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Advances in zebrafish chemical screening technologies

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Abstract

Due to several inherent advantages, zebrafish are being utilized in increasingly sophisticated screens to assess the physiological effects of chemical compounds directly in living vertebrate organisms. Diverse screening platforms showcase these advantages. Morphological assays encompassing basic qualitative observations to automated imaging, manipulation, and data-processing systems provide whole organism to subcellular levels of detail. Behavioral screens extend chemical screening to the level of complex systems. In addition, zebrafish-based disease models provide a means of identifying new potential therapeutic strategies. Automated systems for handling/sorting, high-resolution imaging and quantitative data collection have significantly increased throughput in recent years. These advances will make it easier to capture multiple streams of information from a given sample and facilitate integration of zebrafish at the earliest stages of the drug-discovery process, providing potential solutions to current drug-development bottlenecks. Here we outline advances that have been made within the growing field of zebrafish chemical screening.

Within the last decade there has been a steady increase in the use of zebrafish (*Danio rerio*) larvae to assess the effects of chemical compounds. This model system possesses several features that make it ideal for *in vivo* compound testing, for example: small size (embryos and larvae can be dispensed in microtiter plates), high fecundity (to obtain large sample numbers), transparency (enabling noninvasive imaging) and permeability to small molecules (allowing for drug administration by immersion). The innovation of maintaining embryos and larvae arrayed in a 96-well plate has brought zebrafish to the forefront of chemical screening technology. In a relatively short time, zebrafish chemical screening has evolved from visual observation of arrayed embryos [1] to an advanced system in which individual larvae can be moved in and out of multiwell plates for manipulation and high-resolution imaging [2] and methods for quantifying fluorescent reporters/dyes over time at high-throughput volumes [3]. This review will outline the many advances that have arisen in screening chemical libraries in zebrafish larvae, with an emphasis on the assays being utilized to discern discrete pharmacological effects. By tracking how these assays have developed, we hope to give an idea of the future direction of zebrafish chemical screening, and what aspects and/or issues will need to be addressed. From this we develop two major themes; the need to collect/convert assay data into quantifiable results (i.e., to deal with large numbers of data points) for high-throughput screening (HTS); and a progression

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toward dynamic/temporal assays that assess multiple time-points, which may elucidate more subtle phenotypes. Recent tests have shown that zebrafish perform very well when existing human drugs are tested for conservation of phenotypic effects (e.g., rest/wake cycle [4], seizure activity [5], psychoactives [6] and heart rate [7]). In terms of drug development, zebrafish have been used to provide toxicity testing and/or biological validation for a select group of '**hit**' compounds. However, attaining true HTS volumes will allow zebrafish to be used for large-scale unbiased drug-discovery screens, placing whole organism screening at the beginning rather than the end of the drug-development process. This review will follow the principal stages of a zebrafish chemical screen, and thereby discuss the advances and issues concerning pre-assay development, choice of assay and data analysis.

Zebrafish development & pre-assay issues

While chemical screening techniques have been performed and/or developed in adult zebrafish [8–10], the vast majority of screens utilize embryos (i.e., from 0 to 2 days post-fertilization [dpf]) or young larvae (2–7 dpf). Eggs are fertilized externally and encased within a protective chorion, from which they naturally hatch at 2–2.5 dpf; prior to this, chorions can be removed by treatment with a protease to facilitate drug exposure. From 2–4 dpf, hatched larvae naturally lie in a lateral position that is highly amenable to imaging. At 5 dpf swim bladders become inflated and larvae begin actively swimming. Once they become highly motile larvae typically need to be immobilized for accurate visualization, which can be done simply by adding anesthetic (e.g., tricaine) to the medium. However, larvae at ≥5 dpf often float upon anesthetization, and position themselves in a ventral-dorsal orientation; screens are thus typically performed prior to this developmental stage. Later, at approximately 12 dpf larvae attain better buoyancy control and tend to sink upon being anesthetized. Use of pigmentation mutants (*roy orbison*, *albino*, *nacre*) can be used to maintain transparency and thus facilitate imaging of internal tissues at larval and even adult stages [11,12].

Of critical importance for conducting chemical screens is the large amount of eggs that need to be produced within 0.5–3-h time windows, depending on the assay. While pair-wise crosses of large numbers of adult mating pairs is sufficient for most applications, HTS requires a greater degree of embryo production **(Table 1)** that can be achieved through more 'industrial' approaches. Zebrafish husbandry companies have responded to this need by producing large volume breeding units capable of producing tens of thousands of eggs per day (e.g., Mass Embryo Production, Aquatic Habitats, iSpawn and Techniplast), and a similar system has been produced and tested independently [13]. Following embryo production, the next issue is arraying thousands of embryos into multiwell plates. While this can be done manually, automated systems that perform this step have been developed for large scale projects. The Complex Object Parametric Analyzer and Sorter (COPAS, Union Biosciences) can select embryos on the basis of length, optical density and/or fluorescence expression, and sort a desired number of individuals into individual wells of a microtiter plate; a software addition enables regionally-defined readouts of fluorescence expression per each individual embryo/larvae [14]. Furthermore, this system can be used to exclude dead embryos prior to sorting, which are markedly more opaque than living counterparts and can thereby be identified by optical density. However, the COPAS requires a large number of eggs/embryos/larvae to operate effectively. Thus, this system is not practical for chemical screens which have limited sample numbers (e.g., eight fish) across many different conditions (thousands of drugs). Other automated systems have been developed for placing single embryos into microtiter wells [15], one of which also automates chorion removal [16], however these systems have no apparent ability to sort based on fluorescence or to provide morphological information.

After arraying, embryos/larvae need to be exposed to chemicals and assayed after a defined treatment regimen, or upon attaining a desired developmental stage. While zebrafish larvae are generally permeable to small molecules, solubilizing agents can be added to screening media to ensure drug penetration and solubility; for example, dimethyl sulfoxide. Although a concentration of dimethyl sulfoxide as high as 1% has been routinely used in the field [17], several reports have shown that this can produce morphological defects, behavioral changes, and stress responses [18,19]. Accordingly, solubilizing agents should be employed with caution. Any number of robotics systems for liquid handling can suffice for arraying chemical compounds into multiwell plates either prior to or after arraying embryos. By combining robotics with the COPAS, multiple steps of the chemical screening process can be automated, including embryo and chemical compound dispersion, data collection and analysis [20]. As thousands of samples need to be assayed, some death may occur at any step during this process. To account for these events, automated quality control techniques to identify dead and/or malformed larvae within microwell plates have been developed; these exploit the visual differences between living, dead and/or malformed embryos [21,22].

Zebrafish chemical screening assays

Four major classes of whole organism chemical screening assays are commonly used: morphological screens, behavioral screens, fixed time-point/labeling assays and fluorescence assays. These classifications have been defined solely for the purposes of this review, and are not meant to be definitive. These assays all involve intact embryos; for a review of transcript/proteome-based analytical platforms see Love *et al.* [23]. Furthermore, excellent reviews of the design [24], protocol details [17,25] and pharmacological 'hits' [26] that have arisen from zebrafish chemical screens have been published, therefore these topics will not be emphasized here.

Selection of assay type is usually defined by the question being asked. If a 'yes/no' result is anticipated, visual assessment without data quantification may suffice. However, for screens involving high sample numbers or to avoid observer bias, automated quantification of data and/or automated data analysis is better suited to the rigors of HTS. In addition, the suitability of the assay for large-scale chemical screening should be evaluated using statistical methods specifically designed for this task. As an example, the **Z-factor and Z'-factor** are equations that compare averages and standard deviations between negative and positive controls [27]; the resultant value is used to predict how well the screen would perform if scaled up to HTS levels and can be used as a metric to optimize screen parameters if necessary. Typically, whole organism assays are inherently variable, thus it is critical to define an appropriate sample number (or 'n') to minimize false positive and false negative rates. One effective strategy is to perform a pilot test with a large number of samples using control compounds known to produce desired effects. Resampling statistics, for example comparing random subsets between each condition (or 'bootstrapping'), can then be used to define the least number of samples required to obtain statistically significant differences between treated samples and vehicle controls. Optimally, such strategies reduce the amount of time and energy wasted on false discovery, however, if large sample sizes are required then practical matters (e.g., how much compound is available) also need to be taken into account.

Morphological screens: chemical genetics & toxicology

Visual observation of arrayed fish treated with chemicals is the simplest possible assay, and can be performed easily by researchers at a dissecting microscope. This approach was utilized in initial screens for compounds that affect the development of wild-type embryos [1,28,29]. Subsequent morphological screens utilizing wild-type zebrafish assessed specific processes such as organogenesis [30], fin regeneration [31] or BMP signaling (using a

'dorsalization' phenotype) [32]. The use of living zebrafish essentially made these assays *de facto* **toxicity/teratogenicity** screens as well, which have gained considerable interest within the biotech community, and will be discussed more in depth below. However, the zebrafish community has also developed a number of mutant and transgenic lines with disease-relevant phenotypes that can be utilized for chemical screening purposes. Chemical suppression of such mutant phenotypes (a '**chemical genetics**' approach, reviewed in Wheeler and Brändli [33]) is a powerful method that can give insight into the effect of a compound on specific disease-relevant pathways. An early, commonly cited example of this approach is an elegant screen in which a zebrafish mutant (*gridlock*) that lacks peripheral blood flow was utilized to screen a set of compounds for those that restore blood flow [34]. Similarly, the heart rate of zebrafish can be visually assessed by 1.5 dpf, and has been used to screen for drugs that affect heart rate [35]. This assay was later extended to a voltagegated potassium channel mutant that exhibits a long QT syndrome phenotype (*breakdance*); a 2:1 atrioventricular block that can easily be assessed visually [7]. Other examples of chemical genetics screens have used 'static image' phenotypes (as compared with the motion of the heart or blood flow), such as suppression of a dorsally curved body morphology in a screen for inhibitors of histone deacetylases [36], and a screen that utilized dystrophin mutant models (*sapje* and *sapje-like*) of Duchenne muscular dystrophy along with a simple birefringence assay to isolate compounds that restore normal musculature [37]. All of these examples involve human observation (i.e., phenotypes that can, or must, be scored by a researcher at a microscope, which limits throughput). Furthermore, when data are largely qualitative in nature, screens become almost entirely dependent on researchers making accurate judgment calls about the nature of the output, a highly subjective procedure in some cases. To improve throughput and remove subjectivity, advances in **high-content imaging** (HCI) techniques have provided ways to automate visual data collection and image analysis. Computational methods can be used to yield numerical outputs from morphological data, which enables cross-screen comparisons much more effectively than qualitative measurements.

HCI entails the rapid, automated capture of a collection of images, such as those from each well of a multiwell plate. These images can then be used for quantification of parameters, such as the effects from compound exposure in a chemical screen. Peravali *et al.* described an example of this powerful technique: in their version HCI is utilized to identify and capture high-resolution regions-of-interest (e.g., the head of a larvae) following the collection of low resolution images of the overall body plan of arrayed larvae [38]. From images such as these, morphological landmarks can be used to identify and align the overall structure, which facilitates automated morphometric measurements. Untreated fish are used to define baseline morphometric parameters and thereby reduce fish features to numerical data sets. Comparison to treated fish using unbiased statistical methods rather than qualitative observations allows subtle phenotypes that may not be noticed in a visual screen to be elucidated. Likewise, chemical genetics screens that involve the suppression of a definable morphometric phenotype, such as the dorsal curvature seen in the mutants used in Cao *et al.,* can be easily automated [36]. However, it should be noted that in terms of pattern recognition the human visual system is thought to be superior to computational methods, thus, despite lower throughput, it may be beneficial to retain human observation as an aspect of morphological screens.

An area where HCI can be extremely useful is toxicology screens, which largely rely on morphological observation [39,40]. In these screens, wild-type zebrafish are exposed to varying concentrations of chemical compounds with the intent of revealing any undesirable effects [41]. Several biotech companies (e.g., Phylonix, Evotec and Biobide) specialize in zebrafish toxicology screens as a high-throughput service to prescreen chemical libraries for toxic effects, thereby streamlining the drug-discovery process by eliminating compounds

prior to more exhaustive and costly experiments in mammalian models. As with other morphological screens, toxicology screens have largely been performed manually; however, images collected from HCI can be used to assess several toxicology parameters in a quantitative fashion and with greatly increased throughput. Seizing on this, GE Healthcare has developed the IN Cell Analyzer imaging system [101] and accompanying software to automate morphometric measurements, which can be utilized to determine toxicity-related parameters. Intriguingly, other measurements that can be derived from localized pixel intensity (e.g., brain necrosis [41,42] or hepatotoxicity [43]) or other easily measured parameters (e.g., myotome/somite morphometrics [44]) could be added to this software package to create a full suite of HCI-compatible toxicological parameters. As discussed later, such analyses could be done in tandem with other data collection techniques outlined elsewhere in this review to allow toxicology screening to be folded into a more comprehensive drug-discovery process.

Behavioral assays

Recent methods have been developed to move chemical screens beyond morphological criteria and into the realm of behavior. Zebrafish swimming kinetics is a commonly used parameter in behavioral studies, for instance in assays to quantify the effects of single compounds [45]. This approach has become very popular within the last few years, largely due to the commercial availability of devices that record and quantify swimming behavior (Ethovision and ViewPoint). Low-throughput screens (i.e., using six- or 24-well plates), employing swimming behavior as an assay of seizure liability [46] and avoidance behavior [47], have been developed. Methods to assess and quantify larval swimming behaviors (e.g., mean velocity, active velocity and percent time moving) in a 96-well format have also been described, which have greatly increased the throughput of this type of assay [48]. Farrell *et al.* determined that these parameters become consistent starting at 6 dpf, providing an important time frame for future assays. Rihel *et al.* developed an automated rest/wake behavioral assay that quantified several parameters (rest bouts, rest latency and waking activity) to create a ‘behavioral profile’ for each assayed drug [4]. These profiles were utilized to cluster drugs according to common effects, a process that can be used to elucidate potential side effects, differences in effectiveness, and combinatorial properties of large classes of compounds; importantly, many drug-induced phenotypes were conserved from zebrafish to mammals. Similarly, Kokel *et al.* analyzed the reaction of grouped embryos (8–10 per well) to high intensity light (the photomotor response) to quantify 14 features, which were subsequently compiled to create a behavioral ‘barcode’ [6]. Important factors affecting the throughput of behavioral screens can be gleaned from these assays. First, behavioral assays are typically variable and thereby require replicates for each assayed compound; Rihel *et al.* analyzed ten larvae per drug, while Farrell *et al.* determined that replicates of four to eight larvae were required to achieve suitable Z-factor scores for their assay. Second, the amount of time required to collect data can set practical limitations: the amount of time to assay a single 96-well plate can vary from approximately 1 h (Kokel *et al.*), to 4 h (Farrell *et al.*), or even multiple days (Rihel *et al.*). This factor limits these assays to mid- and low-throughput, respectively. Thus, in the absence of tens to hundreds of data collection apparatus, such approaches are not yet compatible with HTS. Nevertheless, these assays have begun to provide inroads into the power of behavioral screening as a means of providing ever more sophisticated chemical compound characterizations in zebrafish, an area that has clear significance to drug development and which extends well beyond assessments for morphological malformations. While the translational relevance of these HTS assays is yet to be determined, a formidable toolset of neurological techniques has been developed for the zebrafish system and can be employed in secondary assays to assess neural activity at systems, cellular, and molecular levels [49].

Fixed time-point/labeling assays

While behavioral assays yield information on a given system at large, they do not give any information on a cellular or tissue level. Methods for labeling organs/tissues/cells are commonplace in the zebrafish field, and have been used to expand the repertoire and power of chemical screening assays. Procedures involving either fixation of samples, or labeling compounds that compromise biological function, represent 'fixed time-point' assays. The simplicity of these screens makes them very popular, although they often rely on qualitative assessments. The amount of information gained from fixed time-point assays is limited when compared with temporally dynamic screening methods, yet they do have clear advantages. Labeling methods can yield sample sets that can be repeatedly analyzed, and possibly even archived. The simplest of these methods is histochemical staining. For instance, in Shafizadeh *et al.* small molecules were screened for those that induce hemolytic anemia using o-dianisidane staining of erythrocytes [50]. Others have used large-scale immunolabeling protocols to label mitotic cells [51,52], neurons [53] and endothelial cells [54], the latter included a whole-mount ELISA assay to quantify angiogenesis. *In situ* hybridization (ISH) of endogenous mRNA has also been utilized in chemical screens. While time-consuming, ISH has the advantage of being able to label any genetically definable cell type, and reagent availability/quality is not limiting (as compared with antibody labeling). ISH of blood cell types has been utilized to screen chemical libraries for suppressors of acute myelogenous leukemia [55] and compounds that activate hematopoietic stem cell proliferation [56], the latter of which elucidated a compound that was moved into a Phase I clinical trial [102]. While such assays have typically been qualitative in nature, efforts to quantify images of fixed and labeled larvae have been developed; labeled cells include motor and sensory neurons [44], apoptotic cells [53] and mitotic cells [57]. Intriguingly, the IN cell Analyzer has recently been employed to automate quantification of ISH results [58], an innovation that could greatly increase the throughput of this approach. Similar to morphological screens, advances in image quantification greatly increase the amount of data that can be generated from each well. Furthermore automation of immunolabeling and ISH screens through the use of mesh-bottomed microwell plates greatly increases throughput [17,59], ensuring that these approaches will continue to be employed in the future.

Incubation of treated larvae in vital dyes is often a simple protocol that requires little processing compared with fixed embryo assays. In addition, a quantifiable signal is often quickly available, an advantage that increases throughput. Chemical screens that utilize hair-cell specific vital dyes [60–62], and the commonly used acridine orange stain of apoptotic cells [41] have been performed; as these dyes are fluorescent, they can be quantified by analysis of pixel intensity in captured images [41,60]. Other vital dye assays do not label specific cell types but have been used in screens as a reporter of biological processes, such as fat content [63,64]. Similarly an assay involving chemiluminescent substrate conversion to reveal cytochrome P450 (a major class of drug-metabolizing enzymes) activity has been developed for use in toxicology studies [103]. However, dyes/substrates often interfere with ongoing cellular processes, thus disrupting the capacity to perform analyses on the same individuals over time. In contrast, transgenic fish expressing fluorescent reporter proteins enable many of the same methods as vital dyes, yet without a need for fixation or exogenous reagents. For example, the cytochrome P450 assay has recently been replicated using a fluorescent reporter line [65], and a transgenic line expressing GFP in hair cells was used to used to screen for compounds that affect regeneration of this cell type [66]. These lines facilitate quantification of fluorescence-based signals over successive days, thereby enabling the assessment of phenotypes that develop over extended periods. Similarly, methods that facilitate temporally open-ended platforms can be used to monitor the progression or regression of symptomatic readouts in disease models. Due to the inherent strengths of this

approach, we will spend the remainder of the assay section of this review on a description of screens that utilize transgenic expression of fluorescent reporters.

Fluorescent reporters: morphology screens II & reporter-based quantifications

Expression of fluorescent proteins in specific cell types is now commonplace in the zebrafish community, with thousands of transgenic lines now available (Zebrafish Information Network [104]). Many of the advantages of the zebrafish model system are brought to the forefront with this approach: ease of transgenesis [67] and larval transparency result in an ideal organism to assess the effects of small molecules on labeled cell types. For example, our laboratory has generated transgenic lines expressing PhiYFP (Evrogen) in pancreatic beta cells [3], which are easily distinguished in larvae arrayed in a 96-well plate **(Figure 1)**. The output generated from fluorescence-based screens is highly quantifiable and in many ways superior to the assays detailed above; for instance, a greater degree of detail regarding subtle cellular phenotypes can often be achieved. Furthermore, through expression of multiple, different-colored fluorescent proteins within the same transgenic fish, several cell types or organs can be assayed simultaneously. The only drawback of this method is the requirement for ‘nonstandard’ (and sometimes expensive) laboratory equipment, at the very least a fluorescence dissecting microscope capable of distinguishing various fluorescent proteins. This singular drawback is outweighed by the many benefits afforded by fluorescent transgenic lines, and the variety of screens they enable.

There are many examples of chemical screens that employ transgenic expression of fluorescent reporters in zebrafish. Expression of GFP in the heart was used to screen for drugs that effect heart rate, and enabled simultaneous quantification of multiple larvae in individual wells [68]. Rovira *et al.* used transgenic fish expressing two fluorescent reporters to concurrently assay for drugs that affect both pancreatic cell proliferation and Notch signaling [69]. This assay utilized ‘Side View’ multiwell dishes (Physical Sciences Inc.) that enable lateral observation of freely behaving larvae at 5 dpf and above. Fluorescent zebrafish have also been utilized in multiple screens for potential cancer therapeutics, including screens for antileukemia drugs [70] and anti-angiogenics [71]; each of these studies employed manual screening techniques that were followed up with verification in murine models. A GFP-based reporter of FGF signaling has also been developed and used in a manual screen [72,73] for compounds that activate this pathway (through inhibition of a negative feedback regulator); this screen was subsequently automated using HCI techniques [74].

Similar to its use in morphology screens, HCI of fluorescent larvae has enabled quantitative data collection and increases in throughput. Tran *et al.* describe a screen for anti-angiogenic compounds using transgenic larvae expressing GFP in blood vessels; in this assay the image acquisition step was automated, but regional isolation in each image is manually performed [75]. Fully automated assays using similar transgenic lines have recently been reported [20,21]; in these assays the vasculature and other larval landmarks are used to orient each image for subsequent quantification of blood vessel length. Similar methods have been developed to map transgenic expression of fluorescent proteins in multiple stereotypical regions within zebrafish larvae [76], an important advance for quantification of image-based chemical screens, including toxicology screens [77]. Along these same lines, d'Alençon *et al.* have developed an assay for screening compounds that modulate inflammation [78]. In this study, HCI was used to reveal the localization of GFP-labeled leukocytes in response to chemically-induced hair cell ablation; both image acquisition and data quantification were fully automated. Finally, a HCI-mediated platform that analyzes disseminated fluorescent human cancer cells in zebrafish larvae has been recently developed [79]. Interestingly, incorporation of an automated microinjection system (as in Carvalho *et al.* [14]) could

increase the throughput of the outlined xenotransplantation procedure and make this an even more powerful system to elucidate novel anticancer drugs.

While image-based assays yield a great deal of morphological detail and quantifiable data, oftentimes initial assays may only require a quantification of the overall fluorescent signal within treated larvae. Accordingly, Walker *et al.*, recently utilized standard HTS instrumentation (a microtiter plate reader) to quantify changes in fluorescent reporter activity over time using several disease-relevant assays: cell loss and regeneration (in acute Type I diabetes and retinitis pigmentosa disease models); accumulation of reactive oxygen species; and changes in activity of the Notch signaling pathway [3]. Furthermore, quantification of different-colored fluorescent reporters can be done concurrently, enabling the simultaneous assessment of multiple cell types. The main advantage of this screening platform, termed ARQiv (for automated reporter quantification *in vivo*), is the marked increase in throughput. Adaptation of pre-existing HTS instrumentation essentially allows industry standards for high-throughput volumes (e.g., a minimum of 50,000 units per day) to be achieved. This becomes very important for temporally dynamic assays (as exemplified by behavioral assays, above). By maintaining throughput processing at rates of approximately 3 min per 96-well plate (per reader), the inherent loss in throughput incurred by longitudinal evaluations can be offset. Importantly, Walker *et al.*, were able to establish methods that succeeded in achieving HTS-ready Z-factor values (>0.5). This platform is also extremely versatile, thus the number and types of assays that could be applied approximately matches *in vitro* HTS counterparts. Finally, because it utilizes readily available and relatively inexpensive equipment, ARQiv is easy to deploy in academic research environments. Purely quantitative output, however, may not provide subtle details at which HCI excels.

An elegant fluidics/HCI-based screening platform that also facilitates longitudinal assays provides an even more versatile platform for assessing subtle cellular phenotypes [2]. This system, termed VAST (for vertebrate automated screening technology), extends methodologies to zebrafish that this group originally developed for large-scale chemical/genetic screens in worms (*Caenorhabditis elegans*) [80]. In the VAST system larvae can be maintained in a 96-well dish, yet are moved in and out of wells through fluidic valves. Outside of the well, larvae are imaged using a high-speed confocal microscope; as this occurs within a capillary tube the larvae can be oriented to any position. This system also enables several optical manipulations of larvae, such as localized activation of fluorescent reporters, ion channels and caged compounds, and cell ablation by laser micro surgery. Current throughput estimations (~10 s/subject) for a recent multithread parallel processing update to this system bring VAST close to HTS volume capacities [81]. Although it is unclear how VAST performs with regard to HTS-relevant statistical analyses applied in other HCI studies (e.g., Z-factor, as in Vogt *et al.* [21]), this platform does solve several issues that hamper chemical screening in zebrafish (e.g., automated orientation). VAST thus provides the most sophisticated HCI system currently available for whole organism chemical screening. If such a system were to become commercially available it would undoubtedly facilitate more widespread adaptation of zebrafish to drug development.

Issues remaining to be resolved to advance zebrafish chemical screening

A principle factor affecting chemical screening in zebrafish is the maintenance and viability of microtiter plate-arrayed larvae. This becomes particularly important for temporal assays that require data collection at multiple time-points, especially if larvae need to be anesthetized each time. In addition, larvae after 5 dpf generally need to be fed in order to maintain overall health; lack of feeding would result in non-drug-related phenotypes to arise. These issues create a need for media replacement, especially given the small volume within each well. The VAST system solves many of these issues by automating the process of moving larvae in and out of microtiter wells. However, simpler materials-based solutions

can also be pursued, such as the use of multiwell insert systems with mesh bottoms (similar to those used in automated ISH screens [17]) that enable feeding and media exchange by moving inserts between microtiter plates loaded with media containing food, anesthetic and so on. Currently available inserts are made with autofluorescent polystyrene materials, limiting their compatibility with fluorescent data collection. **Microfluidics** techniques are being pursued to facilitate zebrafish chemical screens, including a credit-card sized multiwell dish with microfluidic capabilities for media replacement [82]. However, some developmental defects were observed with the current iteration of this system due to small well size, an issue that could be resolved by adapting the system to industry standard 96-well and 384-well sized formats.

Following (or during) the screening assay, the captured and quantified data need to be analyzed to determine 'hits' that will be further validated and/or re-screened with secondary assays (both of which can be rapidly done in the zebrafish model [31,83]). If the readout is a binary 'yes/no' answer, defining what constitutes a 'hit' may be a relatively simple process. However, as throughput increases and larger quantities of data are produced per screen (more than can be assessed manually), automated data analysis systems will need to be established whereby a defined phenotypic threshold is set that flags compounds that have desired effects. These thresholds can be empirically determined by the researcher, or determined by statistical analysis from pilot screens. Statistical metrics for hit selection have been defined (and expertly described with examples by Zhang [84]) and include: the z-score (for assays of single plates), the t-statistic (to compare data across plate replicates) and strictly standardized mean difference (SSMD), applicable to assays with or without replicates. The z-score and SSMD parameters largely use the mean, median and standard deviations of data sets to define thresholds for hit definition. 'Robust' versions (z*-score, SSMD*) of these parameters replace standard deviations with the median of absolute deviations; as the median of absolute deviations is less affected by 'outlier' data points, which in practice often include positive hits, these equations better account for distortions in the overall mean caused by variability, resulting in a more accurate threshold for hit definition. As the quantified readouts from zebrafish chemical screens (and other *in vivo* screens) often includes many such outliers and can be quite variable – especially when compared with *in vitro* biochemical screens – these parameters are quite useful to distinguish true chemical hits. [27]

Future perspective

As throughput increases, one of the challenges will be to extract as much information as possible out of each well/condition. This will be particularly true for screens in which samples are kept viable for long periods and evaluated over successive time-points. This can be achieved by increasing data collection within a particular assay (e.g., using compound transgenic lines that express complementary fluores-cent reporters to assess effects on different cell types [69]). A general push toward automation in zebrafish chemical screening suggests that different assay types could be used within the same screen, provided that data collection techniques do not interfere with one another. For example, a behavioral assay could be followed with HCI to correlate behavioral performance to morphology at gross (e.g., toxicological) or cellular levels (using labeling techniques). Similarly, using complementary label/reporter systems, several fixed-time-point assays could easily be combined. As the data generated from automated screens become more quantitative, a database that compiles these results would be highly valuable for comparing results across laboratories or assays, especially given that many screens use a common set of publicly available chemical libraries (see **Table 1** and reference [33]). Such a database has been recently proposed to analyze the massive amount of data being generated from zebrafish behavioral screens [85]. Similar databases would also enable informatics techniques to

successfully predict targets based on chemical similarity [86]. Finally, methods facilitating true HTS capacities will allow large-scale unbiased chemical screening approaches in zebrafish. In turn, this will allow whole organism screening to be positioned at the beginning rather than the end of the drug-discovery process, providing potential solutions to current bottlenecks in the development pipeline that have lead to huge cost increases in bringing drugs to market over the past 15–20 years.

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Key Terms

Hit: Chemical identified through an initial screening process that produces a desired physiological effect.

Z-factor and Z'-factor:

Statistical measures of assay 'quality' used to determine if an assay will perform well when scaled-up to high-throughput screening volumes. The Z-factor accounts for variation associated with sample and control data; for the Z'-factor, variation between positive and negative controls is compared.

Toxicology/teratogenicity:

Whole organism screening process for defining lethal/deleterious effects of a given substance.

Chemical genetics:

Phenotype-driven screening process to identify chemicals that produce a desired visualizable effect in living cells or animals.

Key Term

High-content imaging:

Rapid, automated, high-resolution imaging platforms providing cellular, and even subcellular, detail of screened samples.

Key Term

Microfluidics: Miniaturized assay platforms that attain precise control over fluid dynamics using geometrically defined silicone ‘chip’-based microenvironments.

Executive summary

Zebrafish development & pre-assay issues

- External fertilization enables facile chemical exposure and evaluation of compound effects.
- Zebrafish embryos/larvae can be maintained in microtiter multiwell plates for high-throughput screening (HTS).
- Large volumes of eggs necessary for HTS can be generated using commercially available mass production husbandry units.
- Sorting devices automate dispensing of embryos/larvae into microtiter multiwell plates for HTS.

Zebrafish chemical screening assays

- Morphological screens: chemical genetics and toxicology:
  - Gross morphological assessment is a straightforward means of assessing compound effects;
  - A variety of morphological assays are available for more specific analyses; for example, organogenesis, regeneration and heart rate;
  - Zebrafish provide *de facto* toxicity/teratogenicity screening in a vertebrate species;
  - Key advance: high-content imaging (HCI) enables automated high-resolution image collection.
- Behavioral assays:
  - Reproducible behavioral assays, such as motor activity, provide information about compound effects that cannot be provided by *in vitro* or cell culture assay systems;
  - Throughput limitations are inherent due to temporal analysis requirements, currently this can only be surmounted with multiple data collection units operating in parallel;
  - Key advance: videomicroscopy and tracking of swimming behavior performed in a 96-well format, greatly increasing throughput.
- Fixed time-point/labeling assays:
  - Fixation allows researcher more flexibility in terms of scheduling sample processing, sample analysis and repeated measurements;
  - Allows visualization of weak signals and/or markers that lack other detection methods. Limited to examining changes over time across populations versus in individuals;
  - Key advance: labeling protocols (e.g., *in situ* hybridization) can be automated in 96-well format.
- Fluorescent reporters: morphology screens II & reporter-based quantifications:
  - Transgenic zebrafish expressing fluorescent or enzymatic reporters can easily be generated and visually monitored in a typical academic laboratory;

- Reporter expression levels can be quantified using either HCI or plate-reader technologies;
- ARQiv (automated reporter quantification system *in vivo*) system combines zebrafish with existing plate reader technology and is capable of true HTS volume capacities;
- Vertebrate automated screening technology allows researchers to automate dispensing, orientation, imaging and optical manipulations of zebrafish larvae using robotic fluidics;
- Fluorescent reporters can be examined over successive time-points in individual larvae providing a means to follow disease progression, or more importantly regression, following treatments;
- Methods for whole organism HTS will allow unbiased large-scale chemical screening at the beginning, rather than the end, of the drug-discovery process.

Issues remaining to be resolved to advance zebrafish chemical screening

- Long-term maintenance in microtiter plates is not yet compatible with optimal health of arrayed larvae.
- Microtiter plate designs that facilitate stereotyped orientations of anesthetized larvae would improve of HCI-based and ARQiv data collection.
- Statistical data analysis methods that automate ‘hit’ identification are needed to match increases in data collection throughputs.

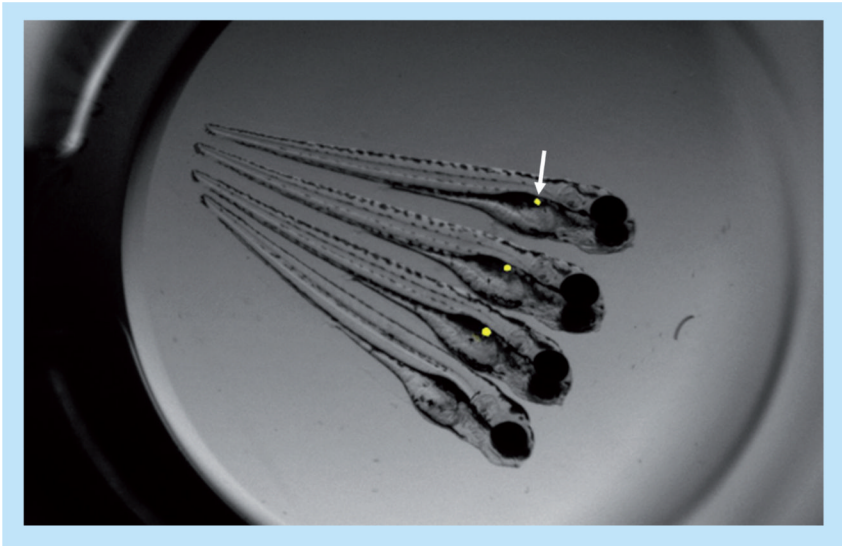

Figure 1. Expression of fluorescent reporters in zebrafish larvae arrayed in a single well of a 96-well plate

Three transgenic larvae (*Tg[ins:PhiYFP-Eco. NfsB,sst2:TagRFP]lmc01* at 4 days post-fertilization) co-expressing PhiYFP (Evrogen) and nitroreductase in beta cells of the pancreas (arrow) are shown [3]; for comparison a single nontransgenic larva is included at the bottom. At this stage larvae naturally settle into a lateral view that is highly amenable to imaging and/or plate reader-based quantification.

Table 1

Selected zebrafish chemical screens, with estimates of the number of larvae used.

Type of screen	Readout	Chemical libraries†	Number of compounds	Replicate‡	Number of samples§	Ref.
Morphology	Heart rate	Prestwick and Chembridge	1200	3	3600	[7]
Morphology	Developmental defects	DiverSet E	5760	1	5760	[30]
Behavioral	Locomotor activity	Prestwick, Spectrum, LOPAC and Enzo	5648	10	56480	[4]
Behavioral	Locomotor activity	Prestwick, Spectrum and DiverSet	13,976	9	125784	[6]
Fixed time-point	YO-PRO-1 dye-labeled hair cells	NINDS	1040	1	1040	[62]
Fixed time-point	ISH of lymphocytes	Spectrum	2000	5	10000	[55]
Fluorescence	GFP-labeled hair cells	NINDS and Enzo	1680	3	5040	[66]
Fluorescence	GFP-labeled pancreatic cells, mCherry Notch reporter	Johns Hopkins Drug Library	3360	2	6720	[69]

†For brevity and ease of comparison, only major libraries are listed.

‡Replicate' refers to the number of embryos/larvae used per assayed drug; either directly stated or inferred from 'materials' sections.

§An estimate of the number of embryos/larvae used for each screen, equal to the 'number of compounds' multiplied by the 'replicate'.