Available online at www.sciencedirect.com
ScienceDirect
Methods 39 (2006) 255–261
METHODS
www.elsevier.com/locate/ymeth

Small molecule screening in the zebrafish

R.D. Murphey, L.I. Zon *

Stem Cell Program and Division of Hematology and Oncology, Children's Hospital, Dana-Farber Cancer Institute, Howard Hughes Medical Institute and Harvard Medical School, 1 Blackfan Circle, Boston, MA 02115, USA

Accepted 7 September 2005

Abstract

The zebrafish is an ideal organism for small molecule studies. The ability to use the whole organism allows complex *in vivo* phenotypes to be assayed and combines animal testing with screening. Embryos are easily treatable by waterborne exposure. The small size and abundance of embryos make zebrafish suitable for screening in a high-throughput manner in 96- or 48-well plates. Zebrafish embryos have successfully been used in chemical genetic screens to elucidate biological pathways and find chemical suppressors. Small molecules discovered by screening zebrafish disease models may also be useful as lead compounds for drug development as there appears to be a high level of conservation of drug activity between mammals and zebrafish. Here we provide the technical aspects of treating embryos with small molecules and performing chemical screens with zebrafish.

Keywords: Zebrafish; Embryos; Chemical genetics; Small molecule; Drug screen

1. Introduction

The concept of chemical space suggests that a small molecule could be synthesized to bind to any biological target. The classical drug discovery approach, sometimes referred to as reverse chemical genetics, is a screen for small molecules that bind *in vitro* to a target of interest. Such screens require a specific target, which is typically only available after years of research and a detailed molecular understanding of a process. An alternative to this approach is to screen an intact biological pathway. A major advantage of this type of screen is that one can find modifiers without having to know all the components of a given pathway.

The method of identifying small molecules that alter the function of a biological pathway, resulting in the induction or rescue of a specific phenotype, is called forward chemical genetics [1–3]. This approach involves screening a large library of compounds to find small molecules that disrupt a phenotype in a biological assay analogous to traditional genetic screens. The cellular target of the interaction can then be identified by biochemical or candidate approaches. A successful example of forward chemical genetics is the isolation of monastrol, an inhibitor of Eg5 kinesin [4]. An advantage of chemical genetics over traditional genetics is the ability to have temporal control over the phenotype. Small molecules can be added or removed by washing at convenient times giving a conditional effect. In addition, if a study relates to human disease, a small molecule may also serve as a lead compound for drug development.

Whole organisms offer several advantages over cell lines for forward chemical genetic screens, providing information on tissue specificity, toxicity, and accounting for bioavailability. Furthermore, cells are not transformed and are in their normal physiological milieu of cell–cell and cell–extracellular matrix interactions. Use of the whole organism can also allow the screening of processes that are not easily replicated *in vitro* such as organ development. The advantages of zebrafish screening over invertebrate model organisms are the ease of waterborne treatment and their closer evolutionary relationship to humans.

A number of laboratories have begun to do zebrafish chemical genetics studies. The Schreiber lab performed a

* Corresponding author. Fax: +1 617 738 5922.
E-mail address: zon@enders.tch.harvard.edu (L.I. Zon).

1046-2023/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2005.09.019

small molecule screen with intact wild-type zebrafish embryos looking for compounds that induced specific developmental phenotypes [5]. After screening 1100 chemicals from the Chembridge DiverSet E library, they found 2% of the chemicals were toxic, while 1% caused a specific developmental phenotype. Chemical inhibitors are also being used in zebrafish to study processes such as angiogenesis [6] and the Fgf signalling cascade [7].

Screening for modifiers of a zebrafish mutant is another reason for screening embryos. We developed a zebrafish chemical suppressor screening technology using a recessive lethal cell cycle mutant that has a fourfold increase in the number of mitotic cells as detected by pH3-staining [23]. A chemical suppressor screen of the zebrafish *hey2* mutant (gridlock), was also recently completed [8]. Two structurally related compounds of the 5000 small molecules tested were found to suppress the gridlock phenotype. They were shown to act by upregulating expression of vascular endothelial growth factor (VEGF). Such chemical suppressor screens could be applied to any zebrafish model of human disease [9].

There are also disadvantages to screening in zebrafish. Zebrafish screening is typically limited to a few thousand compounds per week. The amount of labor involved in a whole embryo zebrafish screen is greater than cell line screens because of the fish husbandry and lower overall throughput. For these reasons zebrafish may often be better used as a secondary screening platform after a higher throughput primary screen. A number of preclinical assays have been developed for using zebrafish in the drug discovery process, such as toxicity testing [10].

A major concern about using the zebrafish to find small molecules is that screen hits will not be active in mammalian systems. A recent study exposed zebrafish to drugs known to prolong the QT interval of the cardiac contraction cycle in humans [11]. It was determined that 18 of 23 small molecules were active in the zebrafish by water treatment, with four more showing activity after microinjection into the embryo. Thus waterborne exposure and microninjection both can allow small molecules to permeate the embryo and in this case the drug targets were conserved.

Drug metabolism is an important factor in the conservation of drug activity across species. Many groups are studying the conservation of drug-metabolizing enzymes in the zebrafish. The phase I biotransforming cytochrome p450 (CYP) monooxygenases are an important means of drug metabolism. The dioxin inducible CYP1A gene is well characterized in the zebrafish and can be induced in many tissues of the embryo [12]. Constitutive and xenobiotics-induced expression of a zebrafish CYP3A gene is also conserved [13]. It should be noted that even mammalian model systems are not always good predictors of specific drug metabolism in humans [14].

In addition to zebrafish embryo screening, chemical screening can also be done in juvenile or adult zebrafish. There are many imaginable screens that could only be studied in older animals, such as a chemotherapeutic screen. Hits from such a screen may more directly translate to lead compounds for drug development. Adult fish can be treated by waterborne exposure or by intraperitoneal injection. Waterborne exposure of adult fish requires a much larger amount of chemical because of the necessary increase in volume. This also leads to a large amount of chemical waste. Other major restrictions on adult screening are throughput and animal facility space. Because of the potential danger of chemicals to other fish, screen fish need to be treated outside of a zebrafish system with manual water changes. Adult chemical screens, though theoretically possible, have not yet been realized.

There have been a number of studies addressing the conserved function of small molecules in the adult zebrafish compared to mammals. Direct water treatment can lead to oral absorption by the gastrointestinal tract through water ingestion [15]. Warfarin and dexamethasone have been shown to be active in adult zebrafish by aqueous exposure [15,16]. Teleosts have been widely used in toxicological studies to determine the risk of neoplasia from environmental hazards. Trout and medaka have been extensively studied as carcinogeneis models [17,18]. Zebrafish are also susceptible to cancer after exposure to known carcinogens [19,20]. These data suggest that aqueous exposure is a feasible route of drug administration in zebrafish.

In this chapter, we will discuss the details of performing a small molecule screen in the zebrafish. This will include a number of types and variations of embryo screens. A distinction is made between single genotype chemical screens and homozygous lethal mutant suppressor chemical screens. Screens for suppression of a homozygous lethal mutation are distinct in requiring heterozygous incrosses to generate embryos. This leads to multiple genotypes being present in a single well. Single genotype screens can describe wild-type, transgenic, or mutant screens that only use embryos of a single genotype. It is possible that a homozygous lethal mutant may be sortable before chemical treatment in a therapeutic screen. In this case if would fall into the category of single genotype screens. Appendix A illustrates an example of a pH3 antibody staining, single genotype screen.

2. Description of methods

2.1. Embryo chemical treatment medium and culture

Zebrafish are maintained according to Westerfield [21]. Zebrafish embryos are raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, and 0.16 mM $MgSO_4$). For zebrafish embryo chemical experiments 1% DMSO is used as a vehicle for small molecules to permeate the embryo. Chemical screening medium is composed of the standard embryo medium (E3) supplemented with 1% DMSO, 20 μM metronidazole, .05 U/ml penicillin, 50 ng/ml streptomycin, and 1 mM Tris, pH 7.4.

Embryos are collected and stored in a 28.5 °C incubator. Clutches should not be combined until immediately before distribution. Any dead or unfertilized embryos should be

cleaned out in the first few hours of development and periodically thereafter to prevent developmental delay of the whole clutch.

2.2. Aliquoting of chemicals and matrix pooling

Small molecule libraries can be developed or acquired by collaboration or purchase. The concentration of novel compound libraries will be in mg/ml or molarity. The best arrangement for known drug libraries is by specific potency, but this is not always how they are arrayed. The relatively large volume required for embryos will often limit the dose used in a zebrafish screen. In a single genotype screen the volume can be as low as 100 μl in a 96-well plate, and in a homozygous lethal mutant suppressor screen it should be 300 μl in a 48-well plate. This distinction is because of the increased number of embryos required for a homozygous lethal mutant suppressor screen (Section 2.6). For a novel chemical library the screening concentration used has been between 1 and 5 μg/ml or 5 and 20 μM [5,8]. This will give a low toxicity rate yet ensure many chemicals reach an active dose.

Chemical libraries are often stored frozen in DMSO in 384-well stock plates. Chemicals can be manually transferred, but automatic liquid handling systems are faster, ensure more accurate volume transfers, and avoid pipetting errors. Compounds should be transferred using a low volume liquid handling system from the stock plates into dilution plates containing screening medium. This diluted stock can then be aliquoted with a Tecan liquid handling robot (Tecan, Durham, NC) into the 48- or 96-well standard tissue culture plates along with additional screening medium if necessary to bring wells up to the total volume.

Individual chemicals can be added to each well, but to improve throughput we employed a matrix pooling strategy. Depending on the number of wells in the stock plate and the screening plate, an optimal number of compounds per pool can be established. If each compound is represented in two pools, an individual hit can be detected without having to test each individual chemical in the pool (Fig. 1). Screening with 8 by 10 matrix pooling uses fivefold less embryos than single compound screening with the additional benefit of testing each compound twice (Table 1).

Pooling can greatly increase throughput, but can complicate identification of individual hits and increase the rate of toxicity. In the case of novel small molecule libraries, most chemicals will have no biological activity so toxicity is less of a problem. On the other hand, pooling known bioactive libraries leads to a very high rate of toxicity (Zon lab, unpublished). It is undesirable for the rate of toxicity to lead to multiple toxic compounds within the same pool, which can occur frequently with a toxicity rate of higher than 2% of the individual chemicals. The choice to pool should be determined empirically by piloting a few hundred compounds of a given chemical library.

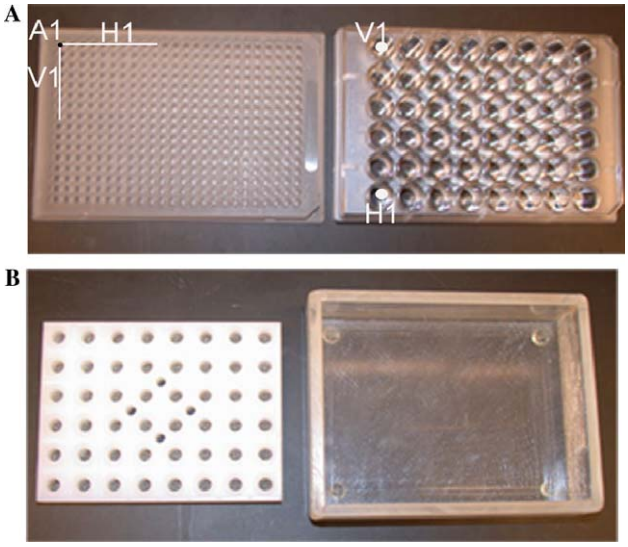

Fig. 1. Methods of increasing screening efficiency. (A) 8 × 10 matrix pooling strategy. Putative screen hits (white circles) in a vertical pool (V1) and a horizontal pool (H1) of a 48-well screening plate (right) point back to one active compound from the 384-well stock plate (left) that is present in both pools (black circle, A1). (B) Staining grids. Mesh-bottomed wells (left) allow easy transfer of embryos between solutions in reservoirs (right) during whole mount immunostaining and RNA *in situ* hybridization.

Table 1
Influence of screen and library type on weekly chemical throughput with 5000 embryos available each week and 8 × 10 matrix pooling

	Single genotype	Recessive lethal mutant
Novel library (pooling)	5000	1000
Known library (no pooling)	1000	100

2.3. Timing of chemical exposure

One of the most important considerations for a chemical screen is the timing of chemical treatment. The decision should take into account the developmental timing of the phenotype being assayed. We typically treat from 4 to 24 hpf for an early developmental phenotype or else starting around 24 hpf. If it is possible to start treatment at 24 hpf, this has the advantages of having less developmentally related embryo death and less chemical toxicity due to the slower rate of cell cycling. Embryos are more readily handled in their chorions, making it easier to distribute them before 48 hpf. Treating before 4 hpf can be difficult because of the relatively high rate of embryo death at this time due to abnormal development. Cleaning out unfertilized eggs and malformed embryos is an essential part of screening due to the small volume necessary to have a high dose of chemical. The death of a few embryos will contribute to the poor health, delay and often death of all embryos in a well. To verify that a given treatment window is appropriate; a positive control chemical is advantageous to verify that the timeframe of the screen is compatible with the desired outcome.

2.4. Distribution of embryos

Often the limiting factor to throughput in embryo screens is the sheer number of embryos needed. Single mating or mass mating of 50–100 pairs of fish can yield approximately 5000 embryos weekly. The age, health and genotype of the adult fish can lead to variability in this number. Depending on the type of screen and pooling strategy, this may be enough embryos to screen up to a few thousand compounds a week (Table 1).

The embryos must be at approximately the same developmental stage at the time of pooling. Pooling of clutches is useful to minimize any unintended family effects of particular parents, unhealthy clutches, or incorrectly genotyped fish. Laying can be synchronized by setting up pair-wise or mass matings with male and female fish separated by a divider. The next morning the dividers are removed simultaneously, allowing the fish to mate. If dividers are not available, fish can be kept in complete darkness with a cover from the time they are set up until the following morning to prevent premature laying. Embryos should be collected and examined before pooling. Initial examination of clutches will allow any prematurely laid, dead or unfertilized embryos to be removed. Just before beginning chemical treatment of the embryos, they should be cleaned out again. All embryos are pooled into a tissue culture dish or 50-ml conical tube before distribution.

Zebrafish embryos are distributed manually as they are not easily pipetted by liquid handling robots. After the chemicals have been aliquoted and the embryos are at the appropriate stage of development, E3 is decanted from embryos and as much liquid is removed as possible with a transfer pipette. Pressing the transfer pipette tip to the bottom of the tube or dish allows most liquid to be removed without aspirating the embryos. Embryos are allocated to each well by scooping them with a small chemical weighing spatula and tapping the spatula gently against the top of the well so that the embryos fall in without the spatula touching the liquid. If embryos stick to the side of the well above the level of liquid, a pipette tip can be used to push them into the medium. Recently, an embryo-sorting robot has been developed that is capable of distributing zebrafish embryos into 96-well plates (Union Biometrica). It might soon be possible to use this robot for chemical screening.

2.5. Single genotype chemical screens

In the case of single genotype or wild-type screens, 3–5 embryos should be allocated per well in 96-well plates. Screens beginning treatment at 4–6 hpf will experience a small degree of death due to a low rate of abnormal development at this stage. When treating embryos closer to 24 hpf, there is little death after distribution such that only a single embryo is absolutely necessary. However, including a few embryos per well is still recommended to identify partial effects that may only affect some of the embryos. This is possible if the target of a compound is highly polymorphic or if the screening dose is close to the minimum effective dose.

After the embryos are distributed, the 96-well plates are wrapped in aluminum foil to protect light sensitive chemicals and placed in a secondary container to prevent accidental chemical spills. Embryos are incubated at 28.5 °C. A couple of hours later, dead embryos should be cleaned out from each well to prevent a toxic bystander effect in the small volume of the well. Removing dead embryos can most easily be done by bending a long glass Pasteur pipette at a 90° angle and using it under the microscope, rinsing out the pipet between wells. The plates are then returned to the incubator for the remainder of the chemical treatment.

2.6. Homozygous lethal mutant chemical suppressor screens

An alternative to the single genotype screen is a suppressor screen of a homozygous lethal mutant. This requires breeding heterozygous parents and treating embryos before mutants are identifiable. This creates the problem of multiple genotypes of embryos in each well. By Mendelian ratios, each clutch should contain approximately 25% homozygous mutants, 50% heterozygous mutants, and 25% wild-type embryos. As a consequence of this, an increased total number of embryos is required in each well in order to have sufficient homozygous mutant embryos tested with each chemical. This is due to the false-positive hit rate occurring when no homozygous mutants are aliquoted into a given well by chance. With 20 embryos per well and a Mendelian recessive inheritance, there is a 0.3% chance of a well having no mutants. If compounds are pooled in the pooling scheme described (Section 2.2), a hit requires detection in both pools of 20 embryos each. Therefore, the false-positive rate for identification of complete suppressors is 0.001%. This decreases the throughput by a factor of 4–5 and makes a pooling strategy or screening in duplicate necessary to reduce the high false-positive rate. Screening is otherwise the same as in a wild-type screen, but performed in 48-well plates due to the larger number of embryos per well.

2.7. High-throughput assays

The most amenable assays to high-throughput screening in zebrafish embryos are those that can be performed on living embryos in plates. This may be done with a visible phenotype, a vital dye or a reporter transgene. The embryos may need to be dechorionated to allow faster dye penetration or to increase the intensity of a reporter. Embryos can be dechorionated directly in the screening plates by adding half the screening volume of a 5 mg/ml pronase solution to each well. After 10 min, the plates are gently shaken until the embryos come out of their chorions. Next a transfer pipette fitted with a 10 μl tip is used to remove as much of the pronase/chemical mixture as possible leaving the embryos in the well. The embryos should then be rinsed three times in fresh E3 to remove residual pronase and

chorions. Care must be taken not to over expose the embryos to pronase or they may be damaged.

Other commonly used screening assays are antibody staining and RNA *in situ* hybridization. These assays will require the embryos to be dechorionated as above and then fixed. The embryos can be fixed in the plate by aspirating off the E3 and adding a screening volume of fixative to each well. Parafilm or an adhesive plate cover is used to prevent evaporation, and the embryos are then fixed at 4 °C overnight or as recommended by the staining protocol. To increase the throughput of staining procedures a 48- or 96-well staining grid made of acetone-resistant plastic with a wire mesh bottom can be used (Fig. 1B). Grids can be manufactured at relatively little cost. Embryos must be moved into the grid with a transfer pipette one well at a time. The grid is then placed into 11- by 8.5-cm reservoirs containing 20–30 ml of the appropriate solution. To change solutions, the grid can be lifted out of one reservoir and placed into another reservoir with the next solution. Refer to Appendix A for an example of an antibody staining screen.

2.8. Hit verification and secondary assays

After small molecule putative hits are discovered in a screen, the first step is to repeat the assay to confirm the results. This is essential in homozygous lethal mutant suppressor screens with a high false-positive rate, but is also important to eliminate any possible mistakes in aliquoting the library, such as a plate swap or flip. If pooling was used, deconvolution of the pooling matrix must be done to identify the well location of the actual hit. This can be problematic if there is only a positive reading in one of the pools in a pooling matrix. In the case of a homozygous lethal mutant suppressor screen this may indicates a false positive, but it may also be due to toxicity or a drug interaction in the other pool. When this occurs, we have found it valuable to retest the compound that was present in the positive pool and the toxic pool.

Once the well of origin is verified, the structure of the small molecule should be examined by liquid chromatography/mass spectrometry (LC/MS) and/or nuclear magnetic resonance (NMR). Small molecules may be unstable when stored in chemical libraries and over time an impure mixture of substances may be found in a given well, including degradation products not known to be present. Thus, the actual chemical structure of the active compound in the assay may not be the original compound. We have seen examples of unexpected structures in commercial libraries (data not shown).

After acquiring small molecule hits in zebrafish systems, it is often desirable to perform secondary assays to determine specificity of the compound. Depending on the assay, it may be important to determine if hits are active in mammalian systems. Assays that can only be tested *in vivo* will have to be tested in a mammalian model system such as the mouse. For cell autonomous assays mammalian cell lines can be tested. In the case where a chemical is not active in mammalian cells, testing in zebrafish cell lines may be helpful. If the chemical is active in zebrafish but not mammalian cell lines, that drug may be interacting with a zebrafish-specific target or may be metabolized or transported differently in zebrafish cells. Lack of activity of compounds in zebrafish cell lines suggests that they are organism specific, possibly requiring whole organism metabolism to become active. If a small molecule is of great interest, a structure activity relationship and target identification could be pursued.

Acknowledgments

We thank Christian Straub, David Hayes, James Follen, and Caroline Shamu for help with small molecule screening, Timothy Mitchison and Randy King for helpful advice, and Howard Stern for help developing assays and screening. We also thank Cassandra Belair and Bruce Barut for expert laboratory management. This work was supported by NIH Grants, 1R01 DK55381 (L.I.Z.), 1R01 HD044930 (L.I.Z.). L.I.Z. is an Investigator of the Howard Hughes Medical Institute.

Appendix A. An example of a mitotic index screen in zebrafish embryos

The following protocol (Fig. 2) was repeated weekly, giving a throughput of 1280 compounds per week for a combination homozygous lethal mutant suppressor screen and wild-type screen. Here we describe the methods used in the wild-type screen. We assayed changes in cell cycle profile by antibody staining for the mitotic marker, phosphorylated histone H3 (pH3). The screen used 48-well plates with a volume of 300 μl per well and matrix pooling as described below. If we had not been doing a homozygous lethal mutant suppressor screen at the same time, we could have increased throughput by using fewer embryos per well and 96-well plates.

(1) Fifty pairs of fish were setup in the evening separated by dividers.
(2) Chemicals were diluted into screening medium. First, 80 μl of screening medium was aliquoted into each well of four 384-well plates using a Tecan robot. The Chembridge Diverset E chemical library (courtesy of the Institute of Chemistry and Cell Biology, Harvard Medical School) was arrayed at 5 mg/ml in dimethyl sulfoxide (DMSO) in 384-well plates with the last 4 columns empty, thus containing 320 compounds per plate. We transferred 1 μl of each compound into each well containing screening medium by performing 10 transfers of a 384-well pin array for each of the four 384-well dilution plates. Given the plate geometry of this library, 8 by 10 matrix pools were created such that each compound was represented in a vertical pool of 8 chemicals and a horizontal pool of 10 chemicals. The diluted chemicals were transferred from the

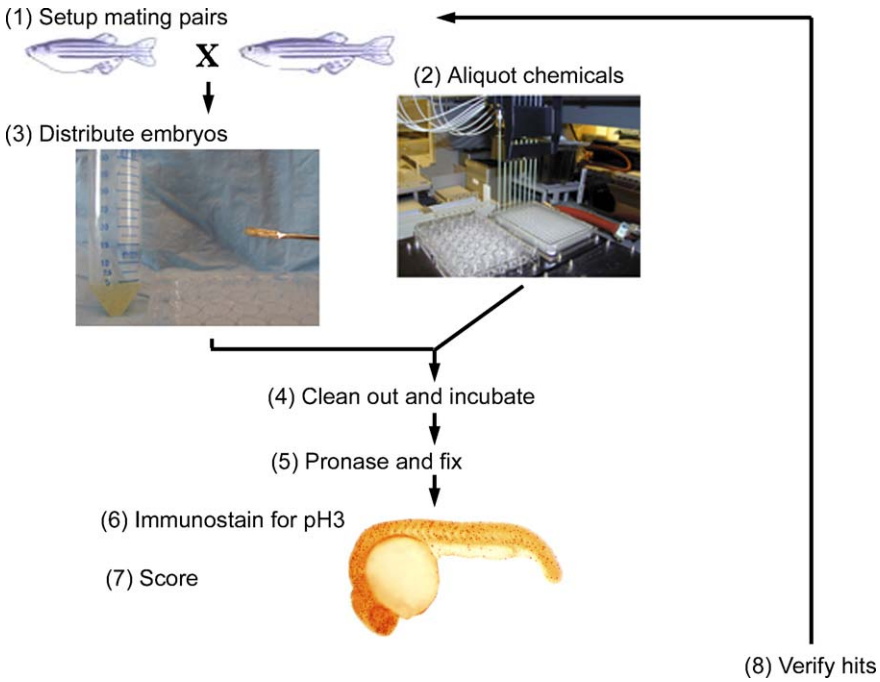

Fig. 2. Flow diagram of a zebrafish pH3 screen. A visualization of the eight steps for this example screen. Refer to Appendix A for details.

384-well dilution plates to flat bottom 48-well plates, using a Tecan liquid handling robot. For vertical pools, 30 μl was transferred from each of 8 wells with an additional 60 μl of screening medium, resulting in a total volume of 300 μl in each well of the treatment plates. For horizontal pools, 30 μl was transferred from each of 10 wells. Each compound is thus tested in two distinct pools. Individual active compounds are identified by deconvoluting the pooling matrix. For example, if a hit is identified in pools V1 and H1, the active compound would be A1 (Fig. 1A).

(3) Embryos were collected and exposed to the chemicals at 50% epiboly. First, each clutch was examined under a dissecting microscope; and all dead, delayed, or deformed embryos were discarded. Embryos were pooled in a single 100-mm tissue culture dish and distributed approximately 20 per well.

(4) The 48-well plates were incubated at 28.5 °C. One to two hours later, we cleaned out any dead embryos from each well. The plates were then incubated at 28.5 °C overnight.

(5) The next day, embryos were dechorionated by adding 150 μl of a 5 mg/ml pronase solution to each well. After 10 min, the plates were gently shaken until the embryos came out of their chorions. The liquid was then aspirated using a transfer pipette fitted with a 10 μl tip leaving the embryos in the well. The embryos were washed once in fresh embryo medium and this was also aspirated as above. Embryos were fixed by adding 300 μl of 4% paraformaldehyde (PFA) in PBS to each well. The plates were next sealed with Parafilm to prevent evaporation, and embryos were allowed to fix at 4 °C at least overnight. Fixation was never longer than one week as this was detrimental to pH3 staining.

(6) The embryos were moved with a transfer pipette to 48-well staining grids to perform pH3 immunostaining. All incubation steps were done on a shaker at low enough speed to not cause embryos to rise out of the grid. The embryos were washed once in PBS before being permeabilized in −20 °C acetone for 7 min. Acetone was dropped onto floating embryos to submerge them. Next a water wash and two PBST washes were performed before embryos were transferred to blocking medium and incubated for 30 minutes. Blocking medium consists of 70% PBST, 20% block solution [10% block reagent (Roche) in maleic acid buffer], 10% heat-treated lamb serum, and 1% DMSO. 1:750 polyclonal anti-pH3 antibody (Santa Cruz Biotech) was added to the blocking medium and incubated overnight at 4 °C. For overnight antibody incubations, the reservoir was sealed with Parafilm to prevent evaporation. In the morning, embryos were washed four times in PBST for five minutes each. Samples were transferred into 1:300 peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson Immunology) in blocking medium for two hours. Four 15-min PBST washes were performed before diaminobenzidine (Sigma) development. Two tablets of DAB were dissolved in 30 ml PBS plus 24 μl of 30% hydrogen peroxide added immediately before use. Samples were incubated in DAB for five minutes and washed once in PBST and transferred to 4% PFA. After staining was complete, embryos were moved with a transfer pipette back into 48-well plates that had been pre-coated with 100 μl of 1% agarose in PBS. The agarose forms a meniscus that keeps embryos

in the center of the well where they are easier to score. Embryos can be stored indefinitely in PFA in plates sealed to prevent evaporation.

(7) Embryos were scored under a dissecting microscope for change in baseline levels of pH3 staining. Hits were noted, and individual chemicals were identified by deconvoluting the matrix pooling system.

(8) Putative hits were verified by individually re-testing chemicals on a large number of embryos. Confirmed hits were examined by LC/MS to verify the chemical structure. We were interested in determining the proportion of chemicals that would be active in cell lines. As a secondary assay we treated zebrafish AB9 [22] (ATCC number CRL-2298) and NIH/3T3 (ATCC number: CRL-1658) cells with the confirmed hits and assayed for pH3 levels.

References

[1] T.J. Mitchison, Chem. Biol. 1 (1994) 3–6.
[2] S.L. Schrieber, Bioorg. Med. Chem. 6 (1998) 1127–1152.
[3] R.K. King, Chem. Biol. 6 (1999) R327–R333.
[4] T.U. Mayer, T.M. Kapoor, S.J. Haggarty, R.W. King, S.L. Schreiber, T.J. Mitchison, Science 286 (1999) 971–974.
[5] R.T. Peterson, B.A. Link, J.E. Dowling, S.L. Schreiber, Proc. Natl. Acad. Sci. USA 97 (2000) 12965–12969.
[6] J. Chan, P.E. Bayliss, J.M. Wood, T.M. Roberts, Cancer Cell 1 (2002) 257–267.
[7] M. Shinya, S. Koshida, A. Sawada, A. Kuroiwa, H. Takeda, Development 128 (2001) 4153–4164.
[8] R.T. Peterson, S.Y. Shaw, T.A. Peterson, D.J. Milan, T.P. Zhong, S.L. Schreiber, C.A. MacRae, M.C. Fishman, Nat. Biotechnol. 22 (2004) 595–599.
[9] K. Dooley, L.I. Zon, Curr. Opin. Genet. Dev. 10 (2000) 252–256.
[10] C. Parng, W.L. Seng, C. Semino, P. McGrath, Assay Drug Dev. Technol. 1 (2002) 41–48.
[11] D.J. Milan, T.A. Peterson, J.N. Ruskin, R.T. Peterson, C.A. MacRae, Circulation 107 (2003) 1355–1358.
[12] E.A. Andreasen, J.M. Spitsbergen, R.L. Tanguay, J.J. Stegeman, W. Heideman, R.E. Peterson, Toxicol. Sci. 68 (2002) 403–419.
[13] H.P. Tseng, T.H. Hseu, D.R. Buhler, W.D. Wang, C.H. Hu, Toxicol. Appl. Pharmacol. 205 (2005) 247–258.
[14] J.J. Bogaards, M. Bertrand, P. Jackson, M.J. Oudshoorn, R.J. Weaver, P.J. van Bladeren, B. Walther, Xenobiotica 30 (2000) 1131–1152.
[15] P. Jagadeeswaran, J.P. Sheehan, Blood Cells Mol. Dis. 25 (1999) 239–249.
[16] D.M. Langenau, A.A. Ferrando, D. Traver, J.L. Kutok, J.P. Hezel, J.P. Kanki, L.I. Zon, A.T. Look, N.S. Trede, Proc. Natl. Acad. Sci. USA 101 (2004) 7369–7374.
[17] G.S. Bailey, D.E. Williams, J.D. Hendricks, Environ. Health Perspect 104 (Suppl. 1) (1996) 5–21.
[18] T.E. Bunton, M.J. Wolfe, Toxicol. Pathol. 24 (1996) 323–330.
[19] J.M. Spitsbergen, H.W. Tsai, A. Reddy, T. Miller, D. Arbogast, J.D. Hendricks, G.S. Bailey, Toxicol. Pathol. 28 (2000) 716–725.
[20] J.M. Spitsbergen, H.W. Tsai, A. Reddy, T. Miller, D. Arbogast, J.D. Hendricks, G.S. Bailey, Toxicol. Pathol. 28 (2000) 705–715.
[21] M. Westerfield, The Zebrafish Book: A Guide for The Laboratory use of Zebrafish (*Danio rerio*), University of Oregon Press, Eugene, 1989.
[22] B.H. Paw, L.I. Zon, Methods Cell Biol. 59 (1999) 39–43.
[23] Stern et al., Nat. Chem. Biol. 1 (2005) 366–370.