Published in final edited form as:

Nat Protoc. 2009 ; 4(10): 1422–1432. doi:10.1038/nprot.2009.144.

Chemical Genetic Screening in the Zebrafish Embryo

Charles K. Kaufman^{1,*}, Richard M. White^{1,*}, and Leonard Zon^{1,2,3}

¹ Department of Medical Oncology, Dana Farber Cancer Institute, Boston, MA, USA

² Division of Hematology/Oncology, Children's Hospital Boston, Boston, MA, USA

³ Harvard Medical School, Boston, MA, USA

Abstract

Chemical genetic screening can be described as a discovery approach in which chemicals are assayed for their effects on a defined biological system. The zebrafish, *Danio rerio*, is a well-characterized and genetically tractable vertebrate model organism that produces large numbers of rapidly developing embryos that develop externally. These characteristics allow for flexible, rapid, and scalable chemical screen design using the zebrafish. We describe a protocol for screening compounds from a chemical library for effects on early zebrafish development using an automated *in situ* based read-out. Because screens are performed in the context of a complete, developing organism, this approach allows for a more comprehensive analysis of the range of a chemical's effects than that provided by, for example, a cell culture-based or *in vitro* biochemical assay. Using a twenty-four hour chemical treatment, one can complete a round of screening in six days.

Introduction

The modern biologist's toolbox continues to expand with an array of techniques aimed at dissecting the basic processes of development and disease. The continually refined approaches of molecular genetics allow for gain and loss of function of a target gene(s), often with a degree of temporal and spatial control (for reviews^{1,2}). Yet in the absence of gene therapy approaches, these techniques are not immediately transferable to therapeutic applications. While the precision of a targeted gene deletion may offer clear advantages for interpreting perturbations, the analysis may be confounded by functional redundancy (for examples^{3,4}).

A chemical genetics approach in which exogenous, typically small molecules are assayed for their ability to alter a biological process of interest has proven complementary^{5,6,7,8,9} (also, see Table 1 and references therein). Temporal control is easily achieved by adding a chemical at a chosen time-point. Once a given chemical treatment produces a relevant effect, this molecule can serve as a ready starting point for drug discovery¹⁰. A given chemical can have pleiotropic effects that may prove a boon in altering a multilayered process, but may indeed prove a burden when seeking to identify the key target/mechanism of action¹¹.

Here we describe a framework for chemical genetic screening in the embryo of the zebrafish, *Danio rerio*, built on our lab's experience using this modality^{12–14}. Zebrafish are small (100

Correspondence should be addressed to L.Z. (zon@enders.tch.harvard.edu).

*These authors contributed equally to this work

Competing Financial Interests Statement

The authors declare that they have no competing financial interests.

Author Contributions Statement

CKK and RMW contributed equally to this work and worked under the guidance and direction of LZ.

+ fish per 20 L tank), reproduce rapidly (100's+ embryos per week per adult female), and develop quickly (most organs formed by 24 hrs), thus offering the advantages of an *in vivo* high throughput, highly scalable system typically associated with cell culture. Biological processes can be assayed in the native context of a complete, developing organism that is transparent, develops externally, and is genetically highly manipulable (for review ¹⁵).

In 2000, Peterson and colleagues¹⁶ presented the initial developmental chemical genetic screen in zebrafish. 1,100 chemicals were assayed in wild type embryos for their effects on the development of the central nervous system, the cardiovascular system, pigmentation, and the ear. By visually examining embryos daily for 3 days, the authors identified chemicals that produced defects in each of these structures. This included, for example, compound 33N14 or MoTP which prevented pigmentation in the zebrafish body and has since been used in studies of melanocyte biology^{17,18}.

Chemical genetic screening in the zebrafish has since been used to identify chemical suppressors of defined genetic mutations. In 2004, Peterson and colleagues screened 5,000 chemicals searching for compounds that suppress the aorta malformation phenotype in the *grl* mutant zebrafish caused by a mutation in the transcription factor *hey2*¹⁹. By assaying for restoration of blood flow to the tail of 48 hour post-fertilization mutant embryos following chemical treatment, the authors identified two compounds, GS3999 and GS4102, that upregulated vascular endothelial growth factor (VEGF) expression and resulted in restoration of blood flow. Soon after, Stern et al reported a screen of 16,320 compounds aimed at identifying suppressors of the cell cycle mutant *crb* that results from a mutation in the *bmyb* oncogene¹². A new compound, termed persynthamide, was identified that suppressed the mitotic accumulation phenotype of *crb* as judged by whole-mount immunohistochemical staining for serine-10 phosphorylated histone H3.

Two more recent reports highlight the use of *in situ* hybridization as a read-out for a chemical genetic screen. In order to identify regulators of hematopoietic stem cell (HSC) formation and homeostasis, North et al¹⁴ screened ~2,500 compounds for their ability to alter HSC numbers during embryogenesis in the zebrafish as judged by *in situ* of HSC markers *runx1* and *cmyb*. 10 compounds that affect the prostaglandin pathway were identified with enhancement or suppression of the prostaglandin pathway increasing or decreasing HSC's, respectively (see Fig. 2a–c, reproduced with permission here to demonstrate example results of ISH-based screening). Follow-up work recently showed a critical interaction of prostaglandin signaling with Wnt pathway activation in the vertebrate hematopoietic stem cell compartment²⁰. Finally, Yeh et al²¹ recently used an *in situ*-based approach to screen a library of 2,000 bioactive compounds for modulators of an oncogene's effects on differentiation of hematopoietic progenitor cells. By leveraging the molecular genetics tools available in the zebrafish, the authors use a transgenic zebrafish with a heat shock inducible transgene for the AML1-ETO oncogene that when expressed in embryos causes a decrease in erythropoietic lineage cells (as read out by decreased GATA-1 *in situ*) in favor of granulopoietic cells. Two compounds, dicumarol and nimesulide, restore GATA-1 expression, and the second is shown to also act through the prostaglandin and Wnt pathways.

Experimental design

In addition to the discussion above, we have highlighted operational details of published chemical genetic screens in zebrafish (Table 1) and below introduce what we believe are some key considerations to be formally determined when embarking on a chemical screen. The procedure described herein outlines an approach for an *in situ*-based chemical screen in which embryos are treated with chemicals for ~24 hours, but it can be easily modified to accommodate differing chemical treatment times and/or readouts.

Aim of the screen

What developmental process are we hoping to alter? What pathway are we expecting to target? Prior screens have successfully broadly targeted the development of an entire organ system (e.g., the central nervous system¹⁶) or more precisely aimed at affecting specific signaling molecules (e.g., the mitotic regulator *bmyb*¹²).

Zebrafish - general husbandry, mating, embryo stages

Both simple pair matings and group matings (3 females/2 males) can be used to generate embryos. Group matings will yield, on average, 200–500 embryos per lay but will lead to a wider distribution of embryo stages. Mixing embryos which have a wide distribution of developmental stages may confound later attempts at scoring for chemical effects as a given chemical may only produce an effect during a narrow window of developmental time¹⁶. Familiarity with the staging of zebrafish embryos based on morphology will facilitate sorting of synchronized embryos²². Finally, antibiotics can be added at the discretion of the researcher, but for short assays as presented here, it is likely unnecessary.

Read-out

How will we identify putative “hits” in our screen that affect the process of interest? In order to assay for the effects of possibly thousands of chemicals, a read-out should ideally not only accurately reflect a perturbation in the desired process but should also be rapid, reproducible, scalable, and sensitive. Pros and cons of various read-out methods applied to date in the literature and/or our lab are described in Table 2, along with theoretical concerns related to each (indicated by “?”). For example, visual examination of embryos for structural alterations following chemical treatment requires no reagent development but may be limited in the range of structures that can be monitored. ISH allows for visualization of the expression of, in principle, any gene product but may require significant preliminary reagent development including cloning of the specified cDNA and optimization of the ISH protocol for the corresponding probe. The use of this method has been previously described by North et al in which alterations in *in situ* staining for *runx1/cmyb* corresponds to alterations in numbers of HSC's (see Figure 2a–c)¹⁴.

Timing/duration of chemical exposure and Toxicity

The time frame for chemical treatment should precede and, if possible, overlap with the interval during which the targeted process or pathway is active. In our experience, extended exposures to chemical treatment (i.e., > 24 hours) or treatment prior to 6 hours post fertilization (i.e., roughly 50% epiboly²²) significantly increases the death rate of embryos as compared to negative control embryos. By noting the death rate of embryos in the negative control wells (typically limited to one, or at most two, out of ten embryos), one can ascertain that a given compound is toxic at the tested concentration when the majority of embryos in the treated well are dead.

Depending on the number of compounds being screened in a given round, one should consider examining for embryo death and, if time permits and it does not interfere with timely processing of the embryos, for evidence of teratogenicity or developmental delay prior to pronase treatment and chorion removal (Fig. 1a–c). Dead embryos will appear opaque and can be identified quickly with minimal delay to the workflow (Fig. 1c). Importantly, these dead embryos will be removed during pronase treatment and subsequent washing, leaving an empty well. Toxic compounds should be retested at a lower dose in subsequent testing.

For read-outs that require embryos to develop to later developmental stages, consider adding a “wash out” step to remove chemicals if it is plausible that an effect produced by an early, transient chemical exposure will persist during embryonic development.

Choice and Use of a Chemical Library

A full discussion of chemical library design and the finer points of chemical biology are beyond the scope of the current discussion (for an introduction, see ²³). Nonetheless, in the Reagents Section, we highlight chemical libraries or their up-to-date counterparts used to date in chemical genetic screens in the zebrafish. When choosing chemical libraries with which to screen, one should consider how characterized the compounds are within the library. Using more well-characterized compounds, including so-called “known bioactives” for which some mechanistic information may already be available, should facilitate follow-up and molecular characterization of a chemical’s effect but may bias towards less novel mechanisms. Additionally, pooling of chemicals has been used to decrease the number of individual observations which are needed to screen a library in its entirety¹², but this may result in unpredictable interactions between multiple chemicals within a pool and has also led to an unacceptable level of embryo death.

Most commercially available libraries come in 384 well format with chemicals dissolved in 100% DMSO. A typical range of concentrations of these stock chemicals is 5–10 mM. In this protocol, 1 μ l of stock solution is added to 100 μ l of E3 medium and then added to embryos in 200 μ l of E3 medium. This provides automatic mixing and avoids having to use pipettes to mix in the presence of embryos, which can be damaging to the embryos. The final volume of 300 μ l corresponds to a concentration of chemical between 16–33 μ M and a final concentration of DMSO of 0.3% (vol/vol).

Controls

A negative control that contains the vehicle alone (e.g., DMSO) should be included on each plate. The vehicle can have biological effects unrelated to the chemical being screened, and inclusion of such a negative control allows for monitoring of embryo staging and for degree of *in situ* staining. While not always available, a positive control(s) should also be included as well. Ideally, this would be a chemical that produces the desired read-out in treated embryos, but use of a morphant, for example, may provide a substitute.

Automation of ISH

In situ hybridization (ISH) of whole zebrafish embryos, as described by Thisse and Thisse²⁴, is a robust and commonly used procedure. To perform ISH on embryos that are still in their chorions (~<48 hpf), a protease called pronase is added to the embryos to soften the chorion and allow for chorion removal during subsequent wash steps. These wash steps also prevent overdigestion with pronase which can damage embryos and lead to degradation during subsequent ISH steps. Removal of the chorion is necessary to allow the ISH reagents and washes to access the embryo. Once embryos have reached the desired stage, they are fixed in a 4% PFA solution, optimally for one night or for up to 2–3 days before the quality of the ISH begins to diminish. Embryos are then dehydrated by adding methanol and can be stored at –20° C in this state for months. Each *in situ* antisense probe for each gene of interest must be individually optimized. Traditionally, the 3’ prime end of a gene including its 3’ UTR is used to generate the antisense digoxigenin-labeled RNA probe used for ISH, although full length cDNA’s may be successfully used. A final probe concentration of 1 ng μ l^{–1} serves as a good starting point for ISH, but lower probe concentrations may be sufficient for, for example, highly expressed genes. Finally, alkaline phosphatase conjugated anti-digoxigenin Fab antibody fragment is used for detection of the ISH probe via production of a purple precipitate (catalyzed by the alkaline phosphatase). In order to avoid formation of nonspecific purple precipitates, it

is important to allow for adequate equilibration in the appropriate alkaline (pH 9.5) Tris buffer prior to addition of the substrate reagent, as described below.

Use of an *in situ* robot, such as the Biolane HTI (see Equipment), allows for automation of the ISH protocol. We describe the use of 48-well flat bottom plates for chemical treatment and 48-well mesh bottom plates for the automated ISH. One robot can handle a total of four 48-well plates at a time. In our experience when using smaller format plates (e.g., 96-well), the death of one embryo in a well has led to the death of all embryos in that well. In addition, liquid exchange in individual wells during the automated ISH protocol was less efficient (possibly related to the type of mesh bottom plates which were used). Using fewer embryos per well, different treatment schemes, or alternate mesh bottom plates (e.g., Millipore cat. # MANM10010, which have recently become available again) may allow for the use of smaller format plates, as has been recently described²¹.

Importantly, the time required for proteinase K digestion can vary significantly in the robot compared to benchtop ISH. We have found that a lower concentration of proteinase K in the robot system (i.e., $\sim 1 \mu\text{g ml}^{-1}$ compared to $10 \mu\text{g ml}^{-1}$ for manual ISH) often gives improved results. This will have to be individualized for each probe, but general guidelines are as follows:

Developmental stage	Proteinase K treatment ($1 \mu\text{g ml}^{-1}$)
<1 somite:	30 sec
5 somite stage	1 min
24 hpf	2–5 min
36–72hpf	10–30 min

Hpf, hours post fertilization

Scoring of chemically treated embryos

Each chemically treated well must be compared to the control wells on the same plate. The robotic ISH results can be quite variable from day to day, so the internal controls on each plate help to account for variations in staining on that particular plate. Within a given well of 8–10 embryos, it is likely that 1–3 will demonstrate alterations in probe staining simply due to biological or technical variability. A phenotype that occurs in more than of all embryos in the well is more likely to represent a true positive result. A quantitative scoring system is very helpful, as this will naturally reveal the spectrum of phenotypic effects and subsequent follow-up can then be prioritized based on that score.

Following Up “Hits”

Most chemical core facilities will offer the capacity to “cherry pick” interesting compounds from the stock plates for repeat testing. If possible, this should be done at a 1–2 log dose range (i.e., 1–100 μM). For those chemicals that “repeat” in the secondary assay, larger quantities of chemical can generally be ordered through Sigma, Alexa, or similar companies. It has been generally noted by several groups involved in chemical screens that it is not uncommon to have something “hit” in the stock plates and then fail to repeat when new chemical is ordered. This is usually due to an error in the identity of the compound on the stock plate, although freeze-thaw cycles and oxidation from exposure to ambient air may alter the make-up of the source compounds in a screening library. Mass spectrometry can be performed on the initial source compound used in a screen and then compared directly to a fresh, reordered stock for chemicals that will be followed up. Periodic spot-checking of chemicals that did not produce observable effects would also be prudent for libraries that will be reused for multiple screens.

Assaying for the biochemical effect of a given chemical will likely be highly specific to the developmental process or signaling pathway being assayed. Still, developing a dose-response curve across a range of concentrations for a candidate molecule may prove useful in helping to optimize follow-up assays. Identifying a lowest effective dose may help to limit toxicity and off-target effects during downstream analysis.

Finally, chemoinformatics approaches can aid in identifying compounds that are structurally related to a positive “hit” compound²⁵. Related compounds that are better characterized may provide hints as to the mechanism of action of a novel “hit” or can be useful to independently verify the specificity of the observed biological effect. A large number of resources exist for this purpose, and we provide links to several example web-based applications in Table 3.

Overall Timing

A typical “bioactives” library has between 1000–2500 chemicals. If wild type embryos are being used in the screen, it is entirely feasible to screen ~500–1000 chemicals per week utilizing 2–3 people. Increased time and numbers of embryos may be required if screening for effects on mutant embryos (e.g., assaying for suppression of a phenotype in homozygous mutant embryos generated from heterozygous adults in which only one fourth of embryos will be informative).

Advantages and Limitations of Chemical Genetic Screens in the Zebrafish

Screening in a complete organism provides a succinct read-out of the spectrum of effects a chemical has on many cell- and tissue-types. Using the zebrafish provides this read-out in a vertebrate organism whose size and life cycle are amenable to high throughput approaches.

The complexity and interrelatedness of developmental processes in a complete organism may cloud the distinction between direct and downstream effects of a chemical treatment. Many biochemical and signaling pathways are conserved from fish to humans, but this is not always the case. Appropriate disease models or assays may be lacking as well. Finally, target identification remains a general challenge in the field of chemical genetics.

Alternatives to Chemical Genetic Screens in the Zebrafish

Cell culture-based screens or *in vitro* biochemical assays likely remain faster and more scalable than whole organism screens and may be better suited for some applications. For particularly novel or poorly understood processes, classical approaches of reverse genetics (e.g., mutagenesis-based screening) may prove to be a better initial approach for establishing links to a specific gene(s).

While we have presented an ISH-based read-out in this protocol, alternatives utilizing fluorescence-based read-outs may be adapted for high throughput use in the future²⁶.

Materials

REAGENTS

Zebrafish, *Danio rerio*. Strain choice (e.g., use of a specific mutant strain) will depend on the design of a screen.

CAUTION - Use of the organism will require IRB approval at a researcher’s institution.

Phosphate Buffered Saline (PBS) (10X solution, Fischer Scientific, cat. no. BP3994)

NaCl (Fisher Scientific, cat. no. S271-1)

KCl (Fisher Scientific, cat. no. S217-3)

CaCl₂ 2H₂O (Fisher Scientific, cat. no. C79-500)

MgSO₄ (Fisher Scientific, cat. no. M65-500)

Pronase (Roche, cat. no. 11-459-643-001, see REAGENT SET-UP)

Paraformaldehyde, granular (Electron Microscopy Sciences, cat. no. 19210, see REAGENT SET-UP) CAUTION – Avoid contact with skin/eyes and inhalation to avoid irritation or possible damaging fixation of the skin/eyes or respiratory tissues.

Tween 20 (American Bioanalytical, cat. no. AB02038-01000)

Proteinase K, recombinant PCR Grade, solution (Roche, cat. no. 03 115 828 001)

Formamide (Invitrogen, cat. no. 15515-026)

SSC (sodium chloride-sodium citrate buffer) (20X, American Bioanalytical, cat. no. AB13156-04000)

Heparin (Sigma, H3393)

BCIP/NBT Color Development Substrate (Promega, cat. no. S3771)

Tris (American Bioanalytical, cat. no. AB02000-05000)

Anti-Digoxigenin-AP Fab Antibody fragments (Roche, cat. no. 11 093 274 910)

BSA, bovine serum albumin (Sigma, cat. no. A2153-10G)

RNA probe. Prepared as per Thisse and Thisse²⁴.

Methanol (Fisher Scientific, cat. no. A456-4) CAUTION Methanol liquid and vapor is flammable. Skin and eye irritant. Use in a fume hood and wear gloves and lab coat when handling.

Lamb Serum (GibcoBRL, cat. no. 16070 096)

Below are chemical libraries that have been used individually or in various combinations in previous chemical genetic screens in the zebrafish:

Chembridge DIVERSet. 50,000+ compounds. Version E with 16,320 compounds used in previous screens no longer available. Web: www.chembridge.com. Contact: sales@chembridge.com

SpecPlus Collection. 960 compounds. Available as part of the Spectrum Collection which includes the NINDS Custom Collection (see below). Web: <http://www.msdiscovery.com/spectrum.html>. Contact: compounds@msdiscovery.com

NINDS Custom Collection II. 1040 compounds. Web: <http://www.msdiscovery.com/ninds.html>. Contact: compounds@msdiscovery.com

BIOMOL ICCB Known Bioactives Library. 480 biologically active compounds. Catalog # 2840-0001. Web: <http://www.biomol.com>. Contact: 1-800-942-0430.

Sigma LOPAC¹²⁸⁰. 1280 pharmacologically active compounds. Prod. No. LO1280. Web: <http://www.sigmaaldrich.com/chemistry/drug-discovery/validation-libraries/lopac1280-navigator.html>

EQUIPMENT

48 well flat bottom plastic plates (Falcon, MULTIWELL 48 well, #353078). In situ hybridization robot (Biolane HTI, Hölle & Hüttner AG, Tübingen Germany) 48 well mesh bottom plates (Hölle & Hüttner, part #33003) Plastic trays for one or two 48 well plates (Hölle & Hüttner, part #10243 or 10244)

REAGENT SET-UP

E3 buffer (embryo water)—Prepare a 50 X stock in advance. 14.61 g NaCl, 0.63 g KCl, 2.43 g mM CaCl₂ 2H₂O, and 1.99 g MgSO₄, mix well in a flask in 1 L of deionized water. Can be stored at room temperature (~20–25° C) for months. Dilute to 1X in deionized water just prior to use.

Pronase—Prepare a stock of 100 mg ml⁻¹ in deionized water. Freeze at -20 C. This is stable for months. Just prior to use, thaw at 37 C, and make sure to mix thoroughly to redissolve as pronase tends to form a precipitate on thawing. Dilute 40 fold in E3 to 2.5 mg ml⁻¹.

Phosphate buffered saline with Tween 20 (PBT) – Dilute 10X PBS to 1X with double distilled water or equivalent. Add Tween-20 to a final concentration of 0.1% (vol/vol). This is stable at room temperature for months.

4% PFA (wt/vol)—In a chemical fume hood, dissolve PFA in 1X PBS in an Erlenmeyer flask, ideally on a combination stir plate/hot plate to provide mixing with a stir bar and heating. DO NOT ALLOW TO BOIL. Allow to cool to room temperature prior to use. This should be made fresh or, alternatively, can be frozen at -20 C; thaw just prior to use. Do not refreeze PFA stocks, as this reduces the quality of fixation.

Hybe (+)—For 1 L, mix 25 ml of 100% formamide, 12.5 ml 20X SSC, 50 µl of 50 mg ml⁻¹ heparin, 1 ml Tween 20 in 1 L final volume of double distilled water. Can be stored at -20° C for months. For Hybe (-), leave out heparin.

Heat inactivated lamb serum—Place at 55o C for 30 min. Store at -20° C (stable for months).

Blocking Solution—100 mg BSA, 1 ml heat inactivated lamb serum mixed in 50 ml PBT. Make fresh. For antibody incubation, add 1:5000 (vol:vol) dilution of anti-dig antibody to blocking solution.

Pre-staining Buffer—Mix 10 ml 1M Tris pH 9.5, 5 ml 1M MgCl₂, 2 ml 5M NaCl, and 100 µl Tween 20, and bring up to 100 ml with double distilled water. Make fresh.

Staining Buffer—Add 225 µl NBT and 175 µl BCIP to 50 ml pre-staining buffer. Make fresh. Keep covered with foil as this is light sensitive.

Stop solution—In a fume hood, add concentrated HCl dropwise to 10X PBS until pH reaches 5.5 as assayed by a pH meter or pH paper. This stock is stable in a tightly sealed bottle at room temperature for months. Dilute to 1X with deionized water prior to use and add Tween 20 to final concentration of 0.1% (vol/vol). CAUTION Avoid contact with HCl or breathing HCl fumes as this may cause severe burns.

We provide volumes (approximate) required for ISH solutions using the Biolane HTI (Hölle & Hüttner AG) robot. These can be calculated per 48 well plate as follows:

Day 1 ISH

Methanol:Phosphate buffered saline plus Tween 20 (PBT) 2:1 = 60ml

PBT: 420 ml

4% PFA (wt/vol): 60 ml

Hybe +: 60 ml

Proteinase K: 60 ml

RNA probe in Hybe (+): 50 ml

Day 2 ISH

Hybe (–): 90ml

2X sodium chloride-sodium citrate buffer (SSC): 90 ml

0.2X SSC: 210 ml

PBT: 390 ml

Blocking solution: 60 ml

Anti-digoxigenin antibody in blocking solution: 50 ml

Day 3 ISH

Pre-staining buffer: 50 ml

Staining buffer: 50 ml

PROCEDURE

Zebrafish mating set-up. (Day 0) TIMING 10–30 min

1. On the night prior to collecting embryos, arrange male and female zebrafish in pairs or in groups consisting of 2 males/3 females separated by a divider.

CAUTION Use of zebrafish will require IRB approval at a researcher's institution.

Embryo collection, staging, and distribution (Day 1). TIMING 2–3 hours

2. Place the mating pairs or groups in fresh water (volume will vary depending on cage size, but should be sufficient to provide ~ 2 cm of water depth above the bottom of the mating cage) and remove the divider to allow the fish to spawn.
3. Carefully stage embryos from each collected clutch, and pool those that are within an acceptable developmental range (i.e. 10–20 minutes apart typically). Using a plastic transfer pipette, place ~8–20 into each well of a 48-well flat bottom plate. The amount of liquid placed in each well is not critical here – it will be adjusted in the next step.

TROUBLESHOOTING

Normalization of well volume, preparation of chemical library plates, and addition of chemicals. TIMING 30 min to 1 h plus time allotted for embryos to develop to the stage at which chemical will be added

4. All fluid must be removed from the embryo-containing wells in order to add back a fixed volume. Lean each 48 well plate vertically against a support (typically a pipette box),

and the embryos will fall to the bottom of the well. Gentle tapping helps them to fall. Using either a custom built or commercially available multiwell suction device placed at the top of the well, the liquid can be removed without any loss of embryos. Using a multiwell or repeat pipettor, add back 200 μ l of embryo water (E3) into each well.

5. On the day of screening, remove 1 μ l of each stock solution from the master chemical plate and place into a recipient 48 or 96 well “transfer” plate containing 100 μ l of E3 containing 1% DMSO (with or without 1% penicillin/streptomycin). Mix using either a liquid handling robot or manually. Ensure that at least 1–2 wells per 48 well plate contain only DMSO as a control. CAUTION Gloves should be worn when handling chemical transfer plates and treated embryo plates.

6. Allow the embryos, now in 200 μ l of E3, to grow in the 48 well plate until they have reached the proper developmental stage for the given assay. At that point, add 100 μ l of chemical from the “transfer” plate to the wells containing the embryos in 200 μ l of E3. Place the embryos back in the incubator to continue to develop until assayed.

Chemical removal, chorion removal, and fixation of embryos. DAY 2 (assuming ~24 hour chemical treatment) TIMING 1 hour + overnight

7. For all of the following steps, the 48 well plate can be vertically leaned against a support to ensure embryos fall to the bottom of the well. Remove the medium containing the chemicals from the wells using the multichannel suction device. Add 750 μ l of E3 to each well to wash away any residual chemical

TROUBLESHOOTING

8. Remove all 750 μ l of E3 from each well, and add back 500 μ l of pronase (2.5mg ml⁻¹) using a repeat pipettor. Place each plate at 28.5 C for 10 min. Remove all of the pronase solution, add 750 μ l of E3, and immediately remove with the suction device. Repeat this E3 wash for a total of 3–4 $\times$ 5 min washes to ensure all bits of chorion are removed. A final wash in 1X PBT is helpful to reduce sticking of embryos in subsequent steps.

CRITICAL STEP: Over-digestion with pronase can damage embryos and lead to degradation during subsequent ISH.

TROUBLESHOOTING

9. Remove the 1 X PBT and replace with 500 μ l of 4% PFA (wt/vol). Seal the plates with parafilm, and place them at 4 C overnight.

CAUTION PFA is toxic; avoid skin contact and inhalation of fumes.

CRITICAL STEP: Do not leave the embryos in PFA for more than 2–3 d as this may decrease the quality of the *in situ* hybridizations.

Dehydration. DAY 3 TIMING 10–15 min + overnight

10. Remove the PFA from the wells and replace with 500 μ l of 100% methanol. Seal the plates again with parafilm and place at –20 C until ready for use.

CRITICAL STEP: Embryos should be left in methanol for *at least* 2 h to overnight to ensure good quality *in situ* hybridization.

PAUSE POINT: Embryos can be left for long periods of time in 100% methanol, i.e., 3–6 months.

Embryo transfer and day 1 of ISH. DAY 4 (or later) TIMING 2.5 hours + overnight

11. Add 50–100 μ l of 1X PBT to the methanol in each well to aid in transfer of embryos as this will reduce sticking. Next, transfer each well of embryos to the corresponding well within a 48-well mesh bottom plate using a plastic transfer pipette.

TROUBLESHOOTING

CRITICAL STEP: Embryos are transferred manually between plates at this stage, so great care should be taken to avoid mixing up well numbers during this process.

12. Perform ISH using a Biolane HTI robot following the general methods outlined by Thisse and Thisse¹⁵. Prepare the appropriate volumes of solutions (see Reagent Preparation above) required for day 1 of the ISH protocol. Place each 48-well mesh bottom plate in the robot and initiate the day 1 program as described in Table 4.

13. At the conclusion of day 1 of the ISH protocol, lift the plates from the pre-hybridization solution and place into airtight plastic containers prefilled with 50 ml of RNA probe in Hybe (+) solution, at a final concentration of 0.8–1 ng μ l⁻¹ of probe. Cover the containers tightly and place in a 70 C hybridization oven and incubate overnight.

CRITICAL STEP: Avoid leaving embryos in prehybe (Hybe +) solution for > 2 hours as this will decrease the ISH signal intensity.

CRITICAL STEP: Excessive proteinase K treatment will make the embryos fragile and less likely to survive the ISH treatment intact.

TROUBLESHOOTING

Second day of ISH. DAY 5 TIMING 4 h + overnight

14. Carefully lift the 48 well mesh bottom plate, drain away any excess probe solution, and then proceed to place the plate directly into Hybe (–) solution to start the day 2 ISH protocol in the ISH robot (Table 4).

TROUBLESHOOTING

Alkaline phosphatase staining and preparation for long term storage of embryos. DAY 6 TIMING 1 h to overnight

15. At the end of the day 3 robot ISH protocol, remove all of the PBS from the tray using a suction device, and gently blot the bottom of the plate on a Kimwipe. Place plates in a container with a lid (e.g. Tupperware or pyrex) and add 50 ml of prestain solution (per 48 well plate). Thus, if performing a single 48 well plate, use 50 ml of prestain; for two 48 well plates, use 100 ml of prestain, etc. Gently rock the embryo plates on a swirling rotator to ensure removal of the PBS and equilibration to the higher pH of the prestaining buffer. Prestain the embryos for at least 10 min, and then remove all of the fluid with a suction device.

CRITICAL STEP Ensure that all of the PBS is removed from the tray to aid in equilibration in the pre-staining buffer.

16. Add 50 ml of staining buffer per 48 well plate. Cover the entire tray tightly with aluminum foil. Check plates at least every 1–2 h to avoid overstaining.

CRITICAL STEP Protect the staining buffer from light.

CRITICAL STEP The time required for proper visualization of the probe varies with the gene being studied.

TROUBLESHOOTING

17. When the control wells are adequately stained, remove the staining buffer with the suction device, and then wash 3 times with PBT for ~5 minutes each. Finally, remove the last PBT wash and replace with stop solution (PBT, pH 5.5).

TROUBLESHOOTING

18. Transfer each embryo well back to a 48-well plastic plate with the embryos in stop solution, and then seal it tightly with parafilm.

PAUSE POINT The sealed plates can be stored at 4°C temperature for over 1 year with no degradation of embryos or alterations in probe intensity. Alternatively, embryos can be stored in 4% PFA (wt/vol), but we have found the extra expense of this unnecessary.

Scoring of embryos. **TIMING** Hours to days, depending on ease of recognizing phenotypes

19. Compare ISH staining of embryos from each chemically treated well to the control wells on the same plate.

TROUBLESHOOTING

TIMING

Step 1–3 Zebrafish mating set-up. 10–30 min

Steps 4–6 Normalization of well volume, preparation of chemical library plates, and addition of chemicals. 30 min to 1 h plus time allotted for embryos to develop to the stage at which the chemical will be added

Steps 7–9 Chemical removal, chorion removal, and fixation of embryos. 1 h + overnight

Step 10 Dehydration. 10–15 min + overnight

Steps 11–13 Embryo transfer and day 1 of ISH. 2.5 h + overnight.

Step 14 Second day of ISH. 4 h + overnight

Steps 15–18 Alkaline phosphatase staining and preparation for long term storage of embryos. 1 h to overnight

Step 19 Scoring of embryos. Hours to days.

TROUBLESHOOTING

Please see Table 5 for troubleshooting advice.

ANTICIPATED RESULTS

Experience from our own laboratory has indicated that generalized toxicity of any given chemical library depends strongly on the embryo stage at first exposure. A screen starting at 50% epiboly and running until 24 hpf, using a “known bioactives” library yielded a 7% toxicity rate (unpublished data, see Figure 1a–c for the appearance of embryos). In contrast, a screen that began exposure at 1–3 somites and completed at 36 hpf yielded a 3% toxicity rate¹⁰.

The likelihood of “false positives” is generally correlated with the strength of the phenotype on the initial screen. Scoring for gradations of ISH staining (i.e. “darker” vs. “lighter” staining) will be more subject to technical and biological noise than assaying for complete abrogation or restoration of staining (i.e. “present” vs. “absent”). Those chemicals that affect only half of the embryos in a given plate, and do so weakly, may be difficult to confirm in further assays.

However, these should not be discarded out of hand, since this is often related to a dosing effect, so these can be tried at a higher dose. Based on prior experience, a “hit” rate of ~1–2% is reasonable to expect from any given screen. We have also found that independent scoring by multiple observers is useful.

Finally, we include a previously published example of the ISH-based readout (Fig 2a–c¹⁴). By noting the normal ISH staining of the HSC markers *runx1* and *cmyb* in the control embryo (Fig. 2a), one can see the effect of a chemical that increases (Fig 2b) or decreases (Fig 2c) developing HSC's in the zebrafish embryo.

Acknowledgments

Thanks to Trista North and Wolfram Goessling for use of *runx1/cmyb in situ* figures. Thanks to Sumon Datta and Philip Manos for informative discussions. Thanks to Christian Mosimann and Jill Dejong for critical reading of the manuscript. CKK is supported by NIH grant 5T32CA09172-34. RMW is supported by NIH grant 1K08AR055368.

References

1. Frese KK, Tuveson DA. Maximizing mouse cancer models. *Nat Rev Cancer* 2007;7:645–58. [PubMed: 17687385]
2. Elliott DA, Brand AH. The GAL4 system : a versatile system for the expression of genes. *Methods Mol Biol* 2008;420:79–95. [PubMed: 18641942]
3. Martin BL, Kimelman D. Regulation of canonical Wnt signaling by Brachyury is essential for posterior mesoderm formation. *Dev Cell* 2008;15:121–33. [PubMed: 18606146]
4. Chen CM, Struhl G. Wingless transduction by the Frizzled and Frizzled2 proteins of *Drosophila*. *Development* 1999;126:5441–52. [PubMed: 10556068]
5. Chen JK, Taipale J, Young KE, Maiti T, Beachy PA. Small molecule modulation of Smoothened activity. *Proc Natl Acad Sci USA* 2002;99:14071–14076. [PubMed: 12391318]
6. Frank-Kamenetsky Zhang AM, Bottega S, Guicherit O, Wichterle H, Dudek H, Bumcrot D, Wang FY, Jones S, Shulok J, Rubin LL, Porter JA. Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. *J Biol* 2002;1:10. [PubMed: 12437772]
7. Jiang X, et al. Distinctive roles of PHAP proteins and prothymosin- α in a death regulatory pathway. *Science* 2003;299:223–226. [PubMed: 12522243]
8. Rosania GR, et al. Myoseverin, a microtubule-binding molecule with novel cellular effects. *Nat Biotechnol* 2000;18:304–308. [PubMed: 10700146]
9. Wu X, Ding S, Ding Q, Gray NS, Schultz PG. A small molecule with osteogenesis-inducing activity in multipotent mesenchymal progenitor cells. *J Am Chem Soc* 2002;124:14520–14521. [PubMed: 12465946]
10. Druker BJ, Lydon NB. Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia. *J Clin Invest* 2000;105:3–7. [PubMed: 10619854]
11. Lokey RS. Forward chemical genetics: progress and obstacles on the path to a new pharmacopoeia. *Curr Opin Chem Biol* 2003;7:91–96. [PubMed: 12547432]
12. Stern HM, et al. Small molecules that delay S phase suppress a zebrafish bmyb mutant. *Nat Chem Biol* 2005;1:366–70. [PubMed: 16372403]
13. Murphey RD, Stern HM, Straub CT, Zon LI. A chemical genetic screen for cell cycle inhibitors in zebrafish embryos. *Chemical biology & drug design* 2006;68:213–9. [PubMed: 17105485]
14. North TE, et al. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature* 2007;447:1007–11. [PubMed: 17581586]
15. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 2007;8:353–67. [PubMed: 17440532]
16. Peterson RT, Link BA, Dowling JE, Schreiber SL. Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc Natl Acad Sci USA* 2000;97:12965–9. [PubMed: 11087852]

17. Yang C, Johnson SL. Small molecule-induced ablation and subsequent regeneration of larval zebrafish melanocytes. *Development* 2006;133:3563–3573. [PubMed: 16914496]
18. Yang C, Hindes AE, Hultman KA, Johnson SL. Mutations in *gfpt1* and *skiv2l2* cause distinct stage-specific defects in larval melanocyte regeneration in zebrafish. *PLoS Genet* 2007;3:e88. [PubMed: 17542649]
19. Peterson RT, et al. Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation. *Nat Biotechnol* 2004;22:595–9. [PubMed: 15097998]
20. Goessling W, et al. Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. *Cell* 2009;136:1136–1147. [PubMed: 19303855]
21. Yeh JJ, et al. Discovering chemical modifiers of oncogene-regulated hematopoietic differentiation. *Nat Chem Biol* 2009;5:236–243. [PubMed: 19172146]
22. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn* 1995;203:253–310. [PubMed: 8589427]
23. Mayer TU. Chemical genetics: tailoring tools for cell biology. *Trends Cell Biol* 2003;13:270–277. [PubMed: 12742171]
24. Thisse C, Thisse B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc* 2008;3:59–69. [PubMed: 18193022]
25. Engel T. Basic overview of chemoinformatics. *J Chem Inf Model* 2006;46:2267–2277. [PubMed: 17125169]
26. Vogt A, et al. Automated image-based phenotypic analysis in zebrafish embryos. *Dev Dyn* 2009;238:656–663. [PubMed: 19235725]
27. Molina GA, Watkins SC, Tsang M. Generation of FGF reporter transgenic zebrafish and their utility in chemical screens. *BMC Dev Biol* 2007;7:62. [PubMed: 17553162]
28. Yu PB, et al. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nat Chem Biol* 2008;4:33–41. [PubMed: 18026094]
29. Owens KN, et al. Identification of genetic and chemical modulators of zebrafish mechanosensory hair cell death. *PLoS Genet* 2008;4:e1000020. [PubMed: 18454195]

Figure 1. Chemical Toxicity

After chemical treatment and prior to fixation for ISH analysis, one can identify (a) normally developing zebrafish embryos (shown here at ~ 24 hpf) and those showing (b) developmental delay (marked with *) and marked aberrant development (bracketed) or (c) death. Bar (a–c) = 0.5 mm. IRB approval is required for use of the zebrafish in such experiments.

runx1/cmyb **Figure 2. Anticipated Results**

Previously published example of *in situ* hybridization (ISH) to hematopoietic stem cell (HSC) markers *runx1* and *cmyb* demonstrating (a) normal staining of the HSC's in a negative control embryo (arrows mark endothelial cells and arrowheads mark haematopoietic clusters) and both (b) increased staining from a chemical which increases HSC's and (c) decreased staining from a chemical which decreases HSC's. Reprinted by permission from T. North and W. Goessling and Macmillan Publishers Ltd: Nature, Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. **447**, 1007–11, copyright 2007¹⁴. Bar (a–c) = 0.5 mm. IRB approval is required for use of the zebrafish in such experiments.

Figure 3. Flow Diagram

Overview of the steps and timing for a chemical genetic screen using zebrafish embryos with ISH as a readout.

Table 1

Chemical Genetic Screens Performed in the Zebrafish Embryo

Paper and Aim of Screen	Screen Read-out	Chemical exposure	Library	Outcome
Peterson et al, 2000 ¹⁶ <ul style="list-style-type: none"> proof of principle for chemical genetic screen in whole organism 	visual exam of central nervous system, cardiovascular system, pigmentation, and ear in wild type treated embryos	8–16 cell stage to 1, 2, 3 dpf, 1 μ M	1,100 chemicals selected from Chembridge Corporation, DIVERSet E	Multiple chemicals: 32N8 - enlarged hindbrain ventricle; 33M20 - elongated notochord; 32N5 - sawtooth hindbrain projections; 32P6- cardia bifida; 31J6 – condition similar to partial AV block; 31B4 - pigmentation block in PRE and melanocytes; 33N14 (MoTP) - pigmentation block, melanocytes only; 31N3 - blocks otolith formation
Peterson et al, 2004 ¹⁹ <ul style="list-style-type: none"> suppression of mutation in transcriptional repressor hey2 (called <i>grl</i>) which causes aorta malformation 	restored blood flow to tail at 48 hpf. in <i>grl</i> mutants	50% epiboly to 48 hpf, 2 μ g ml ⁻¹	5,000 chemicals from Chembridge Corporation, DIVERSet E	2 chemicals suppressed <i>grl</i> phenotype: GS3999 and GS4102, linked to VEGF pathway effect
Stern et al, 2005 ¹² <ul style="list-style-type: none"> identify <i>bmyb</i> pathway interactors 	suppression of mitotic arrest in <i>crb</i> ^{-/-} embryos based on whole mount immuno- histochemical staining against serine-10-phosphorylated histone H3	4–6 hpf to 24 hpf	16,320 from Chembridge Corporation, DIVERSet E, screened in pools of 8 and 10 chemicals at 62.5 μ g/ml per chemical	Persynthamide, suppressed <i>bmyb</i> pathway mitotic defects
Murphey et al, 2006 ¹³ <ul style="list-style-type: none"> identify chemicals which affect the cell cycle 	alterations in number of mitotic cells as measured by whole mount immuno- histochemical staining against serine-10-phosphorylated histone H3	4–6 hpf to 24 hpf, average 20 μ M	16,320 from Chembridge Corporation, DiverSet E	19 chemicals that increased number mitotic cells, 10 which decreased number mitotic cells
North et al, 2007 ¹⁴ <ul style="list-style-type: none"> identify modulators of hematopoietic stem cell (HSC) formation and homeostasis 	alterations of <i>runx1</i> and <i>cmyb</i> (HSC markers) by <i>in situ</i> on WT embryos at 36 h.p.f.	3 somites to 36 hpf	NINDS Custom Collection (1040 compounds), SpecPlus Collection (960 compounds), BIOMOL ICCB Known Bioactives(480 compounds)	10 compounds known to affect prostaglandin pathway, enhancement of this pathway led to increased HSC numbers
Molina et al, 2007 ²⁷	decreased expression of dsEGFP under control of <i>dusp6</i> promoter (FGF responsive gene, serving as FGF reporter)	24 hpf embryos, 6–8 h in chemical	7 compounds (indolinones) structurally similar to SU5402, a	two compounds, previously known to target VEGF signaling,

Paper and Aim of Screen	Screen Read-out	Chemical exposure	Library	Outcome
<ul style="list-style-type: none"> identify inhibitors of FGF signaling 			previously characterized FGF inhibitor	also inhibit FGF signaling
Yu et al, 2008 ²⁸ <ul style="list-style-type: none"> to identify BMP pathway-specific inhibitors 	dorsalization of WT embryos by gross morphology at 12, 24, and 48 hpf, follow-up with <i>in situs</i>	4 hpf, 5–10 μ M	Chembridge Corporation, 5,580 synthetic compounds; Microsource Discovery Systems, 1840 known bioactive small molecules; Sigma-Aldrich, 150 known bioactive small molecules	dorsomorphin, shown to be BMP-pathway inhibitor
Owens et al, 2008 ²⁹ <ul style="list-style-type: none"> identify chemicals which protect hair cells of the lateral line from aminoglycoside- induced toxicity 	preservation of hair cell integrity following neomycin treatment as visualized with vital dyes	Pre- treatment of 5 dpf embryos for 1 h, followed by neomycin treatment for 1 h, 10 μ M	10,690 compounds from Chembridge Corporation DiverSet E; initially in pools of 5 compounds, then singly for positive hits	compounds PROTO-1 and PROTO-2 protected hair cells from neomycin induced toxicity
Yeh et al, 2009 ²¹ <ul style="list-style-type: none"> identify chemical modifiers of an oncogene's (AML1-ETO) effects on differentiation of hematopoietic progenitor cells 	preservation of GATA-1 <i>in situ</i> staining (marker of erythroid cells) following induced expression of AML1-ETO (an oncogene which causes GATA-1 decrease)	Treat 12–16 hpf embryos hetero- zygous for Tg(hsp: AML1-ETO) for 1 h, followed by 1 h heat shock, collect embryos 1.5 h later	2,000 known bioactive compounds from the SPECTRUM library, Microsource Discovery Systems	dicumarol and nimesulide (a modulator of the prostaglandin pathway) restored GATA-1 expression without affecting heat shock induced expression of the AML1-ETO transgene

Abbreviations: dpf – days post fertilization; hpf – hours post fertilization; WT – wild type

Table 2

Screen Design: Read-out Selection

Read-out	Pros	Cons
Visual inspection for morphological changes	<ul style="list-style-type: none"> No reagent development required Flexible Embryos may be assayed at multiple time points if not fixed May be possible to increase sensitivity with staining/vital dyes²⁹ 	<ul style="list-style-type: none"> Dependent on skill of observer May be labor intensive May miss transient effects due to rapidity of zebrafish development? Limitations on assayable structures?
<i>In situ</i>	<ul style="list-style-type: none"> Once fixed at specified time point, embryos stable indefinitely Highly scalable, proven technique in zebrafish²⁴ Possible to assay for any transcript? 	<ul style="list-style-type: none"> Requires reagent development and optimization Automation requires investment (i.e. robot purchase)
Antibody staining	<ul style="list-style-type: none"> Once fixed at specified time point, embryos stable for long periods Scalable Possible to assay for any gene product? 	<ul style="list-style-type: none"> Requires reagent development and optimization Automation requires investment (i.e. robot purchase) Fewer antibodies available for zebrafish?
Promoter: reporter Transgenic Line	<ul style="list-style-type: none"> Gene-specific read-out with minimal processing of embryos Embryos may be assayed at multiple time points if not fixed 	<ul style="list-style-type: none"> Development and characterization of reporter line Sensitivity of assay? May miss transient effects due to rapidity of zebrafish development?

Table 3

Example Web-based Chemoinformatics Resources

Web site	Information Provided by this Resource; Free/Site license
www.discoverygate.com	Information on published literature and patents; Site license
shoichetlab.compbio.ucsf.edu/~keiser/sea/	Relates protein targets based on chemical similarity of their ligands; Free
pubchem.ncbi.nlm.nih.gov	Information on all publicly available molecules; Free
chembank.broad.harvard.edu	Public access repository of screens done at the Broad Institute and elsewhere; Free
www.broad.mit.edu/cmap	Database connecting chemicals to phenotypes to gene expression; Free
www.ingenuity.com	Relates defined biological pathways to chemicals which perturb the pathway; Site license

Table 4

Example ISH Robot Program

Day 1 ISH	
REAGENT	TIME (hours:minutes:seconds)
Methanol:PBT 2:1	0:5:00
Methanol:PBT 1:2	0:5:00
PBT	0:5:00 × 4 washes
Proteinase K	Variable, depending on embryo stage
4% PFA (wt/vol)	0:20:00
PBT	0:5:00 × 2 washes
Pre-hybe/hybe (+)	1:00:00
Total time:	2:05:00

Day 2–3 ISH	
REAGENT	TIME(hours:minutes:seconds)
Hyb (-)	0:5:00
75% Hyb-/25% SSC (vol/vol)	0:15:00
50% Hyb-/50% SSC (vol/vol)	0:15:00
25% Hyb-/75% SSC (vol/vol)	0:15:00
0.2X SSC	0:30:00 × 2 washes
75% SSC/25% PBT (vol/vol)	0:10:00
50% SSC/50% PBT (vol/vol)	0:10:00
25% SSC/75% PBT (vol/vol)	0:10:00
PBT	0:10:00
Block solution	0:30:00
Anti-dig antibody 1:1000	16:00:00
PBT	0:15:00 × 4 washes
Total time:	20:00:00

Abbreviations: PBT – phosphate buffered saline plus Tween-20; PFA – paraformaldehyde; SSC – sodium chloride/sodium citrate buffer

Table 5

Troubleshooting

Step	Problem	Possible Cause	Solution
3	Heterogeneity of embryonic stages	Overcrowding of collected embryos prior to plating Multiple spawning events prior to collection Sick embryos	Keep ~100–200 embryos/petri dish while staging Collect embryos over shorter period of time, i.e., 10 min Remove embryos that are unlikely to survive gastrulation prior to plating
7	Embryo death during chemical incubation	May be expected toxicity of given chemical Embryos treated too early in development Poor initial embryo quality leads to excess number of dying embryos/well	Identify if chemical in question is known to be embryonic lethal Adjust assay to allow for later exposure if possible Try alternative parents that may produce higher quality eggs; remove dying embryos during incubation to protect remaining embryos
8	Chorions fail to loosen	Chemicals inhibit pronase reaction	Increase time or concentration of pronase; manually dechorionate that well
11	Embryos fragmented during transfer to mesh plates	Embryos are sticking to plastic pipettes during transfer	Add 50–100 μ l of 1x PBT to embryos in methanol prior to transfer
13	Embryos dried out	Failure to tightly seal plate during overnight 70 °C probe hybridization	Utilize tightly sealing Tupperware or other container to hold plate
14	Embryos fragmented after day 1	Excess proteinase K treatment	Must be individualized for each assay; decrease time or concentration
15–16	Blue clumps adherent to embryo	Rapid pH change from PBT to staining buffer	Ensure an adequate prestaining step to equilibrate pH
17	Embryos deeply blue/purple	Excessive time in staining buffer Exposure to light during staining	Utilize negative controls as guide to proper length of stain Tightly wrap containers in aluminium foil during staining
19	Unexpected number of “hits” on a plate	Inherent variability of staining strength during ISH	Critical to utilize the negative control of each plate as the comparator