

Targeting neural circuitry in zebrafish using GAL4 enhancer trapping

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We present a pilot enhancer trap screen using GAL4 to drive expression of upstream activator sequence (UAS)-linked transgenes in expression patterns dictated by endogenous enhancers in zebrafish. The patterns presented include expression in small subsets of neurons throughout the larval brain, which in some cases persist into adult. Through targeted photoconversion of UAS-driven Kaede and variegated expression of UAS-driven GFP in single cells, we begin to characterize the cellular components of labeled circuits.

Important advances in the *Drosophila melanogaster* model system have resulted from the ability to visualize and manipulate discrete tissues, and enhancer trap screens have been important for generating this capacity. Historically, transgenesis in zebrafish has been too inefficient to allow for such screens, but efficient methods using transposons or retroviruses have recently allowed the identification of new expression patterns through gene or enhancer trapping in zebrafish^{1–4}. These studies are especially useful because zebrafish represent a vertebrate system with excellent characteristics for imaging, developmental studies and behavioral analysis.

The limitation of this approach is that the trapped cells cannot be manipulated aside from being fluorescently labeled. More desirable would be a system that allows for expression of various transgenes in trapped expression patterns. In the fruit fly and more recently zebrafish^{5,6}, the yeast transcriptional activator GAL4 has been used to drive transgenes linked to the target UAS of the GAL4 protein. This has allowed studies misexpressing regulatory genes, assorted cell markers and rescue constructs, among others, in a spatially and temporally controlled fashion. Here we present a screen intended to combine the flexibility of GAL4-UAS with enhancer trapping in zebrafish.

Our enhancer trapping constructs are based on the pT2KXIGΔin plasmid⁷, which is derived from the *Tol2* transposable element

(Supplementary Methods online). Each construct carries GAL4 and the transcriptional activator VP16 (ref. 8) linked to a 5' basal promoter, intended to drive tissue-specific expression when inserted near an endogenous enhancer. We detected insertions after raising injected fish (F₀) to adulthood and crossing them to carriers of the UAS:Kaede transgene. Kaede is a photoconvertible fluorophore, which fluoresces either in the green wavelength (without conversion) or in the red wavelength (after conversion with ultraviolet light)⁹. We screened for Kaede expression in F₁ progeny at 3 and 5 days post fertilization (d.p.f.). In total, we screened 161 founders, 146 potentially carrying an insertion of the 1.5-kb heat shock cognate 70-kDa protein (*hsp*) promoter¹⁰ upstream of GAL4-VP16 and 15 potentially carrying a truncated 600-bp *hsp* promoter upstream of GAL4-VP16. We found 65 and 11 founders for the 1.5-kb and 600-bp HSP constructs, respectively. Many founders carried multiple insertions, indicated by two or more distinct expression patterns within the F₁ clutch. As a result, we observed 117 expression patterns for *hsp(1.5 kb):Gal4* and 24 expression patterns for *hsp(600 bp):Gal4*. A subset (19) of the 141 patterns showed expression only in frequently labeled tissues (pineal, muscle and heart) that may represent background expression independent of endogenous enhancers. The remainder of the patterns included unique expression in other tissues. A summary of the expression patterns that are being maintained as stable zebrafish lines is available in Supplementary Table 1 online.

A majority of the patterns included expression in discrete portions of the central nervous system (see Fig. 1 and Supplementary Table 2 online). Although expression resulting from trapped enhancers was often strong, it was generally accompanied by background expression in skeletal muscle and heart. This was true for both promoters, although background in the muscle was generally lower from the 600-bp promoter than from the 1.5-kb promoter. This background expression does not interfere with visual analyses of the trapped patterns, but may pose an obstacle to future experiments using transgenes (for example, ion channels) to interfere with neuronal function. For this reason, it will be highly desirable to identify a basal trapping promoter with little or no background expression.

The use of GAL4 in enhancer trapping raises specific concerns about GAL4 toxicity. We observed lethality for a small proportion (6/141) of our patterns, while the remaining insertions appeared viable. This rare lethality included lines carrying both the 1.5-kb HSP promoter and the 600-bp version, and probably resulted from GAL4-VP16 toxicity, as it was not correlated with Kaede expression.

We and others have observed that expression of various marker proteins after conventional transgenesis is often variegated.

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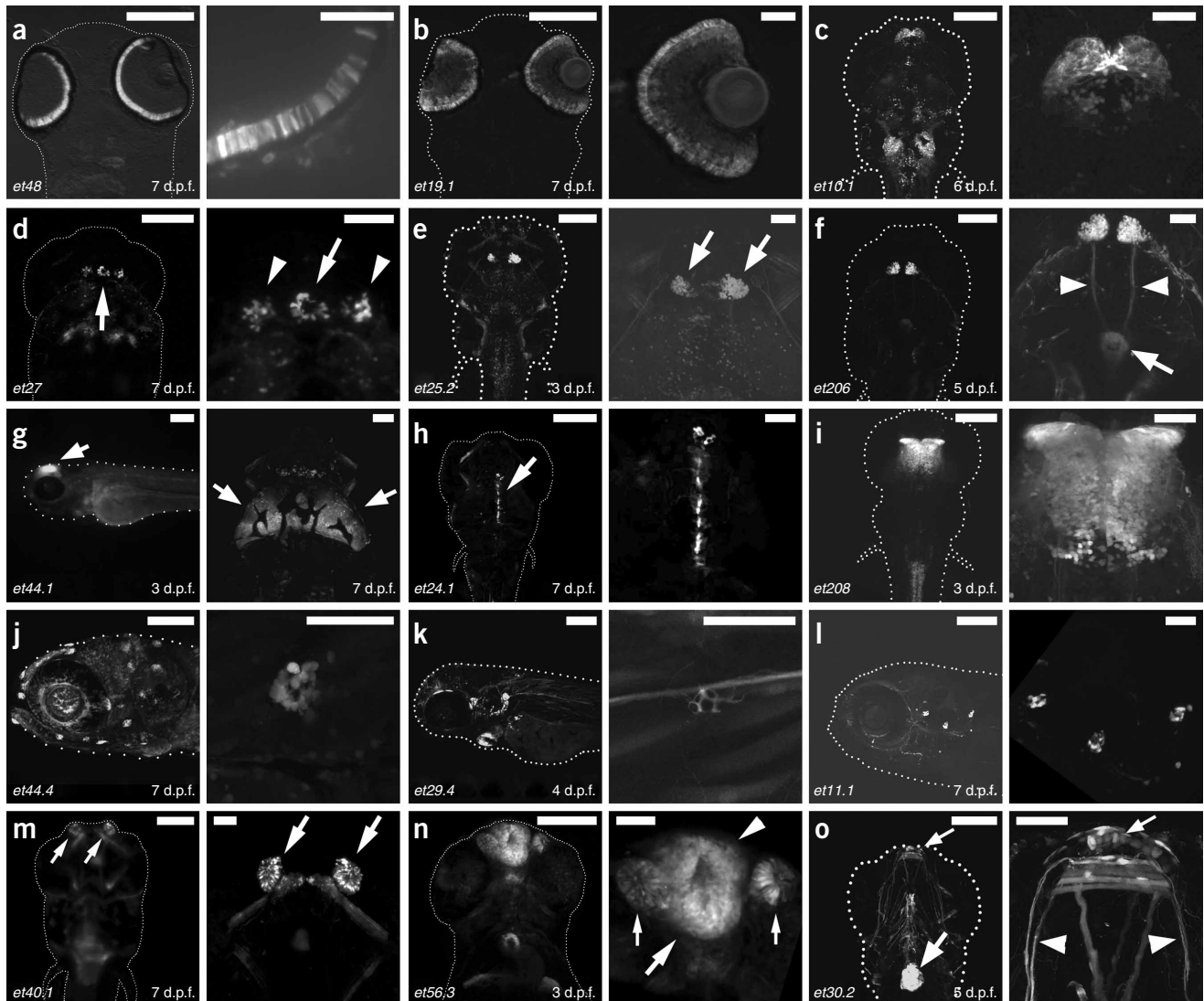


Figure 1 | Larval expression patterns for a selection of enhancer trap lines. **(a)** Horizontal section of *et48* showing expression in the photoreceptor layer of the retina. **(b)** Horizontal section of *et19.1*, with broad expression in the retina. **(c)** *et10.1*, showing specific expression in the pallium. **(d)** *et27*, pineal (arrows) and habenulae (arrowheads). **(e)** *et25.2*, habenulae (arrows) and scattered cells in the tectum (bottom half of the right image). **(f)** *et206*, with expression in many or all habenular neurons, and visible projections (arrowheads) to the interpeduncular nucleus (arrow). **(g)** *et44.1*, lateral confocal stack (left) and dorsal confocal stack (right) showing expression in the tectum (arrows). **(h)** *et24.1*, expression along the dorsal midline (arrow) within the midbrain. **(i)** *et208*, with expression in the thalamus. **(j)** Lateral confocal stacks of *et44.4*, with expression in head neuromasts (a single neuromast is shown in the right image). **(k)** Lateral confocal stacks of *et29.4*, with expression in the lateral line ganglia. Terminals of lateral line ganglion neurons at a neuromast are shown in the right image. **(l)** Lateral confocal stacks of *et11.1*, showing expression in the cristae of the ear. **(m)** *et40.1*, with expression in the olfactory pits (all arrows). **(n)** *et56.3*, with expression in the subpallium (arrow), olfactory bulb (arrowhead) and olfactory pits (small arrows). **(o)** Ventral view of *et30.2*, with expression in the heart (arrow), neurons in the lower lip (small arrows) and unidentified axons (arrowheads). All panels show larvae carrying a *GAL4* insertion and *UAS:Kaede*. Dorsal confocal stacks are shown, unless otherwise indicated. The dotted line indicates the larval outline. For each enhancer trap pattern, a low (left) and a high-magnification (right) image are shown. Scale bars, 200 μm in all left panels and 50 μm in all right panels.

In contrast, we found consistent Kaede expression from zebrafish to zebrafish and from generation to generation in a vast majority of the lines analyzed (see **Supplementary Fig. 1** online for an example). One possibility is that use of the Tol2 vector is responsible for the consistency of the observed expression.

To determine whether our expression patterns result from insertions in or near genes, we identified the insertion sites for several interesting enhancer trap lines. Of 8 insertions analyzed, we found 1 to be flanked by sequence with matches on numerous chromosomes, 1 that matched a sequenced genomic clone but not

an absolute chromosome position, and 6 that matched assembled chromosomal contigs. Among these 6, 3 were inserted into introns of known or predicted genes, and 3 were near such genes (within 12.2 kb). A summary of the insertion sites and their adjacent genes is available in **Supplementary Table 3** online.

We found Kaede to be useful for analyses not possible with standard fluorophores. For instance, photoconversion proved beneficial when characterizing line *et58.1*, which shows expression in blood vessels and in a subset of hindbrain neurons (**Fig. 2**). Axons from these neurons extend to the midline, and also extend rostrally

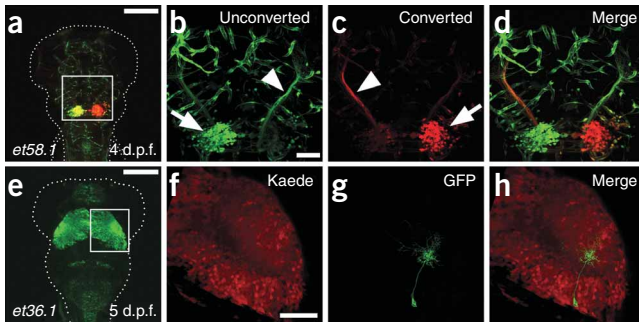


Figure 2 | Analyses of trapped neural circuits. (a–d) *et58.1*, showing Kaede expression in blood vessels and clusters of hindbrain neurons (a). Kaede has been selectively photoconverted in the cell body cluster on the right side of the hindbrain. Unconverted (b) and converted (c) Kaede can be seen in the cell body clusters (arrows), and in axons extending into the contralateral midbrain (arrowheads); both channels are shown in d. (e–h) Triple-transgenic larvae carrying *et36.1:GAL4*, *UAS:Kaede* and *BGUG*. Unconverted Kaede expression for *et36.1*, with strongest expression in the tectum (e). The right tectum (box in e) is shown in f–h after photoconversion of Kaede. Post-conversion Kaede expression shows the full extent of tectal expression (f). Two tectal neurons, presumably periventricular interneurons, labeled with GFP (g). Scale bars, 200 μm in a and e; and 50 μm in b and f.

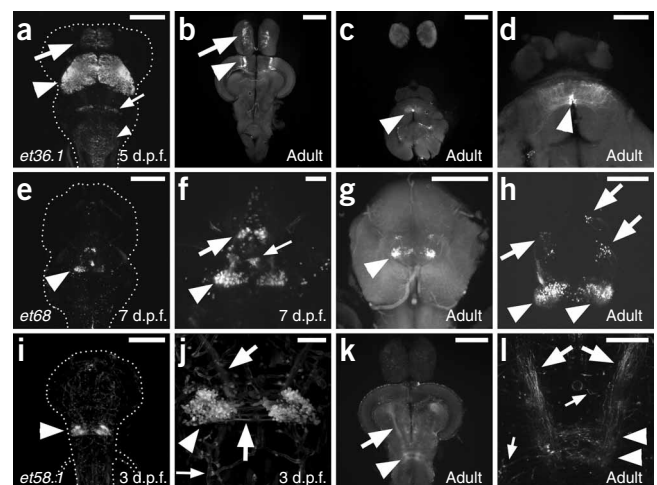
into the midbrain, but it was initially unclear whether these axons cross the midline and whether the rostral projections are from ipsilateral neurons, contralateral neurons or both. We addressed this by photoconverting the cell bodies of neurons on one side of the hindbrain but not on the other (Fig. 2a–d). We observed the red signal in axons crossing the midline and extending into the contralateral midbrain. Few, if any, neurons extended axons ipsilaterally. This morphology is consistent with these cells being the hindbrain neurons that contribute the ventral commissure¹¹.

Previous work has shown that photoconversion of Kaede can reveal the individual morphologies of large, superficial neurons with exceptional clarity¹². We have found this method difficult with neurons that are deeper and in dense clusters. As an alternative approach, we crossed carriers of a *GAL4* enhancer trap and *UAS:Kaede* to carriers of *Brn3c:GAL4*, *UAS:mGFP* (*BGUG*). *BGUG* drives *GAL4* expression in a subset of retinal ganglion cells, but the expression of membrane-bound GFP off of *UAS* is highly variegated, resulting in the labeling of a small subset of cells expressing *GAL4*. One line analyzed in this manner, *et36.1*, shows strong expression in the optic tectum (Fig. 2e). Tectal expression is so dense that it is difficult to resolve single cells. In animals carrying all three transgenes (*et36.1:GAL4*, *UAS:Kaede* and *BGUG*), Kaede is expressed in a majority of tectal cells (Fig. 2f), whereas GFP expression is restricted to a small fraction of those cells (Fig. 2g).

In this example after photoconversion of Kaede, the GFP reporter was observed in two tectal neurons that extend neurites into the tectal neuropil, where they have arbors ramifying through a small portion of the tectum adjacent to the cell bodies (Fig. 2h).

To investigate whether the enhancer trap patterns seen in young larvae are consistent throughout development, we observed adult patterns for three interesting lines. For *et36.1* (Fig. 3a–d), strong larval expression in the tectum was accompanied by weaker expression in the pallium of the forebrain, and in the cerebellum and medulla oblongata of the hindbrain. In the adult brain, strong expression was maintained in the medial portions of the tectum, whereas weaker expression in the dorsal telencephalon may correspond to the larval pallium expression. We observed a clearer correlation between larval and adult expression in *et68* (Fig. 3e–h). This line showed expression in specific subsets of the hypothalamus at 7 d.p.f. (Fig. 3e–f), with four interconnected nuclei combining to produce a dorsally projecting fascicle. We observed a strikingly similar pattern in the hypothalamus of the adult (Fig. 3g–h), with a projection that extended dorsally to the torus longitudinalis (data not shown). Finally, as described above, *et58.1* showed larval expression in blood vessels and in two rhombomeres with axons that crossed the midline and extended rostrally and laterally to the midbrain (Fig. 3i,j). Adults (Fig. 3k,l) maintained similar expression with labeling in the blood vessels, and a pair of rhombomeres

Figure 3 | Expression patterns are stable to adulthood. (a–d) *et36.1* in 5 d.p.f. larva (a) and adult zebrafish (b–d). Larval expression is seen in the pallium (arrow), the tectum (arrowhead), the cerebellum (small arrow) and the medulla oblongata (small arrowhead). Horizontal sections from adult with expression in the telencephalon (arrow in b) and the tectum (arrowheads in b,c). Closeup of the tectal expression in c, with cell bodies (arrowhead) near the midline, and neurites extending into the tectal neuropil (d). (e–h) *et68* in a 7 d.p.f. larva (e,f) and adult (g,h). In larva, expression is seen in the hypothalamus (arrowhead in e). A higher-magnification image (f) shows expression in two bilaterally symmetrical pairs of hypothalamic nuclei (arrow and arrowhead in f). These nuclei are connected with neurites, and they contribute neurites to a dorsally extending fascicle (small arrow in f). The horizontal section in g shows expression in the adult hypothalamus (arrowhead). At higher magnification (h), this expression can be seen to include a pair of large caudal nuclei (arrowheads) and a small number of connected rostral nuclei (arrows). (i–l) *et58.1* in larva (i,j) and adult (k,l). In larva, expression is brightest in the hindbrain (arrowhead in i). A higher-magnification view (j) shows two rhombomeres labeled on each side (arrowhead) with neurites connecting the contralateral rhombomeres and extending rostrally and laterally (arrows). Blood vessels are also labeled in this line (the small arrow in j marks an example). In the adult, expression is also in two pairs of rhombomeres (arrowheads in k and l), which extend their neurites across the midline as well as rostrally and laterally (arrows in k and l). The cell bodies for these projections are located in a more dorsal horizontal section (data not shown). Blood vessels continue to be labeled in adult (small arrows in l). Scale bars, 200 μm in a,d,e,h,i,l, 500 μm in b,c,g,k, and 50 μm in f,j, and are approximate in the panels showing adult zebrafish.



with neurites extending rostrally and laterally to the midbrain's torus semicircularis.

Although the lines presented here have a variety of unique expression patterns, they are far from being an exhaustive catalog of brain regions or cell types. Additional screens will be necessary to generate a more extensive resource for zebrafish researchers. It is equally important that lines are generated for an array of UAS-linked transgenes. Lines like *UAS:Kaede* are useful only for making an initial characterization of expression patterns. Additional tools could include subcellular markers, calcium indicators for reporting neural activity, and ion channels or mutant synaptic proteins for blocking (or stimulating) neural activity. Combined with behavioral assays, activity manipulation could be used to determine the functional importance of the trapped neurons. As we found larval expression patterns to be highly predictive of expression in adult zebrafish, such behavioral analyses could be performed not only on larval behaviors, but on adult behaviors such as aggression, mating, shoaling and learning.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

E.K.S. and H.B. conceived and designed the screen presented here and prepared this manuscript. E.K.S. performed subcloning of trapping constructs, injections, screening, establishment of transgenic lines, cloning of insertion sites and imaging. L.M. and A.A. participated in screening and L.M. established some transgenic lines. L.Z. performed dissection and sectioning of adult brains. N.G. and T.X. generated the *UAS:Kaede* and *BGUG* transgenic lines, respectively. K.A. and K.K. designed and generated the pT2KXIGΔ vector. N.C. performed subcloning of the 600bp *hsp* promoter.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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