# Chapter 4

# Large-Scale Small-Molecule Screen Using Zebrafish Embryos

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## Summary

Zebrafish represents a versatile model organism with many molecular, morphological, and physiological similarities to mammals. Importantly, zebrafish are readily susceptible to perturbations by small molecules, including numerous pharmaceuticals in clinical use. Given these qualities, plus their small size and transparency, zebrafish embryos can be utilized for large-scale phenotype-based screens for small-molecule modifiers of biological processes. Thus, in a manner analogous to classical genetic screens, zebrafish chemical screens have the potential to reveal novel insights into complex biological pathways, as well as to identify lead compounds for novel therapeutics.

**Key words:** Chemical genetics, Chemical screen, Drug discovery, Large scale, Phenotype, Small-molecule library, Zebrafish.

## 1. Introduction

For centuries, man relied on astute, serendipitous observations for discovery of bioactive small molecules for medicinal purposes. In modern times, these chance discoveries have largely been supplanted by large-scale, high-throughput assays for chemical modifiers of purified proteins or cell extracts. Nevertheless, in principle, the phenotype-based approach to small-molecule discovery retains certain advantages. For example, compounds discovered to cause discernible effects on live animals are inherently closer to validation as drug leads than those discovered by target-based in vitro approaches *(1)*. Importantly, since the phenotype-based approach does not depend upon a priori selection

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of a molecular target, it can be utilized to uncover novel or unanticipated biological insights *(2)*.

In the past few years, the zebrafish has emerged as an important model organism for small-molecule discovery *(1, 3, 4)*. Using zebrafish, it is now possible to combine the advantages of phenotype-based small-molecule discovery and modern high-throughput screening capabilities. From our experience, an important advantage of zebrafish chemical screening over other high-throughput platforms is the built-in means to assess specificity, efficacy, and toxicity of small molecules in the context of whole live animals (*see* **Note 1**). Another important aspect of chemical screens using zebrafish, in comparison to other model organisms such as *Drosophila* and *C. elegans*, is the close similarity between zebrafish and mammalian orthologs. Thus, small molecules discovered in zebrafish screens typically have directly analogous effects on mammalian systems, and vice versa *(2, 5–8)*.

Large-scale chemical screens in zebrafish have successfully identified small molecules that modulate numerous developmental

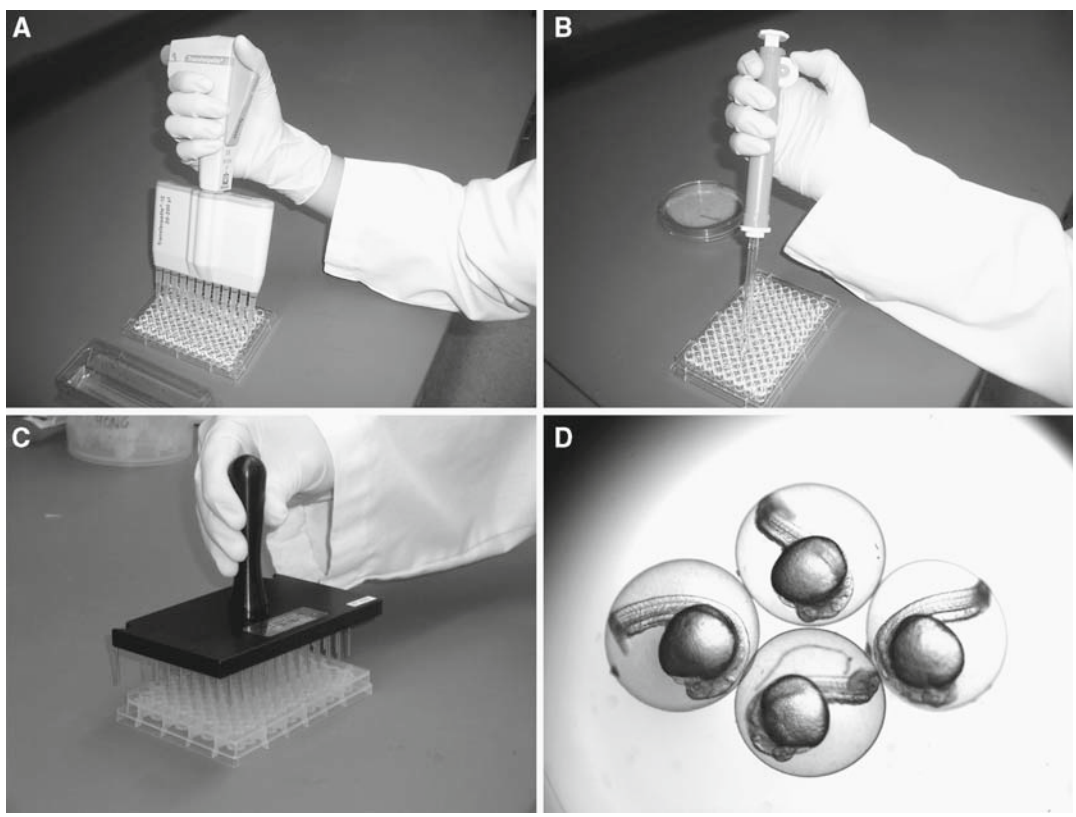


Fig. 1. Basic methodology for chemical screening using zebrafish embryos. (A) Using a multichannel pipettor, 100 μL of E3 medium is transferred to each well of a 96-well assay plate (recipient plate). (B) Using a glass Pasteur pipette and manual pipette pump, embryos are transferred to the recipient plate. (C) Pin-transfer of compounds from the source plate to the recipient plate. (D) Direct visual examination of embryo phenotype using a stereomicroscope.

processes as well as disease models with important therapeutic implications *(2, 5, 9, 10)*. In addition, borrowing from the logic of classical genetic modifier screens, zebrafish have been successfully utilized to discover unanticipated roles of the phosphatidylinositol-3 kinase (PI3K) and the Erk/MAP kinase signaling pathways during artery/vein specification *(2)*. Moreover, the first selective small-molecule BMP inhibitor, which holds significant investigational and therapeutic potential, was recently identified in a phenotype-based screen for compounds that perturb the zebrafish dorso-ventral axis (*13, 14*).

While zebrafish chemical screens can be modified to attain high-throughput capabilities through robotic small-molecule transfer technologies, automated sorting, and phenotyping, the basic methodology is very straightforward: embryos are arrayed in multiwell plates, compounds added by either pin-transfer or by high-throughput liquid transfer methods, and then embryonic phenotypes caused by compounds analyzed (**Fig. 1**). This chapter focuses on basic reagents and methods available to most academic investigators to perform a phenotype-based chemical screening using zebrafish embryos. It is hoped that large-scale zebrafish chemical screens, which require a fertile imagination but relatively modest capital investment, will be broadly adopted for discovery of numerous chemical tools to study biology and for development of new therapies to treat human diseases.

## 2. Materials

One of the most important decisions in planning a zebrafish chemical screen involves the selection of the appropriate fish strain and disease model. One must, for example, consider the potential for informative and meaningful dissection of a particular developmental pathway and/or its general relevance as a disease model. In addition, when planning a screen for chemical suppressors of a mutant or diseased phenotype, penetrance and robustness of the phenotype must be thoroughly evaluated (*see* **Note 2**). We have successfully utilized homozygous viable *gridlock* (*grl*) mutant embryos in our large-scale chemical screens *(2, 5)*. An advantage of a homozygous, viable mutant strain is that, when mutant females are mated to mutant males, all of the progeny will be mutant. In the case of *grl*, the mutant phenotype typically has greater than 95% penetrance. Thus, when three embryos are arrayed into each well, the probability of all three having a phenotypic suppression (wild-type phenotype in mutant embryos) by chance alone is only 1 in 8,000 (0.0125%). Moreover, *gridlock*

mutation causes a distinct loss of tail circulation that can be easily scored using a simple stereomicroscope *(11)*. In contrast, screening for small-molecule suppressors of a homozygous recessive mutation is more challenging, since 75% of embryos from heterozygote crosses will be phenotypically normal at baseline. Zon and colleagues overcame this challenge by placing 20 embryos per well, a format in which only 0.3% of the wells are predicted to contain all embryos with wild-type phenotype *(10)*.

The choice of chemical libraries to screen is another critical matter. Thanks in large part to recent advances in synthetic organic chemistry, one can gain access to hundreds of thousands of small molecules from numerous commercial sources, as well as from the Small Molecule Repository (SMR) of the Molecular Libraries Screening Centers Network (MLSCN; *see* **Note 3**). A major disadvantage of commercial libraries, in comparison to the MLSCN's libraries, is the cost; however, an important advantage of acquiring a library of one's own is that the design and conduct of zebrafish screens can be catered to the researcher's particular interest and expertise (*see* **Notes 4** and **5**).

It is important to remember that zebrafish chemical screening is not a very high-throughput platform. In contrast to molecule-based or cell-based chemical screens, which can screen 100,000 compounds rapidly in an automated fashion, zebrafish screens are limited by the number of eggs laid and by the laborious transfer of embryos into 96-well plates. In our experience, while up to 1,000 compounds can be screened in a given day, one can reasonably expect to screen only about 10,000 compounds in a month. To overcome this limitation, one can modify the screen such that each well contains a pool of compounds *(10)*. A disadvantage of this approach is the potential of dismissing real "hits" due to confounding effects of multiple small molecules (*see* **Note 6**). The last decision involves whether to screen a library of unknown synthetic compounds or of "known bioactives," typically comprising bioactive components of natural products and modern medicines. While known bioactive libraries are typically much smaller than commercial libraries of unknown synthetic compounds, an underappreciated aspect of known bioactives is that they typically have significantly higher "hit" rates than libraries of synthetic compounds, and that "off-target" effects of known bioactives sometimes can be very informative. Thus, given the inherent limitations of a zebrafish chemical screen, it seems prudent to begin with a library of known bioactive molecules (*see* **Note 7**).

### 2.1. Zebrafish Egg Collection

1. Minimum of 20 pairs of adult zebrafish of desired genotype.
2. Fish nets (Aquatic Habitats; Apopka, FL).
3. Breeding tanks (1–2 L capacity), with removable inner container and dividers (Aquatic Habitats).

4. Petri dishes (10 cm) (Fisher Scientific; Pittsburgh, PA).
5. Plastic tea strainer (Fackelmann; Germany).
6. Wash bottle containing embryo water (Nalgene; Rochester, NY).
7. Disposable polyethylene transfer pipettes (Fisher Scientific).

### 2.2. Sorting of Embryos into Multi-well Plates

1. Polystyrene 96-well round-bottom assay plates (Corning COSTAR; Lowell, MA).
2. Glass Pasteur pipette (9 in.) (Fisher Scientific).
3. Manual pipette pump, 10 mL (Bel-Art Products; Pequannock, NJ).
4. E3 embryo medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, containing 0.003% PTU (phenylthio-carbamide, Sigma; St. Louis, MO). PTU can be prepared as a 10× solution by dissolving 0.3-g PTU in 1 L of E3 embryo media. Solutions containing PTU should be protected from light by covering with aluminum foil.
5. Pipettor :12-channel, 20–200 µL (BrandTech; Essex, CT).
6. Disposable polystyrene pipette basin, 50 mL (Fisher Scientific).

### 2.3. Small-Molecule Library

1. Small-molecule library of structurally diverse compounds arrayed in a 96-well format at 10 mM stock in DMSO. In recent screens, we have used DiverSet E library from Chembridge (*see* **Notes 4** and 7). Each master plate is aliquoted into several 96-well polypropylene storage plates (Corning), and stored at –80°C until use.
2. Aluminum sealing tape for 96-well plates (Nunc; Rochester, NY).
3. Multiblot replicator, 96-pin, approximately 100 nL/drop (V & P Scientific; San Diego, CA).
4. Dessication chamber (Fisher Scientific) containing Drierite (anhydrous calcium sulfate, W.A. Hammond Drierite Co; Xenia, OH).
5. Tabletop centrifuge with 96-well plate adaptors (Eppendorf; Westbury, NY).
6. Plastic cover for a micropipette tip rack.
7. Bunsen burner.
8. DMSO (Sigma; St. Louis, MO).
9. Ethanol.

### 2.4. Screening by Visual Inspection

1. Basic incubator, 28.5°C (Fisher Scientific).
2. Stereomicroscope with transmitted light base (Leica Microsystems; Bannockburn, IL) or compound inverted microscope for tissue culture (Fisher Scientific).

## 3. Methods

In classical forward-genetic screens, a single gene can cause myriad phenotypes depending on nature of the mutation. A similar concept holds true for chemical screens. A small molecule found to perturb one particular aspect of the zebrafish embryo when added at one specific dose and time in embryogenesis might have very different effects when added at different doses or times. Thus, by varying the doses of compounds and the timing of compound addition, one can perform several distinct screens using the same small molecule library.

Borrowing further from the analogy to forward-genetic screens, the adage "you find what you seek" applies for chemical screens as well. For example, if one desires to identify small molecules that have vascular effects but do not perturb other aspects of development, one can add chemicals early in development, perhaps at the sphere stage. A compound with strictly vascular-selective effects would be predicted not to interfere with any of the early developmental decisions, whereas less specific compounds might cause gross abnormalities or early embryonic lethality when added at such an early stage. Conversely, if one desires to identify small molecules that have pleiotropic effects, one can add chemicals at the precise time when a particular developmental decision is being made *(2)*.

The main advantage of zebrafish chemical screens over higher-throughput cell- and molecule-based platforms is that in vivo efficacy and specificity can be quickly and directly assessed in a whole zebrafish embryo (*see* **Notes 1** and **8**). By screening at the relatively low dose of 1–2.5 μM, one can enrich for discovery of potent compounds. Such compounds, however, may have significant off-target effects at higher doses. In contrast, at the relative high dose of 20–50 μM, one enriches for compounds with greater specificity (*see* **Note 9**). Lastly, it is important to recognize that once a larva's mouth and gills become functional at 5 days post fertilization, sensitivity to compounds drops substantially, even by 1 order of magnitude *[(6)*, personal observations].

As described in the preceding and subsequent chapters, the screening process can be automated. Since the zebrafish embryo is not an exceptionally high-throughput platform in the first place, the main advantage of adding automation to the screening process lies not in substantially increasing throughput, but in enabling examination of the embryos at unusual hours or at frequent intervals. As in forward-genetic screens, observers trained to distinguish meaningful phenotypic changes remain essential to chemical screens in zebrafish. Also, as in forward-genetic screens, phenotypic analysis can be greatly assisted by the use of transgenic strains expressing fluorescent proteins in an organ-specific manner *(12)*.

### 3.1. Collection of Embryos

1. Mating crosses are set up in the afternoon prior to the day planned for egg collection. Ten to twenty breeding tanks are filled with water from the aquaculture system. Using the fish net, adult fish are transferred to the breeding tanks, each tank containing one male and one to two females in the inner container. Male and female fish will be separated from each other by a divider. Label the cages and put a lid over them to prevent fish from jumping out.
2. In the morning, the dividers are taken out of the breeding tanks and zebrafish allowed to mate and spawn. Over the next 1–2 h, eggs are laid and fall through a grid at the bottom of the inner container and collect in the outer container, where the adults cannot eat them.
3. After 1–2 h, examine each breeding tank for eggs collected at the bottom following a successful mating. Return adult fish back to permanent storage tanks using the fish net. Remove the inner container, and strain the water in each breeding tank through a plastic tea strainer. Eggs will collect in the strainer. Invert the strainer over a Petri dish and, using a wash bottle containing the E3 medium, gently rinse the strainer to flush the eggs into the Petri dish. Average mating success can range from 50 to 100%, and a single mating yields between 100 and 400 eggs. As soon as possible, all unfertilized eggs, which look cloudy white, should be removed using a disposable plastic pipette. Repeat this step as necessary.

### 3.2. Arraying of Embryos in 96-Well Plates

Transfer of embryos to a 96-well plate is the most labor-intensive step in zebrafish chemical screens (*see* **Note 10**). Embryo transfer can be automated using a COPAS XL automated sorter (Union Biometrica; Holliston, MA) but as this requires a substantial investment beyond the means of most laboratories, a manual method is described.

1. Pour about 50 mL of E3 medium containing PTU into a disposable pipette basin (*see* **Note 11**). Using a 12-channel pipettor, transfer 100 µL of E3 medium from pipette basin to each well of a round-bottom 96-well assay plate.
2. Prepare a glass Pasteur pipette by cutting the tip with a glass-cutter to widen the opening and polishing the tip with a flame from a Bunsen burner. Connect the glass pipette to the manual pipette pump.
3. To draw up fluid and eggs into the glass pipette, submerge the tip of the pipette into the E3 medium containing the eggs, and turn the operating wheel of the pipette pump with the thumb. On average, about 20–50 eggs are drawn up each time.
4. Hold the pipette pump upright and agitate fluid collected inside the glass pipette by gently tapping or moving the

plunger up and down with the operating wheel. This will allow eggs to settle near the tip of the glass pipette.

5. Turn the operating wheel of the pipette pump slightly to create a mild positive pressure within the pipette but not enough to expel the eggs. While exerting a positive pressure, touch the pipette tip to the meniscus of E3 medium in each well, and quickly withdraw the pipette. This will draw by capillary action a tiny amount of fluid from the glass pipette to each well, carrying 1–3 eggs along with it. This procedure minimizes the total amount of E3 medium transferred along with the eggs. Repeat this motion as necessary to transfer three embryos to each well. With practice, embryos can be transferred to several 96-well plates in 1–2 h. Alternatively, three embryos can be transferred to each well by actively expelling them. This will result in transfer of variable amounts of medium to each well. To ensure that each well contains roughly the same amount of liquid, E3 medium can first be removed using a multichannel pipette, leaving just the embryos, and then 100 μL of E3 medium put back into each well.

### 3.3. Transfer of Small-Molecule Library

A typical small molecule library is supplied in a 96-well format, with each compound stored in DMSO as a 10 mM stock (*see* **Notes 4** and **7**). While compound transfer can be automated with robotic transfer methods, this chapter will describe methods using manual pin transfer.

1. Thaw at room temperature the desired number of 96-well storage plates containing aliquots of small molecule library (source plates). Take note of the serial or other identification number of the source plates used. To minimize condensation outside the plates, thawing can occur in a desiccation chamber containing Drierite.
2. Gently mix the source plates on an Eppendorf Mix Mate at 300 rpm or by gently swirling to resuspend or dissolve any compounds that might have precipitated out during storage (*see* **Note 12**).
3. Briefly (a few seconds) spin down the plates in a tabletop centrifuge equipped with multiwell plate adaptor.
4. Remove the aluminum sealing tape from source plate.
5. Carefully align the 96-pin multiblot replicator over the 96 wells on the source plate. Slowly lower the replicator and dip the replicator's pins into wells of the source plate. Gently mix contents of wells with the replicator, and then remove the pins from the wells at a slow and even speed (~0.5 cm/s). Removing the pins from the source plate quickly produces larger hanging drops and hence greater amount of liquid transferred. Deliver the compounds on the replicator's pins

to 96-well plate containing embryos and E3 media (recipient plate) by dipping and raising the pins three times through the recipient plate's meniscus. Take care to ensure that the recipient plate is correctly aligned. With a VP409 multiblot replicator, this technique transfers approximately 100 nL of compound to each well. The final concentration of each compound is 10 μM (*see* **Note 13**).

6. Record the identification number of the source plate on the recipient plate.
7. Clean the multiblot replicator by rinsing the pins with DMSO inside a small basin (inverted lid of a pipette tip rack), followed by two rinses in distilled water. Dry the pins by dipping them in ethanol inside an inverted tip lid box, and briefly flaming the alcohol off using a Bunsen burner. Take care not to heat the pins directly in a Bunsen burner, as this will damage the replicator.
8. Repeat **steps 4–6** for each source plate as needed.
9. Cover the recipient plate containing the embryos and compounds with the appropriate lid, and place in the 28.5°C incubator.
10. Cover each source plate containing small molecule library with an aluminum sealing tape. Ensure that the tape forms a tight seal using a rolling sealer. Put the source plates back in a –80°C freezer for long-term storage.

### *3.4. Screening for Effects of Small Molecules by Visual Inspection of Phenotypes*

1. Prior to performing the screen, formulate a specific criterion for what would constitute a "hit."
2. At desired times in development, remove the 96-well plates containing compound-treated embryos from the incubator and examine each well under a stereomicroscope. For better visualization of subtle changes, such as changes in circulatory pattern, a phase-contrast inverted microscope can be used. Fluorescence microscopy can be used to examine perturbation of expression of GFP or DsRed proteins under a tissue-specific promoter. Various methods to automate the screening process are discussed in subsequent chapters.
3. Quickly scan the 96-well plates for any well in which at least two out of three embryos exhibit the prescribed "hit" phenotype. Record the identity of the plate and the well location of each potential "hit." In addition, note the wells containing embryos with any interesting phenotypes even if they are not necessarily a "hit."

### *3.5. Confirmation of a Potential "Hit"*

1. Retrieve the compound library (source plate) from which a potential "hit" was found. Thaw, mix, and spin down the source plate as described above.

2. Remove the aluminum sealing tape, and locate the well containing the compound that produced the "hit" phenotype.
3. Remove a small aliquot (10 μL) of the potential "hit" from the source plate.
4. Using this aliquot, retest the effects of the compound at several doses (1, 5, 10, and 50 μM). For each dose, ten embryos are tested in 0.5 mL of E3 media in a 48-well plate format (*see* **Note 14**). The timing of compound addition for retesting should be identical to that of the original screening.
5. A "hit" is confirmed when the elicited phenotype is reproduced upon retesting of the compound (*see* **Note 14**).
6. Identity of the "hit" compound is determined from the database of small molecules in the chemical library.

## 4. Notes

1. In addition to large-scale chemical screens, whole zebrafish embryos can be utilized for structure–activity relationship (SAR) studies to rapidly identify chemicals with improved potency and efficacy without increased toxicity *(1, 10)*.
2. When considering a screen for chemical suppressors of an inducible phenotype, particular attention must be paid to the background and induced frequencies. In the case of a phenotype caused by a mutant gene expressed under heat-shock control, the condition that induces the phenotype with greatest reproducibility must be carefully mapped out prior to initiating a screen to avoid unacceptably high false-positive rates.
3. The Molecular Libraries Screening Center Network (MLSCN) was established as a part of the NIH Roadmap. MLSCN's Molecular Libraries Small-Molecule Repository (MLSMR) will eventually consist of 500,000 natural products, known bioactives, and a large-diversity set of compounds. While access to MLSCN's compounds is free, screens themselves are performed at 1 of the 10 designated centers. Of these, the Universities of Pennsylvania and Pittsburgh currently have the capability to perform zebrafish screening. Importantly, compounds identified as "hits" from screens done through the MLSCN are considered pre-competitive, and the MLSCN requires that screening results be promptly deposited in PubChem (http://pubchem.ncbi.nlm.nih.gov). Latest information, funding mechanisms, and specific guidelines on submitting an assay method to a particular center within the MLSCN can be obtained from its website

(http://mli.nih.gov/mlscn/). In addition, there are numerous university-based screening programs that provide access to small molecule libraries. For example, The Broad Institute of Harvard and MIT's Chemical Biology Program is open to the larger public research community (http://www.broad.harvard.edu/chembio). Finally, *ChemBank* (http://chembank.broad.harvard.edu) and PubChem (http://pubchem.ncbi.nlm.nih.gov) are two major small-molecule databases open to the research community.

4. Listed here are some commercial vendors that provide small-molecule libraries (this list is not meant to be comprehensive, as the number of vendors and libraries is expanding rapidly): Biomol (http://www.biomol.com), Chembridge (http://www.chembridge.com), ChemDiv (http://www.chemdiv.com), Maybridge (http://www.maybridge.com), Microsource (http://www.msdiscovery.com), Sigma (http://www.sigmaaldrich.com), and TimTec (http://www.timtec.net).
5. Web portals provided by numerous commercial vendors can be used to quickly obtain structural analogs of a primary "hit" for preliminary SAR studies.
6. Only about 2% of small molecules in a typical commercial library cause death or severe developmental delay of zebrafish embryos *(10)*. Thus, if five compounds are pooled per well, death will occur in about 10% of wells. In such a scenario, compounds causing death when pooled can be retested individually at a later time.
7. Libraries of known bioactive small molecules are available from many commercial sources, including Biomol's ICCB Known Bioactives 1 and 2 (480 compounds each), MicroSource Discovery System's NINDS Custom Collection 2 (1,040 compounds) and Spectrum Collection (2,000 compounds), Prestwick's Collection (1,120 of out-of-patent drugs), and Sigma's LOPAC (1,280 pharmacologically active compounds) Collection.
8. For biochemical or cell-based assays, initial "hit" rates can be high. For example, a screen for activators of deacetylase activity of purified Sirt1 found 1,151 initial "hits" out of 2,139 molecules screened (*ChemBank*). For such cases, a number of reiterations of assays as well as alternative tests are necessary to validate initial "hits." By contrast, a screen of over 5,000 compounds found only one that specifically perturbed embryonic dorso-ventral axis (Hong and Peterson, manuscript in preparation).
9. It is interesting to note that 20 and 50 μM are relatively moderate concentrations, and several very important chemical probes, such as cyclopamine, might not have passed muster under such screening conditions.

10. Transfer of hatched larvae can be particularly challenging given their mobility. To screen for small molecules affecting a larval phenotype, one option might to be to transfer embryos to 96-well plates prior to hatching, and add the small molecules at a later time.
11. E3 medium with PTU can be supplemented with antibiotics (2 μM metronidazole, 5 units/mL penicillin, 50 μg/mL streptomycin; diluted from a 100× stock solution) to prevent bacterial and fungal growth *(10)*.
12. After long-term storage at –80°C, some of the compounds in the source library plate may precipitate out of DMSO solution. If some compounds remain out of solution even after 15-min mixing, it may be prudent to proceed with the screen anyway.
13. Final compound concentrations can be changed by varying the amount of E3 in the recipient well or repeating the pin transfer. It is inevitable that some compounds will precipitate out of solution in E3 medium.
14. It is not unusual for a putative "hit" to cause the induced phenotype at a different concentration than that of the screen itself. Moreover, retesting may not reproduce the phenotype in 100% of tested embryos. Multiple retesting, perhaps done blinded, may be necessary to reach a statistically significant difference between treated and untreated embryos. A careful retesting of each "hit" at this early stage can prevent wasting valuable time and effort on false positives.

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