Modern mosaic analysis in the zebrafish

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Abstract

One of the most powerful tools used to gain insight into complex developmental processes is the analysis of mosaic embryos. A mosaic is defined as an organism that contains cells of more than one genotype, usually wild-type and mutant. It is the interplay between wild-type and mutant cells in the mosaic that reveals information about the normal function of the mutated gene. Mosaic analysis has been utilized extensively in Caenorhabditis elegans, Drosophila, mice, and zebrafish to elucidate when, where, and how a gene acts during development. In the zebrafish, mosaic analysis has been used to dissect a number of different developmental processes, including gastrulation movements, mesoderm and endoderm specification, neuronal patterning and migration, axon pathfinding, angiogenesis, and cardiac, retinal, and neural crest development. Mosaic analysis is a particularly effective method for understanding gene function in the zebrafish, a model organism particularly suited to forward genetic, molecular, and classical embryological approaches. These attributes, when combined with the accessibility and optical clarity of the zebrafish embryo, facilitate the real time observation of individual cell behaviors and interactions within mosaic embryos.

Keywords: Zebrafish; Mosaic analysis; Genetic mosaic transplantation

1. Uses of mosaic analysis in the zebrafish

1.1. Determining cell-autonomous vs. non-cell autonomous gene function

Determining whether a gene acts cell autonomously or non cell-autonomously is a classic use of mosaic analysis. A gene is said to act cell autonomously if its function in a particular process is required solely in those cells that exhibit the mutant phenotype. Therefore, if a gene acts autonomously, then mutant cells will exhibit the mutant phenotype when placed in a wild-type embryo and conversely, wild-type cells will behave normally when placed in a mutant embryo. A classic example of cell-autonomy is the function of the spadetail/tbx16 gene in the paraxial mesoderm [1]. In contrast, a gene can be said to act non-cell autonomously if its function is shown to be required in the cells surrounding those that exhibit a mutant phenotype. If a gene functions non-cell autonomously, then mutant cells will be rescued in mosaic embryos by the surrounding wild-type environment and conversely, isolated wild-type cells will exhibit the mutant phenotype in a mutant host.

By thus revealing how genes function to control particular developmental processes, mosaic analysis reveals important information not only about a specific gene’s function, but more generally about developmental mechanisms. For example, non cell-autonomously acting genes indicate that the affected tissue develops in response to external cues. Taken further, mosaic analysis can show where those cues come from, if wild-type cells in a particular position are able to locally rescue a mutant phenotype.

While knowledge of autonomous or non-autonomous activity provides important insights into gene function, assumptions cannot be made about the molecular nature of the protein encoded by that gene. For example, a transcription factor, which by its nature acts cell-autonomously, could appear to act non-cell autonomously if it were primarily responsible for driving the expression of a
non-autonomously acting signaling molecule. Recent examples of this in the zebrafish include the transcription factors, Tbx24, which functions non-cell autonomously to pattern the somites, and AP2, which functions non-cell autonomously to regulate craniofacial development [2,3]. By the same token, even when the identity of a mutant gene is known, mosaic analysis can elucidate the key site of activity for a gene, which may be in only a subset of the cells in which that gene is expressed. Thus, the molecular identification of a mutant gene does not eliminate the value of mosaic analysis for understanding gene function. Indeed, the vast majority of mosaic analyses in the current literature explore the functions of cloned genes.

Although the predictions for autonomous and non-autonomous gene functions in genetic mosaics as outlined above are clear, there are cases where genes appear to function both autonomously and non-cell autonomously with regard to different developmental processes. For instance, the zebrafish cloche gene functions non-cell-autonomously in the initial specification of blood cell progenitors, but cell-autonomously in their subsequent differentiation [4]. Mosaic analysis also showed that the zebrafish pbx4 gene, which encodes a homeodomain partner for Hox proteins, functions cell-autonomously to control the stereotyped migration of one set of hindbrain motor neurons, but non-cell autonomously to control the axon pathfinding of a different set of motor neurons [5]. Furthermore, mosaic analysis has revealed instances in which a gene functions both cell autonomously and non-cell autonomously in a single developmental process. The trilobite (tri)/strabismus (stbm) gene encodes a transmembrane protein in the Wnt planar cell polarity pathway that is required for normal convergence and extension movements during gastrulation [6]. In genetic mosaics, cells lacking tri/stbm fail to elongate or align mediolaterally in a wild-type host embryo, suggesting a cell-autonomous defect. However, wild-type cells also remain rounded and unaligned when surrounded by mutant cells in a tri/stbm host embryo, suggesting a non-autonomous component to tri/stbm function [6]. These results suggest that tri/stbm is likely to be required for the bi-directional signaling between cells that is essential for both establishing and interpreting tissue polarity [6].

1.2. Identifying late functions for genes that are essential for early development

Mutant phenotypes often reflect the earliest functions for a gene; however, genes often play multiple roles during development and adult life. Creating genetic mosaic embryos limits the loss of gene function to specific cells and can circumvent the early lethality of many mutations, since the wild-type cells in the mosaic can rescue the early essential gene function. For mutations that cause early embryonic lethality, this can allow a researcher to study gene function at later stages of development. This feature of genetic mosaics has been heavily exploited in the mouse, where essential early functions of genes in extra-embryonic tissues can be rescued with wild-type tetraploid cells, which contribute only to extra-embryonic tissues (reviewed in [7]). It has been used less in the zebrafish, where it is more difficult to target wild-type cells to a particular tissue in sufficient numbers to fully rescue an early mutant phenotype. The development of a method for targeting cells to the zebrafish endoderm [8] has opened the possibility of specifically rescuing wild-type gene function in endoderm in order to identify later functions in other tissues.

1.3. Testing a cell’s commitment to a particular fate

The same cell transplantation methods that will be described below to generate genetic mosaics can be refined to test the commitment of a cell to a particular fate. In regulatively developing embryos, cell fate is specified through the local cell–cell interactions that normally pattern the embryo. As development proceeds, many cells undergo a progressive restriction of fate, as they become committed to particular developmental paths. By transplanting cells heterotopically between embryos, the resulting chimeric embryos can be used to test the commitment of cells to a specified fate by challenging it with a different environment. The first such use of cell transplantation in the zebrafish established that at the outset of gastrulation, hypoblast derived cells can be respecified and acquire the cell fates typical of their new position, however, by midgastrulation hypoblast derived cells lose pluripotency and become committed to forming mesoderm and endoderm [9]. When testing cell commitment, the number of cells transplanted as well as the position of a cell with in the group can influence its outcome. A cell that lies within the center of a larger community of cells will often maintain its original identity (a “community effect”), while an individual cell or a cell that lies at the edge of a group will often be respecified [10].

1.4. Characterizing the properties of signaling molecules

Secreted signaling molecules can exert their patterning activity locally or over a distance through either direct diffusion or a cell–cell relay mechanism. Analysis of mosaic embryos can be effectively employed to determine the range over which cells can respond to a localized source of an inducing signal. Mosaic expression of ectopic FGF8 induces its transcriptional targets, the ETS genes, pea3, and erm, at a range of 3–4 cell diameters for pea3, and 7–8 cell diameters for erm [11]. The differential activation of these genes suggests that distinct cellular responses are induced by a gradient of FGF signaling. However, these experiments do not distinguish whether FGF acts over this distance by direct diffusion from its source, or whether it is relayed from cell to cell. By making mosaics in which a signal was required to diffuse across a field of unresponsive cells, Chen and Schier revealed that the Nodal-related TGFβ signaling factor, Squint, acts directly as a secreted morphogen to induce different downstream genes in a concentration dependant manner [12]. Chen and Schier, through an
elegant application of mosaic analysis, demonstrated that donor cells containing One-eyed pinhead (Oep), an extracellular cofactor that is cell-autonomously required for the reception of Nodal signals, can respond at a distance to an ectopic source of Squint, even though the intervening host cells lacked Oep and were therefore unresponsive to the Nodal signal [12]. The significance of this result is that it demonstrates that Squint is a secreted morphogen; it acts directly at distant to induce concentration dependent responses. These examples demonstrate that mosaic analysis is a powerful tool that can reveal detailed information about the activity of known genes.

1.3. Identifying maternal functions of essential genes

In the zebrafish, many genes that play a role in early development are expressed both maternally and zygotically. Maternally derived RNAs, which are expressed and stored during oogenesis, are responsible for directing the very early stages of development before the onset of zygotic transcription. The contribution of maternal gene product can sometimes compensate for the loss of zygotic gene function and partially mask its developmental role [13–16]. In cases where the zygotic mutant phenotype is embryonic lethal, the maternal component of a gene’s function can be assessed by creating a mosaic zebrafish with a largely wild-type soma and a mutant germ line, as long as the gene function is not required for the germ line’s development or maintenance. The progeny of such a mosaic female will thus lack maternally deposited RNA [14,17]. By then crossing this female to a male that is heterozygous for the mutation, the relative maternal and zygotic contributions can be determined, as half of the progeny will lack both maternal and zygotic gene function and the other half of the embryos will only lack the maternal function. The making of germline chimeras is an important emerging use of the general transplantation techniques that we describe below, also valuable tools that facilitate this approach have been developed, as we will discuss.

1.6. Using mosaics to examine individual cell behaviors

When combined with the optical clarity and rapid development that makes the zebrafish embryo particularly amenable to microscopic observation, mosaic analysis can be a powerful tool to examine cell movements in a living embryo. Throughout its development, the zebrafish embryo is shaped by complex and coordinated cell migrations, including the morphogenetic movements of gastrulation, neuromere formation, and organogenesis, as well as the directed migrations of germ cells, neuronal precursors, cardiac precursors, and other cell types. Mosaic analysis has been used extensively to characterize the role of genes in cell migration, as in the case of the planar cell polarity pathway components in convergence extension movements. Another emerging use of mosaic analysis has been to analyze the cellular mechanisms that underlie specific cell migrations. During gastrulation, the germ layers are formed as mesendoderm cells internalize at the margin and move under the prospective ectoderm. This local internalization of mesendoderm cells has been interpreted as involution, the turning in and internalization of cells as a sheet [18]. By definition, involution is a property of a coordinated group of cells whose movement requires the coherent organization of cells [19]. Examining the behavior of small groups or individual mesendoderm cells in mosaic embryos has challenged this assumption about the mechanism of mesendoderm internalization in the zebrafish. Maternal zygotic one-eyed pinhead (MZoep) mutant embryos do not form head and trunk mesendoderm, and therefore, cell internalization movements do not occur [13]. When individual wild-type cells are transplanted to the margin of MZoep embryos, they ingress, internalize autonomously, and express markers of the mesendoderm [20]. Similarly, cells that are forced to adopt endodermal fates through the expression of the activated Nodal receptor Taram A, will internalize directly when transplanted to the animal pole, a region of the zebrafish embryo where mesendoderm is usually not found and cell internalization movements do not occur [8]. These transplantation studies demonstrate that the cell movements of mesendoderm internalization can occur cell autonomously and appear to be the property of specified mesendoderm cells, regardless of their location. This strongly argues against involution as the cellular mechanism for mesendoderm internalization in zebrafish embryos.

The mosaic expression of cell lineage markers, such as GFP, can be employed not only to follow the migratory paths of cells in a living embryo, but also to observe changes in cell polarity and morphology during migration. The germinal zone of the upper rhombic lip (URL) has been thought to exclusively give rise to granule cell precursors, which migrate dorsally to the cerebellar anlagen [21]. However, time lapse analysis of URL cells that mosaically express enhanced-GFP revealed that the cells migrate to the midhindbrain boundary, where they then turn and migrate ventrally to the ventral brainstem and contribute to the precerebellar nuclei; demonstrating that cells derived from the URL contribute to other cell fates in addition to the granule cell precursors [22]. These experiments demonstrate that by using mosaic analysis to follow a migratory behavior of a few cells, the morphogenesis of complex tissues can be simplified and understood.

2. General considerations for mosaic analysis in zebrafish embryos

Mosaic analysis in flies, worms and mice has taken advantage of the sophisticated genetic tools that are available in those systems [7,23,24]. In Drosophila, for example, use of tissue specific promoters, the Gal4-UAS expression system, and FRT-mediated mitotic recombination has allowed researchers to control both the timing and location of losses or gains in gene function [23]. Thus far, the lack of
available genetic tools has somewhat limited the applications of mosaic analysis in the zebrafish. As tissue specific promoters, inducible constructs, and transgenic zebrafish lines are developed and become more widely available, researchers will begin to harness the full power of mosaic analysis in the zebrafish. Currently, mosaic zebrafish embryos can be generated through a variety of injection and transplantation techniques. Since DNA is often inherited mosaically after injection at the 1-cell stage, ectopic gene expression can be induced mosaically in transiently transgenic zebrafish embryos generated by injecting a DNA construct that contains the gene of interest under the control of either a tissue specific or a ubiquitous promoter. The mosaicism produced by this technique has recently been used to express exogenous potassium channels and dominant-negative SNARE proteins in single retinal ganglion neurons [25]. Mosaic distribution of RNA and anti-sense morpholinos can be achieved by microinjection into a single blastomere of a 32-cell or later stage embryo. This technique is effective for gain-of-function studies that can evaluate the sufficiency of gene activity or loss-of-function studies that can provide evidence for the necessity of gene function for specific cells fates and behaviors. In these cases, it is important to follow the descendants of the injected cells either with a co-injected mRNA encoding GFP (in the case of mRNA injection) or by using a fluorescein-labeled morpholino in the case of morpholino injection. For example, mosaic inhibition of neurogenin1 (ngn1) by injection of a fluorescently labeled morpholino into a single blastomere of a 32-cell stage embryo was used to place ngn1 downstream of Notch/Delta signaling in neural crest cell specification [26].

Mosaic embryos can also be created by transplanting cells from one embryo, the donor, to another, the host. This particular approach produces a chimera, defined as an organism made up of cells from more than one individual [27]. The chimera is thus a sub-category of mosaic. This transplantation technique is most commonly used to generate genetic mosaics in the zebrafish. Since the creation of mosaic embryos through cell transplantation is widely used, yet technically challenging, we will focus our discussion on this technique.

3. Considerations for creating chimeric zebrafish embryos

In zebrafish, mosaic analysis has generally involved examining cell behaviors in chimeric embryos that are generated by transplanting lineage-labeled cells from a donor embryo into an unlabeled host. Donor and host embryos usually differ in genotype, or in their levels of gene expression due to over-expression of RNAs or injection of anti-sense morpholinos. Often, cells from more than one differentially labeled donor embryo are co-transplanted into a single host embryo, so that one of the donor cell populations can serve as an internal control [1]. Depending upon the desired application, cell transplantation can be performed at either the blastula or gastrula stages, when cells are still uncommitted to a particular fate. There are advantages and disadvantages to each approach. Blastula stage transplants, in which the donor and host embryos are between 1000 cells and dome stage, can be performed on the dissecting microscope using relatively inexpensive equipment and large numbers of embryos can be transplanted at a single sitting. The disadvantage of blastula stage transplants, however, is that the low resolution of the fate map at these stages and the extensive cell mixing that occurs during epiboly means that targeting cells to particular regions of the ectoderm or mesendoderm is difficult. In contrast, gastrula stage transplants allow cells to be targeted to specific positions along the anterior–posterior and dorsal–ventral axes due to the emergence of the fate map at the onset of gastrulation [28,29]. This is useful when determining the function of a gene in a particular tissue. Gastrula transplants are often performed on a fixed-stage compound microscope in order to take advantage of the finer control of cell placement that can be achieved there. Transplants performed on a compound microscope can be slightly more challenging and require more specialized equipment. The set-up of donor and host embryos for transplantation is also more time consuming than for dissecting scope transplants. However many common elements are shared between the two techniques, which will be discussed along with specific instructions for each type of transplant.

4. Preparation of donor and host embryos

4.1. Dechorionation and raising of embryos

Both the donor and host embryos should be proteolytically dechorionated with 0.5 mg/ml pronase (Sigma) as described in the Zebrafish book [30]. Since overexposure to pronase can make embryos too fragile to handle, dechorionation should be closely monitored and washes begun when the chorions begin to collapse. For the best results, embryos should be proteolytically dechorionated prior to the 4-cell stage, since the chorion toughens with age, making it harder to remove without damaging the embryo. After the embryos are dechorionated, they are very fragile and will stick to plastic. Therefore, they should be transferred using a fire-polished glass pipette and kept in either agar-coated plastic dishes (1.2% in Embryo Medium; [30]) or autoclaved glass petri dishes. To improve their health and survival, dechorionated embryos should be raised at low density, 40–50 embryos per dish, in fresh Embryo Medium (EM) with Pen/Strep [30]. Raising the embryos at different temperatures, 25, 28, and 31 °C, to stagger their development will maximize the time window over which transplants can be performed.

4.2. Injection

Lineage tracers should be injected directly into the yolk of dechorionated embryos between the 1- and 4-cell stages.
During these early cleavage stages visible streams of clear cytoplasm move from the yolk into the blastomeres, evenly distributing the injected material. The Zebrafish Book contains detailed instructions for pressure injection into zebrafish embryos [30]. The lineage tracing dyes that are the most widely used to distinguish donor from host-derived cells are the fluorescent dextrans (Molecular Probes), however, other options are available and the choice of lineage tracer depends upon the desired application. Some of the different options for lineage tracers will be discussed in detail in the next section. Generally, injection of 1 nl of a 3% solution of fluorescent dextran is sufficient to allow for the detection of donor-derived cells in mosaic embryos through several days of development. For most experimental purposes, the donor and host embryos used for transplantation should be as closely stage-matched as possible; therefore it is useful to keep in mind that the development of injected embryos tends to be slightly delayed.

For mosaics involving morpholino-injected donor embryos, the morpholino-containing donor cells can best be followed if the morpholino itself is fluorescently labeled. However, co-injection of morpholino and lineage tracer into the yolk of the same donor embryo is equally effective since neither the morpholinos nor the lineage tracer pass between cells after cleavage stages.

In the case of mosaics involving mRNA-injected donor embryos, expressing cells cannot be followed using a co-injected fluorescent dextran because injected mRNA is inherited more mosaically during cleavage stages than is the lineage tracer. As a result, lineage-labeled donor cells may not express the injected mRNA, complicating the analysis. Rather, donor embryos should be co-injected with the mRNA of interest and an mRNA encoding a fluorescent protein (GFP or RFP) since to a large extent the injected mRNAs (and their encoded proteins) will co-segregate in the embryo. Unlike lineage dyes and morpholinos, which diffuse easily between cells during early cleavage stages, mRNAs are less diffusible. Accordingly, mRNAs should be injected as early as possible and targeted to the cell of the early 1-cell stage embryo rather than the yolk to minimize mosaicism in the donor embryo.

5. Selection and preparation of lineage tracers

The choice of the lineage marker that is used to detect donor derived cells in host embryos depends upon whether the donor cells will be imaged using fluorescence microscopy in live embryos, fixed embryos, or if the donor cells will be detected after the embryos are processed for in situ or antibody stain. Important considerations for lineage tracers that will be detected by fluorescence microscopy are how bright the fluorescence is, the photostability of the dye (can successive images be acquired without bleaching), and the excitation and emissions spectrum of the dye (are the appropriate filter sets available). Often, it is desirable to follow cells from more than one donor embryo. In these cases, it is important to choose lineage tracers that have separable emission spectra and that can be detected separately post-fixation. The most commonly used lineage tracers are fluorescent dextrans, tetramethylrhodamine dextran, and fluorescein dextran; however, a wide selection of lineage tracing dyes are available from Invitrogen-Molecular Probes, such as the green fluorescent Alexa Fluor 488 or Oregon Green dextran, which have increased photostability compared to fluorescein dextran and are less phototoxic in cells. It may also be desirable to image the membrane projections of donor cells as they migrate through the host embryo, in these cases the expression of a membrane targeted GFP or RFP could be used as a lineage tracer [31]. If the fluorescence will be detected in embryos after fixation with 4% paraformaldehyde, a lysine-fixable fluorescent dextran such as fluoro-ruby or fluoro-emerald should be used. Generally, even with the lysine-fixable fluorescent dextran conjugates, the fluorescence does not survive in situ hybridization. In this case, another method of lineage detection must be used in order to compare the location of donor-derived cells with domains of gene expression or protein localization. This can be accomplished by using a mixture of 2–3% fluorescent dextran (for visualization during transplantation and in live mosaics), and 2–3% biotin–dextran conjugate that can be detected post-fixation through its high affinity interactions with avidin and strepavidin. Common methods for visualizing biotin-labeled cells utilize either a horse radish peroxidase (HRP)–avidin enzyme complex (ABC kit, Vector Laboratories) to produce a brown precipitate that can be detected by bright-field light microscopy, or a Tyramide Signal Amplification (TSA) kit (Invitrogen-Molecular Probes) that employs enzyme conjugates of strepavidin to produce a fluorescent signal of your choice. One advantage of using these secondary methods of lineage detection is that the signal can be amplified, which may be useful in detecting donor cells many days after transplantation.

The lineage tracing dye used to label donor embryos for transplantation can be prepared by dissolving the selected dextran (Molecular Weight of 10,000 Da) in 0.2 M KCl. The prepared dye must then be filtered through a 0.22 μm spin-filter. The filtered dye is then aliquotted and wrapped in parafilm to prevent sublimation before being stored at −70 °C. Aliquots of the lineage tracer can be stored at −70 °C for well over a year. Once an aliquot is thawed, it should not be refrozen, however the unused portion can be kept at 4 °C for up to a week.

6. Preparation of the transplantation needle

The quality of the transplant needle can greatly influence the success of a transplantation session, so it is worth taking the time to craft a proper needle. Transplantation needles are made from glass capillary pipettes without a filament (either TW100-4 from World Precision Instruments or blood capillaries #53508-400 from VWR) that are drawn to a gentle taper on an electrode puller. The tip of needle is then broken off under a dissecting microscope
using a straight edge razorblade at the point where the inner diameter of the needle is slightly larger than the cells to be transplanted. The razorblade should be held at a slight angle to create a bevel and the break should be as smooth as possible, with no jagged edges. The aperture of the needle tip depends upon the age of the embryos used in the experiment, since cells are larger in younger embryos and get smaller as the embryo ages. For blastula stage transplants the outer diameter of the needle should measure approximately 50–60 μm, for gastrula stage transplants 30–40 μm, and 8–12 μm for single cell transplants at later stages. A needle aperture that is too small could shear the donor cells as they are taken up into the needle, while a needle that is too large is unwieldy and can damage the embryo. After shield formation, the enveloping layer (EVL) becomes more adherent, making it difficult for the transplantation needle to penetrate the embryo. To facilitate needle entry, a barb is pulled on the end of the needle using a microforge (Fig. 1A). First, the tip of the needle is smoothed by bringing it close to the filament of the microforge then the needle is turned so that the leading edge of the bevel can make contact with the filament. Once the leading edge of the needle contacts the filament, it is pulled quickly away to create a barb, or harpoon. To avoid damaging cells, the barb should be straight and the tip of the needle should not curve inward. A barb may also be pulled on the end of needles that will be used for blastula stage transplants, but it is not necessary to penetrate the EVL at this stage and may be a hindrance for single cell transfers.

7. Mounting embryos for transplantation

7.1. Mounting embryos for dissecting scope transplants

The main advantage of performing cell transfers on a dissecting scope as opposed to a compound microscope is that the embryos can be loaded into a transplantation mold, which takes considerably less time than mounting embryos in methylcellulose on depression slides (see below). A plastic template with rows of wedge shaped protrusions (Fig. 2C) is needed to make a mold that will hold the donor and host embryos for transplantation and can be obtained from AL-AN Mfg, Inc. (alanmfgusa@aol.com) or made at any university’s machine shop. To make the mold, the template is floated atop a petri dish that is half filled with molten 2% agarose/embryo medium. Once the agarose has solidified, the template is removed leaving an

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**Fig. 1.** Mounting embryos for gastrula-stage transplants. (A) Ideal shape of a gastrula transplant pipette. A bevel with a sharp tip aids in penetrating the embryo. (B) Immobilizing donor and host embryos in methylcellulose. A strip of methylcellulose is laid down in the well of a depression slide and flooded with a generous amount of embryo medium. Embryos are added to the embryo medium and are rolled onto the methylcellulose using a small loop (C). (D–G) Enlargement of embryos in (B). (D) The donor embryo is oriented to allow easiest access for the pipette, since cells at this stage are still uncommitted. (E–G) Shield-stage host embryos are oriented so that the target region is uppermost. Cells transplanted into the boxed regions will contribute to dorsal hindbrain and cranial neural crest derived from the left (E) and right (G) sides or to the ventral hindbrain and spinal cord (F).
Fig. 2. Microscope set-up for making mosaic embryos. (A) A dissecting microscope transplant rig consists of an oil-filled Hamilton syringe with a micrometer drive (a) connected to an oil-filled reservoir (b) and the transplant pipette (c) via a 3-way stopcock (d). The transplant pipette is mounted on a coarse micromanipulator (e) which is attached to a metal base plate (not shown) via a magnetic foot (f). Transplants are performed on the stage of a stereomicroscope (g) equipped with bottom lighting for optimal optics. (B) A compound microscope transplant rig consists of a similar oil-filled Hamilton syringe and micrometer drive (a and b) but in this case the transplant pipette is mounted on a pipette holder (c) whose X, Y, and Z movements can be controlled either by a coarse (d) or fine (e) micromanipulator. In this example, the micromanipulator is mounted on the body of a fixed-stage microscope, however it is also possible to mount it on the stage of a regular microscope. The embryos, which are immobilized in methyldextrin on a depression slide, are visualized using a 10× objective (f). (C) Transplant mold for immobilizing embryos for stereomicroscope transplants. Dechorionated embryos are dropped individually into wells made by casting this mold into agarose in a 90 mm petri dish. The dimensions of the wells are shown.
agar mold that contains rows of triangularly shaped wells that are just large enough so that each well can hold one embryo. The transplantation mold is then filled with embryo medium containing penicillin and streptomycin and the embryos are loaded with a fire polished glass pipette so that the donor embryos are placed down one column and the host embryos are placed down the adjacent column.

Embryos should be placed into the transplant wells on their sides, and they can be gently repositioned with the transplant pipette as needed during transplantation, as long as care is taken to not puncture the yolk. The position of cells along the animal–vegetal axis influences their contribution to one of the three germ layers, endoderm, mesoderm, and ectoderm [28,32]. Accordingly, cells transplanted close to the margin prior to the beginning of gastrulation will give rise preferentially to endoderm and mesoderm, while cells transplanted toward the animal pole will contribute to ectodermal fates, most commonly surface ectoderm, forebrain, and eyes. Thus a degree of tissue targeting can be accomplished even at blastula stages. The recent development of transgenic lines that allow the dorsal side of the embryo to be visualized before the shield forms will allow donor cells to be targeted to specific embryonic domains before the beginning of gastrulation (see Section 11: “transgenic tools for the generation of mosaics” below). After the cell transfers have been completed, the donor–host pairs can be transferred to the agar coated wells of a 24-well plate to develop further.

7.2. Mounting embryos for transplants on the compound microscope

A higher resolution fate map emerges with the appearance of the embryonic shield, which marks the dorsal side of the embryo. At gastrula stages, the animal–vegetal and dorsal–ventral axes form a coordinate system that has been used to map the location of tissue primordia [28,29]. Performing transplants shortly after the appearance of the shield allows cells to be targeted to broad domains along the anterior–posterior and dorso–ventral axes of the central nervous system [33–35], to the cranial neural crest and epidermal placodes [36,37], and to the oral ectoderm (J. Eberhart and C. Kimmel, personal communication). After the onset of gastrulation it is more difficult to transplant cells to particular domains of the early gastrula stage embryo, since the majority of these cells have internalized by this stage.

Transplanting cells to particular domains of the early gastrula requires the host embryo to be immobilized so that it cannot roll when pierced with the transplant pipette, and higher power optics (100× magnification) to prevent the pipette from nicking the yolk. For these reasons, we prefer to perform gastrula stage transplants on a fixed-stage compound microscope. For compound microscope transplants, embryos are mounted on a depression slide in a 3% solution of methylcellulose (Sigma) dissolved in embryo medium. A vertical stripe of methylcellulose is smeared in the depression of a glass depression slide, which is then flooded with a generous amount of embryo medium (Fig. 1B). One donor embryo can provide enough cells, approximately 300, for up to three host embryos without disrupting its own development. The only committed cell fate in early gastrula embryos is that of the germ cells, which are few in number and located near the margin. The other cells of the early gastrula stage embryo are uncommitted and will acquire the fate dictated to them by the host environment; therefore, the only consideration when orienting the donor embryo is the easy accessibility of the cells. To make it easier to obtain cells without damaging the donor, donor embryos that are slightly younger than the hosts (30–50% epiboly rather than shield stage) can be used without compromising the ability of the donor-derived cells to contribute normally to all tissues.

Using a small loop made by gluing the ends of a short length of 2-lb test fishing line into the end of a capillary tube (Fig. 1C), the donor and host embryos are gently rolled up onto the top of the methylcellulose strip and are then stuffed down into the methylcellulose until secure (Fig. 1B). It is important that during mounting, no damage occurs to the embryo and that the embryo be held tightly by the methylcellulose, so that it does not roll during transplantation. If the transplanted cells are to be targeted to a particular domain, knowledge of the shield stage fate map [28,29] and the position of the target tissue relative to the shield is essential so that host embryos can be positioned correctly. As the host embryos are placed into the methylcellulose, they should be rolled into position so that the targeted region is uppermost, since during transplantation the needle will enter the embryo tangentially to deliver the donor cells without damaging the yolk (Fig. 1D–G). In order to maximize the number of transplants that can be performed in an experimental session, it is helpful to have one person mount the embryos while another person performs the transplants. If only one person is available to do both the mounting and cell transfers, then keep in mind that a prolonged wait time between mounting and transplantation allows the methylcellulose to soften with exposure to embryo medium, prematurely releasing the embryos and allowing them to roll, making targeted cell transfer impossible. Once the cell transfers are completed, the entire slide is placed into a petri dish and flooded carefully with embryo medium containing penicillin and streptomycin. Over the next few hours the methylcellulose will dissolve, releasing the embryos.

8. Selection and setup of transplantation equipment

Successful cell transfers require the precise and stable control of suction and pressure to draw up and expel the donor cells. The apparatus that is used for cell transplantation in the zebrafish blastula (Fig. 2A) consists of a micrometer drive-controlled Hamilton syringe (10–50 μl) attached by a three-way stopcock to a reservoir of mineral
oil and to a micropipette holder through a length of flexible tubing. This apparatus can be constructed from its component parts, as described in the zebrafish book [30], or purchased from Sutter Instrument Company (Manual injector and pipette holder assembly MI-10010). After assembling the transplantation rig, it is filled with mineral oil, taking care to eliminate all air bubbles from the system. The presence of an air bubble will negatively impact your ability to control the suction and pressure. If transplants are to be performed on a dissecting stereomicroscope, then the stereomicroscope used should have fairly high magnification with good optics (at least 80× magnification would be ideal). While fluorescence is useful for determining the position and success of cell transfers, it is not a necessary component of the stereomicroscope, and should not be used during transplantation to avoid damaging the cells. The positioning of the micropipette holder and needle can be controlled by a Narishige manual micromanipulator, as for injections; however, if the base of the stereomicroscope is too wide to allow easy access with the micromanipulator, then an adaptor that attaches to the body of the stereomicroscope can also be purchased from Narishige. The mounting of the micromanipulator must be very stable in order to avoid transferring vibrations to the transplant needle; a magnetic base works well for this purpose.

Cell transfers performed on a compound microscope benefit from the precise control of needle position provided by a three-axis oil hydraulic micromanipulator (e.g., Narishige MMO-203; Fig. 2B). This micromanipulator controls the fine movements of the transplant pipette, while the suction in the pipette is controlled by a micrometer-drive Hamilton syringe similar to the one used for blastula transplants. An upright compound microscope with a fixed stage is preferable for performing cell transfers since focusing such a microscope does not cause the embryo to move relative to the pipette. However, if an adjustable-stage compound microscope must be used, then the micromanipulator can be mounted to the stage rather than to the body of the microscope with the same effect. Adaptors that provide options for mounting the micromanipulator to either the stage or body of compound microscopes from a variety of manufacturers are also available from Narishige.

9. Transplantation

Cell transplants performed on dissecting stereomicroscopes are facilitated by the three-dimensional images, long working distance, and large focal range that are characteristic of these types of microscopes. After the donor and host embryos are arranged in the transplantation mold, the dish should be positioned on the stage so that when the transplantation needle enters an embryo, the needle pushes the embryo against the back wall of its well. Using the micromanipulator, the transplantation needle is lowered into the dish at a fairly steep angle. In our opinion, the transplantation needle should enter the embryo at approximately a 45° angle. Once the tip of the needle is below the surface of the embryo medium, a small amount of embryo medium is drawn into the needle by twisting the micrometer drive controlling the Hamilton syringe. For the most precise control, the interface between the mineral oil and the embryo medium should remain in the thin, tapered part of the needle, but not too close to the end. Gently position the donor embryo with the needle and then draw the needle back and enter the blastula cap of the embryo at the desired position. It should be a swift, hard hit to penetrate the embryo without causing it to roll. The donor cells are then slowly and carefully drawn up into the needle. If the cells are taken up too quickly, they are likely to shear. It is important to avoid taking yolk up into the needle, as it will bind to the mineral oil, which will then kill the cells.

The number of cells that is transplanted depends on the application and on the position to which cells are to be transferred. To test cell autonomy it is necessary to visualize the behaviour of single mutant cells in a wild-type environment, or of single wild-type cells in a mutant environment. But because cells disperse a great deal during gastrulation (particularly in the mesoderm and the posterior CNS), it is possible to transplant over 50 cells and still be able to identify single cells at later stages. Unless the position of the target tissue progenitors in the blastula or gastrula is very well defined, transplanting fewer cells than this will result in many of the hosts being uninformative because they lack donor-derived cells in the desired position or tissue.

After the desired number of cells is taken up, the pressure is reversed slightly to stop the suction and the needle is removed from the embryo. The host embryo is then brought into position by moving the transplantation dish. Small adjustments to the position of the host embryo can be made by gently turning it using the transplantation needle, as long as care is taken to not touch the yolk. When expelling the donor cells into the host embryo, it is important to avoid introducing a large amount of embryo medium or any mineral oil, as this could interfere with development or kill the embryo.

Compound microscope transplants are performed using a long working distance 10× objective (100× magnification). The transplantation needle is positioned by moving it into the focal plane of the embryo. This is accomplished by first focusing on the donor embryo using the 10× objective. The embryo is then moved off to the side, and without adjusting the focal plane; the transplantation needle is brought into the proper position using the controls of the micromanipulator to bring the tip of the needle into focus. The micropipette holder and needle should be positioned at a fairly shallow angle, so that the needle is as close to horizontal as the edge of the depression on the slide will allow. If an embryo is mounted properly in methylcellulose, it will not roll as the transplantation needle enters, unless the embryo is too old to allow the needle penetrate easily. The enveloping layer seems to toughen as embryos age, therefore, gastrula
stage transplants are best performed right after shield formation. After dome stage, the yolk resides just under the cells of the blastoderm cap, which progressively thins as epiboly proceeds. In order to avoid piercing the yolk, the needle should enter the embryo at a focal plane that is just slightly deeper than that of the EVL (Fig. 1D and E). When removing large numbers of cells from the donor embryo, the needle should be moved around as the cells are taken up, in order to avoid accidentally sucking yolk into the needle. If cells from one donor embryo are to be transplanted to multiple hosts, then it is best to enter the donor with the needle only once in order to minimize the possibility of damage. The donor cells can then be transferred to the desired location in up to three host embryos. For some experiments, cells from two or more donor embryos are transferred to a host embryo. In these cases, it is best to take cells from each of the donor embryos into the same transplantation needle before transplanting them to a host embryo. This minimizes damage to the host embryo, and ensures that the cells are transferred to the same area of the embryo, so that their behaviors can be compared. Very little mixing occurs between the donor cells within the transplant needle, therefore, the cells should be transferred to a single host when more than one donor is used. In order to increase the likelihood that the donor cells will contribute to the desired tissue, the donor cells can be expelled while the needle is drawn through the targeted area, as opposed to depositing the cells in a single clump.

10. Germline transplants: a special case

An important goal is to understand the maternal functions of genes that have essential zygotic functions. Since these mutant fish cannot survive until adulthood, it is necessary to transfer the mutant germline into an otherwise wild-type host embryo, creating “germline clones” of mutant cells. Generating germline mosaics involves transplanting primordial germ cells from a mutant donor to a wild-type host embryo. Unlike all other cell lineages in the embryo, the germ cell lineage is specified very early in development by the inheritance of maternal determinants [38,39]. Thus, the first challenge of making germline mosaics is identifying the primordial germ cells (PGCs) in the donor embryo. At midblastula stages the PGCs reside along the margin [40,41], so picking up 50–100 random cells from the margin of a lineage-labeled donor embryo frequently results in the transfer of PGCs. When transferred to the animal pole of an unlabeled host embryo, the primordial germ cells actively migrate towards the presumptive gonad where they can be unambiguously identified at 24 hours of development by their characteristic position, large size, and dye retention due to their slow proliferative rate [14,17,42]. Meanwhile, donor-derived non-PGCs will acquire the fate determined by their location in the host: primarily forebrain and eyes if they were transplanted to the blastula animal pole.

11. Transgenic tools that aid in the generation and analysis of mosaic embryos

As discussed above, the goal of mosaic analysis is often to ask how mutant cells behave in a particular tissue, or to ask whether wild-type cells in a particular tissue can rescue a mutant phenotype. Ideally, cells could be targeted efficiently and exclusively to a specific tissue to address these questions, however this is currently only possible for the endoderm, where donor-derived cells can be targeted by forced expression of an activated Nodal receptor, Taram A [43]. This approach has recently been used to identify an endodermal source of signals that induce sensory neurogenesis and cartilage differentiation in the head [44,45].

As we have also discussed, the alternative way to target cells to particular tissues is to use the embryonic fate map as a guide in transplantation experiments. New transgenic lines are expected to facilitate this approach in the coming years by allowing visualization of embryonic landmarks before they are morphologically distinguishable. Fine targeting of cells along the dorso–ventral and anterior–posterior axes of the embryo has until recently been impossible before the appearance of the embryonic shield on the dorsal side of the early gastrula. However dorsal is specified long before the onset of gastrulation, and a transgenic line in which GFP is expressed under the control of one of the earliest markers of dorsal mesendoderm, goosecoid, has recently allowed more accurate targeting of tissues prior to gastrulation. Using this line, it is possible to target cells to the dorsal mesendoderm as early as 30% epiboly (J. Crump, J. Eberhart, M. Schwartz and C. Kimmel, personal communication) and it should be possible to target cells to other mesendodermal derivatives whose primordia lie at specific positions along the dorso–ventral axis of the margin [28,46–48].

Valuable tools have also been developed that improve the efficiency of making germline mosaics. Injecting the host embryo with morpholinos that knock down the dead end gene effectively eliminate the host germline in a cell-autonomous manner, so that even a single donor-derived PGC can repopulate the entire germline [17,49] (C.M., personal observation). More recently, a transgenic line that allows PGCs to be visualized in live embryos during blastula stages has been developed [50]. When crossed into the mutant whose maternal function is to be determined, this transgene, combined with the dead end morpholinos, will make germline transplants facile because single PGCs can be identified and transplanted into a germline-depleted host.

Transgenic lines in which GFP is expressed in specific tissues or cell types are also valuable tools for the analysis of genetic mosaics, since they allow the visualization of donor-derived cells and tissue-specific gene expression simultaneously and at single-cell resolution in live, developing embryos. Two recent examples from our own work are the use of the pax2.1-GFP transgenic line [51] to elucidate the function of EphA4 in hindbrain boundary formation.
and the use of the isl1-GFP transgenic line [52] to identify a cell-autonomous requirement for the spt5 gene in motor neuron migration [33]. However examples of the use of transgenic lines to inform genetic mosaic analyses are increasing daily. In their effectiveness and elegance these experiments represent the fully realized potential of the zebrafish as a model system for the study of vertebrate development.

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