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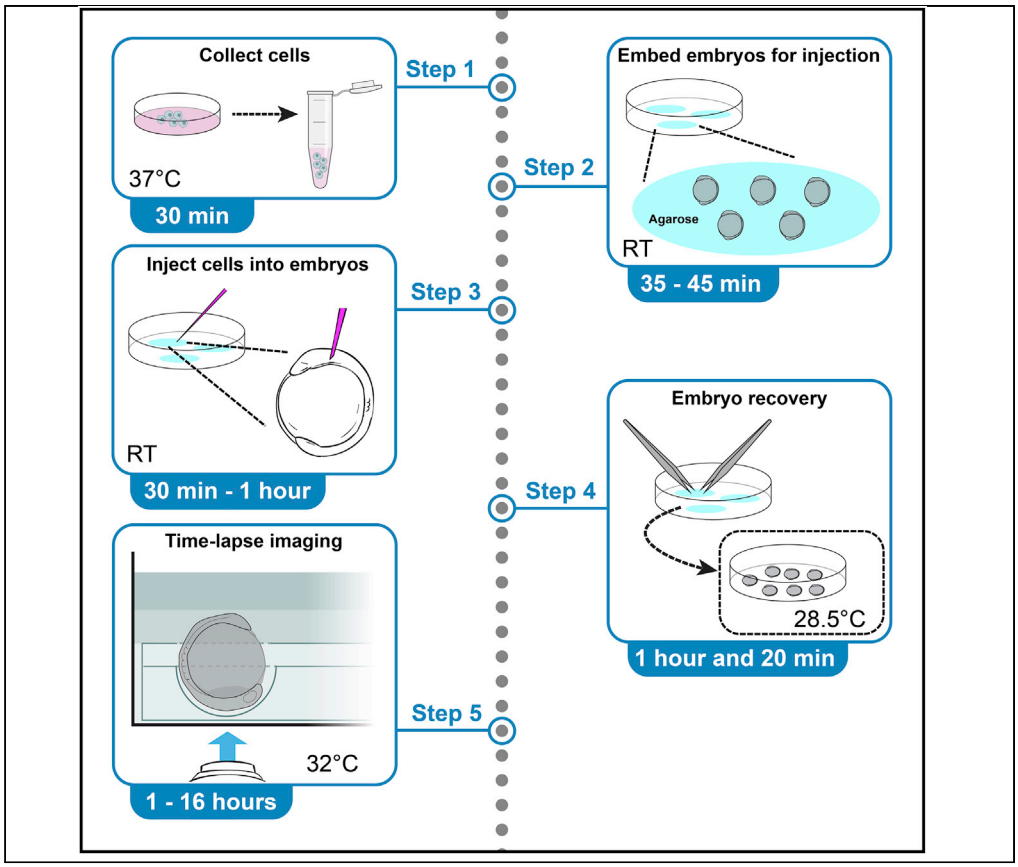
Injection of human neuroblastoma cells into neural crest streams in live zebrafish embryos



Cancer cell behavior is highly microenvironment dependent, but we have a limited understanding of malignant cell-microenvironment interactions *in vivo*. Here, we describe a protocol for xenotransplanting human neuroblastoma (NB) cells into streams of migrating neural crest stem cells in zebrafish embryos, followed by confocal time-lapse imaging and cell tracking. This high-resolution model system facilitates the quantitative spatiotemporal analysis of cancer cell-cell and cell-environment interactions.

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Highlights

Xenotransplantation of human neuroblastoma cells into live zebrafish embryos

Model system to study neural crest and neuroblastoma comigration

Time-lapse imaging and tracking of neuroblastoma cells in an *in vivo* microenvironment

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Injection of human neuroblastoma cells into neural crest streams in live zebrafish embryos

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SUMMARY

Cancer cell behavior is highly microenvironment dependent, but we have a limited understanding of malignant cell-microenvironment interactions *in vivo*. Here, we describe a protocol for xenotransplanting human neuroblastoma (NB) cells into streams of migrating neural crest stem cells in zebrafish embryos, followed by confocal time-lapse imaging and cell tracking. This high-resolution model system facilitates the quantitative spatiotemporal analysis of cancer cell-cell and cell-environment interactions.

For complete details on the use and execution of this protocol, please refer to Treffy et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the injection of human SK-N-AS neuroblastoma (NB) cells into streams of migrating neural crest stem cells in live zebrafish embryos. This protocol can be applied to inject additional human cell lines and patient-derived xenografts into multiple locations within neural crest migratory streams, as described in Treffy et al. (2021). Tracking injected cells requires either staining with a long-lasting dye such as Hoechst 34580 or CellTracker CM-DiI or, preferably, expression of a fluorescent protein. Here, we describe the injection of a stable SK-N-AS cell line that expresses membrane-localized mCherry fluorescent protein.

Prepare embryo mounting materials

Timing: 2 h

Note: We employ an imaging mold designed to hold multiple embryos in place and facilitate automated imaging of the region of interest over time (Figures 1A and 1B). This mold is created using an embryo-shaped template that can be used across multiple embryo orientations and ages (Rajan et al., 2018, 2021) and is composed of 0.9% low melt agarose (LMA) in 30% Danieau solution (prepared from 300% Danieau stock solution; recipe below) plated in a glass culture/imaging cassette (Methods video S1).

1. Prepare 0.9% LMA in 30% Danieau solution (recipe below).
2. Add 200 μL of this LMA solution to one corner of the imaging cassette.

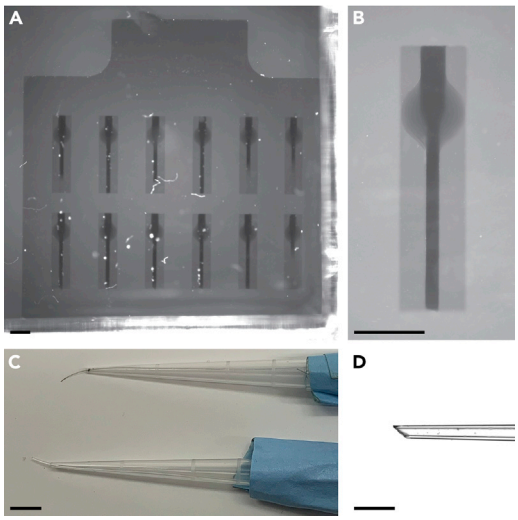

Figure 1. Specialized tools required throughout the protocol

(A) Custom imaging mold designed to hold up to 12 embryos in place.

(B) Close-up view of an embryo slot.

(C) Hair- (top) or fishing line- (bottom) based loops used to delicately manipulate embryos during embedding.

(D) Injection needle pulled from a glass capillary to a fine point and then cut at a 45° angle.

Scale bar: (A and B) 1 mm; (C) 5 mm; (D) 200 μm.

3. Using forceps, place a corner of the mold template on top of the LMA and lower gently to avoid trapping air bubbles.
4. Weigh down the template with approximately 7.0 g (e.g., a vial filled with sand or water) and add additional LMA solution from the sides to fill the area underneath the template. Allow the mold to solidify at room temperature (20°C–25°C) for at least 15 min.
5. Fill the chamber approximately halfway with egg water (recipe below) and leave at room temperature for at least 10 min.
6. Use forceps to gently loosen one edge of the template from the mold but do not remove entirely. Let sit in egg water for another 10 min.
7. Slowly remove the template from the cassette.

⚠ CRITICAL: Shortening the times during steps 5–7 is not recommended, as it can result in imperfections in the underlying agarose that can impact embryo position stability during long-term live imaging.

Note: When wrapped with parafilm, the imaging cassette can be stored at 4°C for up to 2 weeks or until showing signs of dehydration.

Alternatives: We describe the use of a custom mold here, but the protocol can be readily adapted for use without it as well. Briefly, aspirate individual embryos into 0.9% LMA solution, transfer onto a slide, and stabilize and orient using hair- or fishing line-based loops taped inside pipette tips (Figure 1C) as the LMA solidifies.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Agarose	Sigma-Aldrich	Cat# A6013
Agarose, low gelling temperature	Sigma-Aldrich	Cat# A0701
Tricaine/MS-222	Sigma-Aldrich	Cat# A5040
Methylcellulose	Sigma-Aldrich	Cat# M0512
Hoechst 34580	Invitrogen	Cat# H21486
CellTracker CM-DiI Dye	Invitrogen	Cat# C7001
NaCl	Sigma-Aldrich	Cat# 746398
$CaSO_4$	Sigma-Aldrich	Cat# C3771

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Methylene blue	Kordon	Cat# 37344
KCl	Sigma-Aldrich	Cat# 746436
$MgSO_4$	Sigma-Aldrich	Cat# 746452
$Ca(NO_3)_2$	Sigma-Aldrich	Cat# C2786
HEPES	Sigma-Aldrich	Cat# H3375
Phosphate Buffered Saline (PBS)	Sigma-Aldrich	Cat# P5493
0.25% Trypsin-EDTA	Sigma-Aldrich	Cat# T4049
Dulbecco's Modified Eagle's Medium (DMEM) – high glucose	Sigma-Aldrich	Cat# D5796
Fetal Bovine Serum	Sigma-Aldrich	Cat# F2442
Light Mineral Oil	Fisher Scientific	Cat#O121
Pronase	Roche	Cat# 10165921001
Experimental models: Cell lines		
SK-N-AS; use only between passages 2–5	Russo and O'Bryan (2012); Laboratory of John P. O'Bryan, Medical University of South Carolina	N/A
Experimental models: Organisms/strains		
Zebrafish: AB wild-type; both males and females; 3–18 months; 12 hpf	ZIRC	Cat# ZL1
Zebrafish: *Tg(-4.9sox10:eGFP)*; both males and females; 3–18 months; 12 hpf	Wada et al. (2005)	ZFIN ID: ZDB-FISH-150901-15643
Recombinant DNA		
mCherry-Farnesyl5	Laboratory of Michael Davidson, The Florida State University	RRID: Addgene_55045
Software and algorithms		
Imaris	Bitplane	https://imaris.oxinst.com/
Other		
Nunc™ Lab-Tek™ chambered coverglass (imaging cassette)	Thermo Scientific	Cat# 155380
100 mm cell culture treated petri dish	Sigma-Aldrich	Cat# SIAL0167
100 mm non-treated petri dish (for embryo use)	Denville Scientific Inc.	Cat# T1111-20
60 mm non-treated petri dish	VWR	Cat# 734-2794
35 mm non-treated petri dish	Denville Scientific Inc.	Cat# TC9638
6-well cell culture plate	Denville Scientific Inc.	Cat# T1006
Glass Pasteur pipette	MIDSCI	Cat# 357331
1.5 mL microcentrifuge tubes	NEST	Cat# 615001
Dumont Mini Forceps - # 5	Fine Science Tools	Cat# 11200-14
Glass capillaries	World Precision Instruments	Cat# 504949
Nanoject III	Drummond	Cat# 3-000-207
Flaming/Brown micropipette puller	Sutter Instrument	P-87
Confocal laser scanning microscope	ZEISS	LSM 800
Fluorescence stereo zoom microscope	ZEISS	Axio Zoom.V16

MATERIALS AND EQUIPMENT

Egg water		
Reagent	Final concentration	Amount
NaCl	15 mM	4.5 g
$CaSO_4$	8.3 mM	1.125 g
Methylene blue (2.303%)	2.303×10^{-5}%	50 μL
ddH_2O	n/a	Add up to 5 L
Total	**n/a**	**5 L**
Egg water can be stored at room temperature indefinitely. Discard if there is any growth in solution.		

300% Danieau stock solution (Dilute to 30% Danieau solution for use.)		
Reagent	Final concentration	Amount
NaCl	174 mM	10.17 g
KCl	2.1 mM	0.157 g
$MgSO_4$	1.2 mM	0.144 g
$Ca(NO_3)_2$	1.8 mM	0.425 g
HEPES	15 mM	3.57 g
ddH_2O	n/a	Add up to 1 L
Total	**n/a**	**1 L**

Adjust pH to 7.6. Aliquot into 50 mL tubes and store at 4°C indefinitely. Discard if there is any growth in solution.

50× Tricaine		
Reagent	Final concentration	Amount
Tricaine (MS-222)	0.5%	5 g
300% Danieau stock solution	30%	100 mL
ddH_2O	n/a	Add up to 1 L
Total	**n/a**	**1 L**

Add reagents to a glass bottle, add 900 mL water, adjust pH to 7.2, and add additional water to reach a final volume of 1 L. Stir mixture for up to 1 h at room temperature while protected from light. Divide into 50 mL aliquots and store protected from light for up to 1 month at 4°C or at −20°C for up to 1 year.

1% Agarose pads		
Reagent	Final concentration	Amount
Agarose	1%	1 g
300% Danieau stock solution	30%	10 mL
ddH_2O	n/a	Add up to 100 mL
Total	**n/a**	**100 mL**

Melt agarose in 30% Danieau solution and dispense 5 mL into a 60 mm petri dish to solidify. Pads can be prepared ahead of time and stored at 4°C in an airtight container to prevent dehydration. Do not use if the pads appear dehydrated.

Note: The use of 1% agarose pads is recommended to prevent damage to young embryos (<20 h post-fertilization (hpf)) after their protective chorion is removed.

0.9% Low melt agarose (LMA)		
Reagent	Final concentration	Amount
Agarose (low gelling)	0.9%	0.9 g
300% Danieau stock solution	30%	10 mL
ddH_2O	n/a	Add up to 100 mL
Total	**n/a**	**100 mL**

Store at 50°C in an airtight glass bottle to prevent evaporation and alteration of solution composition.

2.5% Methylcellulose		
Reagent	Final concentration	Amount
Methylcellulose	2.5%	5 g
300% Danieau stock solution	30%	20 mL
ddH_2O	n/a	Add up to 200 mL
Total	**n/a**	**200 mL**

Maintain at 4°C for long-term storage. (Manufacturer's guidelines state solution will remain stable for up to 1 year at room temperature.)

Methylcellulose mounting solution		
Reagent	Final concentration	Amount
50× Tricaine	1.5×	60 μL
2.5% Methylcellulose	2%	1.6 mL
30% Danieau stock solution	30%	340 μL
Total	**n/a**	**2 mL**
Make fresh. Incubate at 28.5°C for 20 min prior to use.		

30 mg/mL Pronase		
Reagent	Final concentration	Amount
Pronase	30 mg/mL	1 g
ddH_2O	n/a	Add up to 33.3 mL
Total	**n/a**	**33.3 mL**
Prepare 1 mL aliquots and store at −20°C for up to 1 year.		

STEP-BY-STEP METHOD DETAILS

Prepare embryos for injection

Timing: 10 min

Remove embryonic chorion.

1. Collect embryos at 10–11 hpf (Kimmel et al., 1995) and move to a 60 mm dish.
2. Prepare 0.3 mg/mL Pronase solution by adding 50 μL 30 mg/mL Pronase (recipe above) to 5 mL egg water. Mix thoroughly.
3. Incubate embryos in 5 mL Pronase solution for 5 min at room temperature in a 60 mm dish. Gently agitate the plate every 1–2 min to facilitate chorion removal.
4. Remove Pronase solution and gently wash embryos with 28.5°C egg water 3–5 times to rinse excess Pronase and ensure embryo detachment from the chorion.
5. Transfer embryos to 1% agarose pad. Keep embryos at 28.5°C until ready to proceed with step 15 below.

Collect SK-N-AS neuroblastoma cells

Timing: 30 min

Prepare SK-N-AS cells as a single-cell suspension for injection.

Note: Prior to injection, cells should be approximately 75% confluent in a 6-well plate.

6. Remove media from cells and rinse with 2 mL cell culture-grade PBS prewarmed to 37°C.
7. Add 300 μL 0.25% Trypsin-EDTA and ensure the entire surface of the well is coated evenly.
8. Incubate at 37°C for 1–2 min.
9. Monitor for cell detachment under an inverted microscope. Gentle tapping on the underside of the plate may help remove the monolayer, though excessive shaking should be avoided to minimize cell clumping.
10. Add 3 mL DMEM + 10% FBS to suspended cells, aspirate into a sterile collection tube, and spin down at 168 × *g* for 2 min.
11. Carefully remove supernatant, resuspend cells in 1 mL of DMEM only (no FBS), and transfer to a 1.5 mL tube. Incubate at 37°C for 20–30 min until cells settle at the bottom of the tube.

Note: If cells are not settling, you can also spin them down briefly (168 × *g*, 1 min) prior to injection.

Set up microinjector

Timing: 5 min

These steps describe setting up a Nanoject III microinjection apparatus but are broadly applicable.

Note: The microinjector can be prepared at any point during this protocol.

Note: Injection needles are created from capillaries using a micropipette puller. We used Sutter Instrument's P-87 model with the following unitless parameter values: heat: 475; pull: 70; velocity: 50; time: 80.

12. View pulled capillaries at high magnification with a microscope and snap with No. 5 forceps at a 45° angle. (Figure 1D; Methods video S2).

⚠ CRITICAL: The optimal needle diameter will depend on the specific cells of interest and should be customized accordingly. The opening should be just wide enough to allow smooth passage of individual cells during injection but small enough to avoid major damage to the embryo (see Troubleshooting problem 1). In our experience, injection (as described below) yields a survival rate of approximately 90% within the first hour, after which embryo survival is comparable to uninjected controls.

13. Fill needle completely with mineral oil to eliminate the presence of air that would prevent a seal between the microinjector and needle. Attach needle to the microinjector probe, ensuring that the collet is tightened to obtain a tight seal.
14. Eject approximately half the mineral oil from the needle and ensure there are no air bubbles. Monitor needle briefly for excess mineral oil dripping from the end of the needle. If the seal is adequate, mineral oil will not accumulate.

Embed embryos for injection

Timing: 30–45 min

15. Transfer 2 mL 0.9% LMA to a 40°C heating block.
16. Aspirate 10–15 embryos into a glass pipette. Allow embryos to settle near the bottom of the pipette to displace egg water, then gently dispense into prewarmed LMA.

Note: We recommend coating the glass pipette with 0.9% LMA prior to this step to decrease the risk of embryos sticking to it.

17. Aspirate LMA-coated embryos and deposit into a 100 mm petri dish. Evenly spread the 0.9% LMA into an area measuring roughly 15–20 mm by 10–15 mm to create an agarose pad.
18. Orient embryos rapidly before the 0.9% LMA solidifies: To inject into the midst of migrating neural crest cells, embryos are positioned with their anterior aspect to the left, the dorsal side of the head at the top, and slightly tilted such that the left side of the embryo is angled up to facilitate injections. (Figure 2A; Methods video S3).
19. Repeat steps 16–18 until the desired number of embryos has been mounted.

Note: A 100 mm petri dish can fit 6–8 pads of embryos depending on the volume of LMA used.

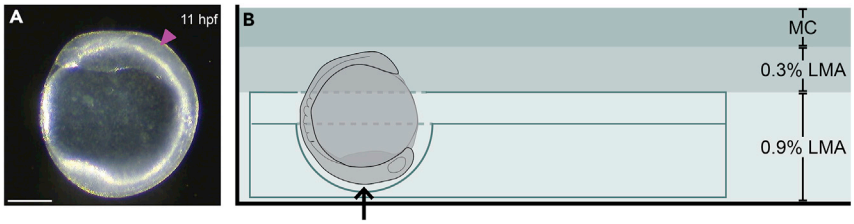

Figure 2. Embryo injection and imaging orientations

(A) 11 hpf zebrafish embryo embedded in 0.9% low melt agarose prior to injection. Arrow indicates the injection site. (B) Schematic depicting embryo embedding for inverted confocal imaging; the region of interest is positioned toward the base of the mold (arrow). MC, methylcellulose; LMA, low melt agarose. Scale bar: 200 μm.

20. Incubate agarose pads at room temperature for 3–5 min and then at 4°C for 2 min.
21. Once solidified, add egg water to keep the embedded embryos and 0.9% LMA hydrated during injection.

Note: With experience, we have embedded up to 40 embryos within a single agarose pad and generated 4–6 pads per dish, resulting in approximately 200 embryos available for injection per dish. However, we recommend starting with 6–8 embryos per pad to practice proper mounting and injection timing.

Inject cells into embryos

Timing: 30–60 min

22. Aspirate 50 μL of cells (from step 11) and place into a 30 mm petri dish. Pipette the droplet up and down to mix and minimize cell clumping.
23. Allow cells to settle on the bottom of the dish for 3–5 min.
24. Aspirate cells into the injection needle (see Troubleshooting problems 1, 2, and 3).
    a. We recommend aspirating a small volume of media before collecting the cells to provide a buffer between the cells and mineral oil.
    b. When aspirating, bring the needle close to the bottom of the dish to obtain settled cells.

Note: Cell density is important for this step to ensure consistency in the number of cells that are injected into each embryo. Using the parameters described in this protocol, we inject 5–10 cells per embryo.

25. Position embryos under the microscope. Ensure that cells are flowing through the needle by expelling a small volume into the egg water.
26. Set microinjector to inject 1 nL at a rate of 8 nL/s. The injection volume can be decreased according to cell density but should not exceed 1 nL per embryo (see Troubleshooting problem 2).
27. Position the needle just posterior to the developing eye, medial to the yolk/embryo interface.
    a. Use a combination of moving the needle on the microinjector and your hands to move the plate to puncture embryos at the appropriate depth (Methods video S4).

Note: You will cause a depression in the embryo as the needle starts to advance. Once the needle enters the embryo, it will relax. At this point, you may need to retract the needle slightly to position at the region of interest and not within the yolk (Methods video S4).

28. Once at the desired depth, inject the cells. (See Troubleshooting problem 4).

Optional: To inject into neural crest cells, we recommend the use of *Tg(-4.9sox10:eGFP)*-positive embryos (labeling neural crest cells with eGFP) for subsequent imaging, as described below. At this stage, you can view injected embryos under a fluorescence microscope to determine if cells were injected into the desired location(s) relative to eGFP-positive cells.

29. Once all mounted embryos have been injected, proceed to the recovery stage. This point marks 0 h post-injection (0 hpi). (See Troubleshooting problem 5).

Embryo recovery and live imaging

Timing: 1–16 h

30. Remove embryos from LMA.
    a. Use a pair of No. 5 forceps to scrape away LMA as shown in Methods video S5.
    b. Aspirate embryos with a glass pipette and transfer to fresh egg water on top of a 1% agarose pad.

⚠ CRITICAL: All agarose must be removed prior to aspirating, or the remaining agarose can damage the embryos.

31. Allow embryos to recover for 1 h at 28.5°C prior to proceeding with downstream applications.
32. Select embryos for imaging.
    a. Discard visibly damaged embryos.
    b. Stage embryos to confirm normal development and use a fluorescence microscope to select healthy embryos that harbor mCherry-positive NB cells in the desired anatomical location(s).
33. Mount embryos for imaging (Figure 2B).
    a. Prepare 1 mL of 0.3% LMA working solution (1:3 dilution of 0.9% LMA in 30% Danieau solution + 1.5× tricaine) and prewarm to 32°C.
    b. Add 500 μL of prewarmed 0.3% LMA working solution onto the embryo imaging mold.
    c. Aspirate up to 12 embryos with a glass pipette, allow to settle near the bottom of the pipette, then drop into the remaining 500 μL 0.3% LMA working solution in the tube.
    d. Transfer embryos and remaining solution onto the embryo imaging mold.
    e. For an inverted microscope, position embryos so that the region of interest is adjacent to the bottom of the imaging cassette. Remove any excess 0.3% LMA (Figure 2B).
    f. Incubate imaging cassette at 4°C for 5–10 min to allow the 0.3% LMA to solidify.
    g. Add 2 mL of 32°C methylcellulose mounting solution on top of the solidified 0.3% LMA.

    Note: Experimental treatments can be added to the LMA and/or the overlying methylcellulose. Please see (Treffy et al., 2021) for examples.

    h. Incubate embryos at 32°C for 30 min prior to imaging, as the embryos may drift slightly as the LMA warms and embryos settle. Recheck orientation before beginning imaging session and adjust z-stack acquisition settings as necessary.
    i. Obtain z-stacks with a 40×/1.1 W objective over 11–14 h, with the incubation chamber maintained at 32°C.

    Note: We prioritized our imaging settings to yield rapid z-stacks (2.5 min per embryo) across 12 embryos, resulting in 30-minute time intervals for each embryo. All data collation, segmentation, and analysis were performed using Bitplane's Imaris software. These choices can be adjusted as desired for specific experimental goals.

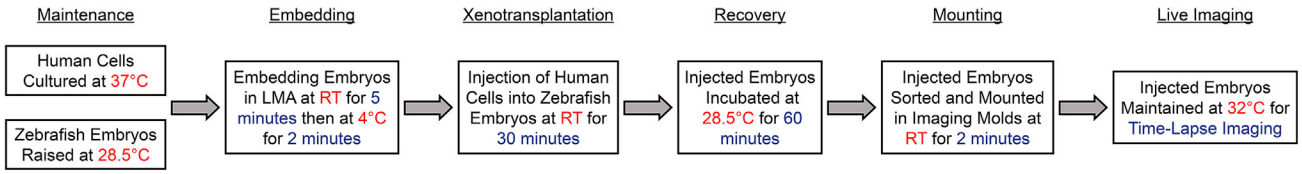

Figure 3. Temperatures throughout protocol
Flowchart illustrates the temperatures used at each stage of the protocol. RT, room temperature.

Note: Please see Figure 3 for a detailed explanation of temperature changes throughout the protocol.

Note: Upon injection of 5–10 cells, we reliably obtain live imaging of 3–8 cells. Due to the low density of injected cells, clumping is not an issue in the embryo. Cells that have entered a neural crest migratory stream travel as individual cells and are clearly distinguishable (Figure 4; also see Treffy et al., 2021). (See Troubleshooting problem 3).

EXPECTED OUTCOMES

Our workflow provides a standardized methodology with which to track NB cell migration and other behaviors *in vivo* as host neural crest cells migrate in the developing zebrafish embryo. These behaviors can be visualized starting immediately after injection (Figures 4A and 4A′) and tracked throughout several hours of embryonic development (Figures 4B and 4C). The technique can be modified for use with various cell lines or primary cells, at different embryonic timing/stages, and/or in the context of genetic or chemical modifications.

LIMITATIONS

This protocol was designed and tested for use in vertebrate embryonic experimental systems (Treffy et al., 2021). As such, the microenvironment will differ to some extent from those found in human embryos and infants. It's also important to note that NB in patients is highly heterogeneous within any given tumor, and additionally, there are a large number of NB subtypes across patients (Jiang et al., 2011; Matthay et al., 2016). Our use of human cell lines and patient-derived xenografts cannot fully recapitulate that landscape of NB tumor diversity.

Additionally, long-term studies of cell survival/tumor formation are limited by the zebrafish adaptive immune system's activation at ~2–3 weeks age (Willett et al., 1999; Langenau et al., 2004). If longitudinal studies of tumor growth/survival are desired beyond those time points, this protocol could potentially be adapted for use in immunocompromised zebrafish.

Finally, it is important to consider the limitations of temperature in our protocol (Figure 3). The rate of zebrafish development changes significantly depending on incubation temperature, with increasing temperatures leading to more rapid embryogenesis (Schirone and Gross, 1968; Kimmel et al., 1995). Meanwhile, a higher temperature (37°C) is used to culture human cells *in vitro* than is optimal for zebrafish embryo growth (28.5°C). We pinpointed 32°C as providing an effective balance between the needs of xenotransplanted human NB cells and appropriate development of host zebrafish embryos, and we recommend the consistent use of internal comparisons and controls (e.g., anterior vs posterior; cranial vs trunk regions) for data analysis as well as careful monitoring of NB survival/proliferation to strengthen confidence in this approach.

TROUBLESHOOTING

Problem 1

Unable to aspirate cells when preparing the injection needle (step 24).

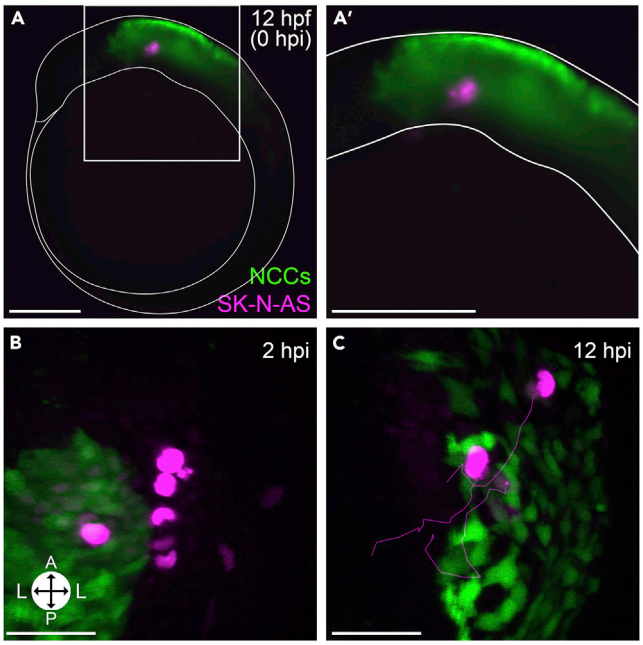

Figure 4. SK-N-AS NB cells injected at 12 hpf and tracked via live confocal imaging

(A and A′) mCherry-expressing SK-N-AS cells (magenta) are shown immediately after injection (12 hpf = 0 hpi) into *Tg(-4.9sox10:eGFP)* zebrafish embryos.

(B and C) SK-N-AS cells (magenta) migrating alongside neural crest cells (green) at 2 hpi (B) and 12 hpi (C). Migration tracks (magenta) are shown in (C) for three SK-N-AS cells.

Scale bar: (A and A′) 200 μm; (B and C) 40 μm.

Potential solution

The needle diameter is likely too small and can be clipped at a higher point. If the needle appears to be large enough for a given diameter of cells, then the needle may be blocked by clumping. To fix this, move the needle out of the cell droplet to manually eject the clump of cells (see example in Methods video S4 at the 25 second mark), or use forceps to cut the tip of the needle off. To help avoid this problem, pipette up and down within the cell droplet to separate the cells prior to filling the needle.

A second possibility is the introduction of air bubbles due to an incomplete seal when assembling the injection setup. You will need to remove the needle from the microinjector and fill it with mineral oil to expel any air bubbles, as outlined above. A complete seal will result in no leakage of mineral oil while the microinjector is idle.

Problem 2

Cells are aspirating out of the needle without initiating injection and/or too much liquid is being injected (steps 24 and 26).

Potential solution

The seal between the microinjector and injection needle is not sufficient and there are likely air bubbles within the needle. Ensure the collet is tightened to maintain a complete seal and remove any air bubbles as described in Troubleshooting problem 1. If the problem persists after removing air bubbles or adjusting the seal, then the diameter of the needle is likely too large, and a new needle should be clipped at a lower point. With practice and use of a single micropipette puller at consistent settings (please see step 12 for our settings), trial and error will quickly yield a working knowledge of the optimum range for needle clipping, customized to the number and type of cells being injected.

Problem 3

Too few/too many injected cells to visualize (steps 24, 26, and 33).

Potential solution

The desired number of cells will vary depending on experimental conditions and goals. If too few cells are injected, you may not be aspirating enough cells. Allow cells to settle near the bottom of the tube following collection to maximize the number of cells aspirated in a given volume in step 22. In step 23, allow the cells to settle near the bottom of the petri dish prior to aspirating into the injection needle. For more buoyant cell types, the incubation times in steps 11 and 23 can be increased to facilitate settling. Alternatively, spinning the cells briefly will concentrate them in a pellet, allowing for more cells to be collected during step 22.

If too many cells are being injected, we recommend diluting your cells prior to step 23 and/or adjusting the microinjector settings to inject a smaller volume. Additionally, cells may clump within the needle itself throughout a round of injections. If a clump is observed near the tip of the needle, you can remove it as described previously (Troubleshooting problem 1).

Problem 4

Embryos moving or getting dislodged during injection (step 28).

Potential solution

It is possible that the 0.9% LMA solution was allowed to set for too long before the addition of egg water, causing the mold to dry out and lose its adherence to the petri dish. Alternatively, the agarose may not have set long enough prior to the addition of egg water. Typically, 5–10 min is sufficient to allow the embryos to set. Petri dishes that have been washed or rinsed from previous use may have particulates on the surface, preventing total contact with the agarose to the dish, and we recommend using a new plate each time for maximum adhesion.

Problem 5

High rates of embryo death following injection (step 29).

Potential solution

Contributors to embryo death may include: contaminants such as chemical residue that could affect embryo health and development; size of injection needle; number of injected cells; air bubbles in injected solutions; large injection volumes; technique for rescuing from agarose post-injection or post-imaging. Meticulous cleaning of any reused components (e.g., petri dishes for embryos) is essential, and the techniques described can be improved with practice. Of particular note, the angle of the needle during injections should be maintained at 45°, which provides a sharp point that is ideal for injection with minimal blunt pressure (Figure 1D).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ankur Saxena (saxenaa@uic.edu).

Materials availability

This study did not generate unique materials.

Data and code availability

No unique datasets or code were generated in this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101380.

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AUTHOR CONTRIBUTIONS

Conceptualization: X.J., R.T., and A.S.; methodology: X.J., R.T., and A.S.; validation: B.A.I., X.J., and R.T.; formal analysis: X.J. and R.T.; investigation: B.A.I., X.J., and R.T.; resources: A.S.; writing – original draft: B.A.I and A.S.; writing – review and editing: B.A.I., X.J., R.T., and A.S.; visualization: B.A.I. and X.J.; supervision: A.S.; funding acquisition: A.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INSTITUTIONAL PERMISSIONS

Zebrafish were treated and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals. All experiments were approved by the University of Illinois Chicago Institutional Animal Care Committee. Approval should be granted by any necessary local or national institutions prior to beginning the experiments outlined herein.

REFERENCES

Jiang, M., Stanke, J., and Lahti, J.M. (2011). The connections between neural crest development and neuroblastoma. In Current Topics in Developmental Biology, First Edition, M.A. Dyer, ed. (Elsevier Inc), pp. 77–127.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. Dev. Dyn. *203*, 253–310. https://doi.org/10.1002/AJA.1002030302.

Langenau, D.M., Ferrando, A.A., Traver, D., Kutok, J.L., Hezel, J.P.D., Kanki, J.P., Zon, L.I., Look, A.T., and Trede, N.S. (2004). In vivo tracking of T cell development, ablation, and engraftment in transgenic zebrafish. Proc. Natl. Acad. Sci. U S A *101*, 7369–7374. https://doi.org/10.1073/PNAS.0402248101.

Matthay, K.K., Maris, J.M., Schleiermacher, G., Nakagawara, A., Mackall, C.L., Diller, L., and Weiss, W.A. (2016). Neuroblastoma. Nat. Rev. Dis. Primers *2*, 1–21. https://doi.org/10.1038/nrdp.2016.78.

Rajan, S.G., Gallik, K.L., Monaghan, J.R., Uribe, R.A., Bronner, M.E., and Saxena, A. (2018). Tracking neural crest cell cycle progression in vivo. Genesis *56*, e23214. https://doi.org/10.1002/dvg.23214.

Rajan, S.G., Nacke, L.M., Dhingra, J.S., and Saxena, A. (2021). Notch signaling mediates olfactory multiciliated cell specification. Cells Dev. 203715. https://doi.org/10.1016/J.CDEV.2021.203715.

Russo, A., and O'Bryan, J.P. (2012). Intersectin 1 is required for neuroblastoma tumorigenesis. Oncogene *31*, 4828–4834. https://doi.org/10.1038/onc.2011.643.

Schirone, R.C., and Gross, L. (1968). Effect of temperature on early embryological development of the zebra fish, Brachydanio rerio. J. Exp. Zool. *169*, 43–52. https://doi.org/10.1002/JEZ.1401690106.

Treffy, R.W., Rajan, S.G., Jiang, X., Nacke, L.M., Malkana, U.A., Naiche, L., Bergey, D.E., Santana, D., Rajagopalan, V., Kitajewski, J.K., et al. (2021). Neuroblastoma differentiation in vivo excludes cranial tumors. Dev. Cell *56*, 2752–2764.e6. https://doi.org/10.1016/j.devcel.2021.09.014.

Wada, N., Javidan, Y., Nelson, S., Carney, T.J., Kelsh, R.N., and Schilling, T.F. (2005). Hedgehog signaling is required for cranial neural crest morphogenesis and chondrogenesis at the midline in the zebrafish skull. Development *132*, 3977–3988. https://doi.org/10.1242/DEV.01943.

Willett, C.E., Cortes, A., Zuasti, A., and Zapata, A.G. (1999). Early hematopoiesis and developing lymphoid organs in the zebrafish. Dev. Dyn. *214*, 323–336. https://doi.org/10.1002/(sici)1097-0177(199904)214:4<323::aid-aja5>3.0.co;2-3.