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aMAZe-ing tools for mosaic analysis in zebrafish

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Genetic mosaic analysis is a powerful technique to analyze the function of cells with varied genotypes in the same animal. By contrast, cell-lineage tracing allows the history of a cell's development to be uncovered. Zebrafish are uniquely suited to merge mosaic and cell lineage studies, and in this issue of *Nature Methods*, Russell Collins and colleagues present the first non-invasive method of generating temporally and spatially controlled mosaics in zebrafish¹.

Unlike flies and other vertebrate models, zebrafish embryos are optically clear, allowing for detailed imaging of live animals. Despite this advantage, techniques for creating genetic mosaics in zebrafish have lagged behind those in the fruit fly and the mouse, owing in part to a lack of genetic tools. Now, the mosaic analysis in zebrafish (MAZe) system allows investigators to create and permanently label mosaic cells with a fluorescent reporter, facilitating both functional mosaic analysis and cell-lineage tracing¹ (Fig. 1a).

Genetic mosaics in zebrafish are most commonly produced by cell transplantation between blastula stage embryos of different genotypes². This technique allows investigators to assess whether embryonic lethal mutations have effects later in development and to determine whether a gene functions cell-autonomously or non-cell-autonomously. Photoactivatable morpholinos have also been used to spatiotemporally control gene expression *in vivo* and can be used to generate functionally mosaic zebrafish by transient gene knockdown³. DNA microinjection into one-cell-stage embryos is also a powerful system used to generate mosaics through the overexpression of transgenes. The additional use of tissue-specific promoters and fluorescent reporters allows the effects of transgenes to be imaged in real time and provides robust methods to perform *in vivo* lineage analysis⁴.

Stable transgenic approaches common in other mosaic models have also been adapted to zebrafish. For example, a heat-shock-inducible Cre-*loxP* system has been used to stably express the constitutively active human *KRAS* (also known as *KRASG12D*) oncogene in various zebrafish tissues to model a diverse array of human cancers⁵. Additionally, an iteration of the Gal4-*UAS* system, in which the yeast transcription activator Gal4 binds in *trans* to an upstream activation sequence (UAS), has recently been modified for use in zebrafish⁶. In this system, KalTA4, a vertebrate-optimized Gal4, activates UAS-driven transgenes in a dose- and tissue-specific manner. KalTA4 has subsequently been used to induce expression of the *Kalloop* transgene. In this approach, KalTA4 is initially produced from a tissue-restricted promoter, which can then activate a second transgene that expresses both GFP and KalTA4. Once *KalTA4-UAS* is activated in a cell, a feed-forward loop called 'kalloop' maintains labeling in

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Mosaic analysis in zebrafish (MAZe) allows lineage tracing and analysis of mosaic animals.

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cells, providing a way to link embryonic expression of a mutant transgene to long-lasting effects in adult zebrafish tissues (Fig. 1b).

Although these techniques are powerful approaches to create genetically mosaic zebrafish and in some instances allow cell-lineage tracing, they have important limitations. Blastula transplants are invasive, technically challenging and can be difficult to spatially target². Light-controlled gene silencing using caged morpholinos is limited to studies in early development and can only achieve partial gene knockdown³. Only a small percentage of DNA-microinjected embryos express the transgene as larvae and adults⁴, and the DNA is often inserted at a high copy number, which may interfere with the early stages of development⁷. Finally, the KalTA4-UAS Kaloop system relies on the continued production of the KalTA4 activator to maintain a feed-forward loop to drive transgene expression and does not permanently mark mosaic cells at the genome level⁶. Off-target expression of KalTA4 would mislabel cells, and it is uncertain how long the feed-forward loop can be maintained. Although these approaches have all had success, new genetic tools are needed to refine mosaic analysis and cell-lineage tracing in the zebrafish.

The MAZe transgenic approach by Collins *et al.*¹ is a new approach to lineage tracing used to genetically mark cells in zebrafish (Fig. 1a). The MAZe transgene consists of an *hsp70* promoter-driven *Cre*, flanked by *loxP* sites, which self-excises under a heat shock. After Cre-mediated recombination, an *EF1 α* promoter induces Gal4-VP16 expression, which in turn activates the expression of a UAS-driven nuclear localized red fluorescent protein (*nlsRFP*). Because the heat-shock is not 100% efficient, only a subset of cells undergo Cre-mediated recombination and subsequently express the *nlsRFP* transgene.

The authors used the MAZe system to visualize muscle cell fusion in living zebrafish embryos and showed that nuclear proteins are dynamically redistributed between the myonuclei of fused fibers. Another advantage of the MAZe system is that Gal4-VP16 activates *UAS* in *trans*, meaning MAZe transgenic zebrafish can be crossed with any zebrafish expressing a UAS-driven transgene to easily examine the function of a particular gene throughout development. Thus, the MAZe system allows for both a true mosaic analysis of transgene-expressing cells in the context of their wild-type counterparts and provides the opportunity to trace cell lineage through expression of a fluorescent marker. MAZe embryos can also be microinjected at the one-cell stage with a tissue-specific *Cre*, bypassing the need for heat-shock and providing spatial control of transgene expression. Taking together the above properties, the MAZe system represents a major step forward in mosaic analysis in zebrafish, and considering the opportunity of imaging developmental and biological processes in live vertebrates, is a valuable addition to the well-established cell-lineage tracing approaches in the fly and mouse.

The future of mosaic analysis in zebrafish will likely involve optimization of the MAZe technique, coupling zebrafish-optimized KalTA4 with permanent genetic cell marking using Cre recombinase-mediated recombination (Fig. 1c). Moreover, developing a MAZe line that relies solely on Cre expression from a second transgene will likely eliminate potential artifacts associated with the leaky *hsp70* promoter. Use of a zebrafish transgenic line that expresses a Cre recombinase fused with an estrogen receptor, which is activated by tamoxifen treatment, will allow for temporal activation of both cell labeling and mosaic analysis.

Finally, the MAZe transgenic approach is limited to transgene overexpression. Developing new models and techniques to assess loss-of-function phenotypes in a mosaic setting will provide unprecedented access to uncover the effects of genetic mutations on development, disease and cancer. Such approaches—commonly known as ‘twin spot’ assays—revolutionized genetic analysis in fly eye development and uncovered how loss of gene expression exerts effects on different cells. In these twin-spot assays, heterozygous mutant animals are stimulated

to recombine their chromosomes during mitosis, producing both a normal wild-type cell and a homozygous mutant cell. This approach relies on tagging both chromosomes with markers to track mutant and wild-type cells and commonly uses FLP recombinase to facilitate mitotic recombination⁸. Developing these reagents in zebrafish would provide powerful techniques to assess gene mutation effects on development. In total, the work by Collins *et al.*¹ is a major step forward for mosaic analysis and cell-lineage tracing in zebrafish and provides a tool to uncover the function of gene pathways in development and beyond.

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Figure 1.

Mosaic analysis in zebrafish. **(a)** Schematic of the current iteration of MAZe, with loxP sites denoted by blue triangles. **(b)** Schematic of the KalTA4-UAS Kaloop system. T2A is a sequence that allows bicistronic expression of two transgenes. **(c)** Future iterations of MAZe may include replacing the EF1 α promoter with a truly ubiquitous promoter and GAL4-VP16 with KalTA4. A second Cre transgene driven by a tissue-specific promoter could be used to spatially target the MAZe transgene, and a tissue-specific and estrogen-responsive Cre (Cre-ER) would allow for spatiotemporal control of MAZe.