Atlas of Zebrafish Development

Robert Bryson-Richardson
Silke Berger
Peter Currie

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Robert Bryson-Richardson
School of Biological Sciences, Monash University

Silke Berger
Australian Regenerative Medicine Institute, Monash University

Peter Currie
Australian Regenerative Medicine Institute, Monash University

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INTRODUCTION

THE NEED FOR AN ATLAS OF ZEBRAFISH DEVELOPMENT

Over the past two decades zebrafish have been established as a genetically tractable model system to investigate many different aspects of vertebrate development. The strengths of this model system stem from a unique combination of embryological manipulability and the optical clarity of the early embryo and larvae, which allows, by simple optical inspection, the visualization of cell biological events within an in vivo context. This is further enhanced by the ability to apply invertebrate-style forward genetics to questions of vertebrate development. While the credentials of the system as a developmental model have long been recognized, it is the application of the zebrafish to a different aspect of biology that has generated a surge in popularity and greatly increased zebrafish laboratory use. Many investigators have now turned to directly modeling human disease states in zebrafish, realizing that many of the same strengths that made it a compelling model for the study of development also allow it to complement existing mammalian disease models (Lieschke and Currie 2007).

Numerous human disease conditions are now being modeled in zebrafish, from addiction syndromes to carcinoma. Remarkably, in the vast majority of instances the results of these studies indicate that the zebrafish models share a similar disease pathogenesis with humans (Darland and Dowling 2001; Langenau et al. 2003; Amsterdam et al. 2004; Carvan et al. 2004; Lockwood et al. 2004; Berghmans et al. 2005; Shepard et al. 2005; Patton et al. 2005). Zebrafish have also come of age as a reverse genetic model system. The ability to specifically knock down and mutate individual loci by a number of different techniques has allowed function of human disease gene orthologues to be directly examined in zebrafish. Antisense oligonucleotides or morpholinos have been utilized very effectively to knock down specific gene function during the embryonic and early larval period. Targeted lesion detection (Wienholds et al. 2003) has produced mutant models of a number of human diseases. This strategy has identified p53 mutant zebrafish that have a predisposition to cancer, rag1 deficient zebrafish with immunodeficiency (Wienholds et al. 2002, Berghmans et al. 2005), and has also been utilized to generate mutations in the APC gene that predispose to colorectal cancer (Hurlstone et al. 2003).

These approaches possess the ability to revolutionize the way that zebrafish biologists approach disease modeling in this system. The commitment of the Wellcome Trust's Sanger Centre to producing a fully sequenced and integrated high-quality genome sequence for the zebrafish has enabled the development of many of the new reverse genetic techniques available to zebrafish researchers as well as facilitating the cloning of randomly generated mutations. Analysis of the zebrafish genome has demonstrated a considerable level of synteny with the human genome, and direct orthologues of human genes can nearly always routinely be located within the zebrafish genome. An ambitious new project that aims to mutate every gene within the zebrafish genome using a combination of the approaches outlined above has also been recently proposed. There is a possibility that a percentage of mutant phenotypes produced by the zebrafish knockout consortium will be phenotyped in a systematic way and catalogued in a database.

Another recent development has raised the stakes considerably for zebrafish as a human disease model. The application of chemical genetics to zebrafish biology has allowed for the first time the simultaneous analysis of a mutant phenotype representative of a human disease and the screening for drugs that will influence this phenotype in vivo (Peterson et al. 2004). The ability to screen in vivo using zebrafish allows the drug discovery process to leapfrog a number of the initial hurdles of toxicity and specificity that plague drug discovery employing in vitro models.

The unique features of zebrafish biology, such as external fertilization, high fecundity, ease of adding putative drugs to larval fish, make this the only fully integrated vertebrate model system for drug discovery. Collectively, these recent advances have heralded a watershed period for researchers utilizing zebrafish as a tool for modeling human disease and indicate a further expansionary phase in its use as a model system given that many of its strengths complement existing mammalian models so fully. However, despite the advances in many areas of zebrafish biology and the generation of sophisticated models of human disease, one area has substantially lagged behind. There is a lack at this current point in time of a detailed anatomical reference for zebrafish. While the first 24 hours of zebrafish development have been well studied, comparatively little is known about the development of larval, juvenile and adult anatomy. Such knowledge is critical for the analysis of developmental processes extending later in development and the accurate modeling of human disease in zebrafish, as these periods are the most likely to generate information that is relevant to human disease pathogenesis. Therefore the aim of this book is to provide

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an anatomical account of the development of the zebrafish from embryo to adult. Our hope is that this resource, in conjunction with the online three-dimensional (3D) anatomical resource FishNet (http://www.FishNet.org.au, discussed in Chapter 2) from which the images in this book are derived, can be utilized as a standard reference text on which to base further analyses that will investigate aspects of normal and perturbed zebrafish development.

EXISTING ANATOMICAL RESOURCES FOR ZEBRAFISH

The major online resource for zebrafish is the zebrafish information network ZFIN (http://www.zfin.org). ZFIN curates information on tools, publications and laboratories involved in zebrafish research, as well as information about the zebrafish strains and DNA stocks held by the Zebrafish International Resource Center (ZIRC). Importantly, ZFIN also contains the first online collection of anatomical images comprising of nearly 80 sections covering the first 5 days of development. Other anatomical resources are scarce for zebrafish and piecemeal in their representation of anatomy. Several specialized zebrafish anatomy atlases exist in book form, including atlases of adult and embryonic brain anatomy. Necessarily these types of atlases are limited by showing only two-dimensional (2D) images in restrictive views. For instance the adult brain atlas (Wullimann et al. 1996), despite its high degree of anatomical complexity, shows 2D brain structure in complete isolation from any other tissues. The embryonic brain atlas is also limited in scope, showing only transverse views of a very limited number of stages (Mueller and Wulliman 2005). Other embryonic atlases and staging series have been published, which collectively approximate the resources currently online at ZFIN (Kimmel et al. 1995; Schilling 2002). All of these resources rely on 2D imaging.

Several online organ-specific 3D atlases of development have now been developed for zebrafish. A 3D atlas of zebrafish vasculature created by microangiography in the Weinstein laboratory shows the formation of the vasculature from 1 to 7 days in a series of 3D renderings (http://uvo.nichd.nih.gov/atlas.html; Isogai et al. 2001; Kamei et al. 2004). This site allows the user to view a series of images at each stage showing the vasculature in 3D utilizing confocal microscopy of specimens labeled using microangiography to chart the forming vasculature. This series of images represents the first example of a 3D developmental model of any organ system in zebrafish. However, the lack of ability to simultaneously visualize both the vasculature and the tissue through which it forms limits the usefulness of the atlas. Furthermore, the use of confocal microscopy to generate 3D models is limited to stages at which the laser light can suitably penetrate the tissue, and necessarily this is restricted to the earliest stages of zebrafish development.

A high resolution 3D anatomical atlas of the zebrafish developing brain is also being developed within the group of Steve Wilson at UCL in London (http://www.zebrafishbrain.org). This atlas relies on the optical clarity of the zebrafish embryo, coupled with a host of transgenic and antibody labelings to highlight discrete neuroanatomical structures within the developing brain. It uses confocal microscopy exclusively to generate highly detailed 3D interactive models of embryonic and larval brain development. Although this will be a unique and important neuroanatomical resource this approach will necessarily be restricted to relatively early stages of zebrafish brain development and will not be applicable to juvenile and adult zebrafish.

A growing repository of 2D section information is being deposited at the Penn State Atlas project (http://zfatlas.psu.edu), which, when completed, will be a significant data set. Although currently mostly unannotated, the high resolution nature of the material will suit those researchers looking at the cellular anatomical level across a wider range of developmental stages. A significant online zebrafish anatomical resource already exists in the form of a curated anatomical nomenclature. Standardized terms for anatomical structures are deposited and curated at ZFIN by the zebrafish research community, and represent a significant framework on which any annotated developmental atlas can now be built. It also represents a mechanism by which names of new or uncertain anatomical structures can be reviewed and approved relatively rapidly. It ensures that any anatomical atlas that uses the nomenclature represents the experience of the wider zebrafish research community, rather than the more limited experience of the individuals creating the atlas. The committee has also used standardized nomenclature to facilitate comparison between species. This book has endeavored to use the standard anatomical terms curated with ZFIN to describe the structures we have annotated in this book.

In summary, the FishNet website and the images from it that we present within this book represent the first full 3D annotated life history anatomical reference, from embryo to adult, of zebrafish. Indeed it represents the first such anatomical reference for any animal model system, covering the complete range of development from early embryo until adult. It fills a current need for a general gross anatomical reference for zebrafish researchers and we hope it will be an important reference text for all individuals who utilize zebrafish as research and teaching tools.

METHODOLOGY AND INTERPRETATION OF THE ATLAS

This atlas aims to provide a reference at the gross anatomical level for the zebrafish, identifying the major anatomical structures in samples covering the range of development from the early embryo until adulthood. The atlas has been generated using the technique of Optical Projection Tomography (OPT, described below). This technique has a number of advantages for the generation of an anatomical atlas. One of the clear advantages is that it is possible to generate a complete three-dimensional (3D) model from a single sample. As a result the sections in each chapter are images from the same sample for all three orientations. Using the OPT approach, however, we are not able to provide cellular resolution for the range of sample sizes encompassing the development of zebrafish from embryo to adulthood. To generate the images we have used in this atlas, we have relied on the autofluorescence of the tissue following paraformaldehyde fixation to generate the contrast of the images. As a result of this approach, structures such as blood, cartilage, and bone appear much brighter than other tissues. Similarly, reflection of light from the eye results in a much brighter appearance. Differences in the level and pattern of fluorescence are necessary to provide the tissue definition in the images but, as a result, users of the atlas may not be immediately familiar with this type of image. Therefore we will provide here not only the contents of the atlas and the methodology but also a brief guide to the interpretation of the OPT images.

STRUCTURE OF THE ATLAS

There are nine chapters of images containing samples at 24, 48, 72, 96, and 120 hours post-fertilization and at 6 mm, 9 mm, 14 mm, and 16 mm total length. Each sample is displayed in the classical anatomical section views of transverse, sagittal, and coronal. Transverse sections are listed anterior to posterior, sagittal right to left, and coronal dorsal to ventral, and are evenly spaced along the axis of sectioning (Figure 2.1).

METHODOLOGY

We produced a series of 18 3D models of zebrafish development from 24 hours post-fertilization until adulthood using the method of OPT as described in Bryson-Richardson et al. (2007). Zebrafish were obtained from either wild-type or golden mutant strains and staged by age for the first 5 days of development and by total length (most anterior point until the tip of the tail) for the remainder of the staging series, due to variability of size with age after free feeding. Samples were fixed in 4% paraformaldehyde overnight at 4°C, and then washed with PBS containing 0.1% Tween 20. Samples were used immediately or progressed through a methanol series for storage in 100% methanol at −20°C. Pigmented embryos were bleached in several changes of 5% hydrogen peroxide in methanol on a lightbox. Samples were hydrated through a methanol series if necessary, washed in water, and then embedded in a 1.5% low melting point agarose gel. An agarose block containing the sample was then attached to a metal mount. The mounted samples were then cleared through a series of 25%, 50%, 75%, and 100% methanol prior to immersion in a 2:1 benzyl alcohol:benyzl benzoate solution. The samples were then scanned on a prototype OPT scanner as described in Sharpe et al. (2002).

OPTICAL PROJECTION TOMOGRAPHY (OPT)

Optical Projection Tomography (OPT) allows the generation of 3D models from cleared biological samples using conventional brightfield and fluorescent staining procedures or, as in this atlas, imaging without labeling utilizing the intrinsic fluorescence of the sample. A series of images is taken of the sample as it is rotated through 360°. These images are projections through the sample that can be used to generate a section image using back projection (Figure 2.2). In transmitted illumination OPT the projection images record the loss of light as it passes through the sample. In the epi-fluorescent OPT imaging used in the atlas the images record the total amount of fluorescence emitted by the sample along the axis of imaging. The 3D models used to generate the atlas were generated from a series of

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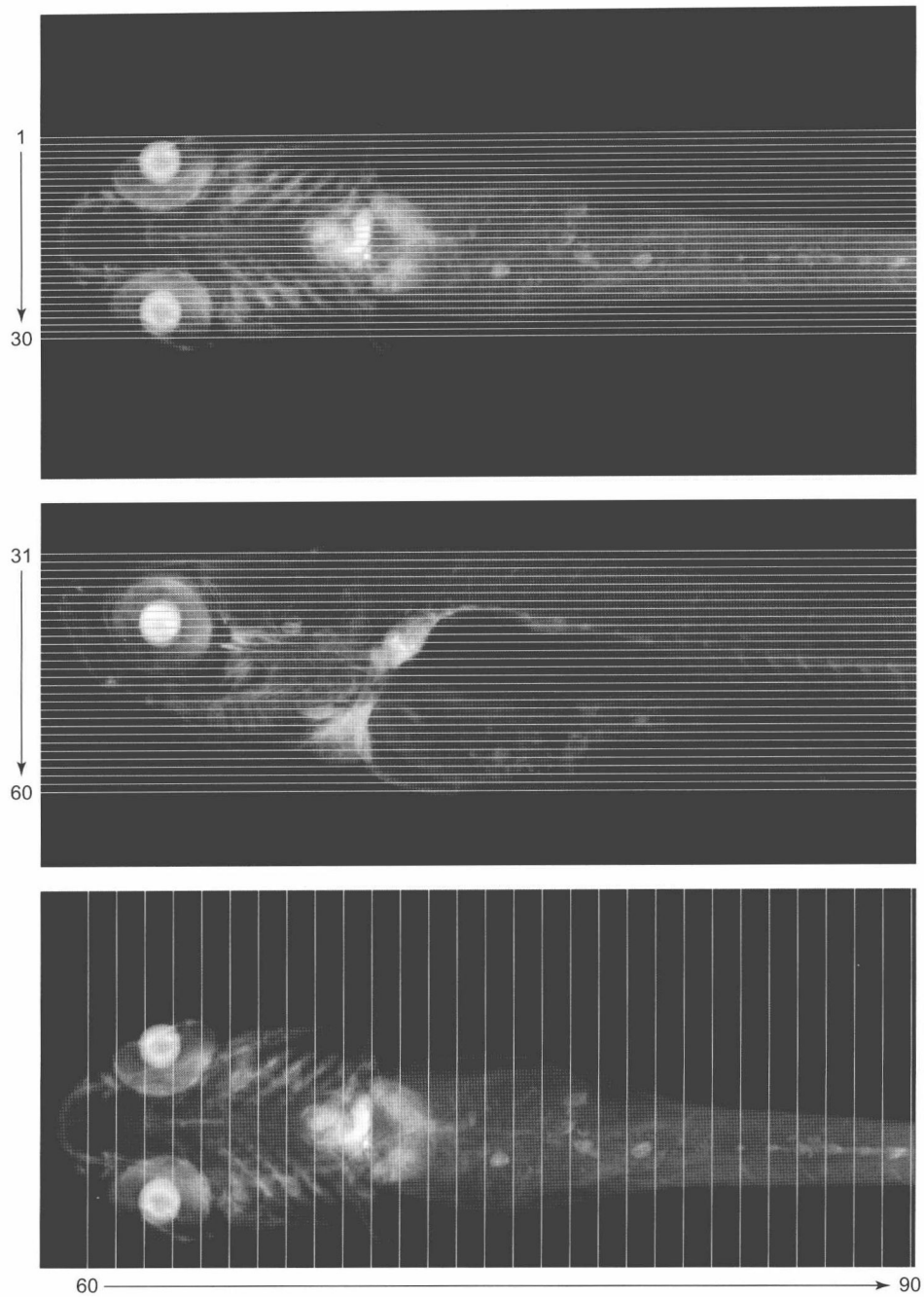

Figure 2.1 Representation of section positions for Chapter 10. In each chapter, sections are evenly spaced and numbered from right to left, dorsal to ventral, and anterior to posterior.

400 images captured over a 360° rotation. Image reconstruction was carried out using filtered back projection (as described by Sharpe et al. 2002). This is very similar in principle to other forms of projection tomography, including magnetic resonance imaging and x-ray computer tomography, the key difference being the use of visible wavelengths to generate the information, as a result of which the sample must be optically clear and have a consistent refractive index throughout, hence the bleaching and clearing described above. In contrast to x-ray and other imaging techniques, however, the resolution is limited by the image-forming optics. The tomographic approach used requires the sample to be in focus for at least 180° of the rotation and therefore the depth of focus should extend at least half-way into the sample. Image resolution is proportional to the numerical aperture of the objective whilst the depth of focus is inversely proportional. Therefore, there is a trade-off between resolution and the thickness of the sample; as a result we are unable to image at a constant resolution throughout the wide range of sample sizes

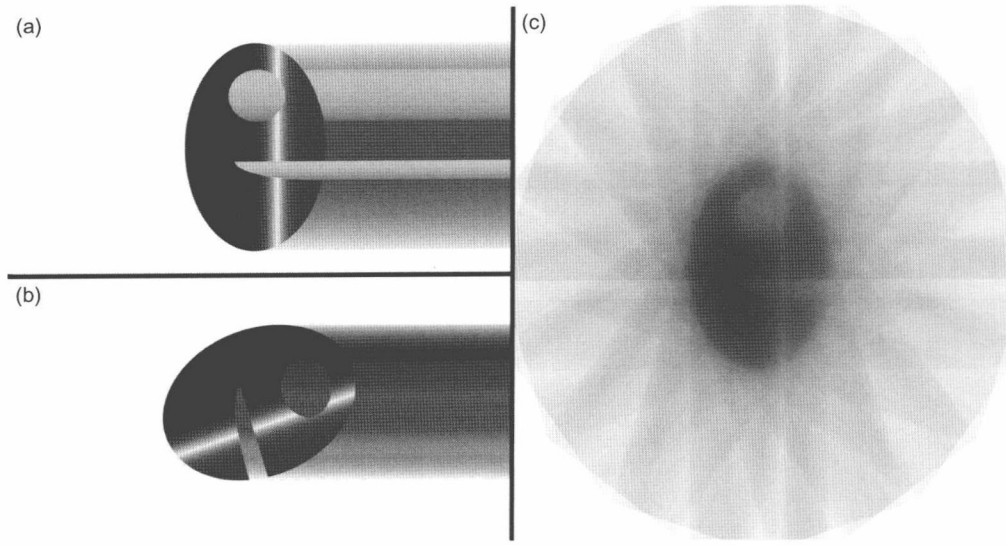

Figure 2.2 Reconstruction of 3D models from projection images. (a) The sample (blue) is illuminated from the left and a projection image formed. Where the sample is thickest less light is transmitted through the sample. (b) The sample is rotated over 360°. (c) Overlapping the projection images reconstructs the shape of the sample, demonstrating the principle of projection tomography.

presented here. A significant advantage of this imaging technique over conventional physical sectioning approaches is that the sample remains intact. Therefore distortion of the sample as a result of the cutting and mounting of sections is eliminated.

INTERPRETATION OF THE ATLAS

As mentioned above, the technique of OPT can generate 3D models of embryonic development; however, the images that are produced have a different appearance to those produced through conventional sectioning and histological staining approaches. In order to assist in the interpretation of the atlas we will present a brief guide to OPT images. The observed differences between tissues are due to varying levels of fluorescence resulting from both natural fluorescence of the tissue and that introduced by fixation. There are various cell components, structures, and chemicals, within tissue samples that are known to cause autofluorescence. Flavins and flavoproteins, for example, emit fluorescence in the wavelengths used for the OPT imaging of the samples presented here but are greatly reduced by fixation and dehydration. Collagen and elastin show high levels of autofluorescence, resulting in the bright appearance of extracellular matrix, blood vessels, and bone in the images. Aldehyde fixatives can react with proteins within the sample to generate fluorescent products. Samples fixed with paraformaldehyde, as used in the atlas, have a much lower level of fluorescence compared to samples after fixation with glutaraldehyde. However, the extremely high level of autofluorescence resulting from gluteraldehyde fixation prevents its use in OPT imaging as the sample appears solid and tissues below the surface cannot be visualized.

In the earliest developmental stages, where contrast between forming tissues is the least, OPT is more informative in relation to shape rather than individual tissue boundaries. However, the earliest stages of development are those that are represented by the largest number of existing anatomical resources, and as the embryo rapidly develops the contrast evident in the images also rapidly increases. In the older samples fluorescent OPT imaging provides sufficient contrast to distinguish the major anatomical structures. The brightest structures in the images are the blood, bone, and lens tissue, which appear saturated in the images (Figure 2.3). The use of fluorescence imaging also establishes very defined boundaries for the air-filled swim bladder and also the ventricles of the brain. Fluid and air-filled structures such as these frequently lose their morphology following physical sectioning but the imaging of the intact specimen using OPT ensures that these structures remain intact and distortion is minimized. More subtle differences in signal between neighbouring tissues allow the distinction of boundaries as illustrated in Figure 2.4. In an individual section, generated by either conventional sectioning techniques or by OPT, it can sometimes be difficult to unambiguously identify structures. A great advantage of techniques such as OPT where we can generate a complete 3D model is that an individual point or structure can be viewed from multiple angles within the same sample. This approach was frequently used in the annotation of the images contained in the atlas and the complete models are available through the accompanying website described in the next chapter.

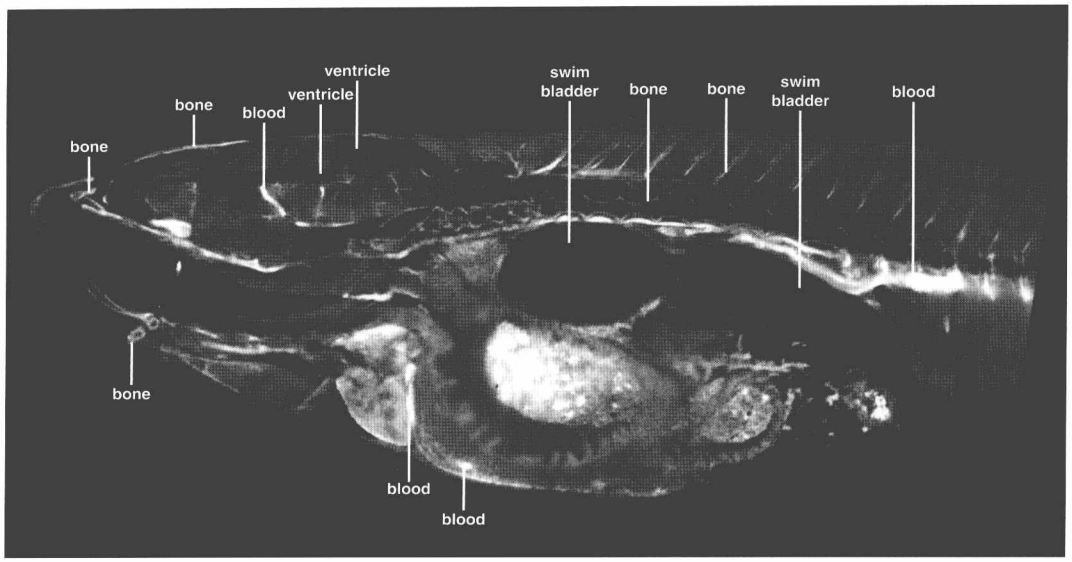

Figure 2.3 Section through a 16 mm fish. The highest levels of autofluorescence are seen in blood vessels and bone.

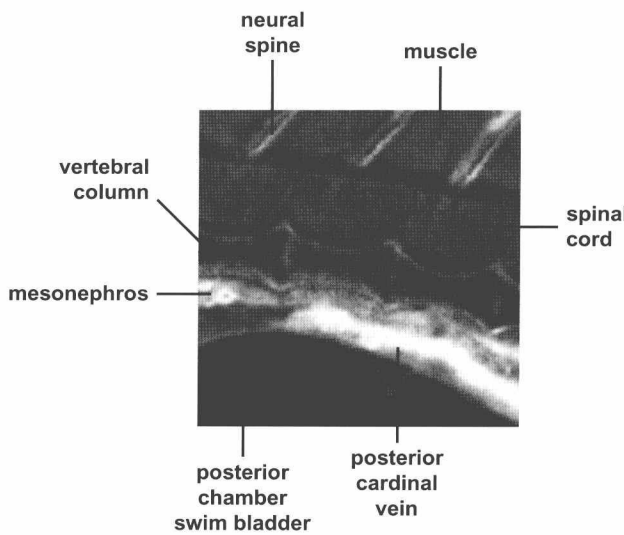

Figure 2.4 Examples of tissue boundaries. Changes in the level of autofluorescence between neighbouring tissue allows the boundaries to be determined. Region of a sagittal section from a 16 mm zebrafish.

FISHNET: AN INTERACTIVE DATABASE OF ZEBRAFISH DEVELOPMENT

The information presented in this book is a condensed version of the total information available at FishNet (http://www.FishNet.org.au). We have previously described the publication of the internet-based resource in detail (Bryson-Richardson et al. 2007) and will therefore not repeat the information here. We will instead describe some of the major features of the website and how it will continue to develop. Additionally, as it is our intention that the two resources will complement each other to provide a complete anatomical reference for the zebrafish research community, we will discuss the pros and cons of each of the digital and print versions both now and into the future, with expected advances in internet publishing technologies.

ADVANTAGES OF THE PRINTED BOOK OVER THE INTERNET RESOURCE

The delivery of an anatomical resource such as this via the internet holds a number of advantages over a traditional, printed version, as explained below. The print reference, however, still has many significant benefits.

Despite improvements in internet access, the time taken for delivery of high-resolution images can still be long, and in order to facilitate browsing and interaction with large datasets image compression, with a corresponding reduction in quality, is still required. Whilst we have made every effort to ensure the image quality at the website remains high we have no such limitations in the delivery of images for the print version. Similarly, whilst display technology is ever improving, it still remains the case that it is often easier to evaluate images in high-quality prints. Alongside the improvements in delivery and display the quality and resolution of our imaging techniques are similarly improving and therefore the print version of the atlas will continue to have benefits. Perhaps the greatest advantage of this print version is its portable nature, allowing its use in the laboratory or aquarium where access to the internet resource may not be possible or appropriate.

ADVANTAGES OF THE INTERNET RESOURCE

The accompanying website to this book adds a number of features not possible in print form. The generation of the 3D models and subsequent section images, described in the previous chapter, has resulted in a wealth of information that cannot be represented in its entirety in print/paper format due to publication costs. In the book we are constrained to presenting a set of selected key stages in the development of the zebrafish and from within each of those stages, several representative section images that will allow the visualization of the gross anatomy. As such, significant temporal and spatial gaps exist in the information presented.

The complete stage series and all sections within each sample embryo, however, have been captured and may be accessed through the website. The increased temporal resolution available online is a significant advantage to the developmental biologist who requires information pertaining to subtle morphological changes throughout development. The interactive nature of the website also allows the virtual sectioning of the 3D models at any of the represented stages in any of the standard orthogonal sectioning planes, and to zoom in and out to different image magnifications. It is also possible to view volume or surface renderings of the complete sample that may be manipulated on screen, which are particularly useful for mental conceptualization of anatomical morphology. The provision of the in-built tools on the website allows the data to be accessed in a user-friendly manner and without the need for specialist software or IT knowledge. Furthermore, the complete 3D models may be downloaded for examination and manipulation locally by the user.

The above describes the existing capabilities of the website, which is not only interactive in its use but also dynamic in its development. We have only begun to explore the possibilities for data presentation in the first release of the website and this dynamic nature will allow the continued improvement of functionality and image quality. There have indeed been several improvements since its original release, including adoption of technology solutions that have significantly improved the speed of image delivery and reduced the effort required for site maintenance. More significant enhancements are in progress, such as development of tools to allow sectioning of the virtual 3D

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embryos in any arbitrary section plane and viewing of multiple angles simultaneously. We are also developing search tools to allow the resource to be searched directly by the use of anatomical names (as opposed to section browsing), again making use of the advantages of internet delivery, and we expect all of these to be made available to users shortly. There are also, as discussed in Chapter 1, significant resources in the form of existing websites for the zebrafish research community. These collective resources house an incredible amount of information and as we develop the site we aim to make it easier for other resources to reference the information contained within FishNet and also improve the connections from FishNet back to these resources. This will allow users to more easily access the collective resources available.

Another benefit of the dynamic nature of the website is the possibility for information to be easily updated, which allows both for addition of further annotations and any modification where necessary. This also allows for the exciting possibility of community annotation by expert users across the wider zebrafish community. It is our hope that in the long term it will be possible for users of the site to contribute their expertise directly into the database, thereby ensuring that individual structures are labeled by the most relevant experts. In the lead up to this community annotation scenario (which will require infrastructure changes to allow for remote editing by researchers across the world), we welcome comments from the community regarding any possible errors, omissions, and changes in current nomenclature which can be updated on the website in a short timeframe. Community annotation efforts would also benefit future versions of the print atlas.

Greater scope still exists in the visualization of 3D images. Currently the manipulation and visualization of large 3D datasets requires specialist software and can require substantial computer processing time. As software and hardware develops it will ultimately be possible to complete many of these tasks remotely through the website, interacting directly with the 3D models, making arbitrary sections, virtual dissections, and adjusting rendering parameters, ensuring that all users can more fully utilize the datasets without the requirements of specialist software, hardware, and expertise.

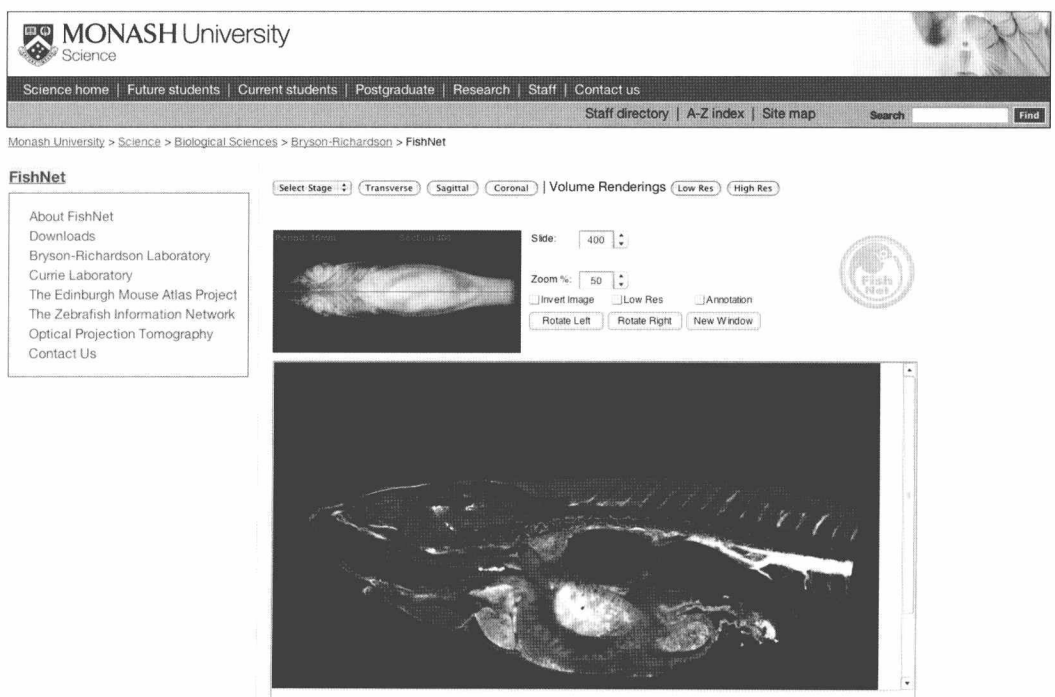


Figure 3.1 The FishNet website. The current interface for the website presents a rendered image of the 3D data set for navigation and a virtual section image. Virtual sections can be examined in any of the three standard section views: transverse, sagittal, or coronal. The section viewed can be adjusted by clicking on the navigation image, typing a number into the slide value box, or by clicking on the up and down arrows. The "Low Res" option allows smaller versions of the images to be viewed, facilitating use of the resource with slow internet connections. Annotated images are evenly spaced throughout the 3D models and selecting annotation will show the closest annotated section to the currently viewed image. In addition to the section images, volume renderings may be viewed. By selecting the "Low Res" or "High Res" buttons in the top right of the screen another window will open containing the rendered model. By clicking on these models and depressing the mouse button the angle at which the models are viewed can be altered.

THE EARLY EMBRYO

24 HOURS

Sagittal Sections

Figure 4.1

Figure 4.2

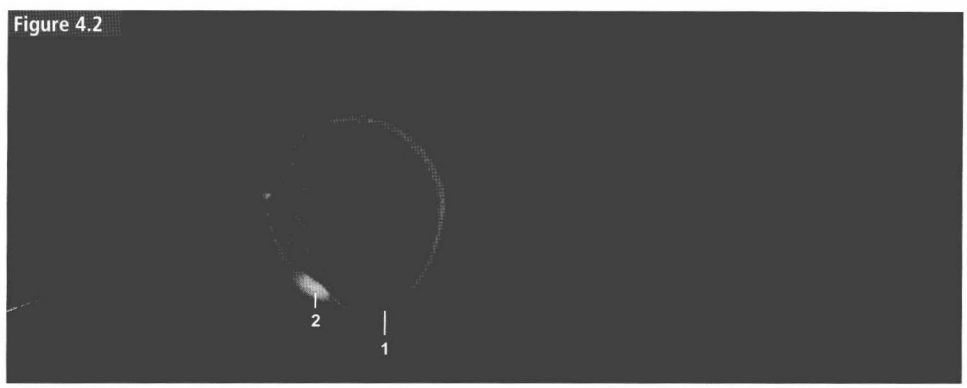
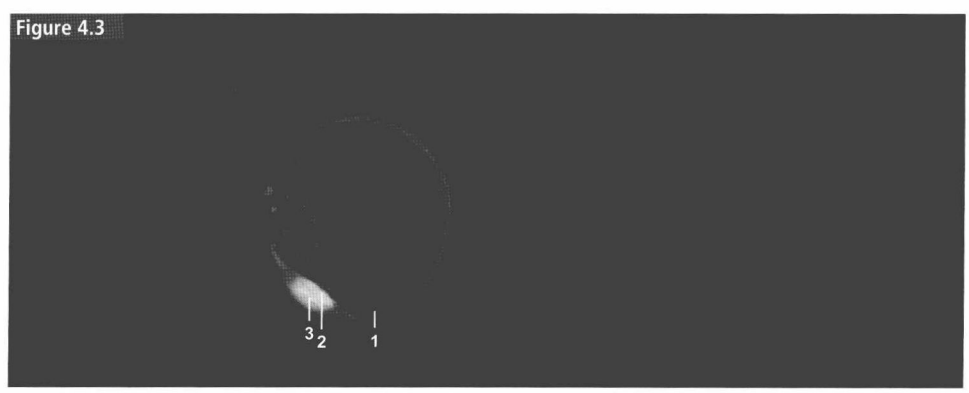


Figure 4.3



1. yolk	**2.** optic cup	**3.** lens

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Figure 4.4

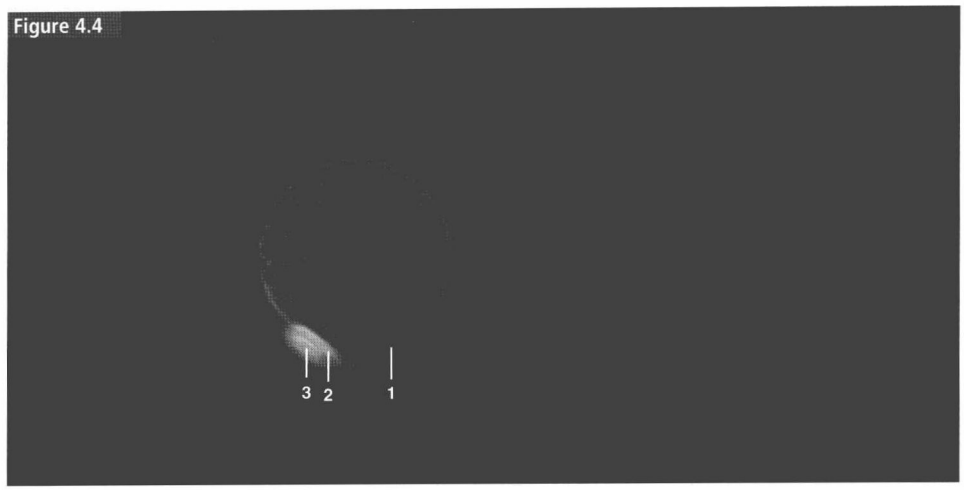
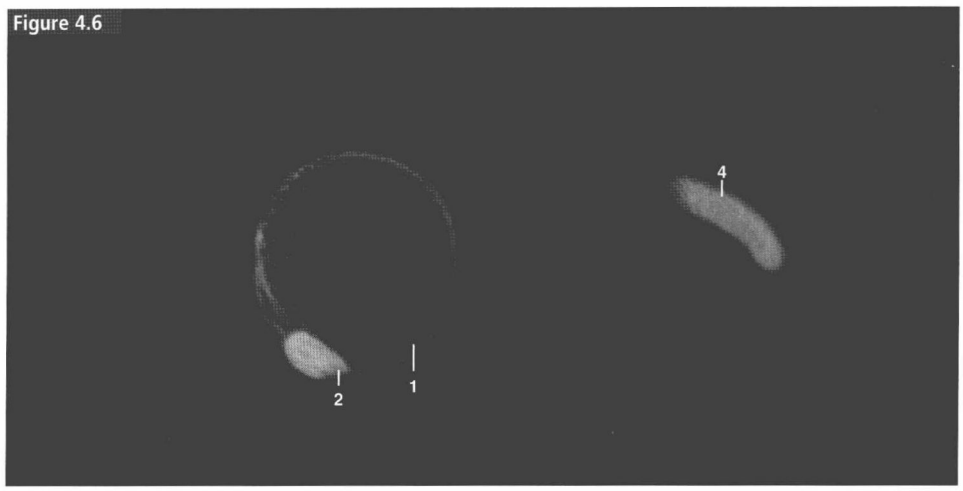


Figure 4.5

Figure 4.6



1. yolk	**2.** optic cup	**3.** lens	**4.** myotome