Exploiting Analysis of Heterogeneity to Increase the Information Content Extracted from Fluorescence Micrographs of Transgenic Zebrafish Embryos

Tongying Shun,¹ Albert H. Gough,^{1,2} Subramaniam Sanker,³ Neil A. Hukriede,^{3,4} and Andreas Vogt^{1,2}

¹University of Pittsburgh Drug Discovery Institute, Pittsburgh, Pennsylvania.

Departments of ²Computational and Systems Biology and

³Developmental Biology, University of Pittsburgh Drug Discovery Institute, Pittsburgh, Pennsylvania.

⁴Center for Critical Care Nephrology, University of Pittsburgh, Pittsburgh, Pennsylvania.

ABSTRACT

Zebrafish embryos are a near-ideal animal model for drug discovery because of their high genetic and physiological similarity to mammals, small size, high fecundity, and optical transparency. The latter properties make zebrafish at larval stages especially suited for high-content analysis and high throughput screening (HTS). However, inherent biological complexity and the inability to screen multiple specimens in a single well present a challenge for HTS because limiting replicates and high variability often prevent assays from reaching the stringent performance criteria demanded of large-scale screening assays. In this report, we present methodology that overcomes these obstacles. We used our previously developed Tg(lhx1a:EGFP)^{pt303} line, which expresses a fluorescent transgene that enables live real-time measurements of kidney progenitor cell expansion. Since transgenes are expressed in specific cell populations, whose localization is precisely controlled, both spatially and temporally, we considered the developing embryo to be a “host” for a cell population, analogous to a well of a cell culture microplate, rather than a single specimen. By adopting this view, parameters routinely used to analyze cultured cells became applicable to characterize and quantify zebrafish transgene appearance beyond the overall intensity or area measurements, which are analogous to calculating well average data. Using the pixel-level distribution of transgene intensity as a proxy to cell-level data, we applied population-based intensity and heterogeneity measurements to quantitatively

describe and characterize transgene expression in each embryo. Subsequent linear discriminant analysis on eight such parameters captured and condensed this information into a single assay parameter that maximizes the difference between positive and negative responses. The improvements in assay performance resulted in the Tg(lhx1a:EGFP)^{pt303} assay achieving HTS compatible assay performance in multi-day variability studies, documenting readiness for HTS of compounds that expand kidney progenitor cell populations.

Keywords: zebrafish, assay development, heterogeneity, high-content screening

INTRODUCTION

It has long been proposed that better experimental models are needed to improve drug discovery productivity.^{1,2} Over the past decade, high throughput screening (HTS) has experienced a shift from simple biochemical or cell-based assays to more physiologically relevant multicellular systems,³ including three-dimensional cellular structures and whole organism models such as zebrafish, *Drosophila*, and *Caenorhabditis elegans*.^{4–6} Zebrafish have become a favorite because they are vertebrates, small in size, highly fecund, and genetically tractable.⁷ In addition, optical transparency during development makes zebrafish embryos uniquely suited for image-based analysis and high-content screening. Our group has developed phenotype- and platform-independent methodology for automated imaging of transgenic zebrafish embryos⁸ that has proven useful in the discovery and characterization of allosteric dual specificity phosphatase inhibitors,^{9–11} fibroblast growth factor pathway activators,¹² antiangiogenic agents,^{13,14} and histone deacetylase (HDAC) inhibitors.¹⁵

Zebrafish have been dubbed “canonical vertebrates”¹⁶ because of their high similarity, both genetically and physiologically, to mammals. Coupled with their logistical advantages such as high fecundity and compatibility with

microplate-based assays, zebrafish hold great promise for drug discovery. Their widespread use in automated screening, however, has been limited by the inherent biological variability¹⁷ of living organisms and limitations of traditional image analysis methods, which rely heavily on thresholding and struggle with images that contain spectrally similar features across multiple scales. Furthermore, sample size is usually limited to a single specimen per well, preventing use of statistical parameters commonly applied to cultured cells in microplates, such as cell population averages and distributions.¹⁸

We have effectively eliminated the bottleneck in image analysis through use of cognition network technology (CNT), an object-based analysis method that permits users to translate human perception into computer algorithms (“rulesets”).⁸ Because CNT emulates human perception, images do not have to be manually preprocessed, aligned, or oriented before analysis, as is commonly done.^{19,20} For example, CNT has enabled the development of a quantitative high-content screening assay for agents that enhance kidney progenitor cell expansion during development.^{15,21} The assay is based on measurements of *Lhx1a* expression, a Lim homeodomain protein that is essential during embryonic development for establishing the kidney field.²² Using CNT and a transgenic zebrafish line that expresses a fluorescent *Lhx1a* transgene,²³ we achieved automated detection and quantitation of GFP-*Lhx1a* in the developing embryo and established kinetics of transgene expression, as well as windows for compound treatment and image analysis. Although the ability to consistently identify and automatically analyze transgenic zebrafish embryos in multi-well plates presented a major breakthrough in our zebrafish drug discovery program, we found that the assay did not routinely meet performance parameters usually demanded of HTS, such as Z-factor,²⁴ robust Z-factor,²⁵ or strictly standardized mean difference (SSMD).²⁶

In this report, we describe the use of multiparameter high-content analysis (HCA) coupled with multivariate linear discriminant analysis (LDA) to extract maximum information from images of *Lhx1a*-EGFP transgenic zebrafish. Our strategy was based on the premise that any single parameter might be insufficient to optimally capture the information content of transgene expression and has three key elements: First, transgene expression is not uniform within the developing embryo, but instead confined to specific, spatially defined cell populations, thus causing characteristic expression patterns. This opened the possibility to include shape parameters to describe appearance. Second, rather than treating the embryo as a single sample (the “one-well one-specimen” paradigm), we regarded the developing zebrafish embryos as a host to transgene-expressing cells.

We posited that upon specifically segmenting the transgene on the pixel level, transgene intensity distributions would become analogous to populations of fluorescently labeled cells plated in microplates. This, in turn, would permit high-content multiparametric evaluation of population statistics, such as intensity distributions, texture measurements, and recently developed heterogeneity indices.²⁷ Finally, we reasoned that it might be possible to quantitatively capture additional information contained in fluorescence micrographs by applying LDA,²⁸ to combine multiple features into one aggregate value, similar to what had been attempted before in cell culture.²⁹ The result is a method that improved assay performance to meet HTS criteria in multi-day variability studies.

MATERIALS AND METHODS

Reagents and Chemicals

Methyl-4-(phenylthio)butanoate (m4PTB or UPHD25) has been described in the literature^{30,31} and was synthesized and characterized as described in Reference.¹⁵

Zebrafish Husbandry and Transgene Generation

All procedures involving zebrafish were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The *Tg(lhx1a:EGFP)^{pt303}* line has been described earlier²³ and was maintained as described.¹⁵

Sample Preparation and Imaging

Heterozygous *Tg(lhx1a:EGFP)^{pt303}* embryos were obtained by natural crossings and kept at 28.5°C. At 256 cell stage (2.5 h postfertilization [hpf]), embryos were arrayed in groups of 10–15 embryos into 12-well plates and treated with vehicle or m4PTB (800 nM) in 2 mL E3 medium containing 0.5% dimethyl sulfoxide (DMSO). Single embryos (eight per condition) were placed in the wells of a 96-well round bottom plate (TTP Labtech, Cambridge, MA) and kept at 28.5°C until 30% epiboly. Plates were imaged on an ImageXpress Ultra high-content reader (Molecular Devices, Sunnyvale, CA) using a 4× objective with maximally open pinhole size in the DAPI and green fluorescent protein (GFP) channel at excitation/emission wavelengths of 350/460 and 488/525 nm, respectively. All images were acquired without binning using identical laser power and gain. The resulting micrographs were 2,000 × 2,000 pixels at a resolution of 2 μm/pixel, covering a field of 4 × 4 mm. Images in *Figure 1* were manually cropped and bitmap stretched in MetaXpress to provide maximum detail and clarity for visual examination of transgene appearance. All measurements were performed on unmodified, 16 bit archived scan images.

Fig. 1. Appearance of eGFP-Lhx1a in the developing zebrafish embryo during development and upon drug treatment. *Tg(lhx1a:eGFP)* transgenic embryos were treated at 3 hpf with vehicle or m4PTB and imaged 13 and 19 h thereafter. **(A)** Fluorescence micrographs at 16 hpf show transgene expression along the trunk and in the head (asterisk) and diencephalon (triangle). **(B)** m4PTB increased transgene expression but also resulted in an ~2-h developmental delay where Lhx1a expressing cells had not yet migrated to their proper locations. **(C, D)** At 22 hpf, positional differences in transgene expressing cells were less pronounced, and increased Lhx1a expression in the trunk and tail (arrows) became the most distinguishing feature. In all panels, dorsal is up and rostral to the left. Scale bar, 300 μ m. DMSO, dimethyl sulfoxide; hpf, hours postfertilization; m4PTB, methyl-4-(phenylthio)butanoate.

Image Analysis and Transgene Quantitation

Archived fluorescence micrographs were uploaded into Developer (Definiens AG, Munich, Germany) and analyzed exactly as described previously, using UV autofluorescence to detect the whole embryo and the GFP-labeled transgene as lower hierarchy subobject.¹⁵ Transgene shape was refined by a closing morphology algorithm and merged into a single object. At this stage, shape parameters were extracted. As a proxy to cell level segmentation, transgenes were “segmented” on the pixel level to extract individual pixel GFP intensity values and calculate population statistics. The final set of features encompassed five parameters extracted from CNT analysis (mean GFP intensity, maximum GFP intensity, total GFP intensity, standard deviation of GFP intensity, and total

transgene area) and three heterogeneity indices (KS_Norm, % outliers, and quadratic entropy [QE]). For a detailed description of those please refer to Reference.²⁷

Linear Discriminant Analysis

LDA²⁸ is a “supervised” learning method that computes the directions (“linear discriminants”) that represent the axes which maximize the separation between multiple classes. In this application there are two classes, MIN (vehicle-treated embryos) and MAX (m4PTB-treated embryos). LDA assumes normally distributed data, features that are statistically independent, and identical covariance matrix for each class. Under these assumptions, the Bayes optimal solution is to predict points if the log of the likelihood ratios is below some threshold. The resulting linear discriminants can be used for dimensionality reduction and sample classification.

Three Heterogeneity Indices²⁷ and five Definiens[®] statistical features for each zebrafish embryo were calculated from pixel-level GFP intensity measurements and used for LDA. We used a set of vectors of 8 features for each sample of a zebrafish (X_i) that had known treatment class (MIN_{*i*} or MAX_{*i*}), to find a linear combination of features that characterized transgene appearance and maximally separated MIN and MAX controls using the *LDA* function in the *MASS* R package.³² The single resulting linear discriminant was then used to classify each zebrafish as MIN or MAX based on a simple

threshold. Day-to-day and plate-to-plate variation in measured intensities, principally due to instrument data acquisition settings and/or labeling, in assays is usually corrected by normalization to the plate control wells. In this study, the linear discriminant was calculated for each plate using the MIN and MAX control wells on the plate. For dose-response experiments, archived images from a prior publication¹⁵ of 14 hpf embryos that had been exposed to vehicle or various concentration of m4PTB for 10 h were reanalyzed with the CNT ruleset. The resulting feature set was subjected to LDA using vehicle and m4PTB (1.5 μ M), respectively, as MIN and MAX classifiers, respectively. Data from three independent experiments were normalized to their respective vehicle

Fig. 2. Transgene expression kinetics using total GFP intensity measurements. Heterozygous *Tg(lhx1a:EGFP)^{pt303}* embryos were treated at the 256 to 512 cell stage with vehicle (DMSO) or m4PTB (800 nM), kept at 28.5°C until 30% epiboly, and imaged on the ImageXpress ultra high-content reader every 2 h for 22 h, and analyzed for GFP expression using a CNT ruleset. Data are the mean of eight replicates ± SEM. Tx, treatment. CNT, cognition network technology; GFP, green fluorescent protein.

controls and averaged. Dose–response curves were generated in GraphPad Prism and analyzed using a four parameter logistic equation.

RESULTS AND DISCUSSION

Characterization of Lhx1a-EGFP Transgene Expression

To develop the EGFP-Lhx1a assay for HTS, we chose vehicle (DMSO) and the HDAC inhibitor m4PTB as minimum and maximum controls. Analysis of assay performance from existing data using integrated GFP intensity as the primary measure of transgene expansion¹⁵ revealed that the assay never quite reached the level usually required for large-scale screening (i.e., Z-factors >0). We therefore decided to investigate reasons for this lack of performance and found it to be a direct result of the developing zebrafish embryo being a complex and highly dynamic system. In our prior studies, the most robust parameter distinguishing vehicle-treated from compound-treated embryos was the integrated (total) intensity

Fig. 3. Describing transgene appearance by shape and intensity features. Images in (A) show two arbitrarily chosen *Tg(lhx1a:EGFP)^{pt303}* embryos treated with vehicle or m4PTB (1.5 μM) and analyzed by the CNT algorithm. (B) Histo-Box plots expose dissimilarities in the distribution of pixel intensities not readily appreciated by visual inspection. (C) Transgene appearance can be captured by multiple features but neither parameter alone provides sufficient robustness for screening. Data in (C) are based on 30 embryos for each condition. Orange and yellow shaded cells illustrate parameters that discriminated between MIN and MAZ controls (S/B > 3, robust Z' > 0, and SSMD > 2).

of transgene expression.¹⁵ In the developing embryo, however, not only does transgene expression change but also cells migrate to predetermined locations. For the *Lhx1a* positive cells, at the onset of gastrulation (stage of cell movements to reposition the cells), *lhx1* is expressed in the germ ring and shield, and these cells will migrate to reposition themselves spatially to give rise to the notochord (axial structure), intermediate me-

soderm (kidney determinants), and neural tissue (expressing in the brain and central nervous system) by 24 hpf.²³

The interplay of expression level and spatial localization changes reflects the complexity of the developing embryo and presents a challenge for assay standardization. To confound matters, compounds that increased transgene expression also caused a slight developmental delay, where cells do not get to

Fig. 4. Correlation matrix to assess feature information overlap. Correlations between five GFP intensity features and three Heterogeneity Indices (KS_norm, QE, and % outliers) from the CNT analysis in Figure 3 were evaluated graphically. The diagonal plots show the histogram of the feature, while off diagonal are scatter plots of the feature pairs. Each data point represents a well on the 96-well microplate. Red lines are robust-regression with Lowess smoothing calculated by the *rlm* function in the R package MASS; dotted red lines denote upper and lower 90% confidence intervals. The green line is a linear regression. The graphical representation serves as a decision-making tool to select features to include or exclude in LDA. LDA, linear discriminant analysis; QE, quadratic entropy.

the correct spatial position in the same temporal pattern as control embryos. In untreated 14–16 hpf embryos, the Lhx1a-eGFP transgene was visible as a characteristic ring around the yolk, with prominent expression in the head (asterisk), dien-cephalon (triangle), and along the trunk (Fig. 1A). In the presence of m4PTB, transgene expression was greatly in-creased, resulting in overcrowding of the kidney field and an ~2-h developmental delay (Fig. 1B). The delay made less of a difference at later time points, and differences in Lhx1a-eGFP expression in the tail (Fig. 1C, D, arrow) became the main distinguishing feature.

The combined developmental and drug-induced changes in transgene appearance at earlier time points explain why trans-gene intensity differences between vehicle- and compound-treated embryos were maximal at 14–16 hpf, but variability was also high. In contrast, at later times in development, differences in trans-gene intensity were less pronounced, but inten-sities remained stable and appearance was more uniform, resulting in lower embryo-to-embryo variability (Fig. 2).

Achieving HTS Assay Performance by Multivariate Image Analysis

While on the surface the dynamics of transgene expression and spatial distribution appeared to be an unsurmountable challenge in assay standardization and optimization, we hypothesized that the complexity and heterogeneous nature of transgene appear-ance would present an opportunity for mul-tiplexed HCA, if the information content encoded by transgene appearance could be sufficiently captured. We therefore developed CNT-based image analysis algorithms that quantitatively extracted multiple parameters encompassing not only embryo average in-tensity measurements but also intensity dis-tributions and transgene shape. Visually, the appearance of the transgenes is quite differ-ent between positive (m4PTB) and negative (DMSO) controls (Fig. 1); however, differ-ences in the distribution of fluorescence in-tensities within the CNT-detected transgenes are not readily appreciated by eye (Fig. 3A).

“Histo-box” plots that we developed for heterogeneity analysis of cell populations²⁷ exposed quantitative differences in the distri-bution of pixel intensities within the trans-

genes (Fig. 3B), which are not optimally described by canonical population statistics (mean and standard deviation), but more usefully described using the Heterogeneity Indices.²⁷

Using a test set of 30 positive and negative control embryos, we calculated and exported 13 intensity and shape parameters (Fig. 3C). We found that five of these parameters rose to the level of screenability when applying noncanonical perfor-mance measures such as the SSMD, which we have found particularly useful in our prior zebrafish screens,¹² and the outlier-resistant, robust Z-prime factor,^{25,33} both of which are preferred alternatives to the Z-prime factor in RNAi screens and in assays with moderate strength controls.^{26,34} However, no single parameter achieved Z-prime values above 0 (= a “doable” assay according to Zhang and Oldenburg²⁴) (Fig. 3C).

Fig. 5. Linear discriminant analysis. Eight features describing GFP intensity distributions within the developing embryo were subjected to LDA. **(A)** Weighting factors (LDA loading) for each feature quantify its relative contribution to the linear discriminant. **(B)** LDA cleanly separates minimum and maximum control populations. **(C)** Comparison of high throughput screening performance parameters using single features versus LDA1 values.

Surprisingly, despite the visual differences between vehicle- and m4PTB-treated embryos, none of the shape-based parameters distinguished positive from negative controls; instead all of the parameters with discriminating power were intensity based.

We therefore reasoned that it might be possible to further improve assay performance by characterizing GFP intensity distributions, including the heterogeneity indices²⁷ as additional features and performing LDA²⁸ to maximize the separation between vehicle- and m4PTB-treated embryos. This had similarly been attempted in a cell-based assay.²⁹ The final feature set comprised eight parameters as follows: five pixel-level GFP intensity statistics (mean, maximum, total, standard deviation, and count) and three heterogeneity measurements (KS_norm, denoting deviation from a normal distribution; % outliers; and QE, denoting differences in the shape of the data distributions²⁷).

We first investigated the extent of correlation between the eight parameters to possibly reduce the feature set by excluding those features that were highly correlated and, therefore, not expected to contribute substantial additional information. *Figure 4* shows that most features were not highly correlated, and the few that were correlated only with a few other features (e.g., area $\propto$ total intensity; mean intensity $\propto$ standard deviation). Because of this, and because all eight features had shown some discriminating power (*Fig. 3*), all features were kept and used for LDA. Because there are only two classes (MIN and MAX), LDA results in a single linear discriminant that maximally separates the two classes. *Figure 5A* lists the individual weights for the feature contributions to the linear discriminant. Note that a weight near 0 would indicate that the feature had minimal contribution to the separation of the two classes. In this case, the most significant feature was *mean.GFP.intensity* with a weight of 5.7.

The second most significant features were *QE* and *Tg.stddev* with similar weights of -3.6 . Both of these features characterize the extent of the distribution, and the similar weights might suggest that they are interchangeable. However, examination of *Figure 4* shows that the relationship between *QE* and *Tg.stddev* was nonlinear, with the *QE* providing better discrimination for distributions with low *Tg.stddev*, while *Tg.stddev* provided better discrimination for samples with a higher *Tg.stddev*. The least significant features were *Percent.Outliers* and *max.GFP.intensity* with weights of -0.23 and 0.23 , respectively (*Fig. 5A*). The similar absolute value suggests that the features have similar weight in constructing the linear discriminant. These two features are essentially measures of the extremes of the distributions and suggest that

these extremes have a relatively small contribution to the discrimination, while the mean and width of the distribution are the most important factors. LDA cleanly distinguished MIN and MAX control groups (*Fig. 5B*) and improved separation compared with each single feature to a point where the assay met the requirement for a doable assay (Z -prime 0.10) (*Fig. 5C*).

It should be noted that because the Z' -factor is not linear, the improvement from the best single parameter (*QE*, -0.3 to LDA, $+0.1$) represents an additional three standard deviation separation between the two populations. Furthermore, the LDA delivered graded responses and recapitulated total transgene expression measurements with similar EC50 values and slightly smaller errors (*Fig. 6*).

Fig. 6. LDA delivers graded responses that mirror those of transgene intensity measurements. Archived images from 14 hpf embryos treated with vehicle or various concentrations of m4PTB were analyzed with the CNT ruleset and the resulting feature set subjected to LDA. Data were normalized to their respective vehicle controls. Each data point represents the mean $\pm$ SEM from three independent experiments. *Upper panel*, LDA values; *lower panel*, total GFP intensity representing transgene expansion.

Assay Validation by Multi-Day Variability Studies

We then performed a 3-day variability assessment similar to that reported by Ghosh *et al.*,³⁵ and encouraged by Iversen *et al.*,³⁶ adapted specifically to zebrafish. In three independent trials, we prepared two half-plates of minimum and maximum controls (DMSO and m4PTB, respectively). Plates were loaded into the ImageXpress Ultra at 11 and 20 h after treatment using a Thermo Fisher CRT robot, scanned in the DAPI/GFP channel, images analyzed with CNT, and extracted features combined by LDA. Figure 7 shows that the assay gave SSMDs of 4.62, robust Z' of 0.44, and Z' values of 0.07 in three independent trials using the LDA1 parameter, documenting

screening readiness. Surprisingly, LDA also revealed that assay performance was even better at the late time point (24 hpf), thus extending the time frame during which plates could be read without loss of performance (Fig. 7). Alternatively, one could envision a screening paradigm where plates are read at both time points, possibly improving hit rates by capturing compounds with different kinetics of Lhx1a induction.

OUTLOOK AND PROSPECTUS

A more widespread use of *in vivo* assays for systematic interrogation of small molecule chemical libraries could substantially improve phenotypic drug discovery. Zebrafish

Fig. 7. Three day variability studies with *Tg(lhx1a:eGFP)* transgenic zebrafish. Two half plates of minimum (DMSO, data points to the left) and maximum (1.5 μ M m4PTB, right) controls were prepared on three different days and imaged at (A) 11 h and (B) 20 h after treatment. Images were processed by the CNT algorithm and extracted features analyzed by LDA. (C) Using normalized LDA1 values as the primary assay performance parameter, the assay gave mean SSMDs of 4.62 and 4.79, robust Z' of 0.44 and 0.50, and Z' values of 0.07 and 0.10 at 11 and 20 h, respectively, over three independent trials. SSMD, strictly standardized mean difference.

have become a favorite among multicellular organisms for systematic discovery of novel therapeutics. While the zebrafish community has made great strides toward HTS, the inherent variability of whole organism-based assays and the inability to screen multiple embryos in single well present limitations toward truly automated HTS and often precludes meeting canonical measures of HTS performance. We have overcome this obstacle by applying multivariate analysis based on pixel distribution of fluorescent transgenes. A novel aspect of this approach was the use of Heterogeneity Indices as additional parameters that characterize the pixel intensity distribution. We believe that for assays such as this, with relatively few complex objects that may be difficult to analyze using traditional image processing functions, analysis of the pixel level intensity distributions provides an alternative that can produce a more robust assay.

The method resulted in a whole organism assay for kidney progenitor cell expansion that met HTS performance criteria and is ready to be deployed for discovery of small molecules that augment the regenerative ability of the injured kidney. How generally applicable the strategy is remains to be seen, but since the method is phenotype-agnostic, it should, in principle, be applicable to any biological system. This view is supported by prior work in cell culture²⁹ and has recently been discussed in a perspective by Singh *et al.*³⁷ Looking forward, our study reinforces the notion that biological complexity represents an opportunity rather than an obstacle in HTS assay development and that heterogeneity should be embraced rather than avoided.

ACKNOWLEDGMENTS

This work was funded by grant 4R01 HD053287, 2R01 DK069403, and 1R01 DK112652 from the National Institutes of Health. This project used the UPCI Chemical Biology Core Facility that is supported, in part, by award P30CA047904 and O'Brien Kidney Center resources supported by 4P30 DK079307.

DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Sams-Dodd F: Target-based drug discovery: is something wrong? *Drug Discov Today* 2005;10:139–147.
2. Lansbury PT, Jr.: Back to the future: the 'old-fashioned' way to new medications for neurodegeneration. *Nat Med* 2004;10 Suppl:S51–S57.
3. Horvath P, Aulner N, Bickle M, *et al.*: Screening out irrelevant cell-based models of disease. *Nat Rev Drug Discov* 2016;15:751–769.
4. Vinci M, Gowan S, Boxall F, *et al.*: Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol* 2012;10:29–49.
5. Deo RC, MacRae CA: The zebrafish: scalable in vivo modeling for systems biology. *Wiley Interdiscip Rev Syst Biol Med* 2011;3:335–346.
6. Kaletta T, Hengartner MO: Finding function in novel targets: *C. elegans* as a model organism. *Nat Rev Drug Discov* 2006;5:387–398.
7. Zon LI, Peterson RT: In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* 2005;4:35–44.
8. Vogt A, Cholewinski A, Shen X, *et al.*: Automated image-based phenotypic analysis in zebrafish embryos. *Dev Dyn* 2009;238:656–663.
9. Molina G, Vogt A, Bakan A, *et al.*: Zebrafish chemical screening reveals an inhibitor of Dusp6 that expands cardiac cell lineages. *Nat Chem Biol* 2009;6:680–687.
10. Vogt A, Codore H, Day BW, Hukriede NA, Tsang M: Development of automated imaging and analysis for zebrafish chemical screens. *J Vis Exp* 2010;pii: 1900. DOI: 10.3791/1900.
11. Kaltenmeier CT, Vollmer LL, Verneti LA, *et al.*: A tumor cell-selective inhibitor of mitogen-activated protein kinase phosphatases sensitizes breast cancer cells to lymphokine-activated killer cell activity. *J Pharmacol Exp Ther* 2017;361:39–50.
12. Saydmohammed M, Vollmer LL, Onuoha EO, Vogt A, Tsang M: A high-content screening assay in transgenic zebrafish identifies two novel activators of fgf signaling. *Birth Defects Res C Embryo Today* 2011;93:281–287.
13. Vogt A, McPherson PA, Shen X, *et al.*: High-content analysis of cancer-cell-specific apoptosis and inhibition of in vivo angiogenesis by synthetic (–)-pironetin and analogs. *CBDD* 2009;74:358–368.
14. Vollmer LL, Jimenez M, Camarco DP, *et al.*: A simplified synthesis of novel dictyostatin analogues with in vitro activity against epothilone B-resistant cells and antiangiogenic activity in zebrafish embryos. *Mol Cancer Ther* 2011;10:994–1006.
15. Sanker S, Cirio MC, Vollmer LL, *et al.*: Development of high-content assays for kidney progenitor cell expansion in transgenic zebrafish. *J Biomol Screen* 2013;18:1193–1202.
16. Fishman MC: Genomics. Zebrafish—the canonical vertebrate. *Science* 2001;294:1290–1291.
17. Gough A, Stern AM, Maier J, *et al.*: Biologically relevant heterogeneity: metrics and practical insights. *SLAS Discov* 2017;22:213–237.
18. Gough A, Shun TY, Taylor DL, Schurdak M: A metric and workflow for quality control in the analysis of heterogeneity in phenotypic profiles and screens. *Methods* 2016;96:12–26.
19. Tran TC, Sneed B, Haider J, *et al.*: Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer Res* 2007;67:11386–11392.
20. Ghotra VP, He S, de Bont H, *et al.*: Automated whole animal bio-imaging assay for human cancer dissemination. *PLoS One* 2012;7:e31281.
21. Skrypnik NI, Sanker S, Brilli-Skvarca L, *et al.*: Delayed treatment with PTBA analogs reduces postinjury renal fibrosis after kidney injury. *Am J Physiol Renal Physiol* 2016;310:F705–F716.
22. Cirio MC, Hui Z, Haldin CE, *et al.*: Lhx1 is required for specification of the renal progenitor cell field. *PLoS One* 2011;6:e18858.
23. Swanhart LM, Takahashi N, Jackson RL, *et al.*: Characterization of an *lhx1a* transgenic reporter in zebrafish. *Int J Dev Biol* 2010;54:731–736.
24. Zhang JH, Chung TDY, Oldenburg KR: A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999;4:67–73.
25. Birmingham A, Selfors LM, Forster T, *et al.*: Statistical methods for analysis of high-throughput RNA interference screens. *Nat Methods* 2009;6:569–575.
26. Zhang XD: A pair of new statistical parameters for quality control in RNA interference high-throughput screening assays. *Genomics* 2007;89:552–561.
27. Gough AH, Chen N, Shun TY, *et al.*: Identifying and quantifying heterogeneity in high content analysis: application of heterogeneity indices to drug discovery. *PLoS One* 2014;9:e102678.

28. McLachlan GJ: *Discriminant Analysis and Statistical Pattern Recognition*. Wiley Interscience, Hoboken, NJ, 2004.
29. Kümmel A, Gubler H, Gehin P, Beibel M, Gabriel D, Parker CN: Integration of multiple readouts into the z' factor for assay quality assessment. *J Biomol Screen* 2010;15:95–101.
30. Zayed SMAD, Mostafa IY, Farghaly M: Über die Eigenschaften von (Arylmercapto) acethydroxamsäuren. *Arch Pharm (Weinheim)* 1967;300:526–533.
31. Minoru U, Takao N, Kazuyuki N: Substituted thiobutyric acid derivatives. *Jpn Kokai Tokkyo Koho*: Otsuka Pharmaceutical Co., Ltd., Japan, 1982:9 pp. JP57058663(A).
32. R: A language and environment for statistical computing [computer program]. Version 3.2 R Foundation for Statistical Computing, Vienna, Austria, 2015.
33. Dürr O, Duval F, Nichols A, et al.: Robust hit identification by quality assurance and multivariate data analysis of a high-content, cell-based assay. *J Biomol Screen* 2007;12:1042–1049.
34. Zhang XD: Novel analytic criteria and effective plate designs for quality control in genome-scale RNAi screens. *J Biomol Screen* 2008;13:363–377.
35. Ghosh RN, Chen YT, DeBiasio R, et al.: Cell-based, high-content screen for receptor internalization, recycling and intracellular trafficking. *Biotechniques* 2000;29:170–175.
36. Iversen PW, Beck B, Chen YF, et al.: HTS Assay Validation. In: *Assay Guidance Manual*. Sittampalam GS, Coussens NP, Brimacombe K, et al., (eds). Eli Lilly & Company and the National Center for Advancing Translational Sciences: Bethesda, MD, 2004.
37. Singh S, Carpenter AE, Genovesio A: Increasing the content of high-content screening: an overview. *J Biomol Screen* 2014;19:640–650.

Address correspondence to:

Andreas Vogt, PhD

Department of Computational and Systems Biology

University of Pittsburgh Drug Discovery Institute

W948 Biomedical Science Tower

Pittsburgh, PA 15260

E-mail: avogt@pitt.edu

Abbreviations Used

CNT = cognition network technology
 DMSO = dimethyl sulfoxide
 EGFP = enhanced green fluorescent protein
 GFP = green fluorescent protein
 HCA = high-content analysis
 HDAC = histone deacetylase
 hpf = hours postfertilization
 HTS = high throughput screening
 LDA = linear discriminant analysis
 m4PTB = methyl-4-(phenylthio)butanoate
 QE = quadratic entropy
 SSMD = strictly standardized mean difference