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Drug Screening of Primary Patient Derived Tumor Xenografts in Zebrafish

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November 28, 2019

RE: JoVE60996

Dear Dr. Cao,

We are pleased to submit our revised manuscript entitled “A Standardized Workflow for Drug Screening of Primary Patient Derived Tumor Xenografts in Zebrafish”, manuscript number JoVE60996.

We appreciated the reviewer’s positive comments and the thoughtful critiques and suggestions. We have revised the manuscript accordingly, as outlined in the reviewer response letter, and believe that the revisions have adequately addressed the issues raised by the reviewers. We have submitted two manuscript documents, a track changes version that is not final and the final, clean manuscript version. The track changes version has been submitted as a supplemental file. We hope the reviewers and you find the revised manuscript appropriate for publication with JoVE.

Sincerely,

Jessica Blackburn, PhD

TITLE:

Drug Screening of Primary Patient Derived Tumor Xenografts in Zebrafish

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KEYWORDS:

zebrafish, patient derived xenograft, high-throughput, drug screen, leukemia, automated

SUMMARY:

Zebrafish xenograft models allow for high-throughput drug screening and fluorescent imaging of human cancer cells in an in vivo microenvironment. We developed a workflow for large scale, automated drug screening on patient-derived leukemia samples in zebrafish using an automated fluorescence microscope equipped imaging unit.

ABSTRACT:

Patient derived xenograft models are critical in defining how different cancers respond to drug treatment in an in vivo system. Mouse models are the standard in the field, but zebrafish have emerged as an alternative model with several advantages, including the ability for high-throughput and low-cost drug screening. Zebrafish also allow for in vivo drug screening with large replicate numbers that were previously only obtainable with in vitro systems. The ability to rapidly perform large scale drug screens may open up the possibility for personalized medicine with rapid translation of results back to clinic. Zebrafish xenograft models could also be used to rapidly screen for actionable mutations based on tumor response to targeted therapies or to identify new anti-cancer compounds from large libraries. The current major limitation in the field has been quantifying and automating the process so that drug screens can be done on a larger scale and be less labor-intensive. We have developed a workflow for xenografting primary patient samples into zebrafish larvae and performing large scale drug screens using a fluorescence microscope equipped imaging unit and automated sampler unit. This method allows for standardization and quantification of engrafted tumor area and response to drug treatment across large numbers of zebrafish larvae. Overall, this method is advantageous over traditional cell culture drug screening as it allows for growth of tumor cells in an in vivo environment throughout drug treatment, and is more practical and cost-effective than mice for large scale in vivo drug screens.

INTRODUCTION:

Xenografting of primary patient cancers or human cancer cell lines into model organisms is a widely used technique to study tumor progression and behavior in vivo, tumor response to drug treatment, and cancer cell interaction with the microenvironment, among others. Traditionally, cells are xenografted into immune-compromised mice, and this remains the standard in the field. However, this model system has several limitations, such as high cost, low replicate numbers, difficulties in accurately quantifying tumor burden in vivo, and the extended time that it takes for tumors to engraft and drug testing to be completed. In recent years, zebrafish have emerged as an alternate xenograft model, with the first being reported in 2005, with green fluorescent protein (GFP)-labeled human melanoma cell lines transplanted into blastula-stage embryos[1,2]. More recently, 2 day post-fertilization (dpf) zebrafish larvae have been used as xenograft recipients to allow for control of anatomic location of injection and for use in high resolution in vivo imaging of tumor interaction with the surrounding microenvironment[3,4].

Zebrafish offer many advantages as a xenograft model. First, adult zebrafish can be housed and rapidly bred in large quantities at a relatively low cost. Each mating pair of adult zebrafish can produce hundreds of larval fish per week. Due to their small size, these larval zebrafish can be maintained in 96-well plates for high-throughput drug screening. Larvae do not have to be fed during the course of a typical xenograft experiment, as their yolk-sac provides the nutrients to sustain them for their first week of life. Furthermore, zebrafish do not have a fully functional immune system until 7 dpf, meaning that they do not require irradiation or immunosuppressive regimens prior to xenograft injection. Finally, optically clear zebrafish lines allow for high-resolution imaging of tumor-microenvironment interactions.

Perhaps the most promising application of zebrafish as a xenograft model is the ability to perform high-throughput drug screening on human cancer samples in a way that is not possible using any other model organism. Larvae absorb drugs from the water through the skin, enhancing the ease of drug administration[5]. Because animals are maintained in 96-well plates, typically in 100−300 µL of water, screens require smaller drug quantities compared to mice. Currently, there are several different methods for standardization and quantification of the effect of drugs on human tumor burden in zebrafish, some of which are more practical than others for scaling-up single drug testing to high-throughput screening. For example, some groups dissociate fish into single cell suspensions, and quantify fluorescently labeled or stained tumor cells by imaging individual droplets of the suspension and quantifying fluorescence using a semi-automated ImageJ macro[4]. A semi-automated whole-larvae imaging method was developed in which larval fish were fixed in 96-well plates and imaged using an inverted fluorescent microscope before realignment of composite images and quantification of tumor cell foci[6]. Both of these assays are fairly labor-intensive methods for quantification, which has made truly high-throughput drug screening in zebrafish xenograft models impractical.

This issue has been addressed by the development of the Vertebrate Automated Screening Technology (VAST) Bioimager and Large Particle (LP) Sampler, a fluorescence microscope equipped imaging unit and automated sampler unit (**Figure 1** and **Table of Materials**), which is a

truly automated method for high-throughput imaging of zebrafish larvae[7–9]. With this unit, fish are anesthetized, sampled automatically from a 96-well plate, positioned in a capillary and rotated into the set orientation based on a preset user preference, imaged, and then either placed back into the same well of a new 96-well plate for further studies or discarded. Combining this imaging technology with zebrafish xenografts may allow for the possibility of personalized medicine that uses high-throughput drug screening of large drug compound libraries against individual patient tumors. Zebrafish xenografts also offer a large-scale and low-cost method for testing both toxicity and efficacy of novel compounds in vivo. Zebrafish can be used as a preliminary screening step before proceeding to mouse xenograft models.

We have developed a streamlined workflow for xenografting primary patient leukemia cells into zebrafish and performing high-throughput drug screens with automated imaging and quantification, which can be applied to any other primary patient tumor cells or cancer cell line. This workflow utilized a fluorescence microscope equipped imaging unit and automated sampler unit to improve upon current standardization and quantification methods and offers an automated alternative to previous, more labor-intensive methods of quantifying tumor mass in vivo.

PROTOCOL:

All procedures described in this protocol have been approved by the University of Kentucky's Institutional Animal Care and Use Committee (protocol 2015-2225). Patient samples were collected under University of Kentucky's Institutional Review Board (protocol 44672). All animal experiments performed following this protocol must be approved by the user's Institutional Animal Care and Use Committee.

1. Thawing primary patient acute lymphoblastic leukemia cells

1.1. Thaw primary patient peripheral blood mononuclear cells (PBMCs) from frozen stock in a 37 °C water bath. Immediately after cells have thawed, transfer cells in their freezing media (90% FBS + 10% dimethyl sulfoxide [DMSO]) to a 15 mL conical tube with slow pipetting, avoiding air bubbles. Add 10 mL of prewarmed 37 °C thawing media (25% fetal bovine serum [FBS] in Iscove's modified Dulbecco's medium [IMDM]) dropwise (approximately 2−3 s per mL) to the cells in the 15 mL conical tube.

NOTE: PBMCs were collected from patient blood samples at the time of diagnosis. The buffy coat was separated by density centrifugation and cells were washed 2x in RPMI 1640 + 10% FBS. Cells were counted and 10^7 cells were frozen per cryovial in 1 mL of freezing media, and stored at -80 °C.

1.2. Centrifuge cells at 100 x *g* for 10 min and aspirate media from the cell pellet. Repeat the addition of thaw media, centrifugation, and aspiration one additional time to remove any residual DMSO.

1.3. Resuspend cells in 5 mL of phosphate-buffered saline (PBS) and remove 10 µL for counting on an automated cell counter or hemocytometer. Add 10 µL of trypan blue to 10 µL of cells removed for counting. Count number of cells per mL and record to later calculate the volume to resuspend cells in for xenografting (see step 2.5).

NOTE: Typically, 500 cells are xenografted per larval zebrafish. For example, 5 x 10^5 cells are needed to inject 1,000 zebrafish. Viability should be >85% to use for xenografting. In this experiment, cell viability was 96% after thawing, assessed by trypan blue staining.

2. Fluorescently labeling cells with DiI

2.1. Centrifuge the desired cell number in 5 mL of PBS at 200 x *g* for 5 min and aspirate supernatant. Stain a minimum of 2 x 10^6 cells in case of clumping or problems loading the needle during injection.

2.2. Make 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) staining solution (5 mL of PBS containing 4 µL per mL of DiI stain, **Table of Materials**) and resuspend the cell pellet in the staining solution.

NOTE: Cell density should not exceed 2 x 10^6 cells/mL when resuspended in the staining solution.

2.3. Incubate cells at 37 °C protected from light for 20 min, vortex gently, then incubate cells on ice for 15 min protected from light.

2.4. Centrifuge cells at 200 x *g* for 5 min and aspirate supernatant. Wash cells with 5 mL of PBS, centrifuge at 200 x *g* for 5 min and aspirate supernatant. Repeat wash, centrifugation, and aspiration one additional time.

2.5. Resuspend cells in 1 µL of PBS per 250,000 live cells and transfer to a 1.5 mL microcentrifuge tube. Keep resuspended cells on ice in the dark and immediately continue to microinjections.

NOTE: This ensures that 500 patient cells are injected into the zebrafish with each injection pump of 2 nL volume.

3. Microinjecting zebrafish larvae

NOTE: Microinjections should be completed within 1−3 h of staining to improve viability of cells.

3.1. Prior to staining cells and injecting, make agarose plates for injecting by pouring 25 mL of 3% agarose in 1x Tris/borate/EDTA (TBE) into a Petri dish and allow it to solidify. Store plates at 4 °C for up to 2 weeks.

3.2. Also prior to staining cells and injecting, dechorionate 2 dpf zebrafish using forceps under a dissecting microscope. For manual dechorionation, pull from opposite ends of the protective

chorion of the zebrafish with forceps until the chorion tears and the zebrafish becomes unenveloped[10].

NOTE: Dechorionation can also be performed by using enzymatic treatment with pronase, as previously described[10]. Casper (roy$^{-/-}$;nacre$^{-/-}$) zebrafish were used for these experiments[11]. Any zebrafish larval strain can be used for xenografting. If pigment interferes with imaging or visualization, propylthiouracil (PTU) treatment can be used to block melanin synthesis if optically clear zebrafish strains are not available[12].

3.3. Prechill needles at 4 °C or on ice to prevent clumping of cells during microinjection. Load 5 µL of stained cells into a chilled nonfilamentous borosilicate glass needle using microloader pipette tips.

NOTE: Microinjector and needle setup methods have been previously published[13].

3.4. Load the needle into the microinjector arm. Bevel the needle tip using a sterile razor blade. Measure the droplet size in mineral oil using a stage micrometer, keeping droplet volume consistently at 2 nL (~0.15 mm diameter) throughout.

3.5. Use 350 µL of 4 mg/mL tricaine-S to anesthetize ~30 dechorionated 2 dpf zebrafish in a Petri dish containing 25 mL of E3 media. After ~1 min transfer anesthetized larvae to a flat-surface injection plate (3% agarose in a Petri dish) and inject larvae with one pump of stained cells at the desired injection site (e.g., the yolk or the pericardium; see **Figure 2A,B**).

NOTE: Injection site should be chosen based on the goal of the experiment. The most common injection site is the yolk. To get cells circulating in the bloodstream, the pericardium, duct of Cuvier, perivitelline space, or retro-orbital space can be used as injection sites. Orthotopic injection sites can also be used, such as the brain.

3.6. Wash larvae off the injection plate into a 10 cm^2 Petri dish (30 larvae per plate) containing E3 media[14] without methylene blue and incubate at 28 °C for a 1 h recovery period. Continue injecting until a desired number of larvae have been injected.

NOTE: Ideally, inject 2−2.5-fold the number of larvae needed for experiments. There will be some die-off of larvae due to stress from injection and the increased incubation temperature. Typically, after practice with the technique, 800−1,500 zebrafish larvae can be injected by a single person within the 1−3 h when stained cells should be injected.

3.7. Move plates of injected larvae to a 34 °C incubator. Do not place the Petri dishes of larvae directly on a metal shelf in the incubator to prevent overheating of the E3 water. For example, place an empty Petri dish between the shelf and Petri dish of larvae to act as a buffer. Remove dead zebrafish larvae after 24 and 48 hours post injection (hpi).

4. Setting up drug screen with xenografted zebrafish

4.1. At 48 hpi, screen zebrafish larvae for fluorescence/tumor engraftment and health (**Figure 2C,D**). Remove any dead or malformed zebrafish and select zebrafish with similar engraftment (**Figure 2C,D**, 1−3 and 1'−3'). Remove unengrafted zebrafish (**Figure 2C,D**, 5 and 5').

4.1.1. For yolk injected fish, remove fish where borders of the yolk cannot be seen around engrafted cell mass (**Figure 2C**, 4) as it makes quantification difficult. For pericardium injected fish, remove fish where injected cell mass encroaches into the yolk sac (**Figure 2D**, 4').

4.2. Add zebrafish to a 96-well plate. To do this, cut the tip off of a 200 µL pipette tip, just large enough for a 4 dpf zebrafish to fit through. Aspirate 150 µL of E3 media with one zebrafish from the plate using a P200 pipette, and add to an empty well of a flat-bottom 96-well plate.

4.3. Dilute the drug to be tested in the required volume of E3 media, at 150 µL per well. Prepare drug at 2-fold the desired concentration. For example, prepare 20 µM of drug in E3 if the desired final concentration is 10 µM, since half of the total volume in each well is comprised of the drug solution. For the DMSO control group, add DMSO at the same volume as the drug.

4.4. Add 150 µL of 2x diluted drug solution to each well containing zebrafish larvae in 150 µL of E3, for a final volume of 300 µL with 1x drug solution per well.

4.5. Incubate the plate at 34 °C. Check for dead zebrafish daily. After 2 days, if desired, refresh the drug by removing 200 µL of liquid from each well of the 96-well plate and replacing with 200 µL of 1x dilute drug solution or DMSO in E3 media.

NOTE: The best results were found after 3 days of drug treatment for this experiment; however, the length of drug treatment can vary between 2−4 days and may need to be optimized based on the experiment being done or drugs being used.

5. Imaging xenografted zebrafish using a fluorescence microscope equipped imaging unit and automated sampler unit

5.1. Prepare 1 L of fresh 4 mg/mL tricaine and 1.5 L of E3 media. Fill media bottle 1 with E3 media and media bottle 2 with tricaine.

5.2. Remove all unwanted fluorescent channels in the imaging software and add the desired channel (DiI for this experiment). Check the desired fluorescent channel as an image will only be taken for the channels with a check mark. Also, select how the images will be taken (z-stacks, automation, loops in series, etc.).

NOTE: For this experiment, the focus was manually set for each fish imaged to obtain the highest number of images with optimal focus.

5.3. Image the DMSO control fish before the drug-treated fish so that the appropriate exposure

time can be set in the imaging software. Once the exposure is set, do not change the exposure time for the duration of the experiment.

NOTE: The focus can either be adjusted manually for each zebrafish to ensure there are no out of focus images, or for fully automated imaging, the same focus can be used between fish with out of focus images being discarded or fish reimaged prior to performing analysis. Furthermore, this experiment could be conducted by using any fluorescent imager followed by quantification of fluorescence using the ImageJ software.

6. Quantifying fluorescence using ImageJ

6.1. Open ImageJ software.

6.2. Go to **File** | **Open** and select the desired .czi file. The software will bring up an import options window.

6.2.1. For stack viewing select **Hyperstacks**, check **Open Files Individually**, check **Autoscale**, and check **Split Channels**. For the color option select **Colorized**.

6.3. Click **Plugins** | **Macros** | **Record**.

6.4. Click **Image** | **Adjust** | **Threshold**. Select image type as **red** in the dropdown menu on the right side of the threshold window. Adjust the minimum threshold until the software is only highlighting areas with fluorescence (**Figure 3A**) and click **Apply.** The software will convert the photo to black and white, with the selected area in black.

NOTE: Use the same threshold for each image in the drug screen to keep results standardized and comparable.

6.5. Click **Analyze** | **Measure**. The software will pull up a results window containing the fluorescent area for that image.

6.6. Click **Create** on the **Macro Recorder** window. This will open up a new window with the code for the macro. Highlight all of the desired images for analysis and open as in step 6.2.

6.7. Select **Run** on the window with the macro. The results window will now contain the area for each image.

NOTE: The image analysis can be done individually without recording and running a macro as well as by repeating the above steps for each image.

6.8. Copy the measured data into a spreadsheet. Average the total fluorescence of all control (DMSO) samples. Calculate the percent difference using the following formula: –[(average DMSO area – experimental area)/average DMSO area] x 100% (**Figure 3B**).

REPRESENTATIVE RESULTS:

Following the protocol described above, zebrafish were xenografted in the yolk and pericardium with primary patient PBMCs that were originally isolated from a T-cell acute lymphoblastic leukemia (T-ALL) patient at diagnosis and banked as a viable, frozen sample. At 48 hpi, xenografted fish were screened for fluorescently labeled tumor cells (**Figure 2C,D**) and treated with chemotherapy (dexamethasone or vincristine) or DMSO. Fish were imaged at 7 dpi, after 3 days on drug treatment using a fluorescence microscope equipped imaging unit and automated sampler unit (**Figure 3A**).

The fluorescent area/tumor burden was measured for each fish imaged using ImageJ and compared between the different drug treatment groups and DMSO (**Figure 3B**). Overall, xenografted fish treated with vincristine showed the largest and most consistent decrease in xenografted cell mass compared to DMSO treated fish. Dexamethasone treated fish showed about half the reduction in tumor area compared to vincristine, but still showed a reduction in tumor area compared to DMSO (**Figure 3**). This mimicked what was seen in the patient, as their leukemia rapidly responded to therapy with a combination of dexamethasone and vincristine. These results demonstrate the ability of zebrafish xenograft models to be amenable to drug screening and automated imaging and quantification, providing a platform for testing various patient samples or cell lines with different drugs or drug combinations.

FIGURE TABLE LEGENDS:

Figure 1: Xenograft drug screen and imaging workflow. Schematic of workflow of xenografting zebrafish larvae and performing drug screen, including imaging on a fluorescence microscope equipped imaging unit and automated sampler unit.

Figure 2: Injection site and representative images of screening xenografted fish. Images of microinjector needle at time of injection into either the yolk (**A**) or pericardium (**B**) of 2 dpf zebrafish larvae. Representative images of screening at 2 dpi depict selection of zebrafish for drug screen (**C,D**). Zebrafish with similar engraftment (1−3 and 1’−3’) should be selected, unengrafted zebrafish (5 and 5’) should be removed. For yolk injected fish, remove fish where borders of the yolk cannot be seen around engrafted cell mass (4) as it makes quantification difficult. For pericardium injected fish, remove fish where injected cell mass encroaches into yolk sac (4’). Scale bar = 0.5 mm.

Figure 3: Drug treatment can reduce xenografted tumor area in vivo. Representative images of zebrafish injected in the pericardium or yolk after 3 days of treatment with DMSO or drugs, either vincristine or dexamethasone. Area of engrafted tumor mass was quantified by setting a fluorescence threshold using ImageJ, selecting all pixels above the set threshold, and measuring the area and mean fluorescence of the selected regions. Pixels above the selected threshold appear in black, while pixels below the threshold appear in white. Pixels were measured in both the yolk and pericardium injected zebrafish images (**A**). Treatment with vincristine led to a decrease in engrafted tumor area compared to DMSO control with n = 4 fish treated per group (**B**). SD = standard deviation. Scale bar = 250 μm.

DISCUSSION:

In this study, we demonstrated a standardized method for thawing and injection of primary patient leukemia cells into zebrafish as a xenograft model. We also established a protocol for high-throughput drug screening of xenografted zebrafish using a fluorescence microscope equipped imaging unit and automated sampler unit. Previously, xenografts have been reported with human cell lines, and quantification of xenografted tumors in a high-throughput manner has been a challenge in the field. This method serves as a basis for studies to utilize a fluorescence microscope equipped imaging unit and automated sampler unit as a way to automate imaging of xenografted zebrafish, with the ultimate goal of performing high-throughput drug screens to predict which drugs a specific patient's cancer may respond to, opening the possibility for more personalized medicine.

Despite the ability to automate much of this protocol, there are still many technical challenges that should not be overlooked. First, it is critical that cells are injected into zebrafish as quickly as possible after staining to prevent cell clumping and cell death. Larvae will need to be dechorionated prior to performing the cell staining protocol. Glass filament needles should also be kept cold prior to loading the needle to reduce needle clogging. Additionally, using embryos produced by healthy adult zebrafish aged 6 months to 1 year is critical to ensure the best viability of xenografted larvae. Finally, xenografted fish should be carefully screened for tumor engraftment, and only those with similar tumor volumes should be used in drug screening to reduce variability in the final results.

Although we used primary patient leukemia PBMCs for our experiments, this protocol can be performed with any tumor type or cancer cell line. For cell lines in culture, adherent cells should be trypsinized and then washed in PBS before proceeding with the staining protocol. It is also important to note that engraftment rates can vary from one sample to the next and between sample types[15]. For example, in our PBMC sample, >90% of circulating PBMCs were leukemic blasts, but this number can vary significantly from one patient to the next, which may affect engraftment rate. Because results are compared to a DMSO control within the same sample type, there is an internal control for engraftment rate, yet this variation should be taken into consideration when deciding how many cells to inject per zebrafish. We have found success when using 250−1,000 cells injected per animal, with 500 being optimal for our studies. While our experiments concluded when larvae were 7 dpf, we would not expect xenografts to survive in the animals for longer timepoints, as the immune system begins to develop at this point, and would likely cause rejection of human cells. Immune compromised zebrafish lines have been created, with *prkdc*$^{-/-}$*;il2rga*$^{-/-}$ zebrafish capable of engrafting human cancer cells[16,17], which may be useful for longer term xenografts or assessing tumor recurrence after drug treatment. However, these immunodeficient lines must be maintained as heterozygotes, so larvae must be genotyped before use. Homozygous fish must also be treated with drugs to deplete macrophages to enable reliable engraftment of human cells, which may complicate drug screening results. Currently, these lines are neither practical nor necessary for large scale drug screening on larvae, which can be completed before the immune system is fully functional at 7 dpf[18].

Our representative results focus on injection of cells into the pericardium and yolk for ease and speed of injection and increased viability; however, cancer cells can be injected into many other anatomic locations and we have had success using this workflow at other sites, including the duct of Cuvier, brain, retro-orbital, and the perivitelline sac. Additionally, it is difficult to estimate how much of each drug the larval fish absorb; if few drugs are used, ideally a toxicity screen at a range of doses (usually 0.1 to 25 µM) will be performed prior to the large scale assay to determine the maximum tolerated dose (MTD). We chose to use the MTD for each drug for our assay, however, 10 µM of drug is commonly used in the zebrafish field as a starting concentration for high-throughput drug screening and is generally well tolerated. Combinations of drugs in pools can be used as an initial screen, as well, to increase efficiency of screening through a large-volume compound library[19].

Although this approach is more automated and efficient than previously reported workflows, this is still a labor-intensive and technically challenging protocol for anyone without prior experience in microinjecting zebrafish. Drug screening in zebrafish xenografts is unlikely to ever reach the ease and efficacy of in vitro screening of compound libraries and lacks some advantages of mouse xenograft models. For example, one major limitation with zebrafish xenografts is that cancer cells cannot be easily retrieved from fish after xenografting at useful numbers for cell banking or most downstream experiments. Even if this were possible, the human cancer cells would have been growing for several days in non-physiologic temperatures and environments and would not be practical for use in later applications. The effects of a slightly lower than physiological temperature on drug kinetics and tumor cell response is also not known, and may produce confounding results. Despite these caveats, zebrafish xenografts do fill a void in being a more practical and cost-efficient method for performing larger scale in vivo drug screens than is possible in mouse xenograft models. Additionally, zebrafish xenografts require far fewer cells for injection than mouse models, so a small amount of patient sample can be spread amongst hundreds to thousands of zebrafish, allowing for drug screens with large sample numbers. With fluorescent labeling, tumor cells can be monitored from the moment they are xenografted into the larval zebrafish, providing some standardization between the animals used in drug screens. Combining these benefits of zebrafish xenografts with the possibility of automated imaging and quantification of engrafted cells opens up many possibilities for making high-throughput drug screening of patient tumors for personalized medicine a reality.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

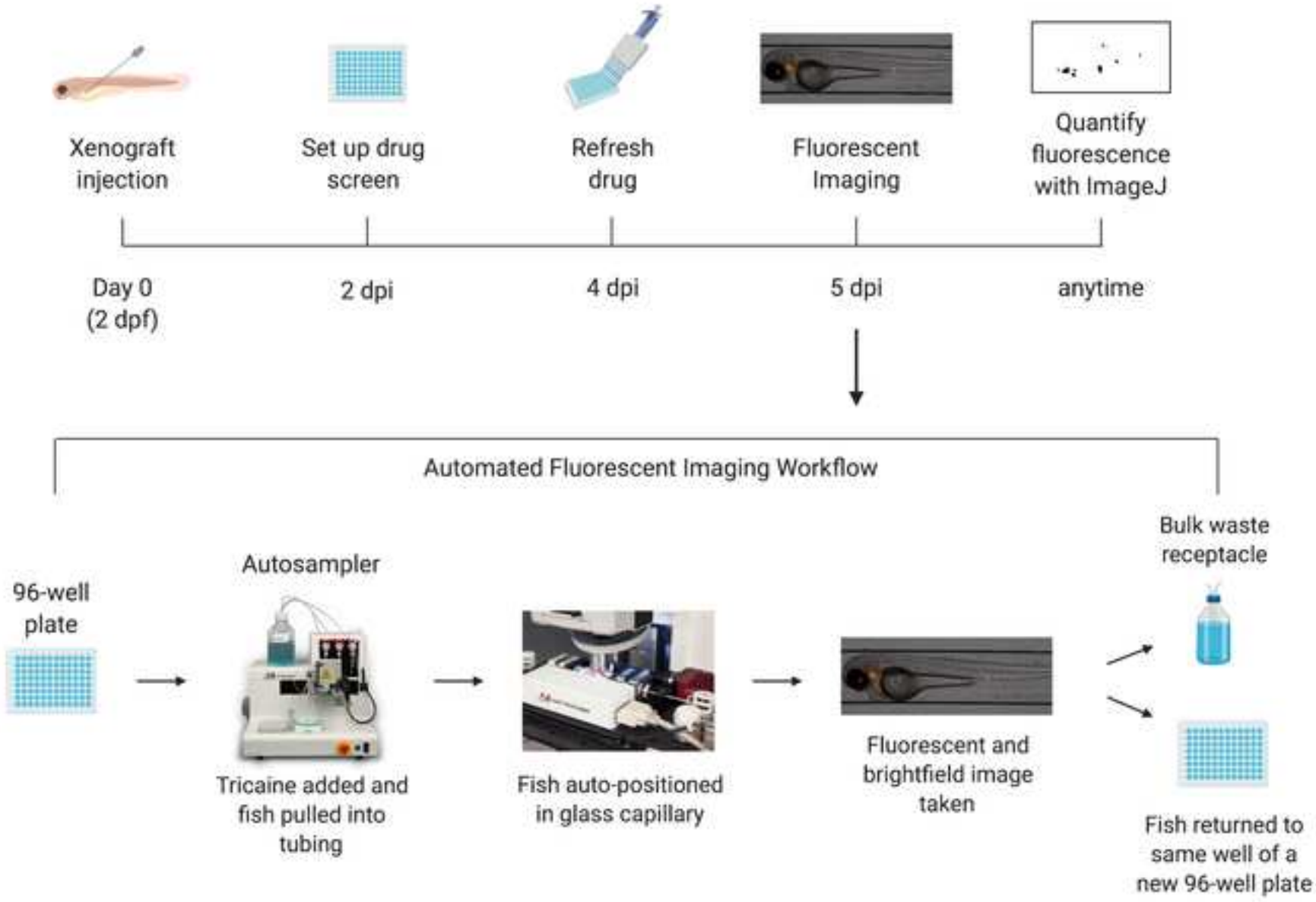


Figure 2

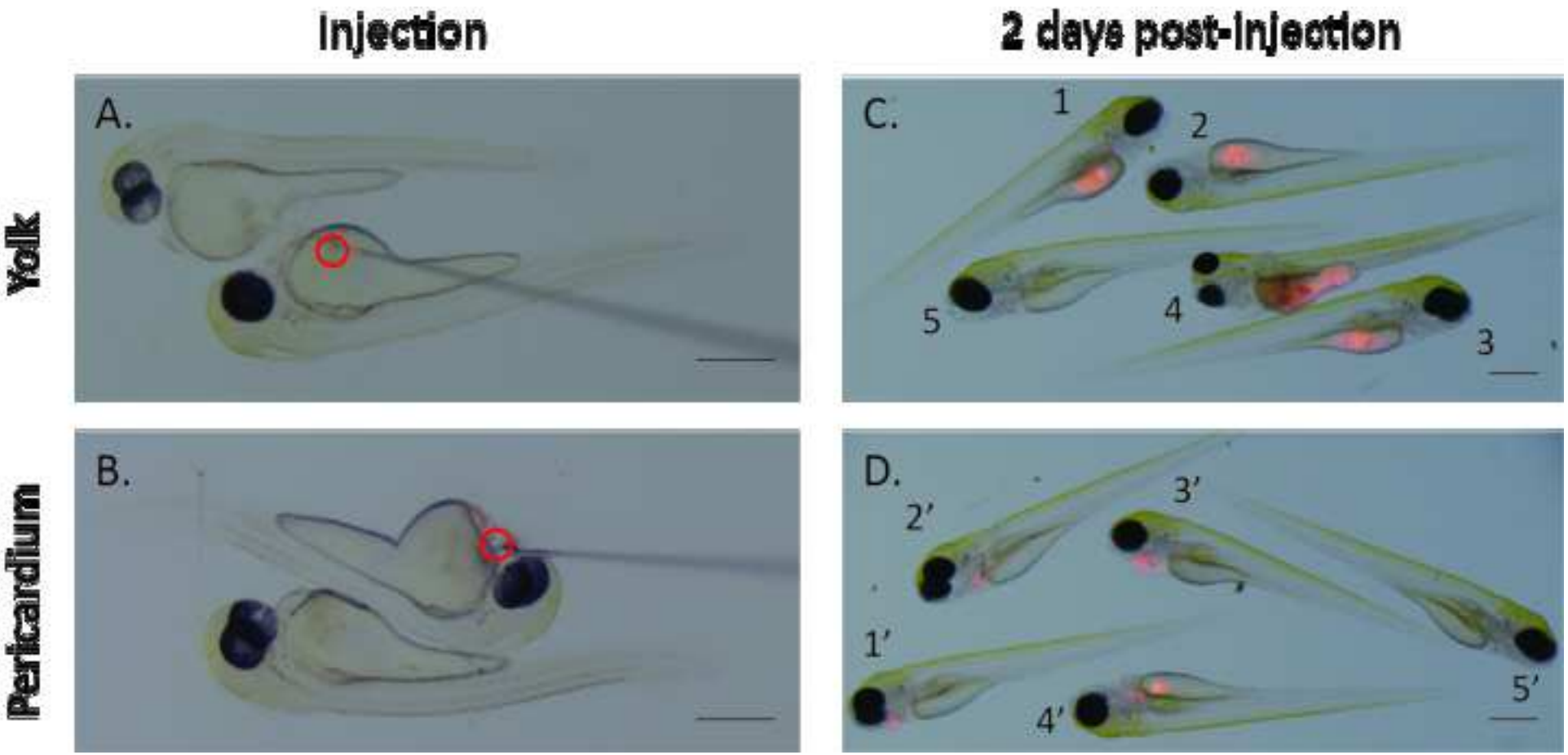
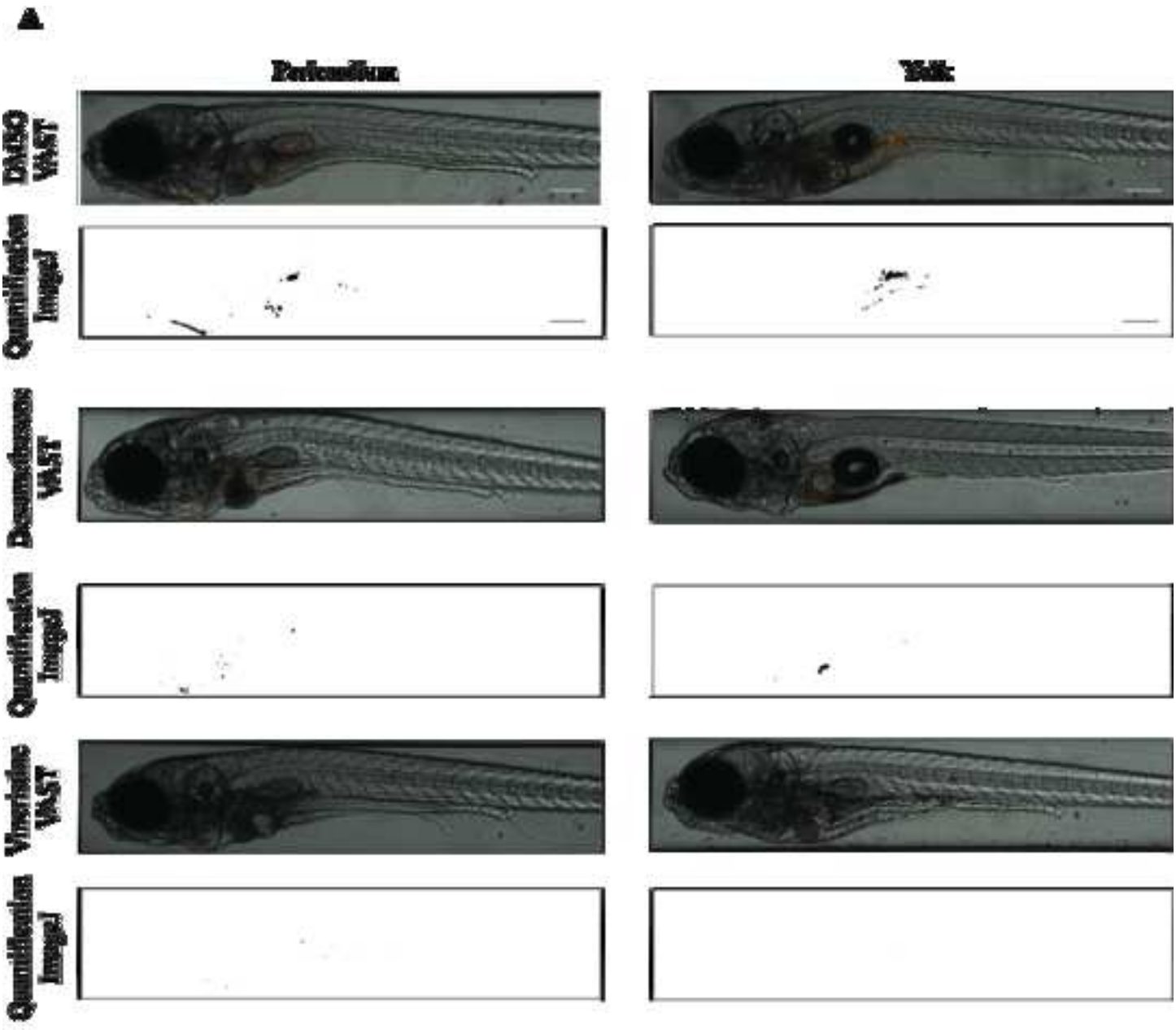


Figure 3

Click here to access/download;Figure;Figure 3 Resubmission 3.psd



Treatment	Average Area (SD)	Average % Difference (SD)
DMSO (n=4)	16.39 ± (4.99)	0.00 ± (30.45)
Dexamethasone (n=4)	7.70 ± (6.46)	-53.06 ± (39.41)
Vincristine (n=4)	1.3 ± (0.79)	-92.07 ± (4.79)

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials resubmission V1.xls

Name of Material/ Equipment	Company	Catalog Number
10x TBE Liquid Concentrate	VWR	0658-5L
96-well plate, flat bottom	CELLTREAT	229195
Agarose	Fisher Scientific	BP160-500
Borosilicate Glass Capillary without Filament	Sutter Instrument Company	B100-50-10
Dexamethasone	Enzo Life Sciences	BML-EI126-0001
DMSO	Sigma-Aldrich	D2438-5X10ML
E3 media		N/A
Femtotips Microloader Tips	Eppendorf	930001007
Fetal Bovine Serum (Premium Heat Inactivated	Atlanta Biologicals	S11150H
ImageJ	FIJI	N/A
Iscove's Modified Dulbecco's Medium	STEMCELL Technologie	36150
Large Particle (LP) Sampler	Union Biometrica	N/A
Methotrexate	Sigma-Aldrich	A6770-10MG
Mineral Oil	Fisher Scientific	BP26291
Phosphate Buffered Saline (1x)	Caisson labs	PBL06-6X500ML
Stage Micrometer (400-Stage)	Hausser Scientific	400-S
Tricaine-S	Pentair Aquatic	TRS1
Trypan Blue	Thermo Fisher	T10282
VAST Bioimager	Union Biometrica	N/A
Vincristine Sulfate	Enzo Life Sciences	BML-T117-0005
Vybrant DiI Stain	Thermo Fisher	V22885

Comments/Description
VAST is compatible with a variety of standard or deep well 24, 48, or 96 well plates
5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4
https://imagej.net/Fiji
automated sampler unit http://www.unionbio.com/copas/features.aspx?id=8
fluorescent equipped microscope imaging unit https://www.unionbio.com/vast/

Editorial comments:
Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have checked over the manuscript for spelling and grammar errors before submitting.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: VAST BioImager, Vybrant, Excel, LP Sampler, etc.

Commercial language has been removed.

3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "VAST BioImager" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

VAST BioImager and LP Sampler have been changed to "a fluorescence microscope equipped imaging unit and automated sampler unit".

4. Summary: Please shorten it to no more than 50 words.

Summary word count = 44.

5. Abstract: Please do not reference Figure 1 here.

This reference has been removed.

6. Please use imperative tense throughout the protocol as if directing someone how to do your experiment. Any text that cannot be written in the imperative tense may be added as a "NOTE".

This has been corrected. Text not in imperative tense has been moved to notes below the protocol steps.

7. 3.2: Please describe how to dechorionated zebra fish.

A description of both manual and enzymatic dechorionation has been added to section 3.2.

8. 3.5: Please specify the injection site here.

We specified in the note after section 3.5 that pericardium and yolk were used as injection sites for this experiment.

9. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Shorter steps have been combined with 2-3 actions per step with 4 or less sentences per step.

10. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

We have highlighted ~2.5 pages of protocol text to be featured in the video.

11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Highlighted text meets these requirements.

12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

All needed details were highlighted.

13. Please do not highlight any steps describing anesthetization and euthanasia.

Anesthesia steps were excluded from highlighting.

14. Figure 1: Please replace "VAST BioImager" and “LP Sampler” with generic terms.

We updated these terms to “fluorescence microscope equipped imaging unit” and “automated sampler”.

15. Please include a scale bar, ideally at the lower right corner, for all microscopic images to provide context to the magnification used. Define the scale in the appropriate figure Legend.

Scale bars have been added to all microscopic images with scales defined in the figure legends.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript by Haney and colleagues focuses on a standardized workflow for drug

screening in a zebrafish model containing xenografted tumors. The authors use a commercial platform (VAST) to image fluorescent reporter zebrafish treated with drug compounds. Factors such as size and intensity of the fluorescent reporter molecule can be quantified along with proper longitudinal orientation of the fish for high-throughput imaging.

The protocol is overall straight-forward in the presentation of the data and techniques, and can be easily adapted by most fish laboratories interested in developing a more high-throughput means of evaluating drug responses. There are some key technical details that are missing that are essential for a methods-heavy protocol paper such as this one. Minor clarity and typo issues also should be addressed before the manuscript is suitable for publication.

We thank the reviewer for careful reading of our manuscript and have addressed their concerns as outlined below.

Major Concerns:
1. A table with the zebrafish lines used fully-reported (citations, and proper nomenclature) for this study is essential. This is needed for the scientist whom is trying to follow this protocol and use these lines as experimental controls.

We have added as a note after step 3.2 that Casper (roy$^{-/-}$;nacre$^{-/-}$) zebrafish were used in this experiment with appropriate citation. This was the only line used for this study; we also noted that any zebrafish strain can be used to carry out this experiment.

2. More details should be given on how the PBMC leukemia patient sample was originally purified. A few sentences and/or a technical citation of this protocol would suffice.

This was added into a note after step 1.1.

3. A zebrafish rag2 mutant (Tang et al., Nat. Methods, 2014) exists for better engraftment of human tumor cells. The authors should cite this paper, and compare engraftment potential in an immunodeficient zebrafish model versus non-immunodeficient zebrafish.

The rag2 mutant zebrafish line and its prkdc$^{-/-}$ counterpart are not able to engraft human cells without pre-conditioning with irradiation. The il2rga-/-;prkdc-/- zebrafish line is able to engraft human cells but must be maintained as heterozygotes and genotyped with each cross to identify the double knockout animals. Animals must also be treated with clodronate liposomes to deplete macrophages for engraftment to occur. These issues make this line impractical for large-scale drug screening in larval fish, as we present here, as larvae do not develop an immune system until 7dpf. These knock-out fish would be useful for xenograft in older fish, and we have highlighted the benefits and drawbacks of these fish in the discussion, lines 384-391.

Minor Concerns:
1. Abstract: There is a space missing between "vivo and drug screen" on the first/title page. Not sure if this is a formatting issue as it does not appear later on in the manuscript body.

We have corrected this issue.

2. It would be highly beneficial to a user of this protocol to know how many trypan blue-positive (e.g. dead) cells were obtained from the thaw and what type of viability percentages the user can expect.

This is a good point, and we have added a note after step 1.6. "Viability should be >85% to use for xenografting. In this experiment, cell viability was 96% after thawing, assessed by trypan blue staining."

3. Please clarify the exact age of the injected zebrafish larvae. It appears to be 2 dpf with the mention of dechorination, but this should be stated explicitly in the methods.

We have clarified in step 3.5 that 2 dpf zebrafish are anesthetized and injected.

Reviewer #2:
Manuscript Summary:
This paper presents a thorough yet concise description of xeno-grafting human ALL into 2 dpf zebrafish larvae, performing in vivo drug treatment, and then quantitatively imaging responses by automated VAST and LP Sampler technologies. It is well-written, easily understood, and the introduction & discussions sections have appropriate depth of content and consideration of limitations and alternatives. There are no significant flaws, and I have only minor suggestions for further improvement.

Major Concerns:
none

Minor Concerns:
1. Line 120: PBMCs were used, but not all cells in this sample (or any other PBMC sample) are malignant. Could the authors briefly comment on this? Injecting 500 cells/larva differs if the sample is 20% vs. 90% ALL blasts. Since control & experimental larvae receive identical cell#, it is internally controlled, but xeno-grafts will vary from one cancer sample to another. Obviously, engraftment rates will vary from sample-to-sample too, so brief mention of both of these considerations seems warranted.

This is a very good point. We have addressed this issue in the discussion, lines 378-381.

2. Line 183: Could 3 dpf larvae (which don't require dechorionation) instead of 2 dpf larvae be used? Maybe with shorter drug treatment time? If not, why not?

This is a good thought, however we wanted to stay out of the window where the adaptive immune system starts to form and can confound results of drug screening. Additionally, the older the fish are, the less drug that will be absorbed through the skin. With regards to shortening treatment time, in our hands, we did not see a significant decrease in xenografted tumor mass in drug treated larvae until 72hr of drug treatment, although this may vary depending on drug used. We have added mention of chemical dechorionation with pronase as an alternative to mechanical dechorionation in step 3.2, and added a note after step 4.5 addressing optimal length of drug treatment.

3. Line 248: How many days were larvae treated with drug (this is mentioned later, but seems like it belongs here too), and how long can larvae be treated in 96-well plates? When do they outgrow them?

Larvae need to be fed after 7 dpf, so drug treatment should not go beyond this timepoint for large scale drug screens for practicality reasons. We have added a range of drug screening options from 2-4 days of treatment in a note following step 4.5.

4. Lines 249-334, Section 5 "Imaging xenografted zebrafish using VAST BioImager and LP Sampler": This entire section is very detailed, perhaps too much so. Most readers likely do not have access to VAST/LPS, so perhaps this level of detail isn't warranted. And if readers do have access to VAST/LPS, maybe they would already be familiar with how to use these instruments? Basically, it's not clear to me whether every click & setting needs to be spelled out. I leave it to the authors to decide...

Thank you for this feedback, this is a good point. We have edited down this section significantly to include only details that are unique to this experimental setup, which may not be included in training when learning how to use this equipment.

5. Line 352: It seems the preceding sentence refers to Figure 3A specifically, not all of Figure 3.

This has been changed to Figure 3A.

6. Line 377: What % of PBMC were T-ALL cells in this particular sample?

This patient had >90% circulating blasts at time of diagnosis. This was added to line 376.

7. Line 383: An example dexamethasone-treated fish in Fig. 3A would be great too.

Examples for dexamethasone-treated fish have been added in Figure 3A.

8. Line 400, Title for Figure 2: this isn't truly a "representative schematic" of xenografted fish. There are real images, so to me, they're "representative images."

Changed to “representative images”.

9. Lines 405-409 are very useful in explaining which fish to use for testing, and which to discard & why. Seems like this belongs in the protocol too.

This has also been added to step 4.1 in the protocol.

10. Line 415: mentions a "yellow outline" in Figure 3, but I don't see this in the figure itself.

Figure 3 legend has been updated to state that “Pixels above the selected threshold appear in black, while pixels below the threshold appear in white.”

11. Figure 1: Image quality is poor, quite grainy--both on my monitor & when printed out. Even the text isn't crisp.

We have exported the figure as a .psd file for print with the highest possible resolution.

12. Figure 2: A scale bar would be useful for non-zebrafish investigators, who may have no idea the size of zebrafish larvae.

Scale bars have been added to zebrafish images in Figures 2 and 3.

13. Figure 3: Text is grainy in this figure too, esp. Fig. 3B.

Text has been re-done to make more clear and exported as a .psd with the highest resolution possible.

Reviewer #3:

Manuscript Summary:
This manuscript describes a standardized workflow for drug screening using zebrafish as a host. The authors provide a detailed workflow with an example using peripheral blood mononuclear cells from T-ALL patient.

Major Concerns:
I have no major concerns.

Minor Concerns:
1) The authors are quantifying areas and not volumes as written in the abstract.

The text has been changed to area in the abstract and in the figure legend for Figure 3.

2) I would tune down the interpretation in the abstract where the authors state that this method allows for "...rapidly screen for actionable mutations using targeted therapies in order to stratify patients better into ongoing clinical trials". I think that this is not realistic perspective for now, especially as the authors only show one example in the paper.

The sentence about stratifying patients into clinical trials has been removed from the abstract.

3) I would also be more cautious with the statement on abstract that relates with ability of this assay to measure the interaction with microenvironment, especially when taking into account that the injection sites (yolk, pericardium) are not the natural environment for hematopoiesis. Also, the described method uses zf larvae and not mature fish, and one can assume that there are marked differences in the microenvironment at different stages of development.

This is a very good point. This statement in the abstract has been changed to read: "…allows for growth of tumor cells in an in vivo environment during drug treatment."

4) Transparency can also be achieved by blocking melanin synthesis with PTU (e.g. see Paatero et al., 2018 "zebrafish embryo xenograft and metastasis assay"; DOI: 10.21769/BioProtoc.3027). Maybe the PTU treatment should be described in the "Protocol", as not everyone has access to transparent zf lines.

This is a good point. This has been addressed in a note after step 3.2 stating "Casper (roy$^{-/-}$;nacre$^{-/-}$) zebrafish were used for these experiments. Any zebrafish larval strain can be used for xenografting, as the fish will not have enough pigment as larvae to interfere with imaging of xenografted tumor cells and quantification. However, if pigment interferes with imaging or visualization, propylthiouracil treatment can be used to block melanin synthesis if optically clear zebrafish strains are not available."

5) What is the zebrafish line that authors are using in this paper?

This has been addressed in the note added below step 3.2. We used Casper (roy$^{-/-}$;nacre$^{-/-}$) zebrafish for this experiment as they are readily available in our laboratory.

6) Protocol 1.6.2. Could authors indicate/estimate how many zebrafish one can inject within 1-3 hours? How realistic is it to inject 1000 zf at one sitting?

This has been addressed in the note following step 3.6 in the injection section of the protocol. The text now states "Typically, after practice with the technique, 800-1,500 zebrafish larvae can be injected by a single person within the 1-3 hours that stained cells should be injected."

7) In Figure 3B, what is the number of injected fish? Four injections per drug or four separate injection groups?

Four injected fish were treated, imaged, and analyzed per group. Data are results from individual animals. Number of fish was added to the figure table in Figure 3B. This data table has been re-done to show average +/- standard deviation.

8) Are there differences between independent tumors in their ability to engraft? How long the human cells survive inside larvae? Maybe the authors could comment on these Qs.

This has been addressed in the discussion, lines 374-381.