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A Simple, Rapid, and Effective Method for Tumor Xenotransplantation Analysis in Transparent Zebrafish Embryos

Monika Verma[1,*], **Michele Rhodes**[1], **Susan Shinton**[1], **David L. Wiest**[1,*]

[1]Nuclear Dynamics and Cancer Program, Fox Chase Cancer Center, Philadelphia, PA, US

Abstract

In vivo studies of tumor behavior are a staple of cancer research; however, the use of mice presents significant challenges in cost and time. Here we present larval zebrafish as a transplant model that has numerous advantages over murine models, including ease of handling, low expense, and short experimental duration. Moreover, the absence of an adaptive immune system during larval stages obviates the need to generate and use immunodeficient strains. While established protocols for xenotransplantation in zebrafish embryos exist, we present here an improved method involving embryo staging for faster transfer, survival analysis, and the use of flow cytometry to assess disease burden. Embryos are staged to facilitate rapid cell injection into the yolk of the larvae and cell marking to monitor consistency of the injected cell bolus. After injection, embryo survival analysis is assessed up to 7 days post injection (dpi). Finally, disease burden is also assessed by marking transferred cells with a fluorescent protein and analysis by flow cytometry. Flow cytometry is enabled by a standardized method of preparing cell suspensions from zebrafish embryos, which could also be used in establishing primary culture of zebrafish cells. In summary, our procedure allows more rapid assessment of the behavior of tumor cells in vivo with larger numbers of animals per study arm, and in a more cost-effective manner.

SUMMARY

We describe a protocol for xenotransplantation into the yolk of transparent zebrafish embryos that is optimized by a simple, rapid staging method. Post injection analyses include survival and assessing disease burden of xenotransplanted cells by flow cytometry.

INTRODUCTION

Analysis of the behavior of tumors in response to genetic alteration or drug treatment in vivo is an essential element of cancer research [1–4]. Such studies most often involve the use of immunocompromised mouse (*Mus musculus*) models [5]; however, xenotransplantation studies in mice are limited in many respects, including limited capacity, extended duration, significant expense, and the requirement for sophisticated imaging equipment to monitor progression of internal tumors [6,7]. By contrast, the zebrafish model (*Danio rerio*) enables

*Corresponding authors: Monika Verma (Monika.Verma@FCCC.edu), David L. Wiest (David.Wiest@FCCC.edu).

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DISCLOSURE

The authors declare no competing interests.

greater capacity, shorter duration, lower expense, and, due to their transparency, simple monitoring of disease progression [8,9].

Zebrafish is a well-developed vertebrate model system with ex-utero development and high fecundity, with individual females producing more than 100 embryos [10]. Moreover, zebrafish embryos are transparent, enabling easy visualization of developmental processes using fluorescence related techniques such as reporters. Finally, the conservation of critical developmental processes make them an ideal model for many types of studies including the behavior of transplanted malignant cells [11,12]. Wild type zebrafish embryos develop melanocytes, which render them optically opaque by two weeks of age, but this has been overcome by generation of *casper* embryos (*roy*^{a9}; *mitfa*^{w2}), which remain transparent throughout life [13]. Because of their optical properties, *casper* zebrafish are ideal as recipients of transplanted tumor cells[14,15,16]. Xenotransplantation of tumor cells into zebrafish has gained importance in past 2 decades [17–21]. Zebrafish embryos have innate immunity; however, they lack adaptive immunity during their larval stage, rendering them functionally immunocompromised, which enables them to serve as effective hosts for transplanted tumor xenografts [22].

Protocols have been developed for tumor engraftment in zebrafish embryos as well as adults that have considered a number of different variables [23–27]. These have explored numerous sites of tumor deposition in zebrafish including injections in yolk, peri-vitelline space, and heart and at different developmental stages [16,28]. The ambient temperature of aquaculture for zebrafish xenografts is also important as zebrafish rearing typically occurs at 28 °C, while mammalian cells grow at 37 °C. Consequently, a compromise temperature must be employed that is tolerated by the fish yet supports tumor growth, and 34 °C appears to achieve both goals [29]. Analysis of the behavior and progression of tumors following xenotransplantation is another major area of focus, and this involves the use of a variety of imaging modalities as well as survival analysis [30]. One of the major advantages of the zebrafish model is the availability of large numbers of study animals to provide immense statistical power to in vivo studies of tumor behavior; however, previous approaches have severely limited this potential because of the requirement of tedious mounting procedures for injections.

Here, we address this limitation through development of a simple, rapid method with which to stage embryos that enables high throughput and monitoring of injection quality using the transparent *casper* zebrafish line. This entails the injection of xenografts into the yolk sac of *casper* zebrafish embryos at 2 days post fertilization (dpf). We observe the survival of embryos following xenotransplantation as part of tumor behavior analysis. We further show the assessment of disease burden after xenotransplantation by making single cell suspensions and analyzing by flow cytometry (Figure 1).

PROTOCOL

Zebrafish maintenance, feeding, and husbandry occurred under standard aquaculture conditions at 28.5 °C, as described [31]. All zebrafish related experiments were done at this temperature; however, following xenotransplantation the animals were cultured at 34 °C

for the duration of the experiment, in accord with procedures approved by the Institutional Animal Care and Use Committee (IACUC).

1. Breeding (3 days before injection)

1.1. Provide dry food (extra feed; 5-6 granules per fish) to fish pairs a week prior to breeding to maximize animal health and increase the number of embryos produced by breeding pairs.

1.2. On the evening prior to embryo harvest, set up breeding animals in breeding tanks with a divider separating male and female fish, using harem matings of 2 females for each male.

NOTE: For experiments with 4 arms of 100 animals per arm, employ 20 breeding pairs per experiment. To determine the number of breeding pairs needed, a good estimate is 50 embryos per *casper* breeding pair. Another option is to employ a more robust, pigmented zebrafish strain and treat with 1-phenyl 2-thiourea (PTU) to prevent pigmentation [31]. In practice, the experiment should be scaled such that one has enough embryos to inject twice the number desired at 1 day post injection (dpi).

2. Embryo collection (days before injection)

2.1. The next morning, remove the dividers, allowing the fish to breed.

2.2. Embryos will be visible in the tanks 20 minutes (min) after removing the dividers.

2.3. Collect the embryos using a sieve in a 90 mm petri-dish containing embryo water made as described in The Zebrafish Book [31]. Briefly, to make embryo water, add 1.5 ml stock salts to 1 L distilled water and methylene blue to 0.1% final. The stock salt solution in made by dissolving 40 g “Instant Ocean” Sea Salts in 1 L distilled water. The ionic composition of embryo water is K^+ (0.68 mg/L), Cl^- (31.86 mg/L), Na^+ (17.77 mg/L), SO_4- (4.47 mg/L), Mg_2+ (2.14 mg/L), and Ca_2+ (0.68 mg/L).

2.4. Allow the zebrafish to continue to breed for an extra hour and collect the resulting embryos.

2.5. Pool the embryos for the experiment.

2.6. In the evening, remove all unfertilized or dead embryos, which are recognizable by their abnormal morphology, and provide fresh embryo water.

3. Embryo maintenance and tool preparation for injections (1 day before injection)

3.1. The following morning, remove any additional dead embryos and provide fresh embryo water.

3.2. Prepare an agarose plate by heating 1.5% agarose in embryo water and pour the heated mixture in the 90 mm petri-plate. One 90 mm dish requires 30-35 mL of the mixture.

3.3. Pull non-filament needles from glass capillaries (Borosilicate) using the needle puller. Needles are pulled (under heat pressure) producing a closed end that will be clipped with

forceps to generate an optimal orifice. The suitability of the needle orifice must be assessed by determining the volume of fluid displaced per unit time (see below).

NOTE: Glass capillaries can be purchased with central filaments or without. Capillaries lacking central filaments are preferred for cell injections.

3.4. Place the needles in a covered 90 mm petri plate in the grooves made using clay (kids modeling clay) until use (Figure 2A).

4. Preparation and labeling of leukemia cells with CellTracker CM-DiI (day of injection)

4.1. Maintain the cells to be transplanted under conditions optimal for their growth. The murine leukemia cells employed here were either sufficient (M82; Rpl22+/+) or deficient (M109; Rpl22−/−) for the ribosomal protein Rpl22, which functions as a tumor suppressor [32].

4.2. Pellet cells in a 50 mL conical tube. Count, then centrifuge at 300 g at room temperature (RT) for 5 min. Discard the supernatant.

NOTE: The number of cells needed will be dictated by the experimental scope and conditions but 10^6 is a good starting point.

4.3. **CM-Dil staining:**

NOTE: CM-Dil staining enables monitoring of the injection bolus.

4.3.1. Make a stock solution of CM-Dil by resuspending a 50 μg vial of CM-Dil in 50 μL DMSO (1 mg/mL or ~1 mM final).

4.3.2. Produce a working solution by diluting the stock (4.8 μl stain/mL) in 1% FBS/HBSS containing any supportive supplements needed by the cells to be used.

4.4. Resuspend cells, at 10^6/100 μL in the working solution of the stain.

4.5. Incubate at 37 °C for 10 min.

NOTE: The staining conditions must be optimized for the cells employed (time, etc). The cells used here required two distinct 10 min incubations at different temperatures to achieve optimal staining.

4.6. Wash with 10 mL 1X HBSS (Hank's Buffered Saline Solution) at room temperature (RT).

4.7. Pellet cells (centrifugation at 300 g for 5 min at RT), decant the supernatant, then resuspend again with 10 mL HBSS and repeat same for a second wash.

4.8. Resuspend the stained tumor cells at 40,000 cells/μL in 1% FBS/PBS and any supportive supplements needed and maintain the cell suspension at 34 °C until injection.

NOTE: Supportive supplements were required for maintenance of the cell lines used in this study (e.g., 1 % FBS and cytokines). The supplements and xenotransplantation may need to be adapted to the particular cell lines under study. PBS was selected here instead of media to avoid any potential toxicity in the yolk.

5. Dechorionation

5.1. Dechorionate the *casper* zebrafish embryos manually at 2 dpf using insulin injection syringes under 2X magnification in a light microscope. Pierce the chorion with one needle while using the other needle to immobilize the chorion.

NOTE: Using pronase during dechorionation is not recommended because it sometimes results in reduced embryo health. Dechorionating the embryos at 2 dpf is preferred because it is easier, and the embryos are less fragile than at earlier times (1 dpf).

5.2. While dechorionating, be careful not to touch the embryos with the needles. Touching or damaging the yolk of embryos by inadvertent contact with the needles might cause death.

5.3. Remove the stripped chorions by changing the embryo water.

6. Setting up the microinjector and needle

6.1. Turn the microinjector and the pump on and set up the conditions suitable for microinjections of cells. An injection pressure of 9-11 psi and time of injection was 0.5 seconds (s) are a good starting point for clipping the needle and setting the orifice.

6.2. Load the tumor cell suspension line (~5 μL) into the microneedle carefully in a single pass, avoiding forming air bubbles, which will disrupt the cell stream inside the needle.

6.3. Cut the end of the needle with forceps (Dumont number 5) to produce an orifice that will support ejection of 10-15 nL of cell suspension per 0.2-0.3 s.

NOTE: The above calculation of nL volume is done using calibration capillaries, where 1 mm = 30 nL. In brief, set the time to 0.5 s, and after every clip of the needle, press inject and collect the volume in the calibration capillary. Then measure the length of the collected volume using the scale under microscope and stop the needle clipping when 30 nL is injected in 0.5 s. Then setup the time of injections as 0.2-0.3 s. (injecting ~10-15 nL of cells)

7. Embryo preparation for injection

7.1. Select healthy embryos under the microscope, culling any with developmental anomalies such as heart edema or a short or curved trunk.

7.2. Anaesthetize the embryos using Tricaine methanesulfonate (MS-222; 0.16 g/L of embryo water) for 1 min in a 90 mm petri-plate.

7.3. Use a glass pasteur pipette to pick up the embryos and arrange 10-15 embryos in a lateral position on the 1.5 % agarose plate (Figure 2B–D).

7.4. Remove excess water using the pasteur pipette, leaving the minimum amount of embryo water needed to keep them alive.

8. Injection procedure

8.1. Check under the light microscope to ensure that the cells have accumulated in the tip of the needle.

8.2. Inject the embryos using the calibrated needle for 0.2-0.3 s (with 10-15 nL corresponding to 400- 600 cells) in the yolk of the embryos.

8.3. Repeat the injection for all the embryos, then collect them in fresh embryo water.

NOTE: Because cells tend to accumulate on the needle tip, the tip will need to be reclipped slightly every 15-20 injections. This will also require resetting the pressure and time with each reclipping to ensure that a similar volume is injected.

8.4. To ensure that the comparison of the behavior of two distinct sets of transferred cells is valid, it is important to monitor the bolus of cells transferred. This is done by sorting the embryos based on CM-Dil staining (in the RFP channel) at 1 hour post injection (hpi), separating those with optimal staining (“good bolus”) from those with inferior staining (“inferior bolus”; Figure 3, yellow arrow).

8.5. The embryos with an inferior bolus can be discarded or used to assess the impact of a different cell dose on disease progression.

8.6. Remove any dead embryos by the end of the day since their death is related to injection trauma rather than tumor growth. Embryos that do not retain cells should also be removed from the analysis since the cells likely leaked out of the injection site.

8.7. Maintain the injected embryos at 34 °C for the duration of the experiment in 90 mm dish with ~60 embryos per plate.

NOTE: After 5 dpf, the yolk will have been consumed by the growing embryos, so embryos must be provided with paramecium food for the duration of the experiment. To ensure proper nutrition, paramecia should be given to the embryos daily from 6 dpf (4 dpi) to 9 dpf (7 dpi). Paramecia are propagated by culturing in flasks under optimum nutrition and temperature conditions, as described [31].

9. Survival analysis

9.1. Monitor the embryos for the next 7 dpi, changing the embryo water every day. Water changes may be reduced to alternate days for convenience if the study involves drug treatment.

9.2. Embryo health should be checked, and death scored for the duration of the analysis.

NOTE: The experimental duration was 7 days for these experiments but may be shorter or longer depending on the aggressiveness of the xenotransplanted tumor.

9.3. CM-Dil fluorescence can be used to assess disease burden (Figure 4A), while the impact of genetic alterations or drug treatments on survival can be determined using Kaplan Meier analysis, and depicted graphically (e.g., with GraphPad Prism; Figure 4B) [33].

10. Single cell suspension of embryos for flow cytometry analysis

NOTE: Disease burden can be assessed by flow cytometry analysis after xenotransplantation; however, doing so requires indelible marking of the tumor cells. Retrovirally or lentivirally-delivered red fluorescent protein (RFP) or mCherry is effective as it provides good signal over the autofluorescence of zebrafish cells, which obscures signal from green fluorescent protein.

10.1. Isolate embryos at the dpi stage of choice. 5 dpi is displayed here (Figure 5).

10.2. Gather 30-40 embryos per condition as a starting point, but the embryo number may differ depending on the stage and aggressiveness of the transplanted cells.

NOTE: Embryos may be subdivided as replicates to provide statistical significance, as shown here (Figure 5B).

10.3. Transfer the embryos to 1.5 mL centrifuge tubes.

10.4. Use 100 mL of calcium free Ringer's solution (recipe [31]) per sample to dissolve the yolk, since low calcium also soften the embryonic tissues, thereby enabling more effective tissue dissociation.

10.5. Pipette up and down intermittently for 5 min to remove yolk using a 200 L pipette tip.

10.6. Pre-heat 0.05 % trypsin/PBS (without phenol red indicator) to 29°C and supplement it with 27 mL of collagenase IV (100 mg/mL) per mL of Trypsin solution. 1 mL of solution will be needed for each sample of embryos.

10.7. Add 1 mL of the trypsin/collagenase solution to each sample of deyolked embryos, and incubate at 29 °C for 30-35 min.

10.8. Pipette the embryos up and down in this solution using a 1 mL pipette tip every 5 min until the structure of the embryos (backbone) is no longer visible.

10.9. Stop the reaction using 200 L of Fetal Bovine Serum (FBS).

10.10. Mix well and incubate at 29 °C for 5 more min to ensure complete inactivation of the trypsin.

NOTE: 29 ºC is employed for the tissue dissociation protocol to prevent heat shock induced death of zebrafish cells, which occurs at 37 ºC; however, if preservation of zebrafish cells is not required, digestion can be performed at 37°C.

10.11. Pellet the cell suspension at 300 g for 5 min at 4 °C and discard the supernatant.

10.12. Resuspend the cell pellet in 4 °C PBS and pellet as above.

10.13. Repeat the wash, then strain the cells through 70 mm cell strainer.

10.1.4 Pellet and proceed with staining for flow cytometry analysis.

NOTE: If culture of primary zebrafish cells is required, wash twice more with 4 °C PBS and resuspend in L15 media (with antibiotics and 10% FBS).

11. FACS staining and sorting of xenotransplanted cells

11.1. Resuspend the cell suspension in staining medium (HBSS with 1 % FBS) and pellet at 300 g for 5 min).

11.2. Resuspend the cell pellet in staining medium with an antibody reactive with the transplanted cells to provide a second marker (in addition to RFP or mCherry) with which to distinguish transplanted cells from zebrafish cells. 50 μL of anti-mouse CD45 (APC-CD45) per sample was employed here at a 1:50 dilution (Figure 5).

11.3. Incubate for 20 min 4 ºC before washing as above with 1 mL of staining medium to remove the unbound dye.

11.4. After discarding the supernatant, resuspend the cell pellet in 200 μL of staining medium containing the vital dye Helix NP Blue (1 μM), which will enable live/dead discrimination.

11.5. Transfer the cell mix to 5 mL round bottom polycarbonate tubes for flow cytometry analysis.

NOTE: Staining a tumor cell control in parallel provides clarity for drawing gates during flow cytometry analysis.

12. Flow cytometry

12.1. Turn on the flow cytometer that is available using a low flow rate (500 events per second or less) to set the parameters.

12.2. Use the tumor cell control (the same cells used for xenotransplantation) to set the voltage for FSC (forward scatter), SSC (side scatter), BV421/CasB (viability), CE-594 (mCherry), and APC (CD45.2) channels.

NOTE: Singly stained samples will be required to establish the compensation settings that eliminate fluorochrome overlap between distinct stains.

12.3 The uninjected embryo sample should be used to establish settings that accommodate both transplanted and zebrafish cells.

12.4. Increase the flow rate up to 8000 events per second and record 1 million events for each sample.

12.5. Analyze the resulting data using an appropriate analysis software, first selecting singlets by plotting FSC-H v/s FSC-A (height v/s area) followed by plot for selection of viable cells. FlowJo software is widely used for such analysis.

12.6. Using the tumor cell control, draw a gate around the transplanted cells by FSC-A v/s SSC-A, then by using the indicator stains, in this case, CD45 and mCherry (Figure 5B).

NOTE: The size gate selected for the tumor will also contain zebrafish cells, which provides the basis for normalization and determination of disease burden.

12.7. Next, analyze the embryo samples with the same gate settings as above. The final plot of tumor stain and fluorescent protein indicator will provide a measure of disease burden (Figure 5C).

NOTE: Here the difference in disease burden was plotted for embryos receiving a good bolus of injected cells vs those receiving an inferior bolus.

12.8. If needed, tumor cells in the embryos can be sorted by flow cytometry for downstream molecular analysis.

REPRESENTATIVE RESULTS

Xenotransplantation

A comprehensive view of the entire experiment and analysis is depicted in Figure 1, spanning from embryo production to the assessment of disease progression by both survival and disease burden analysis by flow cytometry. Our approach brings several improvements that enhance reproducibility and scalability of xenotransplantation, as well as adding a new way to assess disease burden. Success of these experiments is highly dependent upon the health of the transplanted cells as cells that are not healthy and in log phase fail to propagate upon transplantation. The duration of the injection session is also a critical parameter. After tumor cells are prepared, it is critical to complete injection into zebrafish within 3-4 hours. Our approach enables larger numbers of embryos to be injected during this time frame through the simple modification of staging them directly on their side on an agarose plate and injecting in yolk (Figure 2C and D). Moreover, it is imperative that the optimum needle orifice is selected such that enough cells are injected (400-600) but that the orifice is not so large that the embryos are injured. Another consideration is the injection pressure. We find that pressures greater than 12-13 psi disrupt the yolk of embryos, causing death. Finally, another variability inherent to this procedure is the consistency of injection. Cells to be injected settle into the end of the injection needle making precise control in the injection bolus challenging. When the cells are xenotransplanted, all embryos have the potential to receive the same injection bolus but in practice they do not (Figure 3). The number of cells transferred can differ widely depending on the behavior of the tumor cells (e.g., clumping) and the skill level of the operator. We have addressed this uncertainty through CM-Dil staining/mCherry labeling which enables post-injection categorization of animals that have received an appropriate and consistent cell bolus, and those receiving an inferior bolus. The CM-Dil staining, but more effectively marking with a fluorescent protein, has the added

benefit of facilitating the monitoring of disease progression, either by microscopy or by flow cytometry (Figures 4 and 5).

Tumor behavior analysis

Tumor progression can easily be monitored using simple fluorescence microscopy focused on RFP (Fig 4A). Likewise, traditional survival monitoring can be performed by Kaplan-Meier analysis (Log rank and Wilcoxon test) (Figure 4B). Impressively, in contrast to mouse-based xenotransplantation studies where there are typically 8-10 animals per study arm, using our zebrafish method, it is not difficult to achieve study arms with greater than 60 animals each (Figure 4B). This markedly enhances the resolving power of in vivo studies. Finally, we have implemented another approach for disease burden analysis using flow cytometry. This entails disruption of equivalent numbers of embryos and analyzing the tumor cell content of the resulting single-cell suspension by flow cytometry. By combining a tumor specific cell surface marker with the fluorescent protein indicator, the xenotransplanted mice/human cells can be confidently identified by flow cytometry as an approach to assess disease burden (Figure 5). For this purpose, red fluorescent proteins are superior, since the green fluorescent proteins failed to provide signal over the autofluorescence of host zebrafish cells. Here mCherry was employed for cell labeling and monitoring through the course of xenotransplantation for FACS analysis along with CD45. The dual labeling allowed us to measure differences in the tumor burden between good v/s inferior bolus inoculation (Figure 5B and C).

DISCUSSION

Zebrafish xenotransplantation has emerged as a rapid, robust, and cost-effective alternative to mouse studies [12]. Though several approaches to zebrafish xenotransplantation have been reported, our adaptation has resulted in significant improvements. In addition to standardizing parameters around the procedure, these improvements specifically focus on accelerating the rate at which tumor injections can be performed, thus enabling an increase in the number of animals per study arm and using tumor labeling to monitor the quality of injection and post-injection behavior.

While the improvements to this method described here have great potential, the successful execution of this strategy will require a skilled practitioner and optimization for the specific application. We employed leukemia cells. Consequently, the use of solid cancers may bring additional challenges. Such tumors may be prone to aggregation, which would create variability in delivery of the cell bolus; however, even in such circumstances, RFP labeling should enable adequate post injection quality control of the bolus. This is superior to GFP-labeling or green dyes, which are obscured by autofluorescence. Finally, the standardization described here of most parameters impacting success (embryo health, aquaculture temperature, needle orifice, injection pressure, etc.) minimize the variability of this process.

A major consideration for xenotransplantation experiments in zebrafish is the site of injection. Here, we have shown the injections in yolk are quite easy relative to other more technically challenging sites, like periviteline space[34], Duct of Cuvier[35], and intracardial

injection (heart ventricle)[36]. The disadvantage of yolk injection is that it is a vital organ for the growing embryos, so care must be taken to ensure that needle diameter and pressure are carefully controlled so the embryo does not die due to injection trauma. The approach described here mitigates this concern by minimizing injury and discarding any obvious injuries or death by 1 dpi since those issues are unrelated to tumor growth. The final consideration regarding the site of injection is that distinct microenvironments may have greater or lesser ability to support the propagation of xenotransplanted tumors. Consequently, perhaps yolk injections can be performed first before proceeding to more challenging orthotopic injections. The major advantage of yolk injection is that it does not require precise embryo staging and so enables more rapid injection of larger number of embryos, thereby better preserving their health and increasing the statistical power to resolve differences in behavior of transplanted tumor cells.

Post-injection monitoring of disease progression is typically assessed through effects on survival using Kaplan-Meier analysis[37]; however, disease burden testing can also be quite informative. For transplanted cells that remain at the injection site, the tumor burden can be quantified using various microscopy methods, provided the labeling method for the tumor cells is not obscured by autofluorescence[29]. Our CM-Dil stain is easily resolved and unaffected by autofluorescence and so it works well to quantify tumor burden of localized cells. The challenge occurs when tumor cells do not remain at the injection site and disseminate. In such cases, flow cytometry, coupled with indelible genetic marking using red fluorescent proteins, is a very effective way at monitoring disease burden in standardized clutches of embryos since the labeled tumor can be analyzed by using species specific stain different from the zebrafish cells. One shortcoming of CM-Dil is that it is diluted by cell division[38]. Accordingly, adaptation using genetic marking of the tumors using RFP or mCherry carries significant benefit. mCherry expression, coupled with a tumor-specific antibody enables confident identification of transplanted cells among what can be a complex pattern of background signals provided by the host zebrafish cells.

Taken together, our optimized zebrafish xenotransplantation approach and analysis method provides substantial improvement to an already powerful experimental platform.

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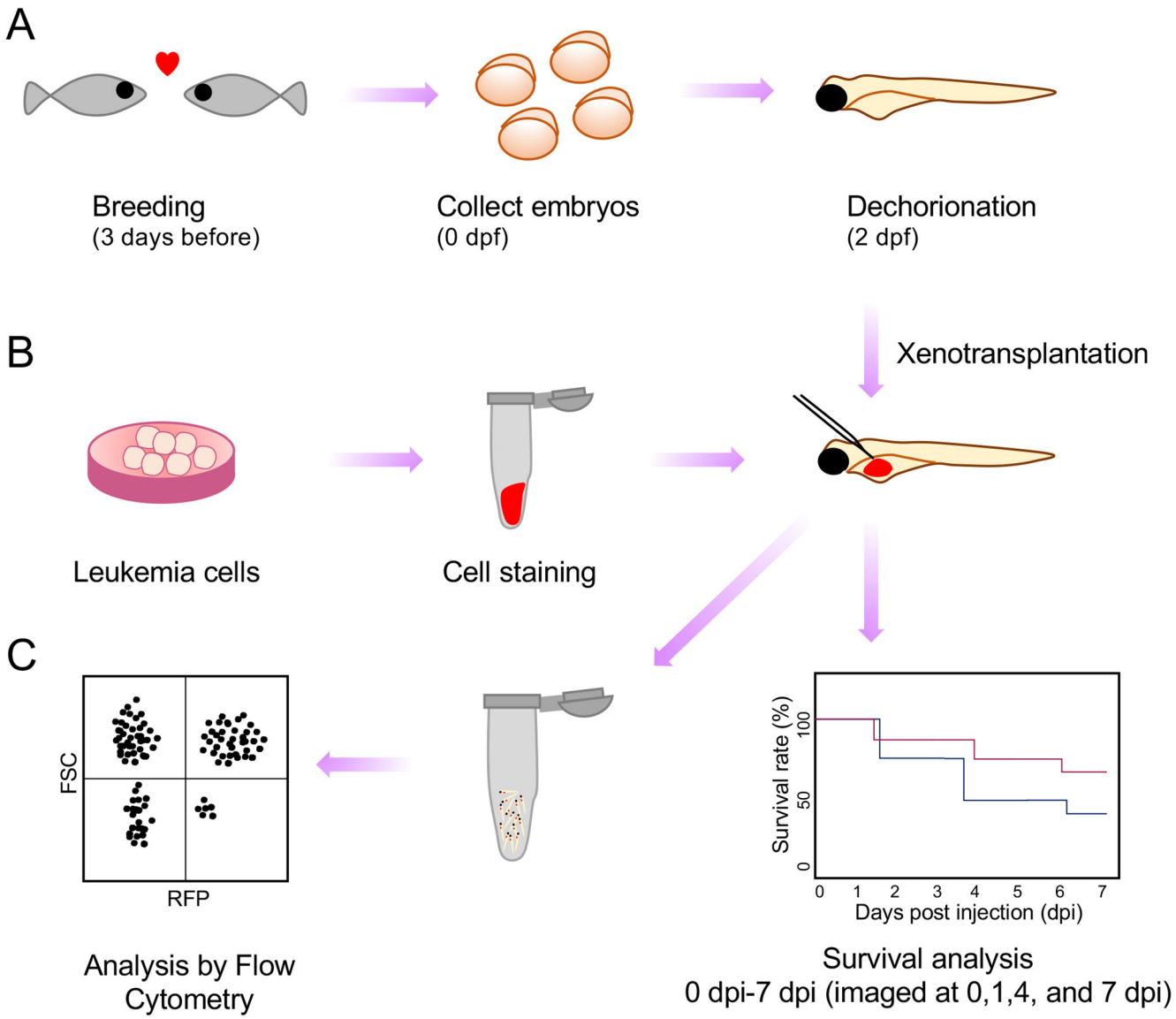

Figure 1: Schematic of the entire xenotransplantation and post injection analysis procedures.

A. Breeding setup, embryo collection and 2 days post fertilization (dpf) morphology is schematized. B. Preparation, staining, and injections of leukemia cells for xenotransplantation in the yolk of zebrafish embryos. C. Post xenotransplantation analyses including survival and flow cytometry.

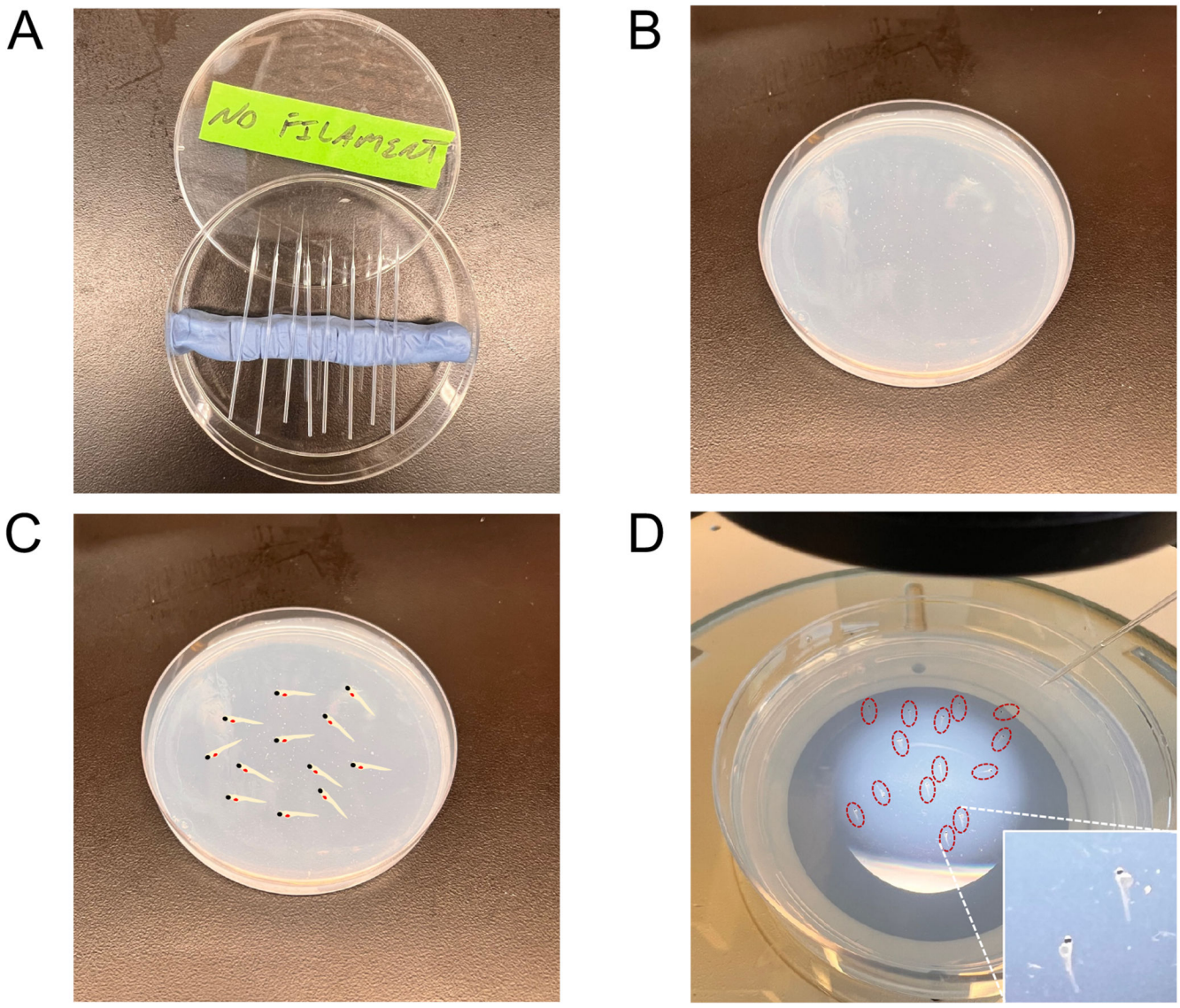

Figure 2: Representative images of the tools used for the injections.

A. Pulled needles in petri plates. B. The agarose plate for embryo staging. C.-D. A plate showing embryos staged (representative diagram in C and real embryos in D) for injections on the embryo loading plate. A higher magnification view of the staged embryos is shown in inset on the bottom right corner of D.

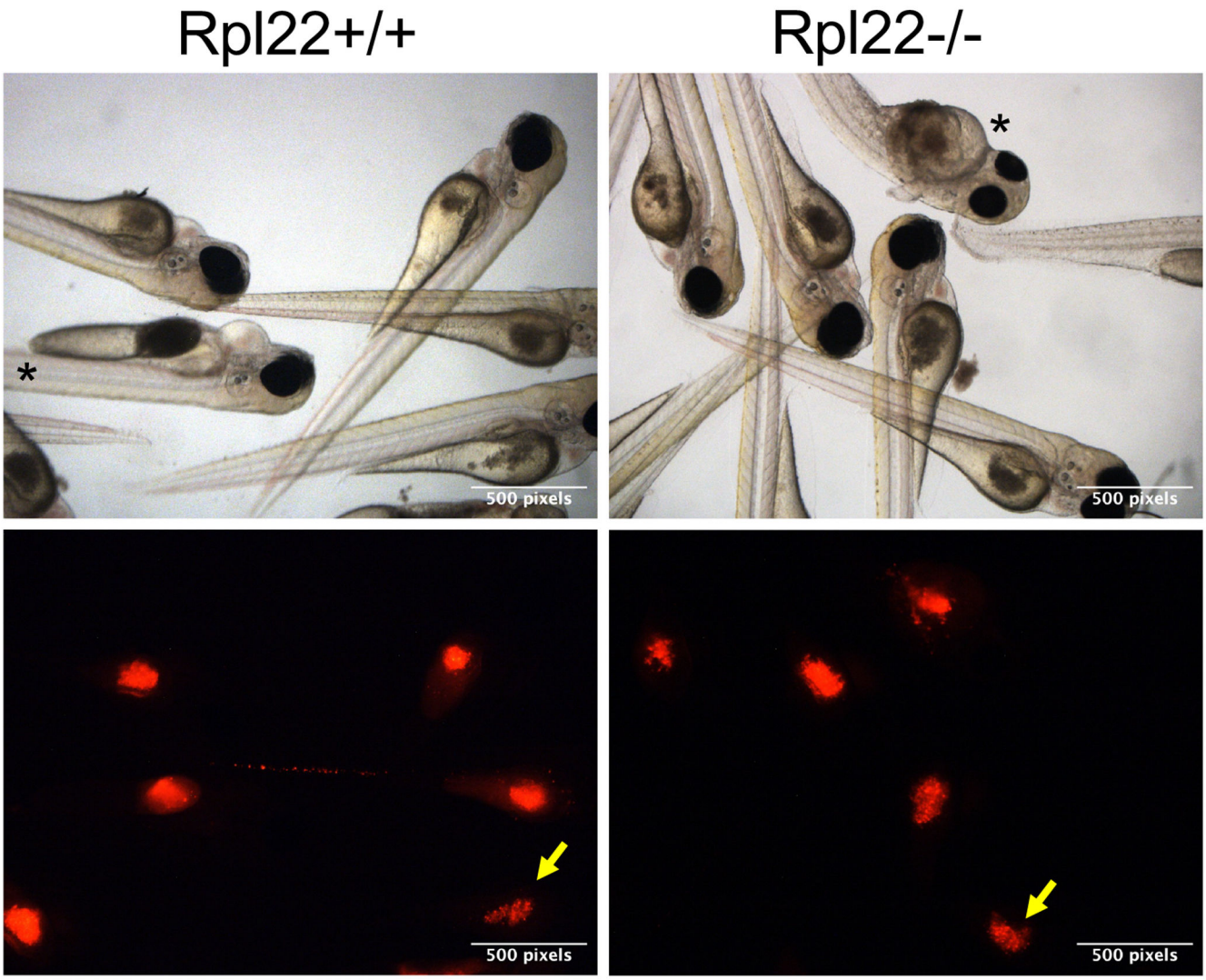

Figure 3: Representative images of xenotransplanted embryos.

Bright field and immunofluorescent images are shown of RFP positive cells in the yolk of *casper* embryos at 1 day post injection (dpi; clutch image). Embryos with an inferior bolus are indicated with a yellow arrow, while those with disturbed morphology are indicated with an asterisk.

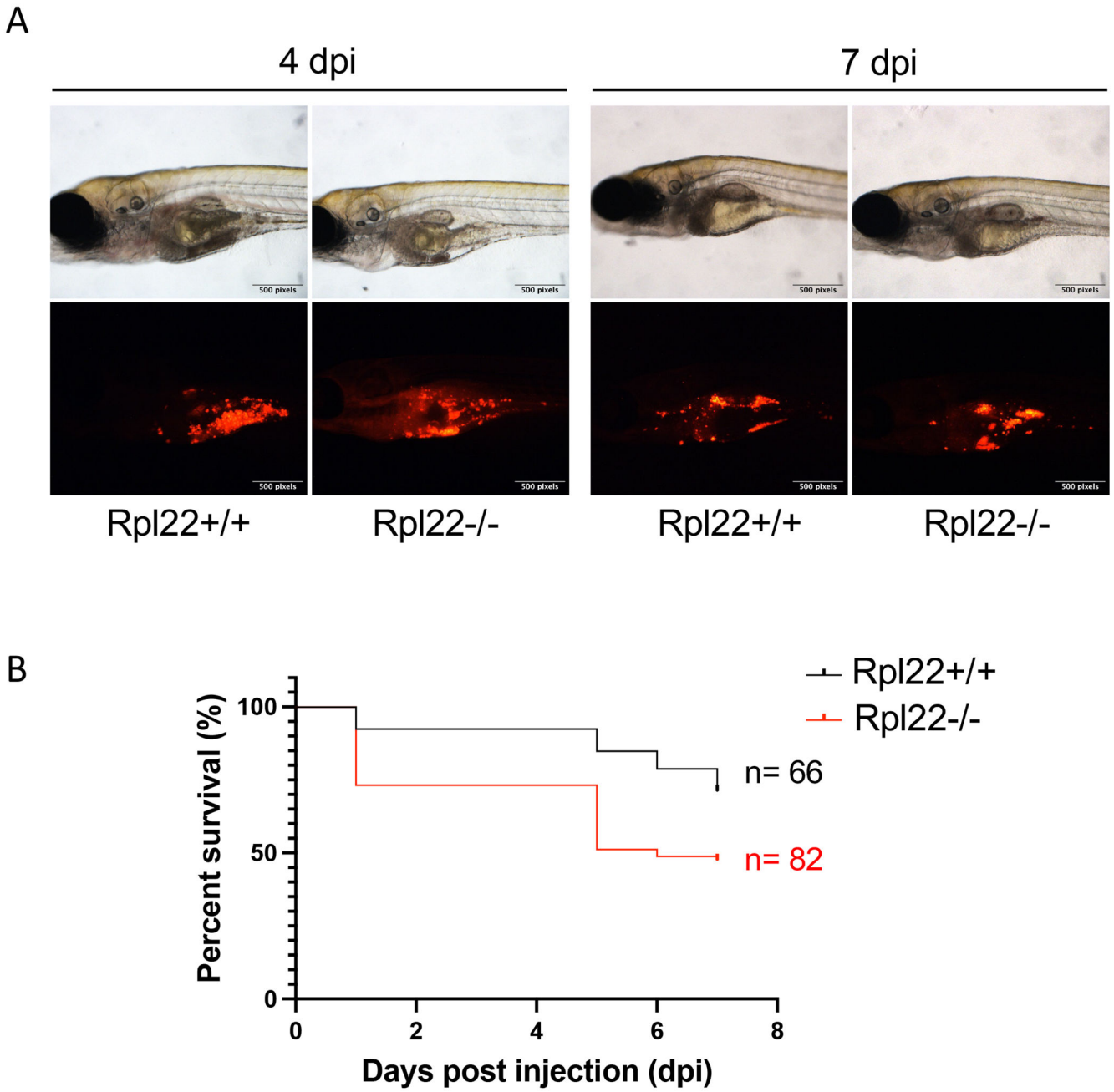

Figure 4: Assessment of disease progression by fluorescence imaging and survival analysis.
A. Representative image of xenotransplanted embryos at 4 and 7dpi. B. The Kaplan Meier plot showing the survival analysis of embryos with two genetically distinct leukemia lines.

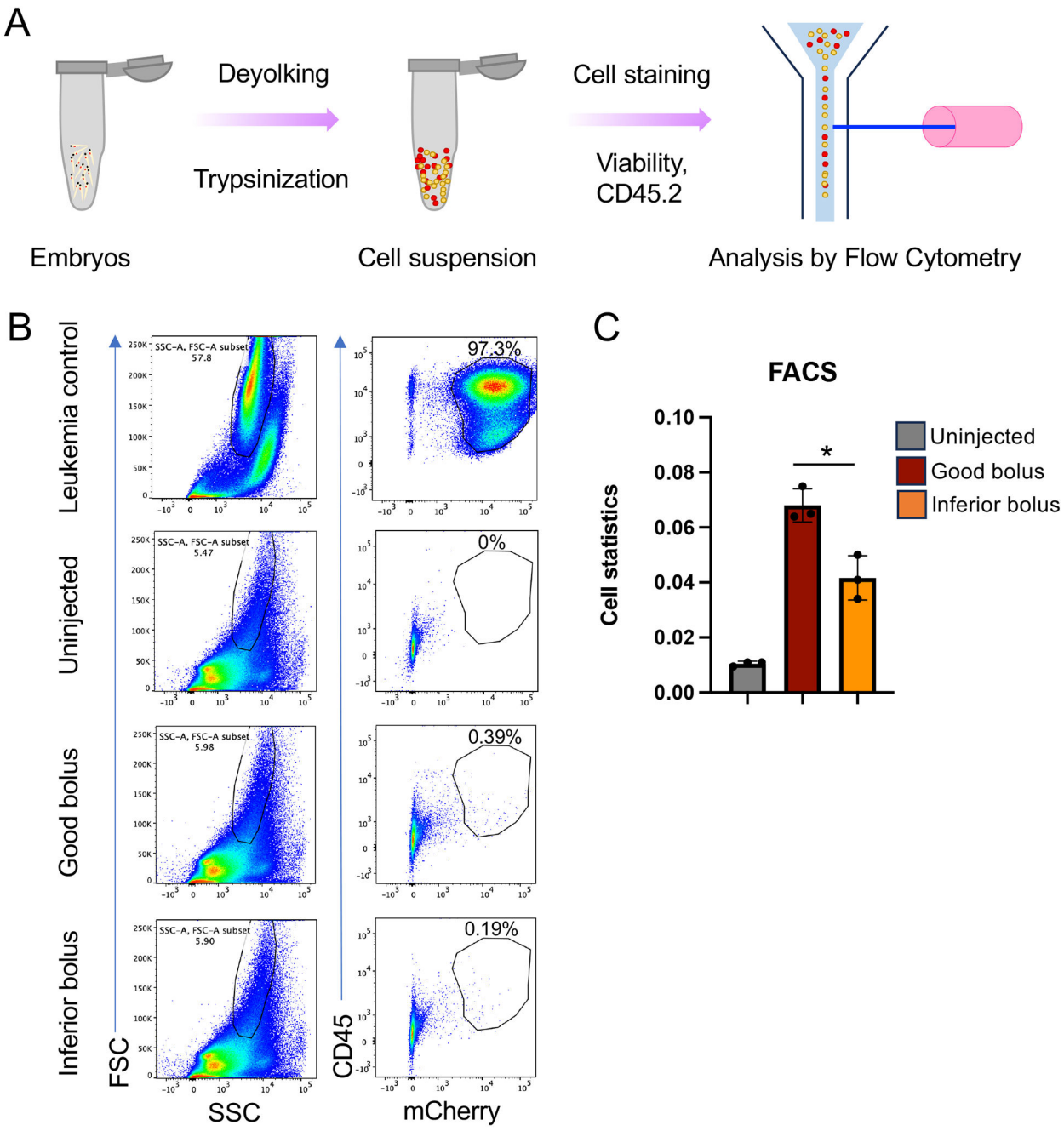

Figure 5: Flow cytometric analysis of disease burden in xenotransplanted zebrafish.

A. Schematic representation of preparation of cell suspension and flow cytometry analysis. Briefly, embryos at 4 dpi are disaggregated into single cell suspensions using trypsin and collagenase followed by flow cytometry. B. Representative plots for the flow cytometry analysis where the left image in each panel is FSC-A v/s SSC-A plot and the right image is CD45 v/s mCherry signals. C. Bar graph showing the cell statistics for xenotransplanted cells as obtained from CD45 v/s mCherry plot for uninjected, good, and inferior embryos

(n= 45, 40, and 40 for each replicate (n=3); p value *=<0.05, calculated using unpaired t-test with Welch's correction in GraphPad Prism 9).