**TITLE:**

Patient-Derived Heterogeneous Xenograft Model of Pancreatic Cancer Using Zebrafish Larvae as Hosts for Comparative Drug Assessment

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**KEYWORDS:**

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**SUMMARY:**

This protocol describes optimization procedures in a virus-based dual fluorescence-labeled tumor xenograft model using larval zebrafish as hosts. This heterogeneous xenograft model mimics the tissue composition of pancreatic cancer microenvironment in vivo and serves as a more precise tool for assessing drug responses in personalized zPDX (zebrafish patient-derived xenograft) models.

**ABSTRACT:**

Patient-derived tumor xenograft (PDX) and cell-derived tumor xenograft (CDX) are important techniques for preclinical assessment, medication guidance and basic cancer researches. Generations of PDX models in traditional host mice are time-consuming and only working for a small proportion of samples. Recently, zebrafish PDX (zPDX) has emerged as a unique host system, with the characteristics of small-scale and high efficiency. Here, we describe an optimized methodology for generating a dual fluorescence-labeled tumor xenograft model for comparative chemotherapy assessment in zPDX models. Tumor cells and fibroblasts were enriched from freshly-harvested or frozen pancreatic cancer tissue at different culture conditions. Both cell groups were labeled by lentivirus expressing green or red fluorescent proteins, as well as an anti-apoptosis gene *BCL2L1*. The transfected cells were pre-mixed and co-injected into the 2 dpf larval zebrafish that were then bred in modified E3 medium at 32 °C. The xenograft models were treated by chemotherapy drugs and/or BCL2L1 inhibitor, and the viabilities of both tumor cells and fibroblasts were investigated simultaneously. In summary, this protocol allows researchers to quickly generate a large amount of zPDX models with a heterogeneous tumor microenvironment and provides a longer observation window and a more precise quantitation in assessing the efficiency of drug candidates.

**INTRODUCTION:**

Precision oncology aims to find the most beneficial therapeutic strategies for individual patient1. Currently, numerous preclinical models such as in vitro primary culture, in vitro organoid culture2, and patient-derived xenografts (PDX) in mice before or after organoid culture are proposed for diagnosis and to screen/assess the potential therapeutic choices3. PDX model generated by the injection of human primary cancer cells into immune-compromised mice, is one of the most promising tools for personalized drug screening in clinical oncology3,4. Unlike the cultured cell line in vitro, PDX models usually preserve the integrity and heterogeneity of the in vivo tumor environment, better mimicking the diversity and idiosyncratic characteristics of different tumor patients, and therefore, may predict the potential medical outcome of patients4. However, the generation of PDX models in mice requires high quality patient samples and months of time to gather sufficient cells and models for multi group experiments, and the cellular/genetic compositions of the xenograft may drift from those of the original patient’s biopsy. The success rate for establishing mice PDX model is also low, making it difficult to be broadly implemented in clinical practice. For the patients carrying rapidly progressed cancers like pancreatic cancer, they may not be able to obtain valuable information from the PDX experiments in time.

In the past few years, zebrafish has been reported to be potential hosts for not only CDX (cell-derived tumor xenograft) models, but also PDX models5-10. As a vertebrate model animal, zebrafish harbors sufficient similarities with mammals in both genetics and physiology, with two significant advantages: transparency and small in size11. Zebrafish is also highly fecundity, and hundreds of inbred larvae can be obtained within a few days from a single pair of adults12. Several studies have employed zebrafish to generate both transgenic and xenograft models of cancer diseases13,14. Compared to mice xenografts, zebrafish xenografts allow tracking at single cell resolution. A certain amount of human tissues is capable of generating hundreds of zebrafish PDX models (zPDXs), while may only be sufficient to generate a couple of mice PDX models15,16. Besides, the zebrafish larvae at 2-5 dpf already develop complete circulatory systems and metabolic organs such as liver and kidney, but not the immune system17, while the remaining yolk sac is a natural 3D medium, ideal for drug screening, drug resistance tests and tumor migration observations6,18-21.

With an ultimate attempt to use zPDX as a screening/testing platform for clinical use, here, we describe an optimized proposal for zPDX model of pancreatic cancer, which allows the in vivo candidate drug assessment within a short time using fewer cells at lower costs. Compared to the previous references about zPDX6,9,10, we introduced several optimizations to make the system more feasible and reliable for clinical personalized diagnosis: 1) pre-sorting different cell groups in the primary tumor tissues and stabilizing primary cells for one week before further experiments; 2) labeling the human cells and enhancing the cell viability in xenograft via lentivirus-based genetic modification; 3) optimizing the zebrafish culture condition in both nutriment supplements (glucose and glutamine) and temperature; 4) quantifying the drug responses of different cell types in a comparative manner. We also made changes to the injection solution by adding several supplementary materials. Altogether, those improvements provide the possibility to quickly generate a more patient-like xenograft in zebrafish hosts that can be used as a reliable tool to assess the response of candidate drugs.

**PROTOCOL:**

All animal procedures were approved and followed the guidelines of the Animal Ethics Committee at Fudan University and all pancreatic cancer specimens were obtained from Fudan University Shanghai Cancer Center. Ethical approval was obtained from the FUSCC Ethics Committee, and written informed consent was obtained from each patient.

1. **Preparing the equipment for the microinjection**
   1. **Preparing the injection plate.**
      1. Prepare a 50 mL solution of 1% agarose dissolved in E3 solution (0.6 g/L aquarium salt in double distilled water + 0.01 mg/L methylene blue). Boil the solution until the agarose dissolves.
      2. Pour 50 mL of the agarose solution into a 10 cm Petri dish and then place the zebrafish embryo fixation mold on the surface. Remove the mold when the agarose solution becomes solidified.
      3. Add 20 mL of E3 solution to the injection plate and maintain it at 4 °C for long-term storage.
   2. **Preparing the injection needles.**
      1. Pull a 10 cm glass capillary with an inner dimension of 0.9 mm into two needles on a needle puller.
      2. Use forceps to cut the end of the needle to create an opening under the microscope.
2. **Preparing embryos for transplantation**
   1. Place 1 to 2 pairs of adult zebrafish in a mating tank at 7-9 pm and collect the fertilized eggs at around 8 am of the next morning.
   2. Transfer the fertilized eggs from the mating tank to a Petri dish containing 40 mL of fresh E3 solution and incubate at 28.5 °C.
   3. After 8 h of incubation in E3 solution, add 0.03% 1-phenyl-2-thiourea (PTU) into E3 solution to inhibit pigmentation. Incubate the embryos in E3 solution with 0.03% PTU at 28.5 °C until 48 hpf. This step can be omitted if using in-bred Casper mutant zebrafish.
3. **Isolation and culture of primary human cells from fresh surgical pancreatic cancer specimen or frozen tissue.** 
   1. Obtain specimens of human pancreatic cancer tissue of the size around 1 cm3 during an abdominal surgery, and immediately transfer the tissue into growth media (DMEM with 10% fetal bovine serum (FBS), 10 μM Y-27632, 100 μg/mL primocin, 10 μg/mL putrescine dihydrochloride, 10 mM nicotinamide and 1% penicillin streptomycin).
   2. Transfer the pancreatic cancer sample into a Petri dish and remove the surrounding necrotic tissue, adipose tissue and connective tissue.
   3. Rinse the cancer tissue for 5-6 times with phosphate buffer (PBS) and cut the tissue into 1 mm3 pieces using scalpels.
   4. Transfer the shredded tissues into 5 mL of HBSS in a 50 mL tube and add collagenase type IV, hyaluronidase and DNase I at final concentrations of 200 units/mL, 100 mg/L and 20 mg/L, respectively. Pipette the mixture up and down to mix well.
   5. Incubate the mixture at 37 °C in a 5% carbon dioxide incubator for 15-20 min. Pipette the mixture up and down a few times every 5 min.
   6. Add 7 mL of DMEM to the tube and centrifuge at 110 x *g* for 5 min at 4 °C when digestion is complete.
   7. Decant the supernatant and re-suspend the tumor mixture in DMEM.
   8. Plate the mixture into a 6 cm Petri dish in 3 mL of full growth media (DMEM with 10% FBS, 20 μg/mL insulin, 100 ng/mL bFGF, 10 ng/mL EGF, 10 μM Y-27632, 100 μg/mL primocin, 10 μg/mL putrescine dihydrochloride, 10 mM nicotinamide, 1% penicillin streptomycin). Separate the cells into two groups.
   9. In group I, add 100x inhibitor of pancreatic cancer fibroblasts into the medium after 48 h to remove the overgrown fibroblasts, leaving the cancer cells as the major cell types;. In group II, the fibroblasts will outgrow the cancer cells within a week.
   10. Culture both cell groups for 1-2 week depending on the cell densities/purities and change the media every three days. The expected cell types in both group I & II can occupy over 98% in proportion in a typic successful experiment.
4. **Labeling the cells with lentivirus expressing anti-apoptosis gene *BCL2L1 (BCL-XL)* and different fluorescent proteins separately**
   1. **Lentivirus production**
      1. Plate 3 × 106 HEK 293T cells with complete DMEM medium (DMEM supplied with 10% FBS) in 10 cm dishes and culture overnight at 37 °C in a 5% carbon dioxide incubator. Replace the medium with 6 mL of serum-free media before transfection.
      2. Prepare solution A: 8 μg of *BCL2L1*-containing lentiviral vectors (pCDH-EF1α-mKate2-E2A-BCL2L1-WPRE or pCDH-EF1α-eGFP-E2A-BCL2L1-WPRE), 2.4 μg of pVSVG, 4 μg pMDL(Gag/Pol), 1.6 μg of pREV and serum-free DMEM in a total volume of 500 µL. Gently pipet the mixture several times, and place it at room temperature for 5 min.
      3. Prepare solution B: 40 µL of PEI (polyethyleneimine) in 460 µL serum-free DMEM. Place it at room temperature for 5 min.
      4. Slowly add solution B into solution A and leave the tube at room temperature for 30 min.
      5. Add the final mixture into the HEK 293T cell culture dish prepared in step 4.1.1 and incubate at 37 °C in a 5% carbon dioxide incubator. After 12 h, add an additional 5 mL of the complete DMEM medium. After 48 h, harvest the medium containing the lentivirus.
      6. Filter the supernatant using a 0.45 μm sterile filter, add the supernatant into a concentration column, centrifuge at 6000 x *g* for 25-30 min at 4 °C. The lentivirus aliquots of 100 µL per tube are made and stored at -80 °C.
   2. **Infection of the primary cells**
      1. Seed the cells (group I & II) to be infected in a 12-well plate with 30-40% density and culture the cells overnight at 37 °C in a 5% carbon dioxide incubator.
      2. Replace the medium with 500 µL of serum-free medium containing 8 μg/mL of polybrene for 4 h. Then, add an additional 100 µL of the lentivirus into the medium (eGFP-E2A-BCL2L1 for Group I or mKate2-E2A-BCL2L1 for Group II). After 12 h, replace the medium with 10 mL of complete medium.
      3. Check the fluorescence markers after 48 h.
      4. Harvest the infected cells and mix them at 1:1 ratio with a final concentration of 106/mL.
      5. Centrifuge the cells at 110 x *g* for 5 min and re-suspend the cell mixture in 50 µL of injection solution (1640 medium with 10% FBS, 0.05% hyaluronic acid sodium salt, 0.05% methylcellulose).
5. **Injecting mixed cell suspension into the zebrafish**
   1. Add 10x tricaine solution into E3 water to anesthetize zebrafish larvae and transfer the larvae (from step 2.3) to the injection plate filled by modified E3 (E3 with 1 g/L glucose and 5 mmol/L L-glutamine).
   2. Fill 25 µL of mixed cell suspension into micro capillaries needle and insert the needle into the micro-injection manipulator.
   3. Set injection pressure and time. Inject 50-80 cells (~8 nL) into the yolk sac of 48 hpf zebrafish.
6. **Culture of the xenografted Zebrafish (zPDX model)**
   1. Transfer the post-xenografted zebrafish larvae into 40 mL of mix solution (E3 solution with 1 g/L glucose and 5 mmol/L L-glutamine) at 32 °C.
7. **Drug administration on the xenografted zebrafish and the assessment of tumor cells/fibroblasts viabilities**
   1. **Determining the optimal concentration of** **gemcitabine/navitoclax.**
      1. Place 10 wildtype zebrafish embryos at 48 hpf into each well of a 12-well plate.
      2. Add different concentrations of gemcitabine or navitoclax in each well and incubate at 32 °C for two days.
      3. After 2 days, calculate the maximal tolerance dosage (MTD) of gemcitabine and navitoclax at which the zebrafish larvae do not shown significant malformation and abnormal behavior, and the working concentrations are set below the MTD.
   2. **Treatment of zPDX models with gemcitabine/navitoclax.**
      1. Place 10 xenografted larvae into each well of a 12 well plate.
      2. Divide the larvae into four groups, treat the control group in E3 containing 0.1% DMSO, and treat the other groups with 5 μg/mL gemcitabine and/or 50 μM navitoclax, and incubate at 32 °C for two days.
   3. **Assessment of the cell viabilities and cellular composition in zPDX models.**
      1. Anesthetize the xenografted larvae post-treatment and place them in 3% methylcellulose.

7.3.2 Image the larvae from the lateral view using a fluorescence microscope or confocal microscope.

7.3.3 Quantify the intensity of red and green fluorescence signals using ImageJ and GraphPad software.

**REPRESENTATIVE RESULTS:**

A schematized outline of the procedure is represented in **Figure 1**. In short,the primary cancer tissue cells were seeded into the complete medium after digestion with or without the addition of pancreatic cancer fibroblast inhibitors. Cancer cells and fibroblasts were enriched as two distinct populations that fibroblasts dominated without inhibitors, and cancer cell growth prevailed after the addition of inhibitors (**Figure 2**). Two lentiviral packaging vectors were constructed, which expressed green or red fluorescent proteins and BCL2L1 as shown in **Figure 3**. The virus-based fluorescent labeling also represented the survival status of the cells. The mixed cancer cells and fibroblasts were injected into the zebrafish yolk sac at 48 hpf and were treated by gemcitabine and/or navitoclax for two days. The cell viabilities in different populations and cellular composition of the xenograft were changed as the responses to the drug treatment (**Figure4**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1:** **Schematic diagram of the generation and drug assessment of the zebrafish xenograft model derived from pancreatic cancer.** Surgically removed tissues from patients with pancreatic cancer are sheared and digested, followed by culture at two different conditions, one of which is added by a fibroblast inhibitor, and the other group is not. After 1-2 weeks, the primary cells were genetically modified to express an anti-apoptotic protein *BCL2L1* and different fluorescent proteins. After that, the two populations were mixed and co-injected into 48 hpf zebrafish larvae to assess the effects of chemotherapeutic drugs.

**Figure 2: Culture and enrichment of pancreatic cancer cells and fibroblasts.** Images of cancer cells and fibroblasts enriched from three different pancreatic cancer patients after 10-days culture. Scale bar, 100 μm.

**Figure 3: Structure of lentiviral vector expressing BCL2L1 and different fluorescent proteins.** Schematic diagram of two lentiviral vectors constantly expressing mKate2-E2A-BCL2L1 or eGFP-E2A-BCL2L1, respectively.

**Figure 4: Effects on xenograft by gemcitabine. (A)** Lateral view of the xenograft in the yolk sac of zebrafish larvae at 3 h post transplantation (hpt). **(B)** Representative images of the xenografts in zebrafish larvae at 60 hpt, treated by DMSO or gemcitabine. **(C)** Representative 3D rendering of the xenografts at 60 hpt. **(D)** Statistics of the fluorescence intensity of the xenograft before and after gemcitabine and/or BCL2L1 inhibitor treatment. N = 10 per group for each assay. Scale bar, 100 μm; NS, not significant; \**P* < 0.05; \*\*\**P* < 0.0001; mean ± SD, with statistical differences determined by one-way ANOVA.

**DISCUSSION:**

Both PDX and CDX models are vital platforms in the field of tumor biology22, and the critical step of a successful inter-species transplantation is to improve the survival of the xenograft. Recently, some studies have shown that transient expression of *BCL2L1 (BCL-XL)* or *BCL2* may significantly improve the viability of human embryonic stem cells in mice hosts without affecting the cell identities and fates23-25. In our manuscript, we labeled the cells with lentivirus constantly expressing anti-apoptosis gene *BCL2L1 (BCL-XL)*, as well as fluorescent proteins. The introduction of *BCL2L1* greatly enhanced the survival time of xenograft human cells in zebrafish to prolong the observation window for the studies, while the fluorescent signals not only label the cells, but also report the living condition of the cells, as we found that the fluorescence fades quickly with the cell death. Meanwhile, we are currently modifying the zebrafish as a better host for human xenografts. On the one hand, the *rag2* and *prkdc* are being knocked out via CRISPR/Cas9 technology to prepare a combined immune-deficient zebrafish, which was similar to combined immune-deficient mice, to make older zebrafish larvae also compatible with human cells and tissues26. On the other hand, the zebrafish was also modified to express human proteins such as IGF1 and INS to better support the survival and proliferation of implanted human cells by using the Tol2 transposon-mediated transgenic technology. Moreover, zPDX also lacks a human-like functional immune system, and the interactions between human stromal cells and the immune system, and in future, we may try co-injecting human immune cells to rebuild a short-term humanized immune environment in zebrafish.

In the mice PDX models, the xenograft conditions were traditionally monitored by direct observation on nodes of visible size, which require a long time to develop. In the transparent zPDX models as comparison, the xenograft can be investigated under microscopy in real time. However, it was difficult to assess the cell number alternations in a single-color population without a proper control, and the idea to introduce two cell population of different fluorescence significantly improves the quantitation of cell viability. By mixing and injecting different cells at fixed ratios, we not only mimic the tumor microenvironment, but also are able to accurately quantify the drug response by comparing the two cell groups. Although the original ratio of cancer cells to fibroblasts in primary tissues is highly different, here we started with 1:1 ratio, and the effects of drug treatment on the same cell composition of a different ratio will be investigated in the future. Besides, the effects of 32 °C instead of 37 °C incubations on the behaviors of human cells also require detailed comparative studies. Lastly, the strategy of the patient-derived heterogeneous xenograft model for pancreatic cancer for comparative drug assessment can also be applied to other types of solid cancers. This approach is particularly useful for PDX using zebrafish larvae as hosts, which are crystal-clear and feasible for analysis at single-cell resolution.

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**DISCLOSURES:**

No potential conflicts of interest were disclosed.

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