# An efficient method to generate xenograft tumor models of acute myeloid leukemia and hepatocellular carcinoma in adult zebrafish

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## ABSTRACT

Zebrafish is emerging as a promising model for the study of human cancers. Several xenograft models of zebrafish have been developed, particularly in larval stages (< 48 h post fertilization) when the immune system of fish is not developed. However, xenografting in adult zebrafish requires laborious and transient methods of immune suppression (γ- irradiation or dexamethasone) that limits engraftment and survival of the tumor or fail to recapitulate specific characteristics of malignancies. Thus, the availability of a simple protocol to successfully engraft adult zebrafish, remains a challenge. The current study addresses this limitation and describes a robust method of xenografting in adult zebrafish. We describe a protocol that involves pre-conditioning of *Casper*, a pigmentation mutant of zebrafish with busulfan that led to a higher rate of engraftment of hepatocellular carcinoma and acute myeloid leukemia cells. To further ascertain the homing characteristics of the injected cancer cells, we transplanted adult zebrafish by two routes of administration and then studied their compartmentalization. This model presents a valuable alternative to rodents to study the biology of these cancers and also a cost-effective platform for evaluation of potential anti-cancer agents.

## 1. Introduction

In the absence of appropriate transgenic models for many malignancies, a xenograft-based tumor model can be a viable alternative to study the disease progression and to test novel therapies. The implantation of tumor cells into immune-compromised animals, especially the murine xenograft [1,2], is a widely-used technique to study mechanisms controlling cancer cell proliferation, survival and metastasis as well as to provide a platform for screening of potential drug candidates [3–8]. However, current techniques of xenografting in mouse rely mainly on using syngeneic or immune-compromised animals such as nude, NOD-SCID or the highly immune deficient NSG mice [2,3] to avoid rejection of the transplanted tumor cells. Such a strategy requires specific genetic strains which often prevents the analysis of immune-tumor cell interactions. For *e.g.*, human tumor cell xenografts grown in immune-compromised strains may fail to model the role of inflammatory response in cancer progression or to predict the efficacy of drug in patients. In addition, the maintenance and propagation of a substantial number of immunocompromised mice to develop xenografts is expensive and challenging.

Two attributes of the zebrafish system makes it attractive as an alternative model for cancer research: 1) transparency in the larval stage and optical clarity of adult strains such as *Casper*, which is a pigmentation mutant [9], thus making it easier to track the transplanted tumor cells. 2) higher fecundity of the animal; on an average a female zebrafish can generate ~ 200 embryos every day, allowing for the rapid scaling of animal numbers for genetic or drug screening at a lower cost. Due to these favorable characteristics, > 20 cancer models based on zebrafish are now established [10], including the highly malignant glioblastoma, melanoma, pancreatic cancers and leukemia [6,11–14]. The first model was developed in 2005, when the highly metastatic melanoma cells were injected at the blastula stage of zebrafish embryo [15]. Several laboratories later validated this pioneering work by testing several mammalian cancer cell lines through different routes of administration and developmental stages of zebrafish [16,17]. Zebrafish also serves, as a useful model in understanding hematological malignancies as the lineages and transcriptional regulators of hematopoietic cells are evolutionarily well conserved [2,18]. Pruvot et al. [7] first reported the transplantation of K562, Jurkat and NB4 human leukemia cell lines in zebrafish larvae at 48 h post fertilization (hpf). Subsequently, other models of myeloid malignancies have been reported [13,14,18,19]. Xenotransplantation methods have continued to improve over time, with approaches to reproducibly quantify cell proliferation more accurately, now available [20]. While larval transplantation models are widely available, generating them typically involves a tedious injection procedure into a large number of larvae [7].

Further these models have limited scope in studying tumor engraftment characteristics long-term [14] and the procedure itself is known to be associated with high mortality [7].

Transplantation in adult forms requires the zebrafish to be immune-compromised. This can be achieved by γ-irradiation which effectively suppresses the immune system for ~20 days [21]. Similarly, treatment of adult zebrafish with dexamethasone is also known to suppress B-cells and T-cells for ~30 days before rejection could occur [12]. The use of isogenic clonal zebrafish strains that have the same MHC haplotype, facilitates long-term engraftment in an immune-competent host. Unlike rodents, there are very few clonal strains available in zebrafish and the fecundity of these clonal strains is relatively low, thus making it difficult to maintain these lines [10,22]. A more recent approach to enable long-term transplantation of the xenograft is to use immune deficient lines such as the recently developed rag2$^{E450fs}$, which is the first immune-compromised model of zebrafish [23].

A major limitation with the current xenotransplantation protocols in zebrafish is the inability to engraft adult fish with high efficiency and for an extended duration [17], except when immune deficient lines are used. In addition, a majority of protocols utilize zebrafish larvae (at 48 hpf) when its immune system has not been established. Alternately, the use of immune ablation with irradiation or dexamethasone has also demonstrated considerable mortality and toxicity [21,24] and thus contributing to a reduced efficiency of the experimental system. Busulfan is a recommended drug used in the conditioning regimen for bone marrow transplantation in case of acute myeloid leukemia (AML) and the second line of treatment for chronic myeloid leukemia [25,26]. Busulfan has been also used in murine models for successful long term (up to 5 weeks) engraftment of hematopoietic cord blood cells and is associated with minimal toxicity [2,27,28]. We thus reasoned that re-purposing this drug, as a myelosuppressive agent in zebrafish would be beneficial. Using this approach, we demonstrate a simple and reproducible method of xenotransplantation in adult zebrafish with a higher rate of engraftment for myeloid leukemia or hepatocellular carcinoma tumor model.

## 2. Methods

### 2.1. Cell culture and zebrafish maintenance

Human leukemic monocyte lymphoma cell line (U-937) cell line was a kind gift from Dr. Vikram Mathews, Christian Medical College, Vellore, India and a human hepatocellular carcinoma (Huh7) cell line was a kind gift from Dr. Saumitra Das, Indian Institute of Science, Bangalore. Cell lines were maintained in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, USA). The cells were grown in the presence of 10 μg/ml each of piperacillin and ciprofloxacin at 37 °C with 5% CO2. The cell lines used in this study were authenticated by short tandem repeat (STR) profiling to ascertain the identity of the cells and to identify any cellular cross-contamination or genetic drift with routine culturing.

The breeding and maintenance of zebrafish were as per established protocols [29]. For our experimentation we used a Casper strain (A kind gift from Dr. Zon's Laboratory, Children's Hospital Boston) [7,14,30]. The fish was maintained at 28 °C in an automated stand-alone housing system (Aquatic Habitats, Pentair, North Carolina, USA) with a 14 h light/10 h dark cycle. Animals were always handled and injected according to the principles of Good Animal Practice. Embryos were maintained at 35 °C after transplantation procedures. The age of embryos is indicated in hours post-fertilization (hpf). The details of reagents and materials used in the study is provided in additional file 1.

### 2.2. Fluorescence labeling of cells

Approximately, 3 to 5 million cells were pelleted at 300 g for 10 min followed by a wash in 1X Phosphate buffered saline (PBS). Cells were stained by 5 micromolar cell tracker (Molecular Probes, Life Technologies, Carlsbad, USA), a lipophilic fluorescent tracking dye for 10 to 15 min at 37 °C. The cells were then centrifuged and washed with PBS, re-suspended in serum-free medium and kept on ice before injection [30]. Labeling efficiency was ~100% as assessed by imaging under the fluorescence microscope.

### 2.3. Cancer cell implantation in zebrafish embryo

Prior to xenotransplantation, the 48 hpf embryos were dechorionized mechanically, using fine forceps. We used ~100 numbers of embryo for the transplantation procedure. The experiments were repeated twice, independently. We anesthetized, 10 to 20 embryos at a time by immersing them in tricaine solution (Sigma Aldrich, Merck Group, Missouri, USA). Subsequently, they were transferred onto a flat 1.5% agarose injection plate using a glass Pasteur pipette and the excess egg water was drained so as to hold the embryos in place during the injections and to maintain them in a moist condition. Embryos were then arrayed onto the injection plate for the xenotransplantation procedure. Approximately, $2 \times 10^6$ labeled cells were resuspended in 100 μl of PBS and used for the injections. The glass needles were filled with the cell solution (~2 μl) and a small amount of solution was then expelled into the yolk sac near the duct of Cuvier using Femtojet injector (Eppendorf, Hamburg, Germany). About, 300 to 500 cells were injected per embryo. The injected embryos were allowed to recover at 28 °C for one hour followed by incubation at 35 °C for the rest of the experiment.

### 2.4. Cancer cell implantation in adult zebrafish

For transplantation procedure in adult fish, we used an immune suppressed Casper strain. For the immune suppression, we administered busulfan in the intraperitoneal region of the zebrafish, at a dose of 20 mg/kg body weight, two days prior to cell transplantation. Each adult fish received ~7 μg of busulfan suspended in a final volume of 2 μl. The dosage of busulfan was selected based on available literature [2,31]. We first anesthetized the zebrafish in tricaine solution (150 mg/l). The state of sedation or the loss of equilibrium was ascertained to be complete, if the fish reclined laterally for > 3 s and did not respond to stimuli. We then administered 2 μl of fluorescently labeled cancer cell suspension containing $1 \times 10^5$ cells into the site near dorsum aorta of the recipient fish using a 5 μl hamilton syringe (Hamilton, Nevada USA). Subsequently, the fish were maintained in a recovery tank for 1 h at 28 °C and then at 34 °C for serial monitoring. To avoid any infections, fishes were placed in normal tank water containing 1% penicillin and streptomycin (Gibco) for 24 h, post-injection.

### 2.5. Imaging

To image adult fish, we anesthetized the fish at day 3, 5 and 15 post cancer cell injection and as described previously [9,18,21]. Casper fishes were used because of the greater transparency of their body, which facilitates *in vivo* monitoring of the tumor. Subsequently, the fish was mounted on the microscope stage and was imaged with a Leica M205FA fluorescent stereoscopic microscope with a Leica DFC310 FX camera (Leica Microsystems, Wetzlar, Germany). For fluorescent imaging, a red channel filter was selected. Images were captured at a uniform setting of 12.5× magnification, 1 s exposure time with 2.5% gain at different time points used in the experiment.

To image the larvae, we anesthetized the transplanted larvae in a petri dish containing egg water with 2.5 mM Tricaine. The larvae were further aligned laterally with a hair loop tool for imaging under the fluorescence stereomicroscope. Lateral view imaging of the larvae was performed since the larvae lie flat on their sides until the swim bladder has inflated (1–5 days post fertilization [dpf]). Beyond this time point,

larvae were imaged in a 96-well dish or by placing the larvae in a drop of egg water. Alternatively, if a position other than the lateral view was required, we mounted the larvae in 1.5% methylcellulose for imaging. Images were captured at a uniform setting of 25× magnification, 1 s exposure time with 2.5% gain at both time points used in the experiment.

### 2.6. Histology

For histological analysis, zebrafish were first anesthetized in tricaine solution and then humanely euthanized, by placing the fish in ice-cold water as described earlier [32]. These fishes (n = 3) were further dissected as per established protocols [33] to harvest the tumor tissue. Subsequently, tumor was washed in 1XPBS followed by an overnight fixation in 10% neutral buffered formalin. The samples were then washed twice in 1XPBS, followed by dehydration in graded ethanol and xylene for tissue clearing and then paraffin embedded. About 5 μm sections were cut longitudinally by manually positioning the paraffin block in their final orientation on a microtome. Tissue sections were collected on a slide and stained with hematoxylin and eosin as described previously [34,35].

## 3. Results

### 3.1. Xenotransplantation

The protocol followed for the xenotransplantation is represented in Fig. 1. The engraftment of human cells in zebrafish can be easily monitored if the cell line is expressing a reporter gene or is transiently labeled with a fluorescent marker. In our study, we used a lipophilic dye to label the cancer cells. This method has been previously described [36] to monitor various cell types in xenograft models [6,8,14,37,38]. Furthermore, we used *Casper* fish to assess the engraftment of the cancer cells as these fish are pigmentation deficient, but are immunologically competent.

In our strategy, labeled cells were initially transplanted into zebrafish larvae or immunosuppressed adults near the dorsal aorta (Fig. 2). Tumor cells were then traced at day 0, 24 or 72hpi in zebrafish larvae or days 0, 7 and 15 after transplanting in adult fish using a fluorescent stereomicroscope. In our observation, this simple technique for transplantation of cells had a reproducible consistency without causing any damage to the cancer cells and also largely preserved the viability of the recipient zebra fish immediately after cell administration.

### 3.2. Tumor engraftment in zebrafish larvae

Several models of AML in zebrafish have been reported employing multiple cell types including K562, NB4, Jurkat cells [7,14,18]. U937 is a highly aggressive leukemic cell line first developed and described by Sundström et al. [39]. U937 cells have been demonstrated to develop myeloid leukemia as early as 4 weeks in NSG (NOD-SCID-γ irradiated) mice [2], and such models have been employed to screen anti-leukemic drugs for their potential therapeutic application in AML [40,41]. Since U937 cells have not been previously utilized for generating a tumor model in zebrafish, we first ascertained its toxicity by microinjection of U937 cells into the 2-day-old zebrafish larvae. We observed that administration of labeled U937 cells in these larvae was reasonably tolerated and ~50% of larvae remained viable at 72 hpi. A set of representative data for the larval injections is shown in Fig. 3. Our data corroborates well with previous zebrafish AML models developed with K562 cells, where a similar pattern of survival was observed [7,19].

### 3.3. AML model of adult zebrafish

The main limitation in developing a tumor model in an adult zebrafish is the functional immune system [42,43], which requires that the animal should be immune-suppressed so as to allow engraftment of transplanted cells. With our protocol employing busulfan as an immunosuppressant, we observed an engraftment in ~87% of fishes (n = 20 out of 23) injected with U937 cells, 3 days after transplantation (Fig. 4). However, the engrafted cell density varied between the recipient fishes possibly due to procedural variation or recipient characteristics. At the day 3-time point (Fig. 4), the injected cells were seen to home around the gastrointestinal area and evidence of tumor growth was seen in this area for the next two weeks. Grossly, the tumor emerged as a moderate enlargement of the peritoneal area (Fig. 4). This feature on homing has been previously described by Mizgirev et al. [44] where the authors used a clonal homozygous zebrafish line CG2, to transplant the leukemic cells in larval zebrafish (5 dpf) and observed the engraftment of tumor within the peritoneal cavity after 10 days of transplantation. Interestingly, the lack of U937 cell migration into specific anatomically defined sites such as thymus of zebrafish may be due to the absence of sufficient cues for engraftment or the absence of an optimal niche in zebra fish.

In our study, the engraftment of U937 cells occurred within 3 days and by day 7, 65% (n = 13/20) of U937 injected fishes survived. Two weeks after xenotransplantation, the overall survival was ~45% (n = 9/20)] (Fig. 5). Further dissection of the U937 cell transplanted adult fish, revealed that the tumor filled the entire intestine and appeared as a homogenous red cell mass under a fluorescent stereomicroscope. Subsequently, the tumor was harvested in PBS and further histological examination was performed. Since this tumor was seen to home to the gastrointestinal area, intestine of mock-injected (control) fishes were also dissected and used for comparison in histological analysis. Hematoxylin and eosin staining of the tumor mass revealed compacted cell aggregates that clustered in the intestine, disturbing the normal intestinal architecture and further confirmed the establishment of U-937 xenograft (Fig. 6).

### 3.4. Comparison of cancer cell homing characteristics in adult zebrafish

We next wished to examine if intraperitoneal homing of the U937 cells was specific to the cell type or the route of cell administration. To understand the former, we administered Huh7 cells through the site near dorsal aorta (n = 14), as done for U-937 cells into the adult zebrafish (n = 23). The homing of cells was visualized using fluorescence imaging. As can be seen in Fig. 7, the Huh7 cells homed into the peritoneal cavity, 7 days after its administration, suggesting that the peritoneal cavity may be the preferred niche for both U937 and Huh7 cells.

In the second set of studies, we performed an intra-cardiac delivery of U937 cells so that the cells are in an active site of blood flow and thus have an opportunity to home suitably as per the cues provided by the host microenvironment. We observed that the intra-cardiac administration of U937 cells had a high mortality rate (~50%, n = 5/10) as the heart is often punctured during injection, leaving the fish prone to infections or a massive blood loss as described earlier [45]. Interestingly, the homing of these U937 cells was found to be in the peritoneal cavity in spite of their administration through the cardiac route (Fig. 7).

### 3.5. Engraftment rate

We further compared the survival rates of the recipient adult zebrafish that had undergone xenotransplantation with either Huh7 or U937 cells by Kaplan-Meier analysis (Fig. 5). In case of adult fish transplanted with U937 cells, we observed a 35% lethality (n = 7/20) within the first week. These findings were similar to previous studies with K-562 cell model of leukemia in zebrafish [19]. Interestingly, xenotransplantation of Huh7 cells in zebra fish had a delayed onset of lethality with approximately 30% (n = 4/14) of deaths occurring in the second week after cell transplantation. As a result, the overall survival rates were variable at the end of two weeks after transplantation (~
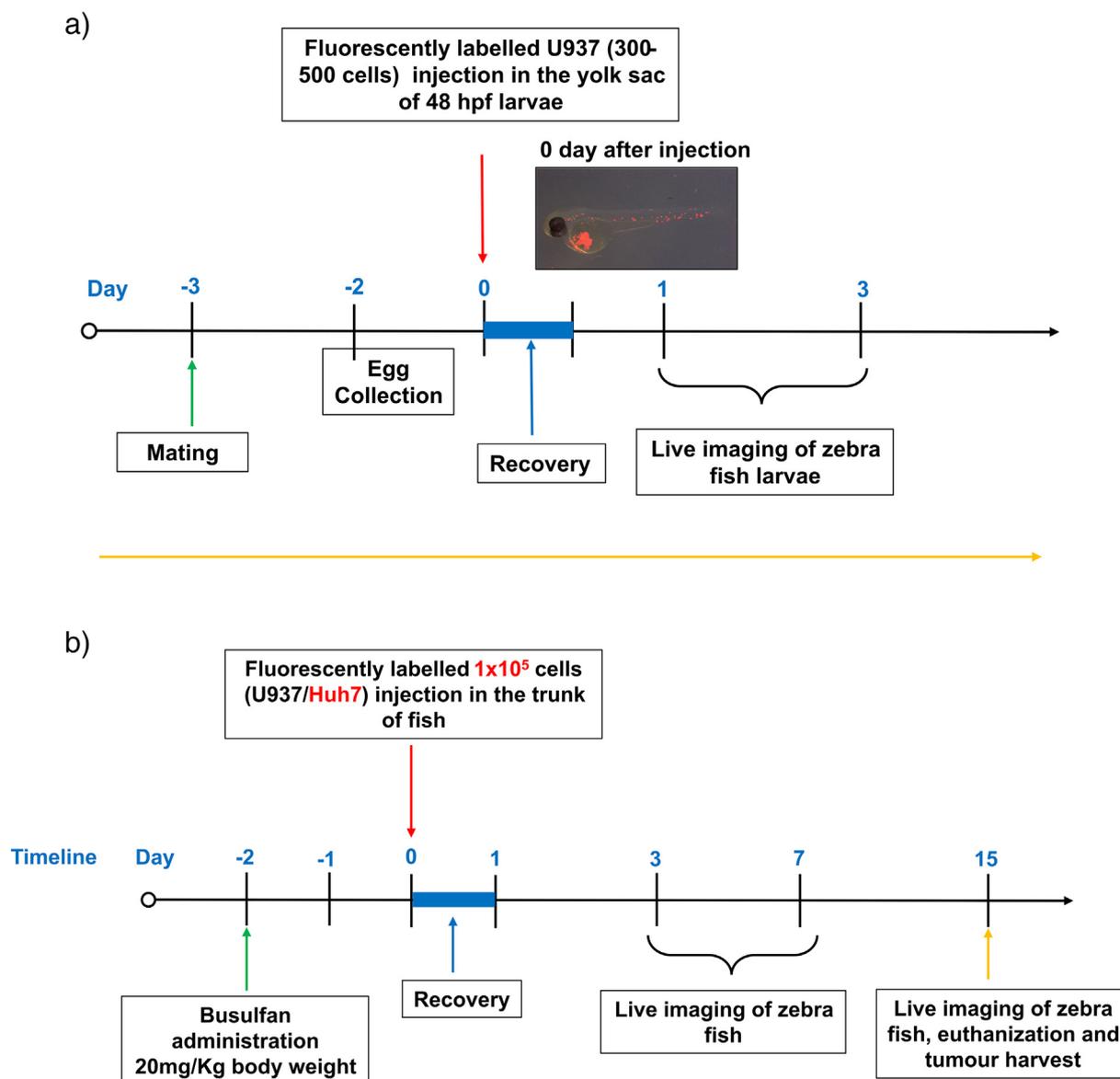
a)



b)



**Fig. 1.** a. Schematic representation of xenograft development in zebrafish larvae: Two days prior to transplantation fertilised eggs were collected by placing male and female zebrafish in mating tank. Twenty four hours before xenotransplantation, embryos were dechorionized manually using forceps. Just before xenotransplantation the dechorionized embryos were anesthetized in 2.5 mM tricaine in embryo medium. Approximately 300–500 cancer cells were injected into the yolk sac of 48hpf embryo. Injected embryos were monitored for engraftment and tumor progression by imaging at 24 and 72 h post injection (hpi). b. Schematic of xenograft development in adult zebrafish. Immune-suppression was achieved by injecting busulfan (i.p.) two days prior to cancer cell administration. Injected fishes were monitored for engraftment and tumor progression by imaging at 3, 7 and 15 dpi. The fishes were monitored daily for any signs of distress and maintained according to established protocols [1].
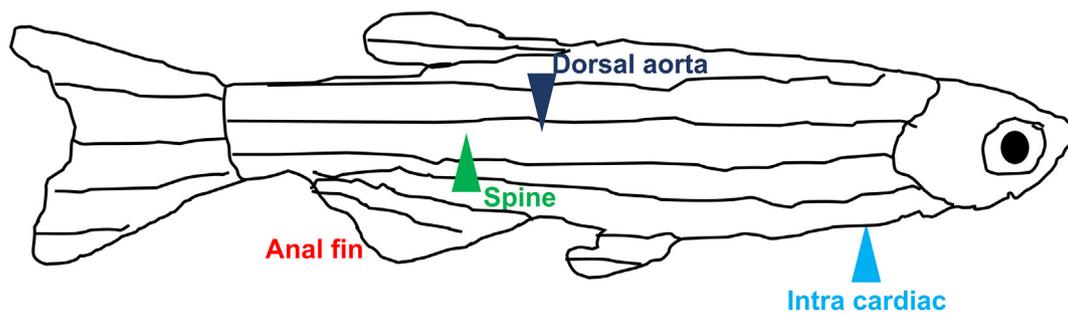


**Fig. 2.** Injection sites for administration of leukemic cells in adult zebrafish. Dorsal aorta site can be identified by bleeding the fish using a pulled glass capillary of 1 mm in size and inserting it near the spine and above the anal fin. Intra-cardiac site can be accessed by placing the fish ventrally in a wet sponge. Heart can be identified by its rhythmic movement.
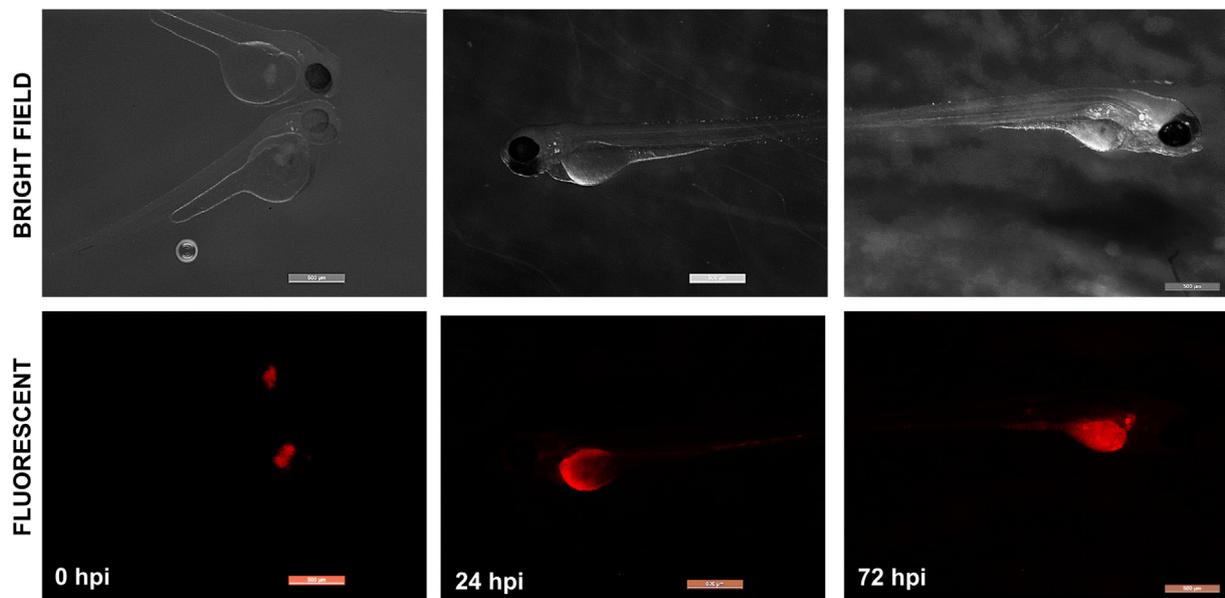
**Fig. 3.** U937 leukemic cell xenograft in the larval stage. Representative bright field and fluorescent images of zebrafish larvae transplanted with human U-937 cells at a dose of 300–500 cells per larvae, at time points 24 and 72 h post injection (hpi) are shown. Images were taken using Leica M205FA fluorescent stereoscopic microscope assisted with Leica DFC310 FX camera (Leica Microsystems, Wetzlar, Germany). Proliferation of the engrafted cells can be seen 72hpi with the presence of fluorescently labeled U-937 cells in the tail region (marked by an arrow).
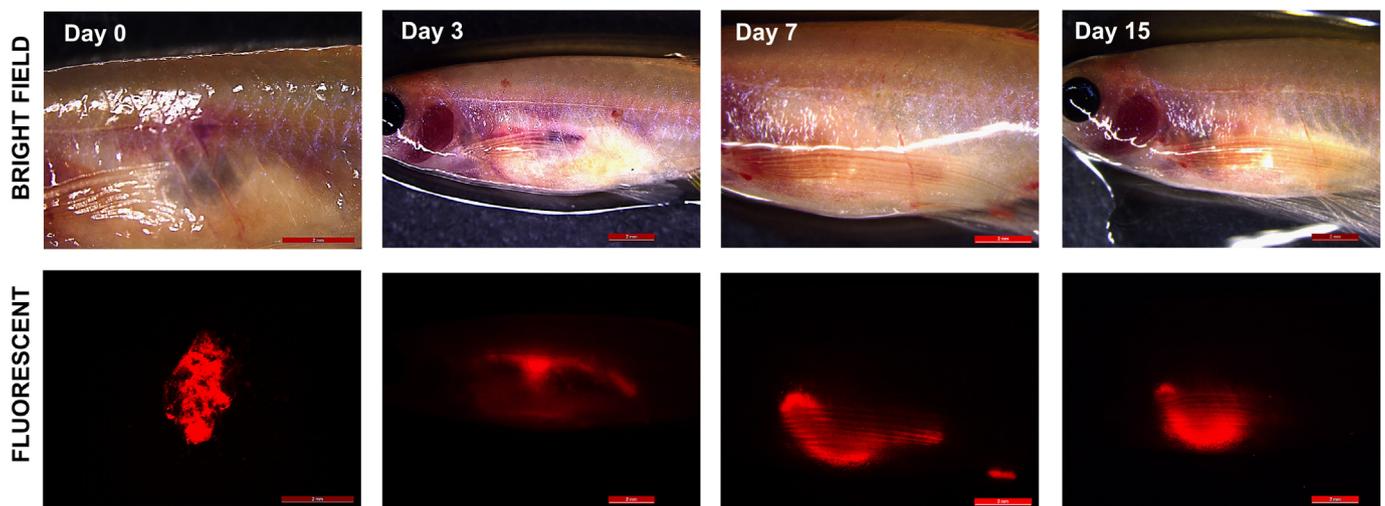


**Fig. 4.** AML tumor model in adult Casper strain of zebrafish. Human leukemic cells (U937) were injected into immune- suppressed Casper zebrafish and images were taken 3, 7, and 15 days post-transplantation in a Leica M205FA fluorescent stereoscopic microscope. The presence of bulky tumor across all time point can be morphologically visualized in the upper bright field panel while the tumor is represented as dense collection of red fluorescent cells in the lower panel. The parameters used for imaging were uniform throughout different time points used (Zoom: 12.5×, Exposure: 1 s, Gain: 2.5×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

45% for U-937 injected fishes and 71% for Huh 7 injected fish). These data suggest that U937 cells generate aggressive acute myeloid leukemia in zebrafish, but it will be feasible to use these models to study the effect of any novel therapies for a medium-term follow up of two weeks.

## 4. Discussion

Xenograft models in zebrafish have been largely developed during the embryonic or larval stages [13,16–18]. Embryo xenograft models are used in short-term assays (< 7 days). These models have been widely used for analyzing tumor metastasis, neovascularization of tumors, therapeutic drug testing or high-throughput drug screening [6,12,14,18,46] for a limited time period (2–5 days post-

transplantation). As far as xenotransplantation in adults is concerned, there is limited data. Zhang B et al. have used adult zebrafish for developing xenograft with cancer stem-like cells (CSCs) and used this model as a platform for anti-CSC inhibitor screening [19]. Transplantation of human cancer cells (adenocarcinoma, MDA-MB-435) in 30 dpf zebrafish was first demonstrated by Stoletov and colleagues in 2007 by injecting cells into the peritoneal cavity in 1-month-old chemically immunosuppressed AB or Tg(fli1:EGFP) zebrafish with dexamethasone [12]. The tumor persisted in this model with a mean viability of 13.6 ± 0.45 days for transplanted fishes. Even tumor models developed in adult fish after irradiation prevents long-term studies since the fish immune system recovers within 20 days of irradiation [21]. Similarly, dexamethasone mediated chemical ablation of the immune system is effective for transplantation of solid tumors but not for
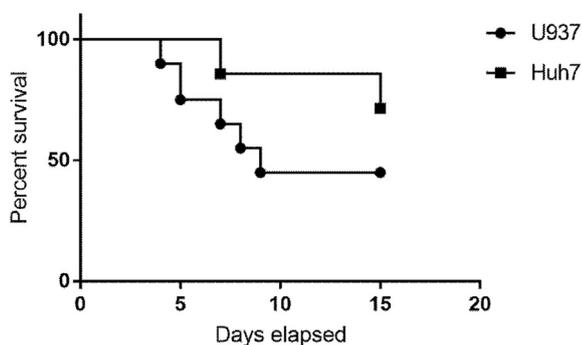
**Fig. 5.** Kaplan Meier survival analysis of adult zebrafish injected with cancer cells. The percentage of cancer bearing adults that survived is denoted as a function of time. Approximately 30–50% of injected fishes die as a result of tumor burden from both the cell lines, although the U937 cell injected fish demonstrate acute fatality within the first week of engraftment in comparison to Huh7 cells.

leukemia [47].

The method described here addresses the current limitation in adult zebrafish xenotransplantation technique by employing a combination of an immune-suppressant (busulfan) that has not been previously tested in zebrafish and the ability to track and visualize the tumor within the fish by employing a *Casper* based pigmentation mutant. This combination had a reproducible rate of engraftment tumor cells in the peritoneal cavity when administered near the dorsal aorta with minimal loss of tumor cells and an extended two-week period of viability in the transplanted fish. However, the experimental follow up in our protocol was for a limited time period (~15 days) and thus future studies are necessary to understand the timeline of recovery of the *Casper* fish immune system and its impact on tumor propagation.

This protocol will enable a researcher to create varied xenograft models, using diverse cell lines for understanding tumor biology, metastasis or potential therapeutic drug screening. However, conditions for the long-term growth of human cells in zebrafish still need to be identified. Presently, pre-clinical studies are dependent on mouse xenografts to evaluate the efficacy of chemotherapeutics [7,14,38].

Investigations in murine models are relatively tedious and expensive [7,18,19,48]. Since the oncogenic signaling pathways between zebrafish and humans [49] are highly conserved [48–50], it is likely that our technique will complement the conventional murine studies to allow rapid screening of novel therapeutics and *in vitro* studies to allow rapid identification of effective anti-cancer agents entering clinical trials. Different groups have utilized xenografts in adult zebrafish [37,51,52] to overcome these shortcomings, but additional time and optimization is necessary for adult xenograft models to become as widely used and standardized as embryonic xenograft platforms.

## 5. Conclusion

This xenotransplantation method is likely to be beneficial for a variety of applications. The superior engraftment of cancer cells in the presence of busulfan will enable multiple xenograft models to be developed using diverse cell lines for either understanding the tumor biology or tumor metastasis. Furthermore, busulfan based conditioning regimen can also simplify the existing patient-derived xenograft (PDX) model of zebrafish. These models are generally established by immune suppression with irradiation and have poor survival rates [21] but are useful for identification and characterization of the chemotherapy resistant and relapse-inducing cancer cells [18,53,54].

## Abbreviations

| | |
|---|---|
| hpf | hours post fertilization |
| dpt | days post-transplantation |
| dpi | days post-injection |
| hpi | hours post-injection |
| SCID | Severe Combined Immunodeficiency |
| NSG | NOD/LtSz-Prkdc^scid Il2r$\gamma$tm1Wjl/J (NOD-SCID-$\gamma$ irradiated) |

**CONTROL INTESTINE 1**

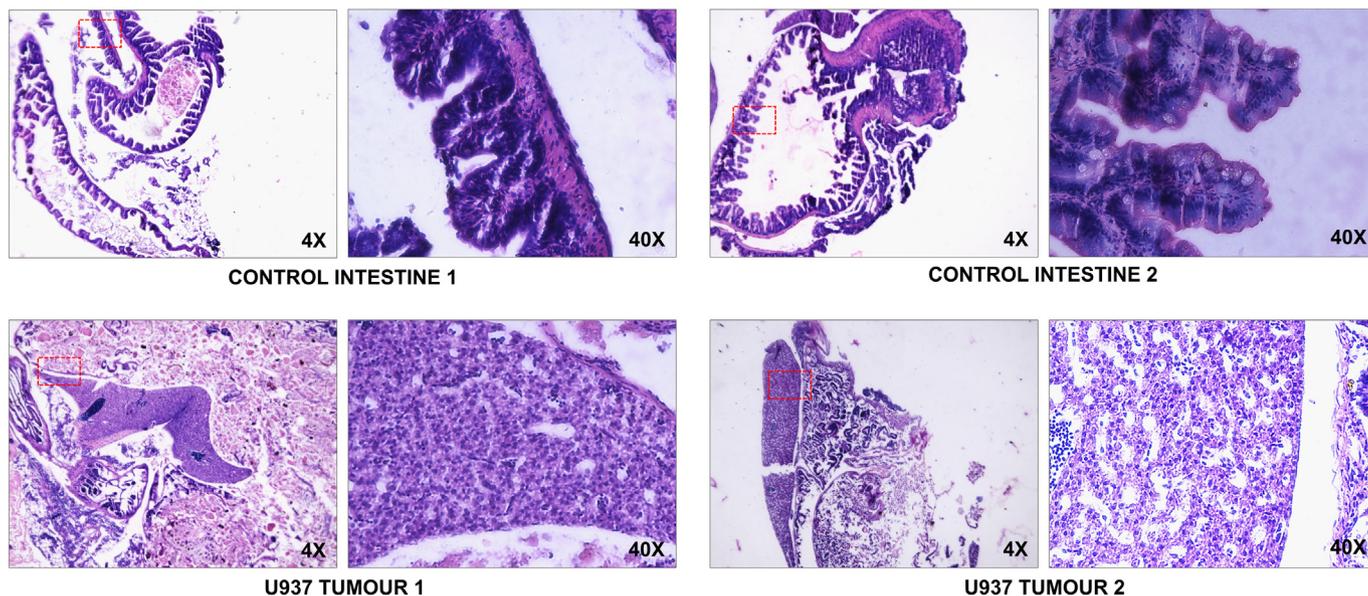**CONTROL INTESTINE 2**

**U937 TUMOUR 1**

**U937 TUMOUR 2**

**Fig. 6.** Histological analysis of AML tumor. Hematoxylin and eosin stained sections of control zebrafish intestine and tumor from zebrafish challenged with U937 cells, from two representative fishes for each group are shown. Top panel shows the normal architecture of zebrafish intestine. Bottom panel depicts tumor growth pattern and infiltrative nature as revealed by the neoplastic cells extending through the intestine and disturbing the normal architecture of the tissue. Magnification used for imaging is depicted in the figure. The framed region in red is magnified and shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
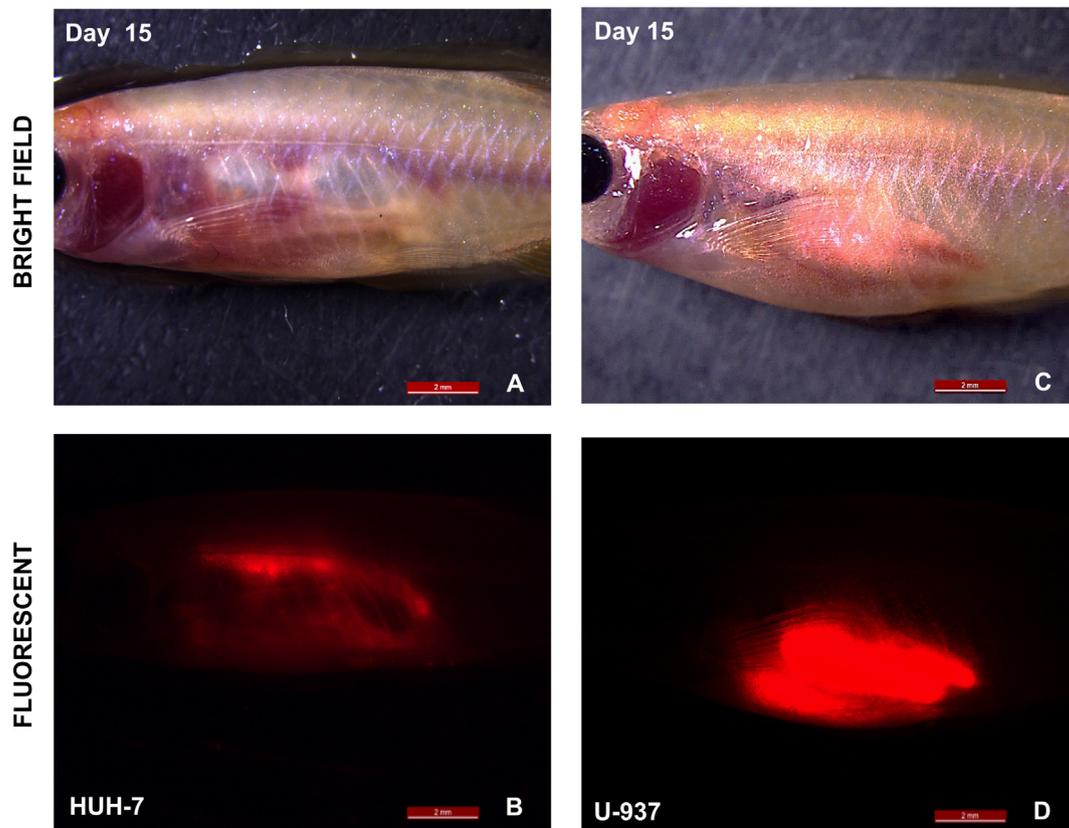
**Fig. 7.** Effect of cancer cell type or route of administration in tumor development in adult zebrafish. Representative image of adult fish transplanted with Huh7 cells demonstrating the xenograft developed 15 days post-transplant at the peritoneal cavity by bright field (Panel A) and fluorescent (Panel B) imaging. An alternative route of intra-cardiac administration of U937 cells (Panel C and D) shows the localisation of these cells to the peritoneal cavity, 15 days post transplantation.

## Availability of data and materials

The datasets generated during the study is available from the corresponding author on reasonable request.

## Author's contribution

NK performed the experiments; NKM performed optimization of larval injection/route of administration in adult fish; PS provided Zebrafish strain and microscopy facility; GRJ conceived and designed the experiments and interpreted the data; NK and GRJ wrote the paper.

## Competing interests

The authors declare that they have no competing interests.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcmd.2018.12.007.

## References

[1] W.-L. Shan, X.-L. Ma, How to establish acute myeloid leukemia xenograft models using immunodeficient mice, Asian Pac. J. Cancer Prev. 14 (2013) 7057–7063, https://doi.org/10.7314/APJCP.2013.14.12.7057.
[2] E. Saland, H. Boutzen, R. Castellano, L. Pouyet, E. Griessinger, C. Larrue, F. De Toni, S. Scotland, M. David, G. Danet-Desnoyers, F. Vergez, Y. Barreira, Y. Collette, C. Récher, J.E. Sarry, A robust and rapid xenograft model to assess efficacy of chemotherapeutic agents for human acute myeloid leukemia, Blood Cancer J. 5 (2015), https://doi.org/10.1038/bcj.2015.19.
[3] T. Troiani, C. Schettino, E. Martinelli, F. Morgillo, G. Tortora, F. Ciardiello, The use of xenograft models for the selection of cancer treatments with the EGFR as an example, Crit. Rev. Oncol. Hematol. 65 (2008) 200–211, https://doi.org/10.1016/j.critrevonc.2007.10.003.
[4] J. Jung, Human tumor xenograft models for preclinical assessment of anticancer drug development, Toxicol. Res. 30 (2014) 1–5, https://doi.org/10.5487/TR.2014.30.1.001.
[5] M. Luconi, M. Mannelli, Xenograft models for preclinical drug testing: implications for adrenocortical cancer, Mol. Cell. Endocrinol. 351 (2012) 71–77, https://doi.org/10.1016/j.mce.2011.09.043.
[6] J.Q. Wu, J. Zhai, C.Y. Li, A.M. Tan, P. Wei, L.Z. Shen, M.F. He, Patient-derived xenograft in zebrafish embryos: a new platform for translational research in gastric cancer, J. Exp. Clin. Cancer Res. 36 (2017) 1–10, https://doi.org/10.1186/s13046-017-0631-0.
[7] B. Pruvot, A. Jacquel, N. Droin, P. Auberger, D. Bouscary, J. Tamburini, M. Muller, M. Fontenay, J. Chluba, E. Solary, Leukemic cell xenograft in zebrafish embryo for investigating drug efficacy, Haematologica 96 (2011) 612–616, https://doi.org/10.3324/haematol.2010.031401.
[8] L. Mercatali, F. La Manna, A. Groenewoud, R. Casadei, F. Recine, G. Miserocchi, F. Pieri, C. Liverani, A. Bongiovanni, C. Spadazzi, A. de Vita, G. van der Pluijm, A. Giorgini, R. Biagini, D. Amadori, T. Ibrahim, E. Snaar-Jagalska, Development of a patient-derived xenograft (PDX) of breast cancer bone metastasis in a zebrafish model, Int. J. Mol. Sci. 17 (2016), https://doi.org/10.3390/ijms17081375.
[9] R.M. White, A. Sessa, C. Burke, T. Bowman, J. Ceol, C. Bourque, M. Dovey, W. Goessling, E. Burns, L.I. Zon, Transparent adult zebrafish as a tool for in vivo transplantation analysis, Cell Stem Cell 2 (2008) 183–189.
[10] R. White, K. Rose, L. Zon, Zebrafish cancer: the state of the art and the path forward, Nat. Rev. Cancer 13 (2013) 624–636, https://doi.org/10.1038/nrc3589.
[11] A.M. Welker, B.D. Jaros, V.K. Puduvalli, J. Imitola, B. Kaur, C.E. Beattie, Standardized orthotopic xenografts in zebrafish reveal glioma cell-line-specific characteristics and tumor cell heterogeneity, Dis. Model. Mech. 9 (2016) 1063–1065, https://doi.org/10.1242/dmm.027235.
[12] K. Stoletov, V. Montel, R.D. Lester, S.L. Gonias, R. Klemke, High-resolution imaging of the dynamic tumor cell vascular interface in transparent zebrafish, Proc. Natl. Acad. Sci. 104 (2007) 17406–17411, https://doi.org/10.1073/pnas.0703446104.
[13] J. Etchin, J.P. Kanki, A.T. Look, Zebrafish as a model for the study of human cancer, Methods Cell Biol. 105 (2011) 309–337, https://doi.org/10.1016/B978-0-12-381320-6.00013-8.
[14] C. Zeiss, Leukaemia xenotransplantation in zebrafish – chemotherapy response assay in vivo.pdf, Br. J. Haematol. 153 (2011) 786–789.
[15] L.M.J. Lee, E.A. Seftor, G. Bonde, R.A. Cornell, M.J.C. Hendrix, The fate of human malignant melanoma cells transplanted into zebrafish embryos: assessment of migration and cell division in the absence of tumor formation, Dev. Dyn. 233 (2005) 1560–1570, https://doi.org/10.1002/dvdy.20471.

[16] M. Konantz, T.B. Balci, U.F. Hartwig, G. Dellaire, M.C. André, J.N. Berman, C. Lengerke, Zebrafish xenografts as a tool for in vivo studies on human cancer, Ann. N. Y. Acad. Sci. 1266 (2012) 124–137, https://doi.org/10.1111/j.1749-6632.2012.06575.x.

[17] A.M. Taylor, L.I. Zon, Zebrafish tumor assays: the state of transplantation, Zebrafish 6 (2009) 339–346, https://doi.org/10.1089/zeb.2009.0607.

[18] B. Zhang, Y. Shimada, J. Kuroyanagi, N. Umemoto, Y. Nishimura, T. Tanaka, Quantitative phenotyping-based in vivo chemical screening in a zebrafish model of leukemia stem cell xenotransplantation, PLoS One 9 (2014) 1–9, https://doi.org/10.1371/journal.pone.0085439.

[19] B. Zhang, Y. Shimada, J. Kuroyanagi, Y. Nishimura, N. Umemoto, T. Nomoto, T. Shintou, T. Miyazaki, T. Tanaka, Zebrafish xenotransplantation model for cancer stem-like cell study and high-throughput screening of inhibitors, Tumor Biol. 35 (2014) 11861–11869, https://doi.org/10.1007/s13277-014-2417-8.

[20] P. Cabezas-Sainz, J. Guerra-Varela, M.J. Carreira, J. Mariscal, M. Roel, J.A. Rubiolo, A.A. Sciara, M. Abal, L.M. Botana, R. López, L. Sánchez, Improving zebrafish embryo xenotransplantation conditions by increasing incubation temperature and establishing a proliferation index with ZFtool, BMC Cancer 18 (2018) 3, https://doi.org/10.1186/s12885-017-3919-8.

[21] D. Traver, A. Winzeler, H.M. Stern, E.A. Mayhall, D.M. Langenau, J.L. Kutok, A.T. Look, L.I. Zon, Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation, Blood 104 (2004) 1298–1305, https://doi.org/10.1182/blood-2004-01-0100.

[22] G. Streisinger, C. Walker, N. Dower, D. Knauber, F. Singer, Production of clones of homozygous diploid zebra fish (Brachydanio rerio), Nature 291 (1981) 293–296, https://doi.org/10.1038/291293a0.

[23] Q. Tang, N.S. Abdelfattah, J.S. Blackburn, J.C. Moore, S.A. Martinez, F.E. Moore, R. Lobbardi, I.M. Tenente, M.S. Ignatius, J.N. Berman, R.S. Liwski, Y. Houvras, D.M. Langenau, Optimized cell transplantation using adult rag2 mutant zebrafish, Nat. Methods 11 (2014) 821–824, https://doi.org/10.1038/nmeth.3031.

[24] Y.M. Khor, T. Soga, I.S. Parhar, Caffeine neuroprotects against dexamethasone-induced anxiety-like behaviour in the zebrafish (Danio rerio), Gen. Comp. Endocrinol. 181 (2013) 310–315, https://doi.org/10.1016/j.ygcen.2012.09.021.

[25] N.D. Bueno, F.L. Dulley, R. Saboya, J.U. Amigo Filho, F.L. Coracin, D.D.A.F. Chamone, Busulfan and melphalan as conditioning regimen for allogeneic hematopoietic stem cell transplantation in acute myeloid leukemia in first complete remission, Rev. Bras. Hematol. Hemoter. 33 (2011) 179–184, https://doi.org/10.5581/1516-8484.20110050.

[26] A. Haddow, G.M. Timmis, Myleran in chronic myeloid leukæmia chemical constitution and biological action, Lancet 261 (1953) 207–208, https://doi.org/10.1016/S0140-6736(53)90884-8.

[27] M. Krevvata, X. Shan, C. Zhou, C. dos Santos, G.H. Ndikuyeze, A. Secreto, J. Glover, W. Trotman, G. Brake-Silla, S. Nunez-Cruz, G. Wertheim, H.J. Ra, E. Griffiths, C. Papachristou, G. Danet-Desnoyers, M. Carroll, Cytokines increase engraftment of human acute myeloid leukemia cells in immunocompromised mice but not engraftment of human myelodysplastic syndrome cells, Haematologica 103 (2018) 959–971, https://doi.org/10.3324/haematol.2017.183202.

[28] J. Hayakawa, M.M. Hsieh, N. Uchida, O. Phang, J.F. Tisdale, Busulfan produces efficient human cell engraftment in NOD/LtSz-Scid IL2Rγ null mice, Stem Cells 27 (2009) 175–182, https://doi.org/10.1634/stemcells.2008-0583.

[29] A. Avdesh, M. Chen, M.T. Martin-Iverson, A. Mondal, D. Ong, S. Rainey-Smith, K. Taddei, M. Lardelli, D.M. Groth, G. Verdile, R.N. Martins, Regular care and maintenance of a zebrafish (Danio rerio) laboratory: an introduction, J. Vis. Exp. (2012), https://doi.org/10.3791/4196.

[30] J. Ren, S. Liu, C. Cui, P. ten Dijke, Invasive behavior of human breast cancer cells in embryonic zebrafish, J. Vis. Exp. (2017) 1–9, https://doi.org/10.3791/55459.

[31] R.H. Nóbrega, C.D. Greebe, H. van de Kant, J. Bogerd, L.R. de França, R.W. Schulz, Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish, PLoS One 5 (2010) 1–16, https://doi.org/10.1371/journal.pone.0012808.

[32] J.M. Wilson, R.M. Bunte, A.J. Carty, Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (Danio rerio), J. Am. Assoc. Lab. Anim. Sci. 48 (2009) 785–789 http://www.ncbi.nlm.nih.gov/pubmed/19930828%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2786934 , Accessed date: 29 August 2018.

[33] T. Gupta, M.C. Mullins, Dissection of organs from the adult zebrafish, J. Vis. Exp. (2010), https://doi.org/10.3791/1717.

[34] J.E. Copper, L.R. Budgeon, C.A. Foutz, D.B. van Rossum, D.J. Vanselow, M.J. Hubley, D.P. Clark, D.T. Mandrell, K.C. Cheng, Comparative analysis of fixation and embedding techniques for optimized histological preparation of zebrafish, Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 208 (2018) 38–46, https://doi.org/10.1016/j.cbpc.2017.11.003.

[35] R.J. Nuckels, J.M. Gross, Protocol Histological Preparation of Embryonic and Adult Zebrafish Eyes, CSH Protoc, (2007), https://doi.org/10.1101/pdb.prot4846.

[36] W. Andrade, T.J. Seabrook, M.G. Johnston, J.B. Hay, The use of the lipophilic fluorochrome CM-DiI for tracking the migration of lymphocytes, J. Immunol. Methods 194 (1996) 181–189, https://doi.org/10.1016/0022-1759(96)00083-X.

[37] M. Guo, H. Wei, J. Hu, S. Sun, J. Long, X. Wang, U0126 inhibits pancreatic cancer progression via the KRAS signaling pathway in a zebrafish xenotransplantation model, Oncol. Rep. 34 (2015) 699–706, https://doi.org/10.3892/or.2015.4019.

[38] F. Tonon, C. Zennaro, B. Dapas, M. Carraro, M. Mariotti, G. Grassi, Rapid and cost-effective xenograft hepatocellular carcinoma model in zebrafish for drug testing, Int. J. Pharm. 515 (2016) 583–591, https://doi.org/10.1016/j.ijpharm.2016.10.070.

[39] C. Sundström, K. Nilsson, Establishment and characterization of a human histiocytic lymphoma cell line (U-937), Int. J. Cancer 17 (1976) 565–577, https://doi.org/10.1002/ijc.2910170504.

[40] K. Li, C. Hu, C. Mei, Z. Ren, J.C. Vera, Z. Zhuang, J. Jin, H. Tong, Sequential combination of decitabine and idarubicin synergistically enhances anti-leukemia effect followed by demethylating Wnt pathway inhibitor promoters and down-regulating Wnt pathway nuclear target, J. Transl. Med. 12 (2014) 167, https://doi.org/10.1186/1479-5876-12-167.

[41] H.S. Radomska, F. Jernigan, S. Nakayama, S.E. Jorge, L. Sun, D.G. Tenen, S.S. Kobayashi, A cell-based high-throughput screening for inducers of myeloid differentiation, J. Biomol. Screen. 20 (2015) 1150–1159, https://doi.org/10.1177/1087057115592220.

[42] A.C.H. Smith, A.R. Raimondi, C.D. Salthouse, M.S. Ignatius, J.S. Blackburn, I.V. Mizgirev, N.Y. Storer, J.L.O. De Jong, A.T. Chen, Y. Zhou, S. Revskoy, L.I. Zon, D.M. Langenau, High-throughput cell transplantation establishes that tumor-initiating cells are abundant in zebrafish T-cell acute lymphoblastic leukemia, Blood 115 (2010) 3296–3303, https://doi.org/10.1182/blood-2009-10-246488.

[43] M.S. Ignatius, D.M. Langenau, Zebrafish as a model for cancer self-renewal, Zebrafish 6 (2009) 377–387, https://doi.org/10.1089/zeb.2009.0610.

[44] I.V. Mizgirev, S. Revskoy, A new zebrafish model for experimental leukemia therapy, Cancer Biol. Ther. 9 (2010) 895–902.

[45] E.K. Pugach, P. Li, R. White, L. Zon, Retro-orbital injection in adult zebrafish, J. Vis. Exp. (2009), https://doi.org/10.3791/1645.

[46] C.J. Veinotte, G. Dellaire, J.N. Berman, Hooking the big one: the potential of zebrafish xenotransplantation to reform cancer drug screening in the genomic era, Dis. Model. Mech. 7 (2014) 745–754, https://doi.org/10.1242/dmm.015784.

[47] M. Astone, E.N. Dankert, S.K. Alam, L.H. Hoeppner, Fishing for cures: the alLURE of using zebrafish to develop precision oncology therapies, Npj Precis. Oncol. 1 (2017) 39, https://doi.org/10.1038/s41698-017-0043-9.

[48] H. Brown, K. Schiavone, S. Tazzyman, D. Heymann, H. Brown, K. Schiavone, S. Tazzyman, D. Heymann, T.C. Ze, Zebrafish xenograft models of cancer and metastasis for drug discovery, Expert Opin. Drug Discovery (2017), https://doi.org/10.1080/17460441.2017.1297416.

[49] K. Howe, M. Clark, C. Torroja, J. Torrance, C. Berthelot, M. Muffato, J.E. Collins, S. Humphray, K. McLaren, L. Matthews, S. McLaren, I. Sealy, M. Caccamo, C. Churcher, C. Scott, J.C. Barrett, R. Koch, E. Al., The zebrafish reference genome sequence and its relationship to the human genome, Nature 496 (2013) 498–503, https://doi.org/10.1038/nature12111.The.

[50] H.M. Stern, L.I. Zon, Cancer genetics and drug discovery in the zebrafish, Nat. Rev. Cancer 3 (2003) 533–539, https://doi.org/10.1038/nrc1126.

[51] M. Dang, R.E. Henderson, L.A. Garraway, L.I. Zon, Long-term drug administration in the adult zebrafish using oral gavage for cancer preclinical studies, Dis. Model. Mech. 9 (2016) 811–820, https://doi.org/10.1242/dmm.024166.

[52] H. Feng, D.L. Stachura, R.M. White, A. Gutierrez, L. Zhang, T. Sanda, C.A. Jette, J.R. Testa, D.S. Neuberg, M. David, J.L. Kutok, L.I. Zon, D. Traver, M.D. Fleming, P. John, A.T. Look, NIH Public Access, 18 (2011), pp. 353–366, https://doi.org/10.1016/j.ccr.2010.09.009.T-Lymphoblastic.

[53] J.S. Blackburn, S. Liu, D.M. Langenau, Quantifying the frequency of tumor-propagating cells using limiting dilution cell transplantation in syngeneic zebrafish, J. Vis. Exp. (2011) 1–5, https://doi.org/10.3791/2790.

[54] T. Farge, E. Saland, F. de Toni, N. Aroua, M. Hosseini, R. Perry, C. Bosc, M. Sugita, L. Stuani, M. Fraisse, S. Scotland, C. Larrue, H. Boutzen, V. Féliu, M.L. Nicolau-Travers, S. Cassant-Sourdy, N. Broin, M. David, N. Serhan, A. Sarry, S. Tavitian, T. Kaoma, L. Vallar, J. Iacovoni, L.K. Linares, C. Montersino, R. Castellano, E. Griessinger, Y. Collette, O. Duchamp, Y. Barreira, P. Hirsch, T. Palama, L. Gales, F. Delhommeau, B.H. Garmy-Susini, J.C. Portais, F. Vergez, M. Selak, G. Danet-Desnoyers, M. Carroll, C. Récher, J.E. Sarry, Chemotherapy-resistant human acute myeloid leukemia cells are not enriched for leukemic stem cells but require oxidative metabolism, Cancer Discov. 7 (2017) 716–735, https://doi.org/10.1158/2159-8290.CD-16-0441.