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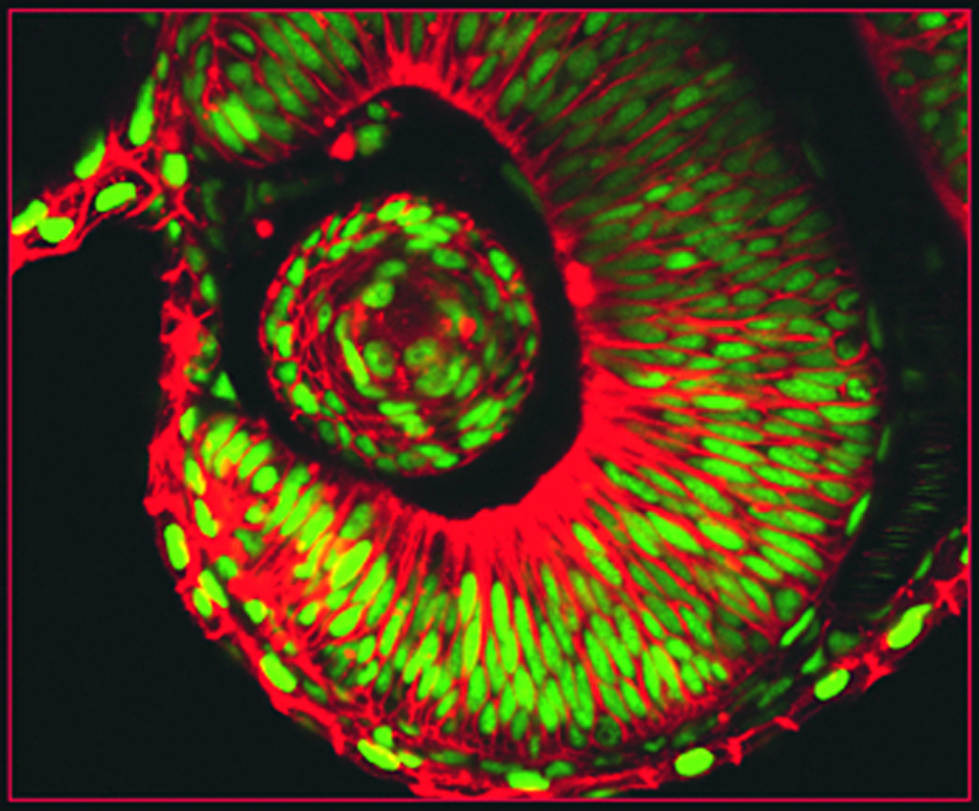

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Methods in Cell Biology

VOLUME 76

The Zebrafish: Cellular and Developmental Biology

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The Zebrafish: Cellular and Developmental Biology

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PREFACE

Research Vessel Nathaniel B. Palmer
Southern Atlantic Ocean, 54° 47′ S, 59° 15′ W
On the Burdwood Banks
20 May 2004

Monte, Len, and I welcome you to two new volumes of *Methods in Cell Biology* devoted to *The Zebrafish: Cellular and Developmental Biology* and *Genetics, Genomics, and Informatics*. In the five years since publication of the first pair of volumes, *The Zebrafish: Biology* (Vol. 59) and *The Zebrafish: Genetics and Genomics* (Vol. 60), revolutionary advances in techniques have greatly increased the versatility of this system. At the Fifth Conference on *Zebrafish Development and Genetics*, held at the University of Wisconsin in 2003, it was clear that many new and compelling methods were maturing and justified the creation of the present volumes. The zebrafish community responded enthusiastically to our request for contributions, and we thank them for their tremendous efforts.

The new volumes present the post-2000 advances in molecular, cellular, and embryological techniques (Vol. 76) and in genetic, genomic, and bioinformatic methods (Vol. 77) for the zebrafish, *Danio rerio*. The latter volume also contains a section devoted to critical infrastructure issues. Overlap with the prior volumes has been minimized intentionally.

The first volume, *Cellular and Developmental Biology*, is divided into three sections: Cell Biology, Developmental and Neural Biology, and Disease Models. The first section focuses on microscopy and cell culture methodologies. New microscopic modalities and fluorescent reporters are described, the cell cycle and lipid metabolism in embryos are discussed, apoptosis assays are outlined, and the isolation and culture of stem cells are presented. The second section covers development of the nervous system, techniques for analysis of behavior and for screening for behavioral mutants, and methods applicable to the study of major organ systems. The volume concludes with a section on use of the zebrafish as a model for several diseases.

The second volume, *Genetics, Genomics, and Informatics*, contains five sections: Forward and Reverse Genetics, The Zebrafish Genome and Mapping Technologies, Transgenesis, Informatics and Comparative Genomics, and Infrastructure. In the first, forward-genetic (insertional mutagenesis, maternal-effects screening), reverse-genetic (antisense morpholino oligonucleotide and peptide nucleic acid gene knockdown strategies, photoactivation of caged mRNAs), and hybrid (target-selected screening for ENU-induced point mutations) technologies are

described. Genetic applications of transposon-mediated transgenesis of zebrafish are presented, and the status of the genetics and genomics of *Medaka*, the honorary zebrafish, is updated. Section 2 covers the zebrafish genome project, the cytogenetics of zebrafish chromosomes, several methods for mapping zebrafish genes and mutations, and the recovery of mutated genes via positional cloning. The third section presents multiple methods for transgenesis in zebrafish and describes the application of nuclear transfer for cloning of zebrafish. Section 4 describes bioinformatic analysis of the zebrafish genome and of microarray data, and emphasizes the importance of comparative analysis of genomes in gene discovery and in the elucidation of gene regulatory elements. The final section provides important, but difficult to find, information on small- and large-scale infrastructure available to the zebrafish biologist.

The attentive reader will have noticed that this Preface was drafted by the first editor, Bill Detrich, while he (I) was at sea leading the sub-Antarctic ICEFISH Cruise (International Collaborative Expedition to collect and study Fish Indigenous to Sub-antarctic Habitats; visit www.icefish.neu.edu). Wearing my second biological hat, I study the adaptational biology of Antarctic fish and use them as a system for comparative discovery of erythropoietic genes. Antarctic fish embryos generally hatch after six months of development, and they reach sexual maturity only after several years. Imagine attempting genetic studies on these organisms! My point is that the zebrafish system and its many advantages greatly inform my research on Antarctic fish, while at the same time I can move genes discovered by study of the naturally evolved, but very unusual, phenotypes of Antarctic fish into the zebrafish for functional analysis. We the editors emphasize that comparative strategies applied to multiple organisms, including the diverse fish taxa, are destined to play an increasing role in our understanding of vertebrate development.

We wish to express our gratitude to the series editors, Leslie Wilson and Paul Matsudaira, and the staff of Elsevier/Academic Press, especially Kristi Savino, for their diligent help, great patience, and strong encouragement as we developed these volumes.

H. William Detrich, III
Monte Westerfield
Leonard I. Zon

These volumes are dedicated to Jose Campos-Ortega and Nigel Holder, departed colleagues whose wisdom and friendship will be missed by the zebrafish community

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PART I

Cell Biology

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CHAPTER 1

Use of the DsRed Fluorescent Reporter in Zebrafish

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Green fluorescent protein (GFP) is firmly established as a fluorescent reporter for the imaging of specific tissues in zebrafish. The employment of other reporters such as DsRed in transgenic zebrafish has made multicolored labeling experiments possible. To date, several DsRed transgenic lines have been generated for lineage labeling, transplantation assays, and commercial applications. Advances in multicolored labeling experiments will depend on the implementation of newly engineered reporters and fusion proteins, as well as on innovative experiments that exploit the power of direct visualization.

I. Introduction

Since the mid-1990s, there has been an explosion in the number of GFP expressing transgenic zebrafish lines reported in the literature. This is primarily true because the ''know-how'' necessary to make transgenics has become

METHODS IN CELL BIOLOGY, VOL. 76

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widespread. In addition, transgenic zebrafish can be used in a wide range of experiments. As a testament to this, nearly every organ system is now represented by tissue-specific GFP transgenic lines. The transgenic catalog is comprehensive, and includes cardiac and skeletal muscle, pancreas, erythrocytes and lymphocytes, vessels, alimentary canal, notochord/floor plate, and central nervous system neurons (Goldman *et al*., 2001; Higashijima *et al*., 2000; Huang *et al*., 2001; Ju *et al*., 1999; Lawson and Weinstein, 2002; Long *et al*., 1997; Motoike *et al*., 2000; Udvadia *et al*., 2001).

Fluorescent proteins have been utilized successfully in mammalian systems, but they are especially useful in the zebrafish. Zebrafish embryos develop *ex vivo* and thus do not require unwieldy culture conditions for microscopic investigation. Furthermore, zebrafish embryos and larvae are optically clear, enabling the observation of even anatomically deep organs throughout the first month of development. These developing tissues are thin enough that they can be penetrated by laser confocal microscopy even in late developmental stages.

Zebrafish expressing fluorescent reporters under the control of constitutive and tissue-specific promoters have been featured in a wide range of experiments. Fluorescent labeling of a specific subset of cells makes it possible to isolate that subpopulation for a variety of purposes. For instance, cells isolated by fluorescence activated cell sorting (FACS) can be transplanted and subsequently followed in living hosts. These cell populations have also been used to generate cDNA libraries and can potentially be used to establish cell lines. In many situations, fluorescent protein expression in living embryos can replace whole-mount *in situ* hybridization of fixed specimens. Manipulations of gene levels by ectopic overexpression and morpholino-mediated knockdown experiments can be assayed in fluorescent embryos, a strategy that permits visualization of morphological or gene expression changes in living animals. GFP can also be used as a sentinel marker in genetic or chemical screens. On the most fundamental level, fluorescence allows us to see morphogenetic processes that were previously invisible.

While most of these advances have been made using GFP, there have been attempts to introduce other fluorescent proteins such as DsRed into the zebrafish, as discussed in this chapter. The combinatorial use of these reporters allows the visualization of multiple tissues with multiple colors, making an already powerful cell biological and genetic system more vibrant.

II. DsRed: History and Properties

The introduction of GFP from *Aequorea* jellyfish in 1994 revolutionized cell biology. The applications of GFP have ranged widely, from tracking gene expression and subcellular labeling to monkey transgenesis (Chan *et al*., 2001) and transgenic artwork (Eduardo Kac's "GFP Bunny"), capturing the imagination of the scientific and public worlds.

In an effort to widen the spectrum of fluorescent proteins, DsRed (originally designated drFP583) was one of several GFP homologs cloned from reef corals (Matz *et al*., 1999). Though the protein is only 23% homologous to GFP, it has several conserved residues in the vicinity of a virtually identical chromophore (Yarbrough *et al*., 2001). With easily separable emission wavelengths of 509 and 583 nm, respectively, enhanced green fluorescent protein (EGFP) and DsRed are suited for dual-color labeling with minimal crossover interference. The original DsRed protein was mutagenized to yield a somewhat faster maturing and more soluble variant that is offered commercially as DsRed2 (Clontech Laboratories, Inc., Palo Alto, CA).

GFP is an optimal genetic fusion tag because of its properties of fast maturation, solubility, and existence as a monomer. In contrast, the obligate tetramerization and slow maturation of DsRed posed formidable obstacles to its employment in fusion protein and gene expression experiments (Baird *et al*., 2000). Also, DsRed maintains high levels of fluorescence for long periods of time after it is produced because of its high extinction coefficient, a property that is both advantageous and yet problematic for short-term gene expression applications. In our experience with DsRed2 in zebrafish embryos, it is detectable approximately 12 hours after the initiation of transcription. As a consequence, we have not been able to use DsRed for the observation or isolation of cells that have activated DsRed transgene expression within a restricted time window. Because DsRed exists predominantly as a stable homotetramer, attempts at using it as a genetic fusion tag have failed. Fortunately, efforts by several groups to develop faster maturing, monomeric DsRed variants have led to the creation of mRFP1, a rapidly maturing (detectable in less than 1 hr) monomeric red fluorescent protein with excitation at 584 nm and emission at 607 nm (Campbell *et al*., 2002). Although mRPF1 has a lower extinction coefficient, quantum yield, and photostability than DsRed, all of which results in lower signal intensity, it represents a significant improvement over DsRed that can and will be further refined. The use of mRFP1 in zebrafish has not yet been reported.

Even before DsRed was widely used in zebrafish for scientific purposes, DsRed fluorescent zebrafish were marketed commercially as pets. These "GloFish™," originally designed to monitor environmental pollution (Knight, 2003), generated considerable controversy because of the potential risks of releasing transgenic animals into the environment. Nevertheless, GloFish™ catapulted zebrafish into the national spotlight and, for better or worse, stretched the boundaries of utility for fluorescent proteins in fish.

III. DsRed in Transgenic Zebrafish

To date, only a handful of transgenic lines have utilized fluorescent reporters other than GFP. This may be attributed to the drawbacks of DsRed or a relative lack of experience using reporters other than GFP in transgenesis. The earliest

report of such use investigated the efficacy of three fluorescent proteins for multi-labeling in zebrafish (Finley *et al*., 2001). Single-, double-, or triple-labeled transiently expressing embryos were produced by injecting GFP, blue-shifted GFP variant (BFP), or DsRed DNA constructs. Finley *et al*. found that simultaneously expressed fluorescent proteins could be detected independently within the same cells, showing that it would be effective to label zebrafish tissues with these markers. Subsequently, this group generated germline transgenic lines that ubiquitously express GFP, BFP, and DsRed in order to demonstrate that the Sleeping Beauty transposase can enhance transgenesis efficiency (Davidson *et al*., 2003).

The first lines expressing DsRed or yellow-shifted GFP (YFP) under tissue-specific control were made for ornamental and industrial purposes. Gong *et al*. (2003) used a muscle-specific *mylz2* promoter to drive GFP, YFP, and DsRed expression in zebrafish. These fish were considered suitable for ornamentation because the promoter drove such strong expression that fluorescence was visible without the aid of ultraviolet (UV) light. The authors proposed that the fish might also be used as a source of recombinant proteins because of the high levels of protein production. Experimentally speaking, these and other commercially available lines might also function as markers in transplant or explant assays since donor-derived cells with high levels of fluorescence would be easily identified.

Recently, the *lmo2* promoter was used to create two transgenic lines, *Tg(lmo2:EGFP)* and *Tg(lmo2:DsRed)* (Fig. 1A,B), which both exhibit embryonic blood and endothelial expression (Zhu and Zon, unpublished data). To distinguish hematopoietic from vascular cell populations in living embryos, *Tg(lmo2:DsRed)* was mated to *Tg(fli1:EGFP)*, a line that labels endothelial cells (Lawson and Weinstein, 2002). In these double transgenic embryos, the vasculature was labeled with EGFP, while the vasculature and blood were labeled with DsRed (Fig. 1C). *Tg(lmo2:DsRed)* was also mated to *Tg(gata1:EGFP)*, an erythrocyte-specific line (Long *et al*., 1997). Since erythroid progenitors and their progeny coexpress *gata1* and *lmo2*, these cells expressed both EGFP and DsRed in this double transgenic embryo (Fig. 1D). Endothelial cells were DsRed+/EGFP−, and could thus be distinguished from fluorescent yellow erythroid cells that expressed both proteins.

From these examples, several points can be made about the combinatorial use of fluorescent proteins. First, an examination of *Tg(lmo2:DsRed)* in the background of GFP expressing vascular and erythroid transgenics demonstrates that lineage-specific cells can be distinguished by fluorescent gene expression even if they are morphologically indistinct. Second, it is theoretically possible to identify or isolate cell populations that are marked by the overlap of gene expression domains similar to a Venn diagram. Third, the interplay between cell types in close physical association can be examined using time-lapse video microscopy. In essence, the techniques that have been used to analyze the interactions between subcellular components within individual cells can be translated onto whole organisms.

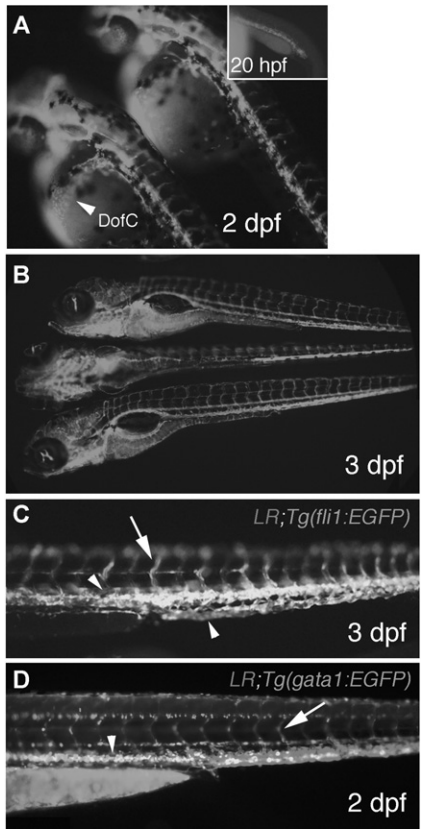

Fig. 1 Visualization of hematopoietic and vascular tissues using DsRed and EGFP transgenic embryos. (A) In *Tg(lmo2:DsRed)* embryos (abbreviated *LR*), DsRed protein is initially detected at 20 hpf (inset); 2 dpf *LR* embryos labeling hematopoietic and endothelial cells in the ducts of Cuvier (DofC). (B) Labeling of the vascular endothelial network of a 3 dpf *LR* embryo. (C) *LR; Tg(fli1:EGFP)* embryos distinctly label hematopoietic (arrowheads) and endothelial cells (arrow) in 3 dpf embryos. (D) In *LR; Tg(gata1:EGFP)* transgenic embryos, green/red erythrocytes (arrowheads) circulate through vessels (arrow) labeled by DsRed in 2 dpf embryos. (Zhu and Zon, unpublished data) (See Color Insert.)

IV. Use of Multiple Fluorescent Reporters in Transplantation Assays

Transplant and chimeric experiments in mice have traditionally taken advantage of Y-chromosomes and the *lacZ* reporter to identify donor cells in recipients. In zebrafish, it is preferable to employ fluorescent markers because they can be used to identify donor-derived cells without sacrificing the recipients. Also

consider that GFP or DsRed expressing tissues can be identified by immunohistochemistry if, for example, visualization of deeper tissues is required. The lack of cell-type specific surface antibodies in the zebrafish has made tissue-restricted fluorescent markers important for the isolation of these subpopulations that would be otherwise inaccessible. Another advantage of using fluorescent genetic tags in zebrafish is that breeding multi-labeled animals is easy, making it possible to devise and create sophisticated reagents in a relatively short amount of time.

Traver and colleagues (2003) pioneered the use of multicolored transgenic zebrafish in the setting of hematopoietic cell transplantation. They used whole kidney marrow (WKM), which is the primary site of adult hematopoiesis and bone marrow equivalent in teleost fish, from double-labeled fluorescent fish to follow multilineage, donor-derived hematopoiesis and early homing events (Fig. 2A–D). Two transgenic lines were used to independently label erythrocytes and leukocytes. The first line, *Tg(gata1:DsRed)*, expresses DsRed under the control of the *gata1* promoter and marks the erythroid lineage over an animal's lifetime (Long *et al.*, 1997; Traver *et al.*, 2003). The second, *Tg(β-acting:EGFP)*, expresses GFP in virtually all blood lineages except erythroid, and was used to mark myeloid, precursor, and lymphoid cells. WKM was collected from *Tg(gata1:DsRed); Tg(β-actin:EGFP)* double transgenic adults and injected into the circulation of wild-type embryos, resulting in the appearance of DsRed or GFP positive cells in the hosts. The red fluorescent cells were erythrocytes. Two morphologically distinct types of green fluorescent cells, seen rolling along vessel lumens, were likely lymphocytes and myelomonocytic cells. GFP expressing cells also homed to the sites of the developing thymus and kidney, whereas DsRed cells were only seen in circulation, suggesting that the GFP fraction contains lymphocyte and progenitor cells. A single fluorescent marker, GFP, was able to resolve two distinct cell types by outlining the shape of small round cells, identified as lymphocytes, and amoeboid cells with pseudopodia, identified as myelomonocytic cells.

The double transgenic kidney marrow was also transplanted into *vlad tepes* (*vlt*), a *gata1*$^{-/-}$ mutant lacking erythrocytes (Lyons *et al.*, 2002), and *bloodless* (*bls*), a mutant with an absence of primitive blood cells (Fig. 2B–D) (Liao *et al.*, 2002). While transplantation into *vlt* resulted in robust reconstitution of DsRed erythrocytes, the reconstitution of GFP+ leukocytes occurred at levels comparable to the wild-type recipient setting. In *bls*, the appearance of both DsRed+ erythrocytes and GFP+ cells in the developing thymus and pronephros was fast and robust, suggesting that there was a relative lack of competing host cells during the first few days of *bls* development. In this context, the independent labeling of two cell types with different colors helped to resolve the differences in engraftment kinetics between two mutant recipients. These results demonstrate an elegant way to identify donor cell types in the context of living transplant recipients. This type of experiment provides a bird's eye view of post transplantation hematopoietic cell homing, proliferation, and differentiation, a view that is impossible to obtain in any other system.

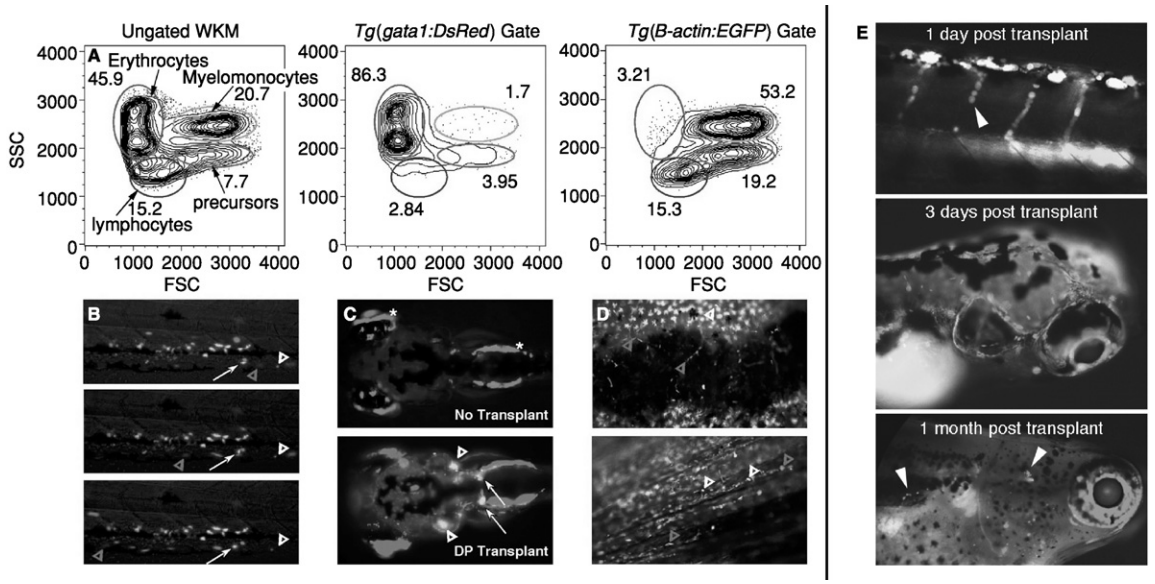

Fig. 2 Use of multiple fluorescent reporters in transplantation assays. Left panel: Transplantation of whole kidney marrow from double transgenic donors allows independent visualization of leukocytes and erythrocytes in recipient embryos. (A) Scatter profile of ungated WKM in a representative *Tg(gata1:DsRed); Tg(β-actin:EGFP)* double transgenic adult (left). DsRed+ cells were contained only within the erythrocyte gate (middle), whereas GFP+ cells were non-erythroid (right). (B–C) Transplantation of 48 hpf recipients showed transient reconstitution of donor-derived erythrocytes and leukocytes. (B) Visualization of the tail vessels in a *gata1*$^{-/-}$ transplant recipient showed a slow-moving, round leukocyte (arrowhead), a larger leukocyte displaying an end-over-end tumbling migration (arrow), and a rapidly circulating erythrocyte (red arrowhead) at 1 day post-transplantation. Each frame is separated by 300ms (20× magnification, anterior to the left). (C) Dorsal views comparing untransplanted (upper) and transplanted *bloodless* (*bls*) recipients (lower). *bls* recipients showed rapid and robust engraftment of the pronephros (arrows) and bilateral thymi (arrowheads) by GFP+ leukocytes by day 5 post-transplantation. Asterisks denote autofluorescence of the eyes and swim bladder in the DsRed channel. (D) *bls* recipients display sustained, multilineage hematopoiesis from donor-derived cells. Upper panel shows robust reconstitution of DsRed+ erythrocytes (red arrowheads) and GFP+ leukocytes (white arrowhead) as observed in the dermal capillaries of a *bloodless* recipient at 8 weeks post-transplantation. Lower panel shows similar multilineage reconstitution as observed in the tail capillaries of another *bls* recipient at 8 weeks (20× magnification). From Traver *et al.*, 2003.

Right Panel: Transplantation of primitive wave hematopoietic progenitors from *Tg(lmo2:EGFP)* embryos into *vlad tepes* (*vlt*) recipients. (E) The EGFP expressing population from 10–12 somite *Tg(lmo2:EGFP); Tg(gata1:DsRed)* transgenic embryos were isolated by FACS and transplanted into 48 hpf *vlt* embryos. One day post transplantation, circulating donor-derived cells could be identified by DsRed and EGFP fluorescence (arrowhead points to GFP+ circulating cell). By 3 days post transplantation, most circulating cells were DsRed+, suggesting that the EGFP+ donor progenitors had differentiated into Gata1^{+} erythrocytes. One month after transplantation, each of the surviving recipients carried approximately 10–200 DsRed+ circulating cells (arrowheads). (Zhu and Zon, unpublished data.) (See Color Insert.)

The transplantation of fluorescently labeled cells can also be used to assay the cell fate of a specific population. For example, the *Tg(gata1:DsRed)* fish were used to evaluate *lmo2* and EGFP expressing primitive hematopoietic progenitors (Zhu and Zon, unpublished data). In this experiment, EGFP+ donor cells were FACS sorted from 10- to 12-somite staged *Tg(lmo2:EGFP); Tg(gata1:DsRed)* embryos and injected into the circulation of 48 hpf *vlt* embryos that normally die within 14 days (Fig. 2E). One day after transplantation, circulating donor-derived erythrocytes could be identified by DsRed and EGFP fluorescence (Fig. 2E). By three days post transplantation, few $EGFP^+$ circulating cells were present and virtually all observable fluorescence was in the DsRed channel, suggesting that the $EGFP^+$ donor progenitors had differentiated into Gata1/DsRed expressing erythrocytes. One month after transplantation, surviving fish contained between 10 and 200 $DsRed^+$ circulating cells. This transplantation experiment showed that donor-derived primitive wave hematopoietic cells and their progeny could be detected in the circulation of *vlt* recipients for more than one month. This experiment demonstrated how one fluorescent label can be used for donor isolation, while another can be used as a marker of donor cells. It follows that the inclusion of even more reporters within a single transgenic animal will increase the versatility of possible experiments.

V. Fusion Protein Reporters

The number of novel fluorescent proteins is rapidly increasing. Thus, a thorough account of these developments is beyond the scope of this chapter. In addition to novel fluorescent proteins, fluorescent fusion proteins that label subcellular structures are being applied to fish. The first example was a transgenic line that labels cell nuclei with a histone-GFP fusion protein (Pauls *et al*., 2001). It is only a matter of time before the tools that had been developed for other animal models are transferred to zebrafish. In *Drosophila*, GFP fused to the C-terminal end of moesin, a protein that localizes to the cortical actin-cytoskeleton, has been used for the analysis of cell shape changes during morphogenesis (Edwards *et al*., 1997). GFP-*moesin* can potentially be used in zebrafish in order to analyze cell shape changes in processes that have specific relevance to vertebrate organogenesis. Now that red fluorescent fusion proteins can be made using mRFP1, multiple subcellular components can be labeled with several colors in the context of the whole organism.

Fluorescent fusion proteins have also played a role in zebrafish disease models. Recently, a zebrafish model of T-cell acute lymphoblastic leukemia was made by driving the expression of a mouse c-Myc-GFP fusion transgene under the control of the zebrafish Rag2 promoter (Langenau *et al*., 2003). The GFP fusion protein was integral in showing that the leukemia arose in the thymus and spread to the gills, retroorbital soft tissue, skeletal muscle and abdominal organs. In this leukemia model, it would be advantageous to ectopically express other genes that can