Computer-aided 3-D reconstruction of serially sectioned mouse embryos: its use in integrating anatomical organization

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ABSTRACT This paper reviews recent work on a project that uses a computer-aided approach for making 3-D reconstructions of serially sectioned mouse embryos (the *digital mouse*). The captured images are aligned using a *warping* program so that almost perfect alignment of adjacent sections is achieved with minimal deformation. The sections that are viewed on the computer screen are in fact computer-generated grey-level images with a resolution of about 10 μm. The reconstructed embryo may then be resectioned in any plane to simulate as near as possible an exact match on the computer screen to the viewer's own material. Individual anatomical domains may then be *painted* in different colors, and these domains may be selected by querying the textual database containing anatomical and other information. Further, it is now possible to generate 3-D images of individual anatomically-discrete components or related sets of components of a particular system in isolation from the rest of the embryo, or, if required, against a 'ghost-like' image of the intact embryo, or specific parts of an embryo. In the article, examples are given of the use of the system in interpreting the vascular, gut and paraxial mesoderm systems, while both the advantages and disadvantages of this approach are also discussed. The eventual aim will be to provide 3-D reconstructions of mouse embryos from fertilization up to 14 days *post coitum* of development. When completed, this project will allow the accurate spatial mapping of gene-expression and cell lineage data onto the digital *Atlas* of normal mouse embryonic development.

KEY WORDS: *3-D reconstruction, 9-day mouse embryo, Theiler stage 14 embryo, anatomical domains, anatomy database*

Introduction

For those who are interested in analyzing the normal sequence of events taking place during the early postimplantation period and throughout organogenesis in mammals, but more particularly in understanding the genetic control of morphogenesis, it is essential that appropriate means be available to allow the detailed morphology of these stages of development to be examined at the gross, histological, cellular and ultrastructural level.

Clearly, the analysis of the gross morphology of recently isolated intact rodent embryos under the dissecting microscope represents the starting point for most researchers who wish to study the factors that influence the normal development, and the mechanism of induction of morphological abnormalities whether experimentally or congenitally produced.

Of the various techniques that are now routinely used in the molecular analysis of development, *in situ* hybridization to mRNA and the analysis of Lac Z-stained whole-mount preparations of transgenic embryos have proved to be an extremely useful and instructive first step towards understanding the genetic factors that influence early mammalian development, as they allow organs where gene activity is occurring to be readily recognized. This approach may then be followed up by the histological analysis of a specific organ, or region(s) of the embryo, in order to determine in more detail where a particular gene product is localized. Such an approach, while entirely logical, does require the researcher to have a detailed knowledge of the normal histological morphology of the region under investigation. It should, however, be said that because current emphasis has shifted from the morphological to the genetic basis of development, this detailed knowledge is not as widespread as it might be.

In the almost complete absence of adequate training in the interpretation of histological sections of early mammalian embryos, the only way that a conscientious researcher can now

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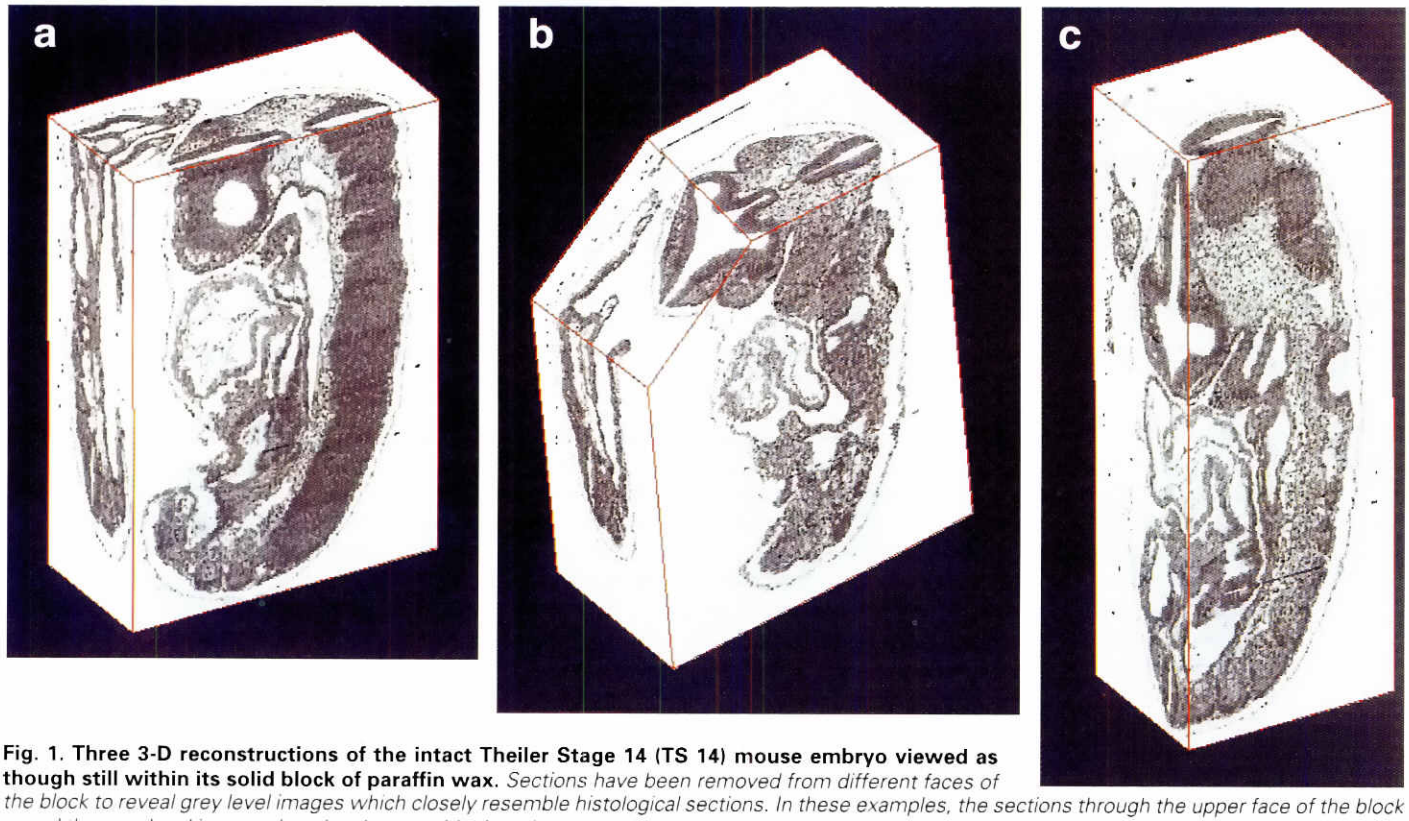

Fig. 1. Three 3-D reconstructions of the intact Theiler Stage 14 (TS 14) mouse embryo viewed as though still within its solid block of paraffin wax. *Sections have been removed from different faces of the block to reveal grey level images which closely resemble histological sections. In these examples, the sections through the upper face of the block reveal the grey level images that closely resemble the orientation of the original transverse sections that were captured, aligned and warped, being the first stage of the 3-D reconstruction exercise.*

gain expertise is to consult either an acknowledged authority in this area or, if the latter is not readily available, the appropriate text-books (for mouse, see Kaufman, 1992; see also, Rugh, 1968; Theiler, 1989; for rat, see Hebel and Stromberg, 1986; for human, see Gasser, 1975; O'Rahilly and Muller, 1987) which provide illustrations of representative sections of embryos of the same, or nearly similar, species, from similar stages of development to those under investigation. The researcher then attempts to match, as best he can, his sections with those illustrated in the text-book. This can clearly be a useful exercise, but is often of only limited value if the slides of the researcher do not mesh with those that are illustrated, and this is usually the case because their material is likely to be sectioned in an oblique plane, rather than in the perfectly transverse, coronal or sagittal plane that should be found in an ideal text-book illustration.

The problem lies not with the histologist, but in the nature of the mammalian embryo at any stage of development beyond the early somite stage, which has a complex and asymmetrical shape, invariably curved in different directions along its embryonic axis. Accordingly, while the orientation of serial sections through such an embryo may be symmetrical, and in the transverse plane in the thoracic region, they will almost certainly section the caudal part of the neural axis, for example, in the distal part of the tail region, in an oblique plane; similarly, other isolated regions of the same embryo may be sectioned in the coronal, or even in the sagittal, plane. Clearly, a more sophisticated methodological approach is essential to assist those that have difficulty in the interpretation of their histological sections.

The digital mouse

The aim of the present review is to consider one computer-based approach in which a lack of familiarity with the detailed morphology of a particular stage of development should not preclude the researcher from interpreting, with a high degree of confidence, the significant features of their serially sectioned material. While this approach would, one hopes, only be necessary as a stop-gap solution, it might encourage those in this position to undertake the essential background reading required to increase their knowledge of early mammalian development. A description of the reconstruction and painting technology that underlies this approach, and a detailed description of the reconstructed 9 day p.c. mouse embryo will be published elsewhere.

This approach, which uses computer programs developed at the MRC Human Genetics Unit in Edinburgh, has now allowed the complete sequence of serially sectioned somite-stage mouse embryos, a subset of which was previously used to illustrate *The Atlas of Mouse Development* (Kaufman, 1992), to be digitized, and the stored images interactively aligned with a 3-D resolution of about 10 μm, thus allowing detailed tissue organization to be seen. Most importantly in the present context, sections can be constructed through this digital embryo which may be cut at any arbitrary orientation or position required. One of the advantages of this technique is that it allows grey-level images to be produced on the computer screen which very closely resemble those seen in the researcher's own material, assuming that both the computer-generated histologi-

cal sections and the material under examination are at similar stages of embryonic development.

An important component of the research presently being undertaken in Edinburgh involves the *painting* of each of the anatomically-defined tissues (i.e. the delineation of their borders) seen in the histological sections, so that when this component of the exercise is eventually completed it should be possible for an enquirer who is scanning through the sections on the screen to request that each of these tissues be delineated and displayed independently, together with its name and some essential information (see below). Once this level of tissue discrimination is achieved, it should then be possible to view, in a 3-dimensional format, each of the constituent components of the embryo either in isolation or in combination with other "background" tissues or organ systems. The eventual aim will be to provide such 3-D reconstructions of mouse embryos from fertilization up to 14 days *post coitum* of development.

This methodology is presently being continuously upgraded with the assistance of a team of computer scientists from a number of countries in Europe. The eventual aim of this component of the project is to produce a graphical, user-friendly anatomically-based database of mouse development which may then be used to store, in readily retrievable graphical form, gene-expression domains, gene-trap and lineage data, etc, and to link this to molecular sequences, and information on gene function. This graphical database will link to a textual database of gene expression which is being compiled by the Jackson Laboratory, Maine, USA (see Ringwald *et al.*, 1994). To quote Baldock *et al.* (1992), "*... once the mouse embryonic anatomy and the expression data have been stored, it will be relatively easy to use this information to investigate the molecular basis of tissue differentiation, the key question in contemporary developmental biology*". At some later date, it may be possible to establish a complementary database from which information on mutants and their phenotypes might also be readily retrievable. This will rely on developments in the modeling of 3-D shapes to enable the recording and comparison of their statistical variation. Visualization and browsing of the mutant and time-varying data will be further enhanced by using 3-D "morphing" techniques to show the developmental process, and by the opportunity to link this database with others available over the Internet.

The anatomy database

As an essential component of this exercise, a systematic anatomical nomenclature has been developed that will, in due course, link directly with the spatial co-ordinates of the digital *Atlas*. It will also provide, in addition to a list of the anatomically-defined tissues present at each stage of development, an indication of the lineage of each of these tissues, and their relationship to each other. The anatomical database covering the entire gestation period of the mouse gradually evolved from the index for *The Atlas of Mouse Development*. A reasonably complete list of all of the major anatomically-defined tissues has now been prepared for each of the 26 Theiler stages (TS) of mouse development, with an indication of at which stage they can first be seen. The terminology used is based on the English version of the *Nomina Anatomica* (and is complemented, where appropriate, by information on the commonest synonyms used).

The number of anatomically-discrete tissues recorded in the index increases rapidly during development, from as few as 6 named tissues at TS 4 (3 days p.c.), to approximately 175 at TS 16 (10 days p.c.), rising to over 800 at TS 26 (17.5 days p.c.). This database of anatomical nomenclature, which provides a description of the standard or normal mouse embryo, is now in the process of being tested.

It is appreciated, however, that embryos at exactly the same *temporal* age are not necessarily uniform in their *developmental* age, and often show some degree of natural variation. The stage at which a tissue first appears often depends on how closely an histological section is examined, and different microscopists may use different criteria for establishing whether a particular tissue is present, or has yet to appear. The staging of tissues, accordingly, has a subjective element to it.

Computer-aided 3-D reconstruction and arbitrary sectioning of mouse embryos

Once all the sections of an embryo have been digitized, the data can be envisaged as forming a 3-D block, or digital mouse: from this, using appropriate programs, it is possible to abstract planes of data in any orientation, and these 2-D images are, of course, equivalent to sections. In the example to be illustrated here, all 307 sections from a Theiler Stage 14 mouse embryo, 30 representative transverse sections from which had formerly been published as Plates 18a and 18b in *The Atlas of Mouse Development* (Kaufman, 1992), were captured and digitized. Using appropriate methodology specially developed for this purpose, the sections were then subjected to an aligning and *warping* program. The required end-point of the processing is a representative mouse embryo which can serve as a standard for mapping gene-expression information (Guest, 1994; Guest and Baldock, 1996).

In Figure 1, a 3-D reconstruction of the intact Theiler Stage 14 embryo is shown as though still within its solid block of paraffin wax. Sections have been removed from different faces of the block, either in conventional orthogonal (i.e. transverse, sagittal or coronal) planes, or in arbitrary planes, to simulate as near as possible an exact match on the computer screen with the viewer's own material, as observed in parallel down their own microscope. The grey-level images seen on the screen closely resemble histological sections, and may be viewed either in black and white or in color if some or all of the individual anatomically-discrete tissues have been *painted* (see below) and color-coded.

The *painting* of discrete anatomical domains

Constant reference to each of the original sections under the microscope is necessary in order to check the accuracy of the *painting*, a tedious exercise. Following sequential images on the screen, all of which have been carefully aligned during the *digitizing*, *stacking* and *warping* exercise, substantially reduces the time taken to do this necessary task.

One, perhaps unexpected, aspect of this digital mouse is that viewing the *painted* images on the computer screen demonstrates that certain histological features which were not easily seen when the original hematoxylin and eosin sections were scanned, now appear more clearly delineated. This is particularly evident when viewing the cephalic mesenchyme much of which appeared to consist of relatively homogeneous originally pink-stained tissue. Once the cephalic mesenchyme had been *painted* one particular color, the neuroepithelium of the primitive brain vesicles another

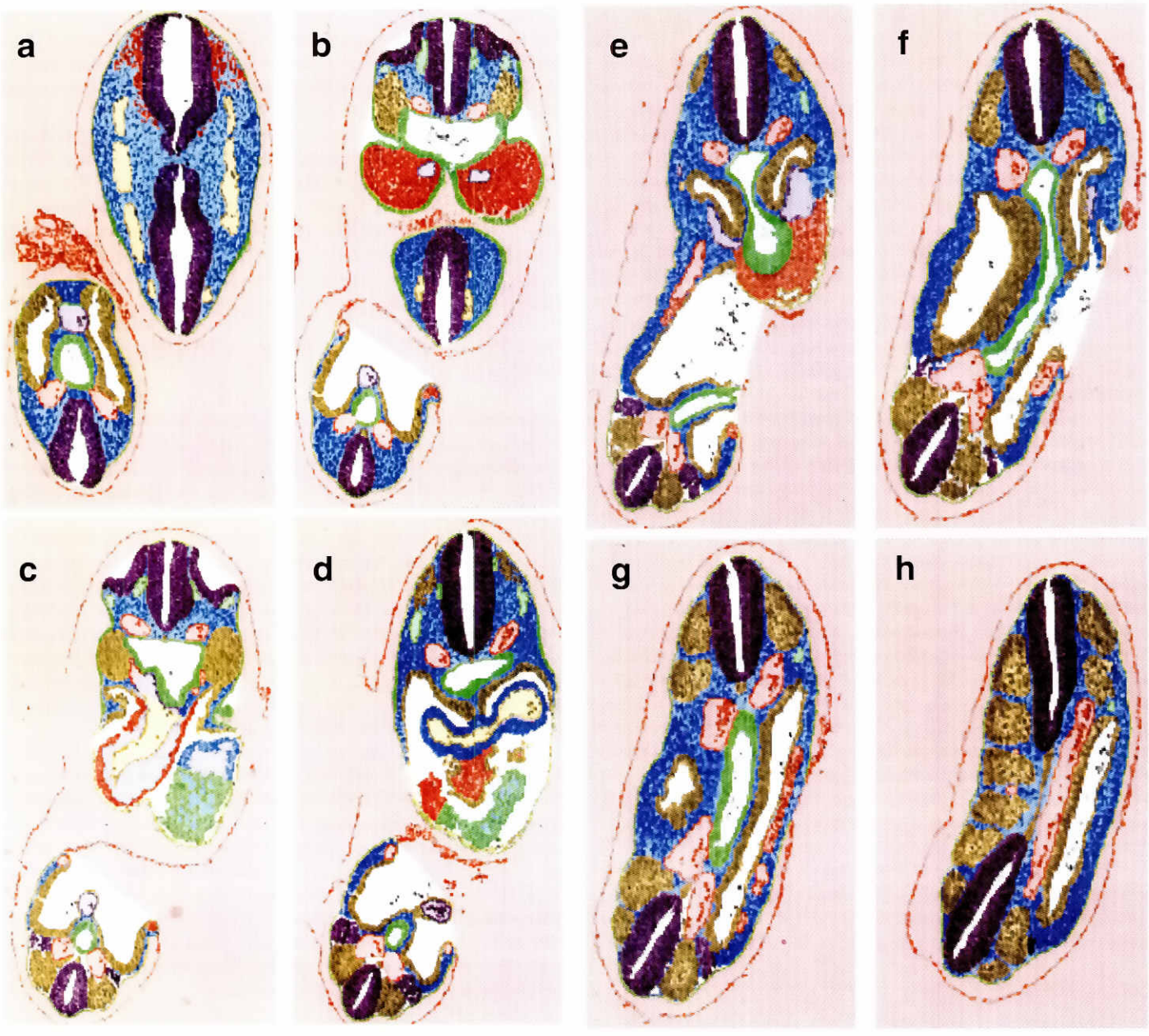

Fig. 2. Eight representative grey-level images that closely resemble the orientation of the original transverse sections that were *captured*, aligned and *warped*. *The individual anatomically-discrete domains have been painted in five different colors, this being the total number of colors available in this prototype program. The grey-level images illustrated correspond to sections 75* **(a)**, *131* **(b)**, *148* **(c)**, *198* **(d)**, *219* **(e)**, *244* **(f)**, *253* **(g)** *and 264* **(h)**, *out of the total of 307 sections that were originally captured".* **(Key to a)**: *blue, mesenchyme; violet, neuroepithelium, endothelium of vitelline artery; red, neural crest, endothelium of dorsal aortae, extraembryonic tissues (amnion, allantois); emerald green, gut endothelium, surface ectoderm, endothelium at origin of umbilical artery; yellow/khaki green, endothelium of primary head veins, coelomic epithelium.* **(Key to b)**: *blue, mesenchyme; violet: neuroepithelium, otic placode (otic pit), endothelium of vitelline artery, endothelium of first branchial arch artery; red, endothelium of dorsal aortae, amnion, endothelium of umbilical veins, first branchial arch (mandibular component); emerald green, gut endothelium, surface ectoderm, endothelium of anterior cardinal veins; yellow/khaki green: endothelium of primary head veins, coelomic epithelium, second branchial arch, notochord.* **(Key to c)**: *blue, mesenchyme, wall of common atrium, cardiac jelly (primitive ventricle); violet, neuroepithelium, otic placode (otic pit), endothelium of vitelline artery, intermediate plate mesoderm (medial: nephrogenic cord; lateral, precursor of pronephric duct), cardiac jelly (common atrium, outflow tract), endothelium of first arch artery; red, endothelium of dorsal aortae, amnion, endothelium of umbilical veins, wall of outflow tract of heart, endothelium of second arch artery; emerald green, gut endothelium, surface ectoderm, endothelium of anterior cardinal veins, wall of primitive ventricle; yellow/khaki green: coelomic epithelium, second branchial arch, somites, endothelium of outflow tract of heart, notochord.* **(Key to d)**: *blue, mesenchyme, wall of common atrium, cardiac jelly (primitive ventricle); violet, neuroepithelium, endothelium of vitelline artery, intermediate plate mesoderm, cardiac jelly (common atrium); red, endothelium of dorsal aortae, amnion, endothelium of umbilical veins, wall of outflow tract of heart, septum transversum; emerald green, gut endothelium, surface ectoderm, endothelium of anterior cardinal veins, wall of primitive ventricle; yellow/khaki green, coelomic epithelium, somites, endothelium of primitive atrium, notochord.* **(Key to e)**: *blue, mesenchyme; violet, neuroepithelium, intermediate plate mesoderm, endothelium of sinus*

color, and the prospective optic region of the prosencephalon a third color, each on the basis of their anatomical features, the location of the various populations of cephalic neural crest cells became emphasized: not only were the trigeminal (V) and facial (VII) crest cell populations clearly seen, but also the trigeminal-derived perioptic crest cells that later differentiate to form the sclera of the eye. Similarly, the *painting* exercise tended to emphasize the morphological features of the neural crest-derived cells that were still in the process of migrating into the first and second branchial (pharyngeal) arches.

Examples are provided here of a series of eight representative transverse grey-level images through the TS 14 mouse embryo, sectioned in a similar plane to those previously illustrated in *The Atlas of Mouse Development* (Fig. 2a to 2h), to demonstrate the value as a teaching aid of the labeled images, where all of the anatomically-discrete tissues have been individually *painted*. Because of the limited number of colors available in the basic *painting* program at one time (only five colors are available in this prototype program), adjacent anatomically-discrete tissues cannot always be discriminated on the basis of their color alone. However, individual anatomical domains may be selected by querying the textual database. A key is provided in the legend for these images that indicates which color was used to "paint" each anatomical domain.

However impressive these color illustrations may appear to be in the figures, the detail seen is even further enhanced when the images are viewed on the computer screen, as it is then possible to alter the intensity of both the grey-level image and the superadded color. By modifying these two parameters independently, it is then possible to highlight the boundaries of the specific tissue under examination, against those of the adjacent tissues.

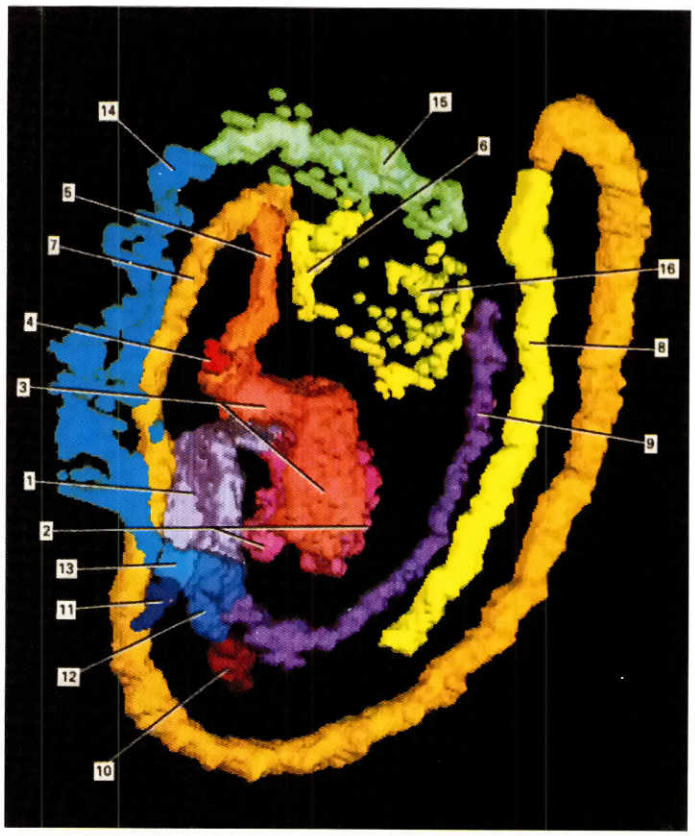

Fig. 3. 3-D reconstruction of the endothelial lining of the heart and right side of the vascular system of the TS 14 embryo. *Heart: (1) primitive atrium; (2) primitive ventricle; (3) bulbus cordis and outflow tract. Outflow vessels (arterial system): (4) second arch artery; (5) first arch artery; (6) internal carotid artery; (7) dorsal aorta; (8) vitelline artery. Inflow vessels (venous system): (9) umbilical vein; (10) vitelline vein; (11) posterior cardinal vein; (12) sinus venosus; (13) common cardinal vein; (14) anterior cardinal vein; (15) primary head veins; (16) vessels connecting branches of internal carotid artery with primary head veins.*

Three-dimensional reconstruction of embryos and anatomically-defined components of embryos that have previously been serially sectioned and the images captured on a computer

The exercise of reconstructing, in 3-dimensions, intact embryos, and isolated components of embryos, that have previously been serially sectioned can be extremely informative. In the example illustrated here, the accurate alignment of the total of 307 transverse sections that comprised this embryo has now allowed it not only to be serially sectioned in any plane (see above), but, following the *painting* of its individual components, has allowed them to be visualized, either in isolation or in the context of other components of this embryo. Further, because of the nature of the *warping* exercise, the 3-D images that are generated using this technique very accurately reflect the configuration of these components as they might have been observed were it technically possible to dissect them out in isolation from the original embryo, but without them being deformed by the dissection process.

The ability to scan not only the grey-level images of the original transverse sections that have been *captured*, but also the computer-generated sagittal and coronal sections, as well as, where appropriate, less standard planes of sectioning, greatly assists the labeling (or *painting*) exercise, as well as facilitating the understanding of complex relationships in the early embryo.

Using serial sections of a Theiler stage 14 mouse embryo with about 15-20 pairs of somites, a reasonably competent viewer

venosus; red, endothelium of dorsal aortae, amnion, endothelium of umbilical vein, septum transversum; emerald green, gut endothelium, surface ectoderm, endothelium of anterior cardinal vein; yellow/khaki green, coelomic epithelium, somites, notochord. **(Key to f)***: blue, mesenchyme; violet, neuroepithelium, intermediate plate mesoderm, endothelium of common cardinal vein; red, endothelium of dorsal aortae, amnion, endothelium of umbilical vein; emerald green, gut endothelium, surface ectoderm, endothelium of anterior cardinal vein; yellow/khaki green, coelomic epithelium, somites, notochord.* **(Key to g)***: blue, mesenchyme; violet, neuroepithelium, intermediate plate mesoderm; red, endothelium of dorsal aortae, amnion, endothelium of umbilical vein; emerald green, gut endothelium, surface epithelium, endothelium of anterior cardinal vein; yellow/khaki green, coelomic epithelium, somites, notochord.* **(Key to h)***: blue, mesenchyme; violet, neuroepithelium, intermediate plate mesoderm; red, endothelium of dorsal aortae, amnion, intersegmental arteries; emerald green, surface epithelium, endothelium of anterior cardinal vein; yellow/khaki green, coelomic epithelium, somites, notochord.*

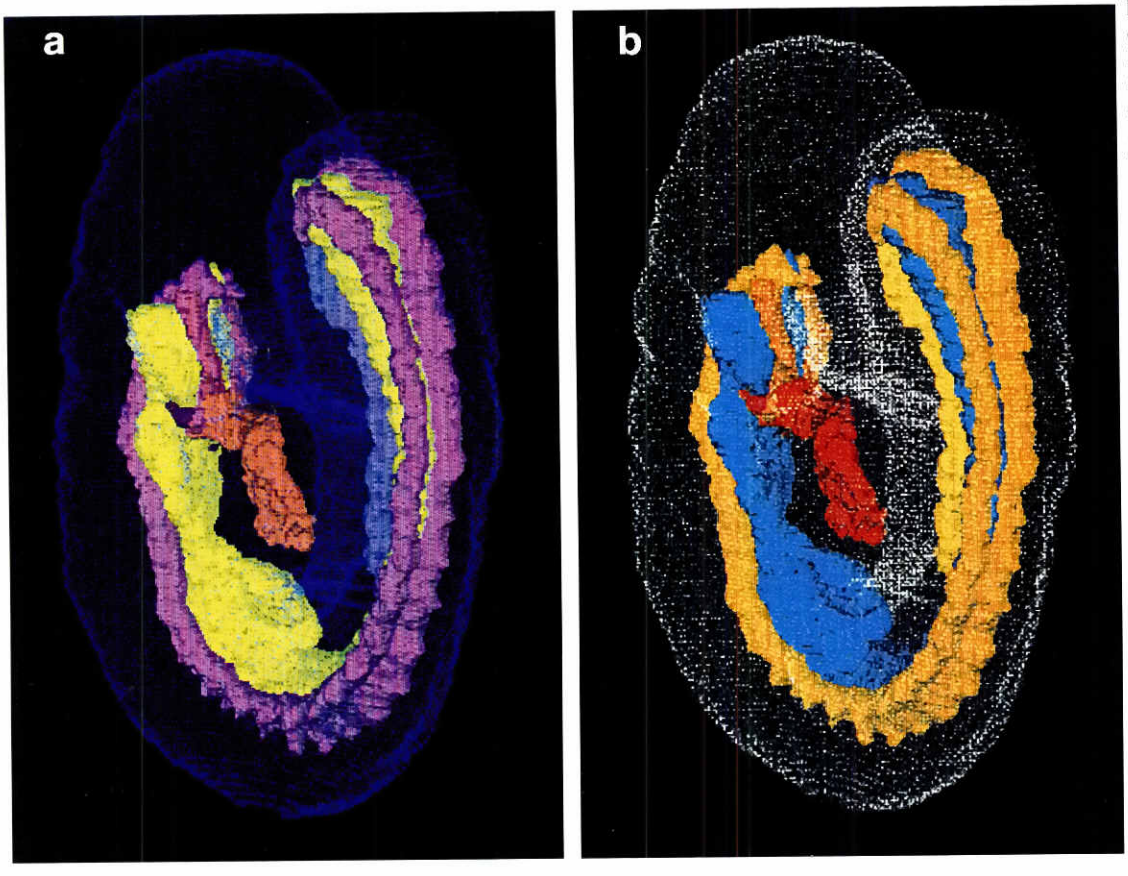

Fig. 4. 3-D reconstructions of the outflow tract of the heart, the first and second arch arteries, the paired dorsal aortae and the umbilical artery, to show their relationship to the primitive alimentary system. *Note in particular the relationship between the first arch artery, which arches over the first pharyngeal pouch before it joins the dorsal aorta, and the proximal part of the second arch artery which passes dorsally below the first pharyngeal pouch but, in this embryo, has yet to reach the dorsal aorta. A dilatation in the pharyngeal region of the primitive foregut just caudal to the second arch artery is the first indication of the second pharyngeal pouch. The proximal parts of the segmental branches of the dorsal aortae are also clearly seen. A faint 'ghost-like' image of the outline of the intact embryo has been included for* **(a)**. *If the density of the outline is increased* **(b)**, *though useful for certain purposes, this tends to obscure the image under analysis.*

Tissue	*Figure 4a*	*Figure 4b*
alimentary tract	*yellow*	*bright sea green*
outflow tract of heart	*pale orange*	*brown/sienna*
second arch artery	*magenta*	*brown/sienna*
first arch artery	*dark pink*	*orange*
dorsal aorta	*pale magenta*	*dark yellow*
vitelline artery	*pale violet*	*yellow*
outline of embryo	*dark blue*	*white*

should have little difficulty in gaining an accurate impression of the 3-dimensional form of, for example, the central nervous system (the cephalic and axial components of the neural tube at this stage of development), from a brief scanning of the serial sections. Equally, there should be little difficulty in assessing the general configuration of the primitive gut tube (though the pharyngeal region of the primitive foregut is becoming increasingly complex even at this stage of development). However, the configuration of other components of the embryo, such as the detailed anatomy of the vascular system, are more complex, and consequently pose much greater problems for the casual viewer. Even the most experienced embryologist who is familiar with this stage of development could only hope to gain a general rather than a detailed picture of such a system from the sections alone. Even for such an experienced embryologist, there are certain features that he is unlikely to be able to accurately reconstruct in 3-dimensions in his own mind during the visual scanning of serially sectioned material e.g. the migration of the neural crest, the relationship between the arterial and venous components of the vascular system, etc.

The use of 3-D reconstructions clarifies many of these problems, and here we consider, as an example, the vascular system. The preparation of 3-dimensional reconstructions of the primitive vasculature of embryos at this stage of development (Fig. 3) reveals that the analysis of the anatomical subdivisions of the heart and its inflow and outflow tracts is not the straightforward exercise one might expect. In relation to the inflow tract, there are no obvious morphological boundaries at this stage that would, for example, allow the distal (ie. caudal) part of the anterior cardinal vein to be distinguished from the proximal part of the common cardinal vein (the duct of Cuvier) which in turn (with the umbilical vein) drains directly into the sinus venosus. While it is possible to draw a reasonably sharp boundary between the sinus venosus and the common atrial chamber, in reality they are continuous structures. All anatomical subdivisions of the vascular system are, of course, artificial, but it is clearly essential to create recognizable subdivisions when considering the developmental fate of its different parts. When these discrete components are appropriately color-coded on the transverse sections, then viewed on, for example, the computer-generated sagittal sections, or on the 3-D reconstructions, it becomes possible to check not only whether the labeling has been accurately undertaken, but also to establish whether it provides an informative representation of the system or anatomical domain under analysis.

In relation to the venous drainage of the cephalic region, it is only when these vessels have been appropriately *painted* and their 3-

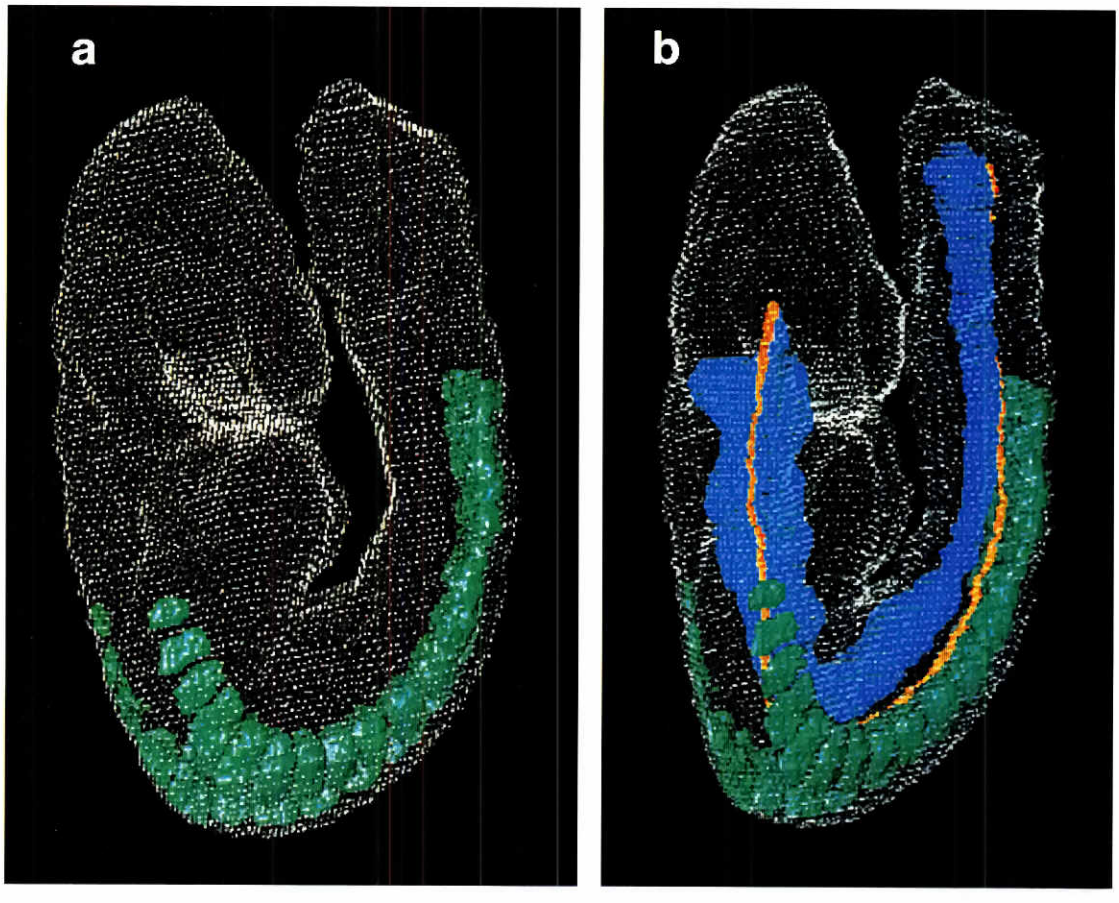

Fig. 5. Right postero-lateral view of a 3-D reconstruction of the somites (green) of the TS 14 embryo displayed against a 'ghost-like' image of the intact embryo. (a) *Slightly more posterior view of this embryo with, in addition, the inclusion of the 3-D reconstruction of the primitive alimentary system (sky blue) and notochord (orange)* **(b)**.

D configuration ascertained can one see that, even by this stage of development, the rostral part of the anterior cardinal venous system is already divisible into two major components: the primary head veins that clearly drain into a single large venous trunk (which corresponds with the internal jugular vein), and the lower and more lateral part of the cephalic region that drains into a smaller venous channel (the precursor of the external jugular vein).

With regard to the outflow tract of the heart, the situation is equally complex, and emphasizes the fact that the earlier descriptive subdivisions of this region into bulbus cordis, truncus arteriosus and, more distally, the aortic sac are difficult to reconcile with the lack of boundaries seen in serially sectioned embryos. Accordingly, the global term 'outflow tract' is now commonly employed to cover certainly the first two of these three subdivisions. The first branchial arch artery is also particularly well seen, as is the proximal part of the second arch artery which has yet, in this embryo, to make contact with the dorsal aorta. If certain components of the vascular system, such as the outflow tract, the first and second arch arteries and the dorsal aortae, and the primitive alimentary system are selectively displayed, the relationship between the first and second arch arteries and the first pharyngeal pouch is particularly clearly seen (Fig. 4a and b). The presence of a faint 'ghost-like' background of the intact embryo is also useful in that it enables the system under investigation to be visualized in the context of the whole embryo (Fig. 4a). Clearly, the viewer would need to balance the density of the outline, otherwise the image under analysis could become obscured (Fig. 4b).

While no difficulty should be encountered in recognizing the somites on individual histological sections, because of their characteristic location and form, it is not technically possible for even the most experienced of embryologists to establish, from even the most careful analysis of serially sectioned material, which is, for example, the fifth, seventh or ninth somites. Once the somites have been reconstructed in 3-D, however, they may be visualized either in isolation against a 'ghost-like' outline of the intact embryo (Fig. 5a), or they may be displayed to show their relationship to the primitive alimentary system and the notochord (Fig. 5b), or in relation to any other system that may be relevant. Furthermore, and in addition to the two views of the embryo displayed in Figure 5a and 5b, the embryo may be rotated and viewed in any other orientation if this helps to establish the relationship between the different structures being studied.

As a further example of the flexibility of this system, it is possible to combine the 3-D reconstruction and analysis of a number of anatomically-discrete systems with 2-D histological sections taken at any level and in any orientation through the embryo. Two examples are provided of this facility, where 3-D reconstructions of the neural tube, the somites and the alimentary system are displayed with a single, approximately transverse, section through the mid-thoracic region and tail. An approximately frontal view of this embryo is illustrated in Figure 6a, and a right-lateral view is shown in Figure 6b. This approach would therefore, for example, allow the determination of a specific somite on an individual histological section. The optic

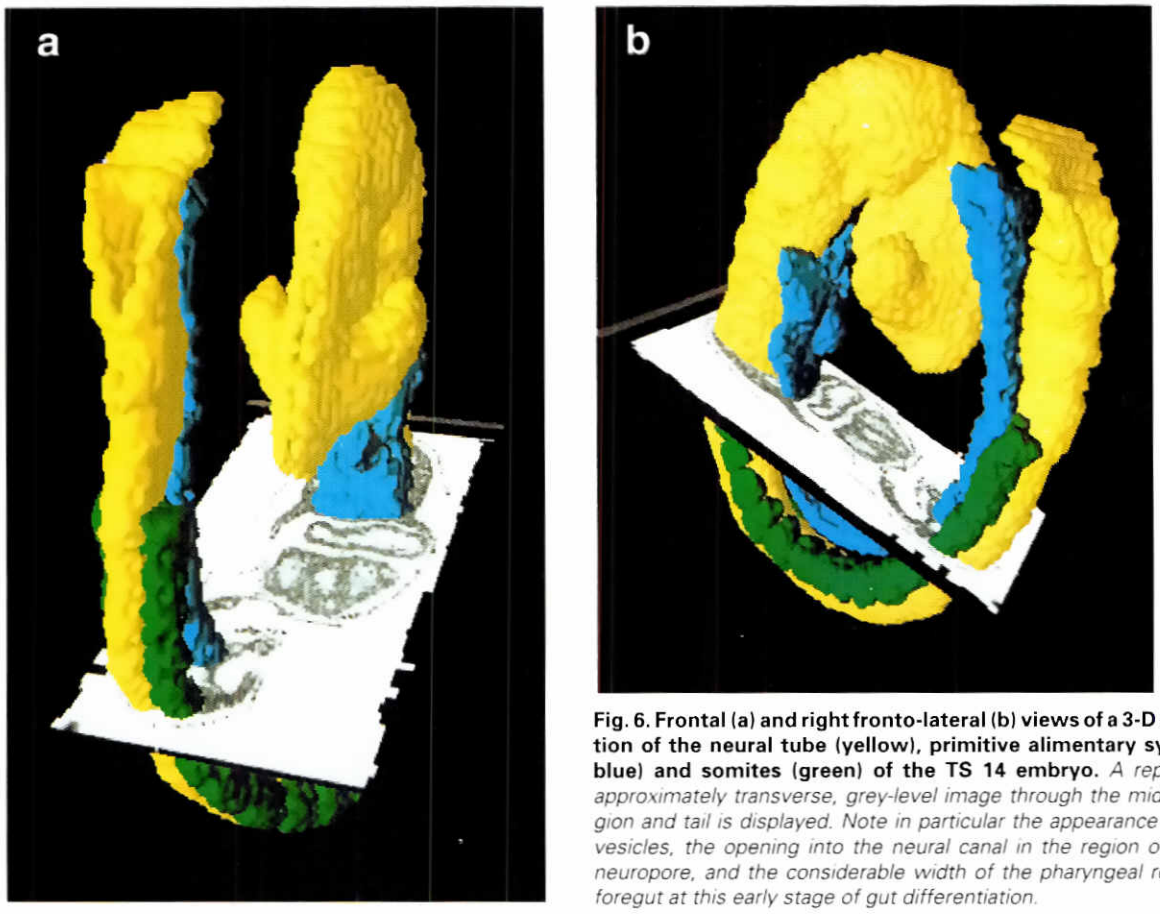

Fig. 6. Frontal (a) and right fronto-lateral (b) views of a 3-D reconstruction of the neural tube (yellow), primitive alimentary system (pale blue) and somites (green) of the TS 14 embryo. *A representative, approximately transverse, grey-level image through the mid-thoracic region and tail is displayed. Note in particular the appearance of the optic vesicles, the opening into the neural canal in the region of the caudal neuropore, and the considerable width of the pharyngeal region of the foregut at this early stage of gut differentiation.*

vesicles and the caudal neuropore are particularly clearly seen, as is the width of the primitive alimentary system at this early stage of its differentiation.

One additional, and quite remarkable feature of the system that cannot be adequately demonstrated here, is that computer graphics technology combined with 3-D glasses allows the viewer to see the system under analysis in three dimensions.

Discussion

The advantages and limitations of the methodology described here

As emphasized in a section above, there are considerable advantages to be gained from the ability to generate 3-D images of individual, anatomically-discrete components, or related sets of components, of a particular system in isolation from the rest of the embryo, or, if required, against a 'ghost-like' image of the intact embryo, or specific parts of an embryo. In the example used to illustrate this point, 3-D reconstructions of the arterial and venous components of the vascular system of the TS 14 mouse embryo are shown as though completely isolated from the rest of the embryo, with each anatomically-defined (and named) component of the system individually color-coded. Their relationship to the 'inflow' tract of the primitive heart (the vessels that flow into the primitive atrium via the two horns of the sinus venosus – such as the common cardinal and umbilical veins) and 'outflow' tract of the primitive heart (the ventral aorta with its various branches) is clearly displayed; furthermore, the detailed anatomy of the first pair of branchial (pharyngeal) arch arteries that flow into the dorsal aortae, and the second branchial arch arteries, which are seen to be growing towards the dorsal aortae, but have yet to make contact with them, is particularly distinct. The latter relationship is normally difficult to discern, even from a detailed scanning of the original histological sections.

It is suggested that, however experienced the viewer, he would not be able to obtain such an accurate impression of the detailed architecture and ramifications of even this relatively straightforward system using standard microscopy. While it may be possible to gain a *reasonably* accurate impression of the overall shape of the vascular system in very early limb-bud stage embryos, it would certainly not be as accurate as the reconstructions illustrated here, nor would the *proportional size* of its various components be as accurately envisaged as that displayed in these computer-generated images.

Two other unrelated examples can also be given that demonstrate the additional perspective provided by these reconstructions. The first relates to the recognition of individual somites. This is a relatively straightforward exercise when each of the somites is appropriately *painted* on the serial sections, and the whole reconstructed in 3-D. This may be of critical importance if gene expression at a particular stage of gestation is confined to a single or a group of somites or where, for example, there is progressive gene

expression in successive somites. This may be the case, for example, in relation to those somite-derived cells that are destined to enter the forelimb, and from which the limb musculature will in due course differentiate. The second is the rhombomere system, though this is not illustrated in this article. When the hindbrain of TS 14 embryos is reconstructed in 3-D, it becomes possible to recognize, and appropriately designate, each of the individual rhombomeres. This is particularly timely now that so much information is accruing in relation to the fate of the cells derived from these specific regions of the hindbrain (Ganju *et al.*, 1994). In contrast, it is certainly not possible to recognize individual somites, and very difficult to determine the boundaries of most of the rhombomeres from even the most careful analysis of transverse serially sectioned material.

The system does, however, have its limitations. Clearly, the first of these must be that the digital *Atlas* is based on the histological features of a single mouse embryo which, while it may be representative of a group, cannot be expected to demonstrate, or even be expected to indicate the incidence of the anatomical variations commonly encountered in the general population, or even between individuals within a single litter. While this may not present a major problem when studying the relatively advanced embryonic stages which are presently being digitized, it may become a significant problem when considerably earlier stages of development are digitized.

Individual embryos which from a detailed examination of their external anatomical features fall naturally into a specific Theiler Stage, will also inevitably vary to some extent in relation to the degree of differentiation of their component systems. Equally they may vary to some extent due to secondary problems associated with fixation or subsequent processing of the material. This is particularly likely to be the case when analyzing cryosectioned material, where the quality of the histology is ofter poorer than that obtained from paraffin- or plastic-embedded material. Similarly, because there are temporal discontinuities between sequential Theiler Stages, it is inevitable that researcher's material will not exactly match those sections displayed in the digital *Atlas*. Under such circumstances, it is essential that those who wish to insert new gene-expression data into a gene-expression database should select the most appropriate digital embryo from the available series, the one that they believe represents the best fit.

The idiosyncratic features of certain inbred strains of mice are also relatively well known and add a further complexity. Difficulties are encountered, for example, in staging early post-primitive streak-stage embryos because of significant strain differences in the timing of the first appearance of the allantoic bud. In (C57BLxCBA) F1 hybrid mice, favored by many researchers, the allantoic bud is first seen at the late streak stage (now termed TS 10.5) (Theiler, 1972; Kaufman, 1992). In the PO outbred strain, by contrast, the allantoic bud does not appear until somewhat later (in fact during TS 11, if the original criteria used by Theiler (1972) to classify these stages is strictly adhered to) (Downs and Davies, 1993) (K. Lawson, personal communication).

Similar examples from adult human anatomy abound: in the renal system, for example, major variations from the normal arrangement are relatively commonly encountered. Such variation clearly poses similar problems when learning human anatomy from a computer package or even many text-books, unless attention is drawn to the range and incidence of the common variations encountered in the population. This should not be a problem if the digital mouse *Atlas* is used to assist in the recognition of anatomical structures in early mammalian embryos, where major variations from the normal arrangement are unlikely to be encountered in most strains of mice. At this early stage in the project, the digital *Atlas* of *normal* mouse embryos cannot be expected to cope with pathologically abnormal embryos, or those isolated from stocks bearing induced genetic mutations. In the future, when more experience is available, it might be possible to expand its use for this purpose. It is likely, however, that this information could be included in a database of mouse developmental anatomy, possibly as an appendix which could include information, for example, on the range of abnormalities commonly seen in strains of mice carrying null mutations.

Gene-expression and other data

When successfully completed, this project will also allow the accurate spatial (i.e. 3-D) mapping of gene expression and cell lineage data onto the digital *Atlas* of normal embryonic development, the latter representing the hypothetical standard mouse embryo for a specific developmental stage. Information so far available clearly indicates that gene-expression domains, especially those involved in pattern formation, frequently fail to match anatomical boundaries particularly in the earlier stages of development. It is nevertheless essential that the "painted" domains are accurately located on the digital *Atlas*, otherwise future researchers will run into insurmountable problems when querying the database to establish whether the specific spatial domains where their own favorite genes are seen to be expressed share domains with others which may have been inserted onto the database many years earlier.

The greatest difficulty that is likely to be encountered in constructing a complete database of gene expression is how to cope with the enormous backlog of information that is already available in the literature, most of which is highly selective in the sense that it will contain information relating to gene-expression domains of specific interest of the individual or group that undertook the original work, but is unlikely to contain more than a passing reference to gene-expression patterns in other regions of the embryo. Information from the early literature is also likely to be particularly difficult to vet, because of the considerable advances in technical expertise in recent times. Some data may have been published on the basis of findings extrapolated from a single section, while other published information may have been derived from the analysis of large numbers of sections obtained from numerous embryos at the same and different stages of development. Difficulties will also inevitably be encountered in determining how to include information about strengths and gradients of gene-expression, particularly at the boundaries of the gene-expression domains. The latter may vary considerably even between adjacent histological sections.

From a technical viewpoint, once this graphical database of gene expression is fully operational, there should be little difficulty encountered in inserting the gene-expression data directly onto the appropriate sites on the grey-level images. The mechanical aspect of the labeling (or *painting*) exercise should present no problems greater than those encountered in the original *painting* of the discrete anatomical tissues – in formal terms, an anatomical and a gene-expression domain are equivalent, although in due

course the gene-expression patterns will encode intensity information. Furthermore, the *Atlas* can be used as the basis for a database of any spatially organized information such as cell lineage, metabolic and cell activity.

Conclusions

While the original justification for the computer reconstruction project outlined in this article was to establish the anatomical basis for a gene-expression database, it now appears that, while this aim is likely to be achievable in the future, its other function, namely as a teaching aid for molecular biologists and others with no formal education in mammalian embryology but more particularly in the interpretation of serially sectioned early mammalian embryos, will be of at least equal importance in the long term. This function is of critical importance, first, because there seems little likelihood that sufficient numbers of individuals will ever be available who are capable of interpreting the histological material from which the gene-expression domains must be delineated. Second, while textbooks in the form of workshop manuals have undoubtedly proved to be an invaluable stopgap, they clearly need to be supplemented by interactive computer-aided methodology along the lines indicated in this article. One of the principal restricting factors at the present time is the inordinate amount of time it takes to *paint* anatomical domains. When this purely technical problem is overcome, there should (at least in theory) be no limit to the number of embryological stages that could be made accessible for researchers and students alike.

Methodology

Reconstruction

The grey-level voxel image was reconstructed from the set of serial sections by a process of *digitization*, image alignment and review, image *warping* and finally *restacking*, to form a 3D grey-level digital image of the histological sections. All stages of this process use proprietory software and hardware systems used at the MRC Human Genetics Unit and is based on the woolz processing system (Piper and Rutovitz, 1985).

The image of each section was *digitized* using a Zeiss Axioplan microscope fitted with a 5x Neofluor objective lens and a Xillix Technologies 1400 digital CCD camera linked to a Sun Microsystems Sparc 10 workstation using an MRC designed camera interface. Each section image was shade corrected in the usual way (Baldock and Poole, 1993) to remove the effect of uneven background, and, after *capture*, each section image was subsampled by a factor of 3 to produce an image with a pixel size of 4.08 μm.

The image of each section was aligned with the previous section using the *registration* options of the image *capture* program. The image of the previous or other section is used to form a 'see-through' overlay through which the current section can be aligned by a combination of rotating the microscope stage and translating the image using the computer mouse. This results in a set of images roughly registered and ready for the subsequent process of *warping* which corrects small registration errors as well as distortions from the sectioning process.

Warping

Sectioning and the subsequent histological processing introduces systematic and random distortions into each tissue section. These have to be eliminated in order to be able to make arbitrary sections through the digitized embryo. To do this, we have developed a technique for *warping* each image so that arbitrary sections through the stack do not show the ragged edges of misaligned image features. The method treats each section image as a thin elastic plate linked at corresponding points on the upper and lower adjacent sections and the stack of images is iteratively modified until the required alignment and distortion correction is achieved. The calculation is performed fully automatically and the final corrected image determined by using techniques based on the Finite Element Method described in detail elsewhere (Guest and Baldock, 1996).

Given that the pixel size of the original digitized section was about 4 μm and the section thickness was about 7 μm, we estimated that the resolution in a section through an arbitrary plane was about 10 μm. In other words, it was possible to see quite fine detail (e.g. an epithelium) in such a section.

Painting

Although the experienced viewer immediately recognizes specific tissues in the digitized embryo, these actually have to be delineated and named to establish a link between the anatomical labeling and the spatial coordinates. *Painting* software has been developed to facilitate manual delineation of structure. Some of this is automatic (e.g. the recognition of sharp boundaries through edge detection), but much of the work has to be done manually. The current version of the *paint* program allows the user to delineate structures in any arbitrary viewing plane with up to 5 different colors, each of which may correspond to a different structure. The program also has a series of different tools that provide 'power-assistance' to the user and speed up the *painting* process. It also provides a window in which a 3D visualization of the *painted* structures can be seen with the option of viewing from arbitrary directions and at an arbitrary scale.

Glossary of technical terms

Capturing images: the process of converting an optical image, via a video camera, to a digital one.

Digitizing images: the partitioning of an electronic image into a set of small domains or *pixels* (in this case, about 4 μm in size) and of assigning a numerical (digital) optical density to each. This gives the 'grey-level' image.

Painting of an anatomical domain: delineating anatomically-discrete tissue boundaries on the digital or 'grey-level' image.

Stacking of sections: the first step in making a digital embryo. This is done by combining all the 'grey-level' images to make a 3-D 'stack.' Assigning a thickness to a 2-D *pixel* converts it to a 3-D *voxel*.

Warping program: histological sections have distortions that arise during processing and that cause the stacked sections to be misaligned. Warping is the electronic process by which these distortions are minimized.

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This paper is dedicated to Denis New, an old friend of MHK, on the occasion of his retirement from paid, but not, we hope, active research.

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