In order to facilitate this exercise, selected scanning electron micrographs have been provided which clearly illustrate the three-dimensional form of intact and dissected embryos, their isolated external components and a variety of their internal organs and organ systems. The majority of the book, however, consists of plates which display intermittent serial histological sections through a selection of embryos specifically chosen to display the sequential but nevertheless continuous changes that take place shortly after implantation, during the period of embryogenesis and organogenesis, and the relatively short period of consolidation which occurs during the latter part of gestation in this species. An additional selection of plates displays, using both scanning electron micrographs and representative histological sections, the sequential changes that take place in relation to specific organs and organ systems, such as the eyes, the gonads, the kidneys, the lungs and the heart, where the detailed changes that occur during their development and differentiation are not easily observed in the relatively low magnification intermittent serial sections.

It is hoped that this book will serve as a laboratory "workshop manual", which provides readers with the means to recognize, with the minimum of difficulty, all of the major structures that are likely to be encountered in their own serially sectioned material. In order to facilitate this exercise, every effort has been made to select micrographs which illustrate the normal features that may be observed in symmetrically sectioned embryos.

While a very wide range of standard and special fixatives and histological stains is available to the embryologist, there are distinct advantages to be gained by using a simple fixative (e.g. Bouin's fixative), and a single, simple staining regimen (e.g. haematoxylin and eosin). The latter gives a minimum, but acceptable, level of shrinkage artefact, associated with an excellent degree of cellular clarity. In those few instances where other fixatives or histological or histochemical stains have been used, generally for a specific purpose, e.g. to demonstrate the presence of primordial germ cells, attention is drawn to the fixative used and the particular staining regimen employed. Similarly, in nearly all instances, the material has been embedded in paraffin wax (Paraplast) and the wax blocks cut at a nominal thickness of either 6 μm, 7 μm or 8 μm. While it might have been useful for those that are new to the field to have access to colour plates, for most purposes when the principal aim of the exercise is to recognize a specific structure, this was considered to be an unnecessary extravagance, and would, without question, have made the book far too expensive and, in all probability, not a commercial proposition.

While some individuals may wish to browse through the plates in order to gain a general impression of the interrelationship which exists between the different components of a specific region of the embryo, backing this up with a detailed account of the same events, most individuals are likely to want to use the Atlas to recognize specific structures or features present in their own material. For this reason, in

addition to a few observations on methodology, the majority of the introductory text consists of an introduction to the plates, complemented by a brief descriptive account of the events that occur during mouse embryogenesis, organogenesis etc. While some readers may consider that the introductory text is too brief, I believe that by its brevity it serves the useful purpose of directing the reader's attention to the plates (with their associated explanatory notes) which are the raison d'etre of this monograph. It is, however, only by frequent reference to the reader's own material, supplemented by reference to the plates presented here, that familiarity with the three-dimensional arrangements within the embryo, and the sequential changes that occur during development and differentiation, can be most readily appreciated. As with any technical activity, it is possible to gain a certain limited degree of expertise by carefully following an instruction manual. However, to gain mastery of the subject, it is essential to try to understand, in this instance, how individual organs and systems relate to each other, both at the tissue and cellular level, during their development and differentiation.

Clearly, simply looking at conventionally stained individual histological sections, or even at serially-sectioned material, has its limitations; it is nevertheless the first and possibly the most critical approach that should be used in post implantation embryological studies, as it provides essential baseline morphological information which can be gained by no other means. Additional more specialized information can be gained, in certain circumstances, by utilizing a variety of other conventional stains, or by using more specific histochemical stains. If applicable, immunohistochemical means may also be employed to visualize certain cells or tissues. However, whatever the information gained, this must still be viewed in its proper context, and for this to be the case, it is essential that the reader should understand that the two-dimensional sections that are being viewed are only useful if they provide the reader with a three-dimensional image, and confer an essentially dynamic view of the events being studied. Most individuals can learn to "read" serially-sectioned histological material, but it is only after this elementary degree of competence is gained, that one can truly appreciate the value of this particular analytical approach.

It has been argued, erroneously in my view, that the analysis of serially-sectioned material is both time-consuming and tedious. This can undoubtedly be the case, but such comments often come from individuals who have never made any concerted effort to try to reconstruct in their minds the three-dimensional architecture of the material they are viewing. It has equally been argued that the analysis of serially-sectioned material provides only a static view of events. This is, of course, in a narrow sense correct, but anyone attempting to understand the dynamic and sometimes rapid changes that are occurring during, for example, embryogenesis or organogenesis, is most unlikely to be satisfied with looking at serial sections through a single embryo, or even through serial sections of a small number of embryos. Where possible, other supplementary means of analysing a particular event should be studied using, for example, cell lineage markers if these are available, or by the production and analysis of interspecies chimeras. Even these seemingly more dynamic approaches can have their limitations, and it would still be essential to analyse the latter material, in particular, histologically to check that the end result of the exercise bears some resemblance to the normal architecture of the tissue or organ system under investigation. Undoubtedly, it would be invaluable if a wide variety of cell-specific lineage markers could be found, as this would provide an analytical approach which would be of immense value in facilitating our understanding of the complex interactions that take place during embryonic development. Certain cell-specific markers are already available, for example, for the demonstration of neural crest-derived tissue, and it is likely that other similar cell-specific markers will soon appear.

Over the last few years, an explosion of information has emerged as a result of the use of molecular biological probes to investigate the genetic control of (particularly) pattern formation during early mouse embryonic development. Even though this field is still in its infancy, it seems certain that it will provide new insights into the morphogenetic interactions that occur during the pre- and early postimplantation period when the overall pattern for all subsequent development is laid down. A descriptive account of the gross changes that take place during the early postimplantation period, during embryogenesis and organogenesis, has largely evolved from the analysis of serially-sectioned material. The various, and almost entirely descriptive, accounts of the early development of the mouse, including that presented in this Atlas, now form one type of data base. This baseline information has been supplemented over the years by the findings from appropriately designed experimental studies, and from the analysis of spontaneously or induced genetic mutations, and from teratological studies. It now seems likely, however, that in order to gain a more detailed understanding of the fundamental mechanisms that control how cells and tissues interact during early embryonic development, we will additionally require the results obtained from an altogether different source of information, namely from the use of genetic probes. Even the initial information that has emerged as a result of the use of this relatively new experimental approach suggests that the interpretation of the findings is likely to be extremely complex, as even single genes or gene complexes not infrequently appear to act in more than one location in the developing organism at any one time, and over variable periods of time. Despite the fact that the interpretation of these findings is likely to be several orders of magnitude more complex than was initially envisaged, this is the direction that must be explored, and will almost certainly produce findings of the most fundamental importance in relation to increasing our understanding of the morphogenetic factors that influence the development of both mice and men.

Even with the emergence of these new technologies, it will nevertheless inevitably still be necessary to establish at the gross histological level the final common pathways by which individual cells or tissues are controlled by individual genes or groups of related, or even unrelated, genes. This type of genetically derived fate map and the morphogenetic maps that will emerge in the not too distant future, are likely to require very sophisticated computer technology to make the maximum sense of the four-dimensional informational input. While the latter may provide a novel perspective from which to view early mammalian development, any attempt at interpretation of this material will inevitably require a very thorough understanding of the events displayed in this Atlas. Old-fashioned and descriptive it might be, but the amount of information that can be obtained from the analysis of serially-sectioned paraffin-embedded material is directly related to the degree of perseverance, perspicacity and intelligence of the observer, who must be just as aware of the limitations of this material as the observer is of its exceptional potential.

To gain the maximum possible information, paraffin histology should, where possible, be complemented by a range of other analytical approaches that are appropriate to the questions that are being addressed. While scanning electron microscopy, in isolation, may be of limited value, it can occasionally provide invaluable information which can greatly facilitate the interpretation of "difficult" histological sections. Similarly, though beyond the scope of this Atlas, transmission electron microscopy can often provide invaluable information in relation to inter- and intracellular structure, and thereby shed important light on the complexities of cellular and tissue function which can only be hinted at from the analysis of paraffin sections. With the recent availability of gene probes and other sophisticated molecular biological methodology, the use of these techniques can often give the researcher the impression that all other approaches are old-fashioned, and consequently incapable of providing new insights into complex problems. This is clearly wishful thinking on their part, and is unfortunately not infrequently propagated by those grant-giving bodies that give the impression that if a technique is novel or trendy, it must be worth supporting, even though at least a proportion of those that use the new methodologies are clearly incapable of fully interpreting their findings.

It is probably worth briefly indicating here why this monograph has had such a long gestation period. The original plan was to produce an Atlas covering only the period from implantation up to about day 12 of gestation. This exercise was almost completed, when the author decided that a less detailed Atlas covering the period from implantation to day 17.5 of gestation would be of greater value to a wider audience. The duration of the project has additionally been increased by a period of at least a year or two, and possibly even more, because all of the work involved in the preparation and labelling of the plates has been undertaken by the author. While the latter was an exceedingly onerous component of the effort involved in the preparation of the Atlas, it was undertaken by the author in order to try to minimize labelling errors which could easily arise had the work been carried out by individuals unfamiliar with the material being labelled.

While every attempt has been made by the author to ensure the accurate identification of structures, only the plates and associated figure legends of the cephalic region of the more advanced embryos have been subjected to detailed scrutiny by others. Dr K.S. O'Shea and Dr Sam Hicks (Department of Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor), because of their familiarity with the features of the embryonic mouse brain, kindly volunteered to look through this material. The latter exercise resulted in an amended, improved and slightly more detailed labelling of this material, though, of course, the author accepts full responsibility for all errors of labelling or identification that may appear both in these plates and elsewhere in the Atlas. For the sake of accuracy, the author would be grateful if all errors in labelling or points requiring clarification could be addressed to him, so that if the Atlas were to be revised at some future date, appropriate changes could be made at that time.

A careful selection has had to be made to keep the total number of plates down to a minimum, in order to ensure that publication costs could be kept as low as possible. This has inevitably meant that certain areas have only received a superficial coverage, while other equally important topics may have been omitted altogether. As with every monograph, the final product is heavily coloured by the interests and expertise of the author. Clearly, with the eventual appearance of this Atlas, other individuals with more specialist interests can now produce their own atlases to fill in these deficiencies.

I would like to take this opportunity to acknowledge those individuals initially in the Department of Anatomy, University of Cambridge, and more recently in the Department of Anatomy, University of Edinburgh, who provided me with technical assistance essential during the preparation of this Atlas. I am particularly indebted to Mr Jack Cable (Edinburgh) for the meticulous care he has taken in the printing of the vast majority of the micrographs that appear in the plates, Miss Susan Bates (Cambridge) who sectioned most of the pre- and early somite stage embryos and the early limb-bud stage material, and Miss Corrine Arnott (Edinburgh) who sectioned all of the more advanced embryos, as well as for providing practical advice regarding the preparation of embryos for histological examination. Thanks are also due to Mrs (now Dr) Sheila Webb for her invaluable support and assistance in maintaining the author's research activities in Edinburgh while he was not infrequently distracted preparing material for the Atlas. The dissections of the palate were the work of Mr Peter AhPin, and diagrammatic reconstructions illustrating the differentiation of the branchial arch arterial system are based on the findings from a research project

undertaken by Mr Paul MacKenney. Mr (now Dr) Jeremy Skepper (Cambridge) and Mr Bob Shields (Edinburgh) assisted in the preparation of the scanning electron micrographs, and Mr Raith Overhill (Cambridge) and Mr Ian Lennox (Medical Illustrations, Edinburgh) assisted in the preparation of the diagrammatic illustrations that accompany the plates, though all of the reconstructions necessary to produce these illustrations and their labelling was undertaken by the author. The typing of the manuscript material was undertaken by Miss (now Dr) Helen Dingwall, Miss S. Macdonald and Mrs June Gillam. I also wish to express my indebtedness to Academic Press, but particularly to Dr Susan King, for their patience and encouragement over the years, and to IRL Press, Oxford, for allowing me to reproduce a limited number of tables, diagrams and micrographs that were used to illustrate my contribution to *Postimplantation Mammalian Embryos* (Copp and Cockcroft, 1990). I also wish to acknowledge the patience of my scientific colleagues (but particularly Dr Anne McLaren) for their unfailing optimism over the years that this Atlas would eventually see the light of day, and Action Research for the Crippled Child who provided microscope equipment to support this project. Finally, I wish to record my indebtedness to my wife and sons for their unfaltering loyalty, patience and consideration during this seemingly endless exercise.

Matthew H. Kaufman

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**Note:**
**Plates are located within the text to which they refer. When this has not been possible, the page where the relevant plate can be found is indicated.**

# Tables and figures

# 1

# Introduction

## 1 INTRODUCTORY REMARKS

In order to understand the events that occur during the early postimplantation period in any mammalian species, it is essential that intact embryos at different developmental stages, carefully removed from the uterus and dissected from within their extra-embryonic membranes, are studied in isolation, as this provides the simplest means of analysing the gross morphological and conformational changes that take place during the period under investigation. This material may be readily isolated and examined in the living state under a dissecting microscope in either phosphate-buffered saline or tissue culture medium, both of which should preferably be supplemented with protein (e.g. 4 mg/ml bovine serum albumin). In recently isolated embryos with more than about 6 pairs of somites present, the heart should show regular contractions, but the rate of the latter may be considerably slower than occurs *in vivo* if the temperature of the solution is substantially below 37°C.

If the embryonic material is to be examined in more detail, it must be "fixed" in one of several ways, depending on the analytical technique to be employed. If the material is to be examined by standard light microscopy, a wide range of fixatives may be employed, though for most purposes either Bouin's solution or 10% neutral buffered formalin is usually adequate. If, however, more sophisticated histological techniques are to be employed, for example if the sectioned material is to be examined to investigate intracellular enzyme activity, then different methods of fixation and/or sectioning (e.g. the use of a cryostat) will almost certainly be required.

In order to gain a detailed understanding of the three-dimensional relationships of the various components of the embryo, it is essential that serially-sectioned material is analysed. For most purposes, the material is embedded in paraffin wax, and sections cut at a nominal thickness of either 5, 7, 8 or 10 μm, whichever is the most convenient. Normally the sections are stained with haematoxylin and eosin, though a wide variety of other stains may be used, for example, if it is necessary to investigate the disposition of connective tissue, or to analyse in detail the various components of the nervous system, when one of the silver staining techniques may be employed.

To complement the analysis of the living embryos and serially-sectioned material, embryos at similar developmental stages may also be analysed by scanning electron microscopy. This latter technique has the principal advantage that it provides an excellent means of viewing the surface contours of an embryo in great detail. However, this approach is only of relatively limited value in itself in the analysis of embryonic material, as under normal circumstances a detailed knowledge of the subsurface structures is also required. However, as amply demonstrated in several of the plates, dissected material may also be scanned, and the resultant products can be extremely valuable for facilitating the interpretation of serially-sectioned embryos. Should the need arise, it is also feasible for material that has previously been examined by scanning electron microscopy to be embedded in epoxy

resin (e.g. Araldite, (Ciba–Geigy (UK) Ltd) and subsequently serially-sectioned. While there are certain technical difficulties involved in this manoeuvre, this dual approach has much to commend it. It is of particular value, for example, in the analysis of embryos with gross morphological abnormalities such as of the cephalic region which are difficult to describe adequately or illustrate by any means other than by scanning electron microscopy because of the small size of the specimens.

Similar fixatives to those used for scanning electron microscopy may also be used if the material is to be examined by transmission electron microscopy. While the latter technique may provide unique information on the ultrastructure of early embryonic tissues, particularly with respect to their subcellular components, any consideration of this technique is unfortunately beyond the scope of this Atlas. However, it is appropriate to mention at this stage that material previously embedded in epoxy resin for ultrathin sectioning for transmission electron microscopy, may also be used to provide individual sections or even serial sections cut at a nominal thickness of from about 0.5 to 1.0 μm, or even up to 2–3 μm in thickness. This approach can provide information with respect to cellular detail, which is not readily seen when similar material is examined by conventional paraffin histology.

A selection of scanning electron micrographs is provided in the Atlas in order to illustrate the gross conformational changes that take place in the external appearance of the embryo, and of certain of its internal organs. This material is supplemented with intermittent serial sections from a representative selection of embryos which have been chosen because they most clearly illustrate the principal features to be seen at each stage of development. In most instances, the embryos have been sectioned in the transverse plane, because these sections are relatively easy to interpret. Furthermore, it will also be rapidly apparent that this plane of sectioning provides maximum information of the type most often required in developmental studies. For certain special requirements, however, material may be optimally sectioned in another plane. A number of representative examples are therefore illustrated where embryos have been sectioned sagittally, principally, though not exclusively, in order to investigate developmental events that occur in the median plane. In nearly all of the material illustrated here that has been sectioned in the sagittal plane, selected intermittent sections are provided which pass parasagittally from one side to the other side of the embryo, through the median plane. These sections particularly emphasize the progressive degree of asymmetry that develops very soon after the early somite "headfold stage" is achieved. In the examination of the cephalic region of mid- and late-gestational stages of embryonic development, for example, it may be of value to section material in the coronal plane, as this provides a particularly useful means of analysing the development of the orofacial structures such as the tongue or palate. This also tends to be the plane of section favoured by the neuroanatomists/neurophysiologists for their analyses of the early post-natal and adult rodent brain.

For the benefit of the reader, and for obvious aesthetic reasons, every effort has been made to illustrate, in relation to the intermittent serially-sectioned material, bilaterally symmetrical histological sections, as these substantially facilitate all forms of analysis. While this can usually be achieved in "unturned" embryos, in partially "turned" and fully "turned" embryos this is not technically feasible, since the lower half of the trunk and the proximal part of the tail region are invariably deflected away from the median plane. The situation is further complicated by the fact that the internal organs and the vascular system rapidly lose their bilateral symmetry. Nevertheless, in all of the embryos illustrated, the sections that pass through the cephalic region retain their bilateral symmetry, and this symmetrical arrangement is still clearly apparent as far as the external features of these embryos are concerned. The material illustrated therefore demonstrates what can be achieved if adequate care is taken in orientating the material before any sectioning is undertaken.

# 2. METHODOLOGY

### Stages in the preparation of early embryos for histological examination

In order to obtain histological sections of the highest possible quality, extreme care must be taken in the handling of the material. The rules that apply for the handling of adult tissues have invariably to be modified, since early embryonic material is extremely delicate and intolerant of extreme changes in its external environment. Consequently, the tissues will not remain intact if they are not handled with great care.

There are clearly many means that have been developed over the years for preparing serial sections of early mammalian embryonic material. The instructions that follows are not absolute, and should be modified according to local conditions. Such variables as the air temperature and humidity, as well as the purity of the chemicals used, can have a dramatic effect during the critical processing stages. In the author's laboratory, the timings of the various stages have been modified over the years by trial and error, so that it is now possible for serial sections of the highest quality to be reliably achieved.

**Table 1 Preparation of paraffin-embedded embryos**

| Process | Solution | Crown–rump length of embryo (mm) <2 | 3–5 | >6 |
|---|---|---|---|---|
| Fixation | Bouin's fluid (saturated picric acid 75 ml, glacial acetic acid 5 ml, 40% formaldehyde 25 ml) | 2–24 h | 2–24 h | 2–24 h |
| Storage after fixation | 70% Ethanol | Variable period | Variable period | Variable period |
| Dehydration and clearing | 80% Ethanol | 1 min | 3–5 min | 30 min |
| | 90% Ethanol | 1 min | 3–5 min | 30 min |
| | 96% Ethanol I | 1 min | 3–5 min | 60 min |
| | 96% Ethanol II | – | – | 30 min |
| | 100% Ethanol I | 1 min | 3–5 min | 60 min |
| | 100% Ethanol II | – | – | 30 min |
| | (1:1) 100% Ethanol:benzene[a] | 1 min | 3–5 min | 15 min |
| | Benzene[a] | Until cleared | Until cleared | — |
| | Xylene | – | – | Until cleared |
| Embedding | Paraplast I | 1 min | 3–5 min | Vacuum embed |
| | Paraplast II | 1 min | 3–5 min | Vacuum embed |
| | Paraplast III | 1 min | 3–5 min | Vacuum embed |

[a] With embryos of up to about 5 mm crown–rump length, benzene is preferable to alternatives such as xylene, toluene, inhibisol, because it facilitates visualizing when the end point of "clearing" has occurred. The other agents appear to cause increased hardening of the tissues, and possible problems with processing because the material is more brittle and consequently difficult to handle. With larger embryos these other agents should be employed instead of benzene for the "clearing" stage. Since benzene is a particularly dangerous chemical, appropriate safety measures must be taken when this agent is being used.

From Kaufman, 1990, with permission.

## Fixation

For most purposes, exposure to Bouin's fixative (saturated aqueous picric acid, 75 ml; 40% formaldehyde, 25 ml; glacial acetic acid, 5 ml) for 2–24 hours, depending on the size of the embryo, and long-term storage in 70% alcohol at room temperature until needed, has proved to be entirely satisfactory. This particular fixative produces acceptable levels of artefactual damage. If, however, the degree of damage to a particular tissue is unacceptable, then a wide variety of other simple fixatives (such as 10% neutral buffered formalin) are available, and their effects on the tissue being analysed should be investigated. In general, the time that the material spends in 70% alcohol is relatively unimportant, though the volume of this solution should be large compared to the size of the embryo. The 70% alcohol will inevitably remove some of the characteristic yellow picric acid staining of the material, and it is advisable that this solution should be changed on at least several occasions. The yellow coloration produced can be useful when the embryo is being orientated in the paraffin wax. The addition of a few drops of eosin to the 90% alcohol stage during the dehydration sequence stains the embryo pink, and also facilitates its orientation in the paraffin wax.

As a general guide, the smaller the embryo, the shorter the duration in Bouin's fixative. Thus embryos with a crown–rump (C–R) length of about 2 mm or less need only about 1 hour in this fixative; 3–5 mm C–R length embryos require only about 2 hours, while 6–10 mm C–R length embryos require about 6 hours, and 11–15 mm C–R length embryos would require about 10 hours up to a maximum of about 24 hours – but certainly no longer in Bouin's solution, as excessive hardening of the tissue occurs which inevitably leads to problems during the sectioning stage. A general guide to the duration of the various stages involved in the processing of embryos of different gestational ages is presented in Table 1.

If special histochemical studies are to be undertaken, then the appropriate reference books should be consulted to establish in the first instance the optimal fixative which should be employed. To demonstrate the presence of *alkaline phosphatase* activity, for example, we have found that early embryonic material is best fixed in 80% alcohol at 4°C for a maximum period of about 2 hours. The duration of exposure to the fixative should of course be modified according to the size of the material to be analysed.

## Dehydration, clearing and embedding of embryos in paraffin wax

The timing of the individual steps during the dehydration sequence has been found to be of critical importance, since overexposure during processing can result in excessive hardening of the tissue and disintegration of the material during sectioning. Extreme care must also be taken during the clearing and mounting of the sections onto slides, otherwise damage

will inevitably occur. To facilitate the processing of this material, a fine wire mesh cassette or carrier may be employed (e.g. wire mesh thin bar grids No. G206, Agar Aids, Stansted, Essex, UK). The different alcohols may be retained in glass Petri dishes, rather than small bottles, as this allows more room for manoeuvre. As the number of changes of solution is considerable, the use of a wire mesh carrier reduces the necessary handling to a minimum, and consequently decreases the risk of damaging the material.

During the dehydration sequence the embryo has to progress from 70% alcohol, through successively 80%, 90%, 96% and subsequently absolute alcohol, then into a 50:50 mixture of absolute alcohol:benzene. For embryos of about 2 mm C–R length or less, it will be apparent from reference to Table 1 that the duration of exposure to each stage should be for 1 minute, and certainly for no longer than 1.5 minutes. For embryos of 3–5 mm C–R length, the duration of each step should be between 3 and 5 minutes. For embryos of 6–10 mm C–R length, the duration of exposure of each step should be as follows: 80% and 90% alcohol, 30 minutes, followed by two changes, each of 30 minutes duration, and finally exposure for 15 minutes in the 50:50 mixture of absolute alcohol:benzene.

Embryos of less than 5 mm C–R length should be retained in benzene until they clear, and then removed immediately into the first wax stage. Since benzene is a particularly dangerous chemical, appropriate safety measures must be taken when this agent is being used. For the larger embryos, xylene appears to be a more satisfactory clearing agent, and material should be retained in this agent until it clears, then immediately transferred into the first wax stage. Paraplast (Monoject Scientific Inc., Athy, Co. Kildare, Ireland) has been found to be a suitable embedding agent.

For embryos of less than 5 mm C–R length it has been observed that adequate penetration with paraffin wax occurs if, during the embedding procedure, the material is left in either a hot oven (at about 60°C) or preferably retained under a hot lamp. Three changes of wax are required. For embryos of 2 mm C–R length or less, they only require to be retained for 1 minute in each wax step. For 3–5 mm C–R length embryos, the duration of each wax step should be 3–5 minutes. The larger embryos require to be processed using a vacuum embedding oven, otherwise incomplete penetration of the wax into the embryo inevitably occurs.

As indicated earlier, the addition of a few drops of eosin into the 90% alcohol stage stains the embryo pink, and greatly facilitates the next step which involves the accurate orientation of the embryo in the paraffin wax.

## Orientation of embryos in paraffin wax prior to sectioning

In the embedding of particularly small embryos, it is advantageous to use a dissecting microscope as this greatly helps in the visualizing of the material. In addition, the availability of a pair of *heated* forceps (e.g. heated forceps with control unit, from Histolab-Cytolab, PO Box 101, Hemel Hempstead, Herts, UK) will also prove to be invaluable if minor adjustments are to be made to the position of an embryo within wax. It has been found that much less damage may be done to the embryo if its orientation is carried out under a heated lamp whose temperature is sufficient to allow the wax to be retained in a melted state throughout these critical stages of the embedding procedure. If the wax cools down and solidifies, then has to be melted down once more (and this may have to be repeated on several further occasions) until the embryo is satisfactorily orientated within the wax block, then damage inevitably results. The rule, then, is that the embryo should be manoeuvred as little as possible both prior to and once it is in the wax.

Rather conveniently, on most occasions, early "turned" embryos that have been isolated from within their extra-embryonic membranes automatically fall onto their side in the wax. It is then only necessary to establish in which direction the embryo is facing, and the location of its head and tail regions. Pins with different coloured heads may be used for delineating these three critical landmarks. When smaller embryos are being sectioned, similar principles apply, so that specific landmarks should be located and delineated as described above.

## The cutting of serial sections

Having carefully orientated the embryo in the paraffin wax, it is next necessary to mount the wax block onto an appropriate "chuck". It is firstly necessary to ensure that the long axis of the embryo is exactly horizontal when the block is mounted in the chuck, so that the sections through the cephalic and trunk regions will be symmetrical. When transverse sections are required, the embryo should be located in the block so that the cephalic region is sectioned first, with subsequent sections being directed towards the thoracic and finally the lower trunk region. When analysing individual sections, but more particularly when photomicrographs are published, it is the convention that on transverse sections the neural axis and dorsal part of the embryo always appears towards the top of the section. In order to achieve this end, it is convenient if the embryo is sectioned on its side in the "block" with its neural axis facing towards the operator, and ventral surface facing away from the operator. The right side of the embryo therefore is directed towards the upper surface of the block. The block should be trimmed to reduce the width of the wax ribbon and reduce the distance between consecutive sections, but taking care to ensure that an adequate gap still remains both between the sections on the ribbon, and between the rows of sections on the slide.

For most purposes, sections are cut at a nominal thickness of 7 or 8 μm. The ribbons of sections are mounted by floating them onto glass slides previously coated with glycerine/albumin placed on a hot plate. The latter may be prepared by saturating 10 ml of

**Table 2 Staining paraffin sections of embryos**

| | | |
|---|---|---|
| Dewax | Xylene | 5 min |
| Rehydration | 100% Ethanol | 5 min |
| | 96% Ethanol | 5 min |
| | 90% Ethanol | 5 min |
| | 70% Ethanol | 5 min |
| | Running tap water | Wash |
| Staining | Haematoxylin (Delafield's or Ehrlich's) | 10 min |
| Differentiate | Acid–alcohol (1% HCl in 70% ethanol) | 15–30 s |
| | Running tap water | Wash |
| Counterstain | Eosin (aqueous) | 5 min |
| Dehydration | Ascending alcohols to 100% ethanol | Rapid changes |
| Clearing | Xylene | 2 changes of 5 min |
| Mounting | Dammar xylene mounting medium | – |

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distilled water with flaked egg albumin. When this has been dissolved, add an equal volume of glycerine, and 0.1–0.2 g of sodium salicylate. A small drop of the albumin/glycerine is put on the slide with an equal volume of distilled water, and these are then mixed well together. This is smeared over the surface of the slide to produce a thin coating, and the excess removed. It is usual to place the first sections to come off the microtome at the top left-hand corner of the slide, with the last sections of the final ribbon towards the bottom right. This convention facilitates the subsequent examination of the tissues. To reduce the total number of slides required, particularly for the larger embryos, precleaned 76 × 39 × 1.2 mm glass slides are usually employed. While sections for histochemistry are usually dried in a 37°C incubator, all other slides are dried in an incubator at 60°C.

### The staining of paraffin sections with haematoxylin and eosin

Prior to staining, the sections are dewaxed in xylene for 5 minutes, then rehydrated through absolute alcohol, 96%, 90% and finally 70% alcohol (5 minutes each stage), then washed in running tap water. The sections should then be stained for 10 minutes in either Delafield's or Ehrlich's haematoxylin, washed in running tap water, and differentiated in acid–alcohol for 15–30 seconds. The sections are then washed once more in running tap water, then counterstained with eosin for 5 minutes. Following a final wash in running tap water, the sections are then dehydrated through ascending alcohols to absolute alcohol, care being taken to make sure that the eosin is not lost into the alcohol solutions. The sections are then cleared in xylene and finally cover slipped using Dammar xylene mounting medium (Raymond A Lamb, UK). The steps involved in staining paraffin sections with haematoxylin and eosin are summarized in Table 2. Although haematoxylin and eosin are probably the most popular staining combination for the analysis of serially-sectioned mammalian embryonic material, a wide range of alternative staining methods is available, particularly for the analysis of specific tissues, such as connective tissue or nervous tissue. For further details, the reader should consult one of the standard manuals of histological techniques (see, for example, Bancroft and Stevens (1982), Culling *et al.* (1985)). For histochemical stains, water-based mountants generally need to be employed.

# 3 GENERAL OBSERVATIONS ON THE STAGING OF POSTIMPLANTATION MOUSE EMBRYOS

### Terminology

The overall changes that take place during the early postimplantation period of all mammalian species are fairly similar, and a knowledge of the embryology of one should be an invaluable guide to the events that occur in other mammalian species. In the mouse, and in other rodents, but not in other mammals, inversion of the germ layers occurs, and attention will be drawn in Chapter 5 to the configurational changes that take place during the process of "turning" in these species. Similarly, substantial differences occur in the fine structure of the placenta between different mammalian species. This also applies to the relationship which exists between the extra-embryonic tissues and the embryo, and their individual functional roles. These aspects of comparative placentation, however, are beyond the scope of this Atlas, and the interested reader should refer in the first instance to one of the specialist texts which deals specifically with this topic (see, for example, Amoroso, 1952; Hamilton and Mossman, 1972; Steven, 1975).

The few reviews, books, and chapters in others, that deal specifically with the embryology of the mouse, namely Otis and Brent (1954), Snell and Stevens (1966), Rugh (1968, reprinted 1990) and Theiler (1972, reprinted with minor changes, 1989) and the rat (Keibel, 1937; Witschi, 1962; Hebel and Stromberg, 1986), are all useful to some degree, but are of only limited value when it is necessary to recognize specific structures, though they are more helpful when it is of value to assess the exact stage of development of an embryo. For this reason, it is hoped that sufficient information will be available in this Atlas for those who need to have a reasonably detailed knowledge of the development and histological features of the mouse, and with certain reservations, those of other related species. In Table 3, a comparison is made between the timing of equivalent stages of *early* embryogenesis in the mouse, rat and human embryos (see also Butler and Juurlink, 1987). A similar staging system also exists for the chick embryo (Hamburger and Hamilton, 1951).

If the staging system proposed by Theiler (1972, reprinted with minor changes, 1989) for the mouse is adopted, which is largely based on the classification of Streeter (1942) in his Developmental Horizons of Human Embryos, a classification which itself has recently been updated by O'Rahilly and Müller (1987), it is then possible to assign all pre- and early postimplantation mouse embryos to specific stages with relative ease. For mouse embryos, beyond about 14–15 days of gestation, and approximately equivalent to human fetal material of 8 or more weeks of gestation, the gestational age alone has been used as the simplest means of staging this material, until a more comprehensive classification system is developed for the more advanced stages of this species.

As will be clearly apparent from the brief synopsis of Stages 6–19 of Theiler's staging system provided in Table 3, all of the critical events described that occur up to about 11.5 days of gestation in the mouse may be readily recognized both in intact embryos and in sectioned material. No account, however, is taken of the changes that occur during, for example, cardiogenesis, nor of the differentiation of other organ systems such as the kidney or the lung, presumably because these are less readily apparent from a superficial examination of the embryo. In practice, it is probably more important to establish whether an embryo is morphologically normal or otherwise, and whether its degree of development is consistent with its gestational age, than assigning it to one specific stage or another. In order to make such an assessment, it is essential to have a thorough knowledge (i) of the normal morphological appearance (in gross terms, and at the histological level) of embryos at the various stages studied, and (ii) to be aware when specific developmental features are first likely to be encountered. The detailed 17-point scoring system that is used in teratological studies for analysing the morphological differentiation of early rat embryos devised by Brown and Fabro (1981; see also Brown, 1990) provides an objective means of evaluating this material, and could easily be adapted for the analysis of similar somite-stage mouse embryos. Similarly, it is also helpful to acquire an appreciation of the normal range of size both of intact embryos and of their component parts at different stages of embryogenesis. While the *crown–rump length* would appear to be a rather crude measurement, it does in fact provide a particularly useful means of evaluating whether a "turned" embryo, which may be morphologically normal or abnormal, displays evidence of moderate or severe degrees of growth retardation. Early "turned" diploid parthenogenetic and diandric triploid mouse embryos, for example, appear on gross inspection to be morphologically normal, but are invariably substantially smaller than normal (diploid) fertilized embryos at similar stages of development (Kaufman, 1983a; Kaufman *et al.*, 1989).

As in all biological systems, a limited degree of variability exists even in embryos encountered within a single litter (possibly by the equivalent of up to 6–12 h of growth between the least and the most advanced embryo). Similarly, embryos from different females that have been isolated at an identical gestational time may vary quite considerably in the stage of development achieved. An appreciation of the normal range is therefore of particular importance, and this can only be achieved from the analysis of large numbers of embryos.

In order to achieve a reasonable degree of uniformity, all of the serially-sectioned embryos analysed in the present study have been isolated from spontaneously cycling (C57BL × CBA)F1 hybrid females that had previously been mated to genetically similar F1 hybrid males. While autopsies were carried out at a variety of different times on days 4.5–12 of gestation, in order to obtain a representative selection of embryos which adequately demonstrate the major morphological features likely to be encountered during embryogenesis and the early stages of organogenesis, the isolation of developmentally more advanced embryos was usually carried out during the morning on each successive day of gestation. Similarly, while the embryos isolated from randomly bred mice or from different inbred or F1 hybrid strains of mice may vary slightly in the degree of development achieved at a specific time postconception, for most purposes such differences are of little consequence during the second half of gestation. As indicated above, this degree of interstrain variation is usually no greater than the difference observed between the least and the most advanced embryos present within a single litter, or between litters isolated from different females of the same strain isolated at a similar time postconception. Furthermore, since the morphological differences observed between individual strains are generally so small, no difficulties should be encountered in the analysis of embryonic material isolated from genetically dissimilar strains of mice to those analysed in the present study.

Whereas in the analysis of human and other mammalian embryos, the *crown–rump* length is a commonly employed parameter, it has recently been