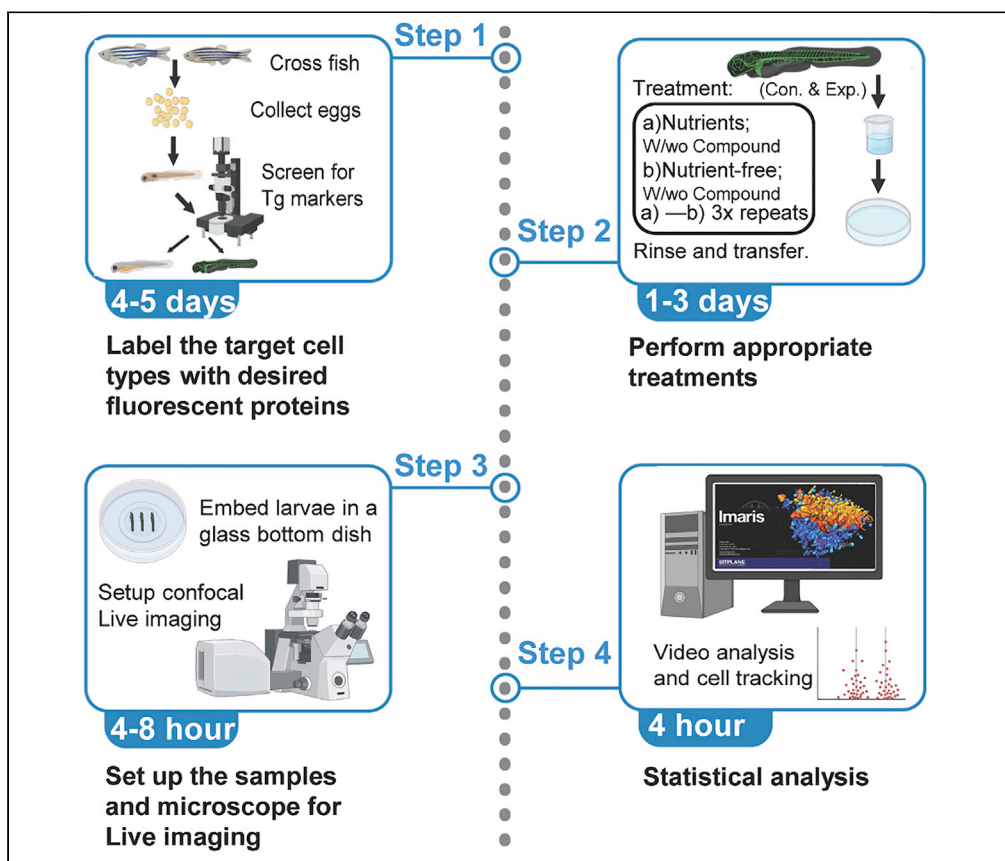# STAR Protocols

Protocol

Optimized protocol for live imaging of overnutrition-elicited interactions between immune cells and β cells in zebrafish

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Highlights

Detailed protocol for inducing β cell death in larval zebrafish with overnutrition

Thorough guide to perform live imaging of macrophages and neutrophils near the islet

A primer on tracking immune cells using Imaris

Here, we provide an optimized protocol to observe the interactions between infiltrating immune cells and islet β cells using live imaging. This protocol is useful for the characterization of cell-cell interactions and for the direct visualization of immune cell migration to the principal pancreatic islet during islet inflammation. We describe the preparation of zebrafish transgenic lines and detail steps for setting up the fish for live confocal imaging.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Optimized protocol for live imaging of overnutrition-elicited interactions between immune cells and β cells in zebrafish

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SUMMARY

Here, we provide an optimized protocol to observe the interactions between infiltrating immune cells and islet β cells using live imaging. This protocol is useful for the characterization of cell-cell interactions and for the direct visualization of immune cell migration to the principal pancreatic islet during islet inflammation. We describe the preparation of zebrafish transgenic lines and detail steps for setting up the fish for live confocal imaging.
For more details on the use and execution of this protocol, please refer to Yang et al. (2022).[1]

BEFORE YOU BEGIN

Before starting the experiment, researchers should have breeders with the desired genotypes and with the cell types of interest labeled with fluorescent proteins (red, green, or other compatible combinations). They should also have all the required materials and equipment ready, such as a LSM 780/880 Laser Scanning Microscope or equivalent, a stereo & zoom microscope and Imaris v 9.2x software, etc. See "key resources table" and "materials and equipment" for the lists of these items.

Institutional permissions

The zebrafish is a vertebrate species. Using zebrafish in research requires permission from institutional animal care and use committee (IACUC) or its equivalent. All experiments performed in this protocol adhered to the regulatory standards set forth by Vanderbilt University and IACUC.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Instant Ocean Salt	https://www.webzoo.net/	AS 1010006
Sodium chloride (NaCl)	Research Products International	S23020
Potassium chloride (KCl)	Research Products International	P41000
Sodium hydroxide (NaOH)	Research Products International	S24000
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Millipore Sigma	MX0070

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$·4H$_2$O)	Millipore Sigma	C1396
HEPES	Millipore Sigma	H4034
Sodium hydroxide (NaOH)	Millipore Sigma	S8045
Agarose, low gelling temperature	Sigma-Aldrich	A9414
Tricaine	Millipore Sigma	Cat# A-5040
Trizma hydrochloride solution	Millipore Sigma	Cat# T2819
Deoxynucleotide (dNTP) Solution Set	New England Biolabs	N0446S
GoTaq® DNA Polymerase	Promega	Cat# M3001
Oligonucleotides		
tnfa genotyping primer forward: ATCTTCAAAGTCGGGTGTATGG		N/A
tnfa genotyping primer reverse: CTCACCACTTCCATCTTGTTGA		N/A
Experimental models: Organisms/strains		
Zebrafish: *Tg(ins:H2BmCherry)*. AB strain. Adults and larvae, mixed gender.	Maddison et al.[2]	vu513Tg
Zebrafish: *Tg(mpeg1:EGFP)*. AB strain. Adults and larvae, males and females.	Ellet et al.[3]	gl22Tg
Zebrafish: *Tg(acta1:dnigf1ra-EGFP)*, referred to as *zMIR*. AB strain. Adults and larvae, males and females.	Maddison et al.[4]	vu530Tg
Zebrafish: *Tg(LyzC:GFP)*. AB strain. Adults and larvae, males and females.	Hall et al.[5]	nz117Tg
Zebrafish: *Tg(LyzC:DsRED2)*. AB strain. Adults and larvae, males and females.	Hall et al.[5]	nz50Tg
Zebrafish: *Tg(ins:H2BmCherry); Tg(mpeg1:EGFP), Tg(LyzC:DsRED2)*. AB strain. Adults and larvae, males and females.	Yang et al.[1]	N/A
Zebrafish: *Tg(ins:H2BmCherry); Tg(mpeg1:EGFP), Tg(LyzC:GFP)*. AB strain. Adults and larvae, males and females.	Yang et al.[1]	N/A
Zebrafish: *tnfa−/−; Tg(ins:H2BmCherry); Tg(mpeg1:EGFP); Tg(LyzC:DsRED2);zMIR*. AB strain. Adults and larvae, males and females.	Yang et al.[1]	N/A
Software and algorithms		
GraphPad Prism 6	GraphPad Software	https://www.graphpad.com/
Imaris v 9.2x	Bitplane	http://www.bitplane.com/
Other		
LSM 780 Laser Scanning Microscope	Zeiss	http://www.zeiss.com; RRID:SCR_014344
Thermocycler	Bio-Rad	T100
Thermocycler	Biometra GmbH	Trio
Stereo zoom microscope (Discovery.V12)	Zeiss	https://www.zeiss.com/microscopy
Stereo zoom microscope	Nikon	SMZ800
Nylon mesh strainer	N/A	https://www.amazon.com/Nylon-Strainer/s?k=Nylon+Strainer
35 mm glass bottom dishes (uncoated)	MatTek	P35G-1.0-20-C
100 × 15 mm Petri dish	Fisherbrand	Cat# FB0875713
Insulin syringe (1 mL)	BD	BD 329424
Transfer pipettes	Fisherbrand	Cat# 137117M
5 ³/₄ Disposable Pasteur Pipettes Borosilicate Glass	Fisherbrand	Cat# 13678200A
Beaker Griffin 50 mL	Thermo Fisher Scientific	Cat# 02540G
Sterile cell strainers	Fisherbrand	Cat# 22363549
Natural convection incubator	Being Lab	Cat# BH15155U
Commercial chicken eggs	N/A	N/A
Digital dry baths/block heaters	Thermo Fisher Scientific	Cat# S37338

MATERIALS AND EQUIPMENT

Egg Water

Reagent	Final concentration	Amount
Instant ocean salt	N/A	0.2 g
Reverse osmosis water	N/A	1 L
Total	N/A	1 L

Note: Store egg water at room temperature (ca.20°C) (maximal time for storage: 6 months).

50X Danieau's solution stock

Reagent	Final concentration	Amount
NaCl	2900 mM	169.4 g
KCl	35 mM	2.61 g
$MgSO_4 \cdot 7H_2O$	20 mM	4.94 g
$Ca(NO_3)_2 \cdot 4H_2O$	30 mM	7.09 g
HEPES	250 mM	59.58 g
NaOH	10 M	2 mL
Reverse osmosis water	N/A	Add to 1 L

Note: Add water to 1 L and stir until dissolved. The pH should be approximately 7.6. If not, adjust using 10 M NaOH or 2 M HCl. Store at 4°C for up to 6 months.

⚠ CRITICAL: When adjusting the pH, wear appropriate personal protective equipment (PPE), including disposable nitrile gloves, safety glasses/splash goggles and lab coats. High concentration NaOH and HCl solution is corrosive and dangerous.

Note: To generate 0.3x Danieau's solution stock, add 6 mL 50x Danieau's solution stock into 994 mL reverse osmosis purified water.

Tricaine Stock

Reagent	Final concentration	Amount
Tricaine	4 g/L	400 mg
Danieau's solution	0.3x	97.9 mL
Tris HCl solution (pH 9.0, 1M)	0.02M	2.1 mL
Total	N/A	100 mL

Note: Adjust pH to around 7 ($\pm$ 0.2). Store 25 mL aliquots of this solution in a −20°C freezer. and protect from light. It is good for 1 year.

⚠ CRITICAL: Wear appropriate personal protective equipment (PPE), including disposable nitrile gloves, safety glasses/splash goggles and lab coats, when handling any amount of Tricaine.

1.5% Low melting agarose (LMA)

Reagent	Final concentration	Amount
Low melting agarose	1.5%	1.5 g

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Reagent	Final concentration	Amount
Danieau's solution	0.3x	96 mL
Tricaine Stock (4 *g*/L)	0.16 *g*/L	4 mL
Total	N/A	100 mL

Note: Add agarose and 96 mL 0.3x Danieau's solution into a 250 mL flask or bottle and mix well. Heat up the mixture in a microwave oven for 30 s. After melting the agarose, mix well and divide them into four 50 mL glass bottles. After the agarose cools down to 60°C (touchable by bare hand), add 1 mL Tricaine Stock (0.4%) into one glass bottle mixture and mix well. Distribute 750 μL aliquots from one bottle into 1.5 mL microcentrifuge tubes and keep the tubes on a Block Heater (42°C). We usually make 25 aliquots each time. and use them within 2 weeks. Store the other 3 glass bottles in a refrigerator (around 4°C) until use. It is good for 1 year.

⚠ CRITICAL: Avoid repeated heating up the low melting agarose (1.5%) that contains Tricaine with a microwave oven, this will lead to water evaporation and loss of Tricaine potency.

STEP-BY-STEP METHOD DETAILS
Preparation of the desired fish lines for live imaging

⏱ Timing: Approximately 3–6 months

1. Generate breeders.
   a. Raise the following breeder lines to adulthood: 1) *Tg(acta1:dnigf1ra-EGFP)*[vu530] (referred to as zMIR; henceforth); *Tg(ins:H2BmCherry)*[vu513] (Maddison et al., 2015)[4], 2) zMIR; *Tg(ins:H2Bm-Cherry); Tg(mpeg1:EGFP),*[6] 3) *Tg(LyzC:DsRED2),* and 4) *Tg(LyzC:GFP).*

   Note: These lines can be identified under a fluorescent microscope by their fluorescent markers at 4 days postfertilization (dpf) by their specific expression patterns (Figure 1).

      i. Use a transfer pipette to carefully move the desired larvae individually into a Petri dish.
      ii. Raise them to adults in the fish facility.
   b. Raise the above breeder lines that are also *tnfa+/−*. PCR genotyping is needed to determine the *tnfa* genotypes.

   ⚠ CRITICAL: If the desired stage of larvae is older than 6 days post fertilization (dpf), larva should be fed with *Paramecium* or 1% egg yolk medium twice daily. Starvation during development may result in misleading outcomes and conclusions.

Generate fish with the desired fluorescence lineage markers

⏱ Timing: 1 week

2. Set up appropriate breeders to generate progenies with the desired genotypes.

   Note: We use 2 distinct breeder lines for each imaging experiment. In this protocol, we use *Tg(ins:H2B-mCherry)* to label β cells; *Tg(mpeg1:EGFP)* to label macrophages; *Tg(LyzC:DsRED₂)* or *Tg(LyzC:GFP)* to label neutrophils. For imaging 2 cell types, each breeder line carries one cell type marker in addition to the desired mutation(s) or transgene(s). For imaging
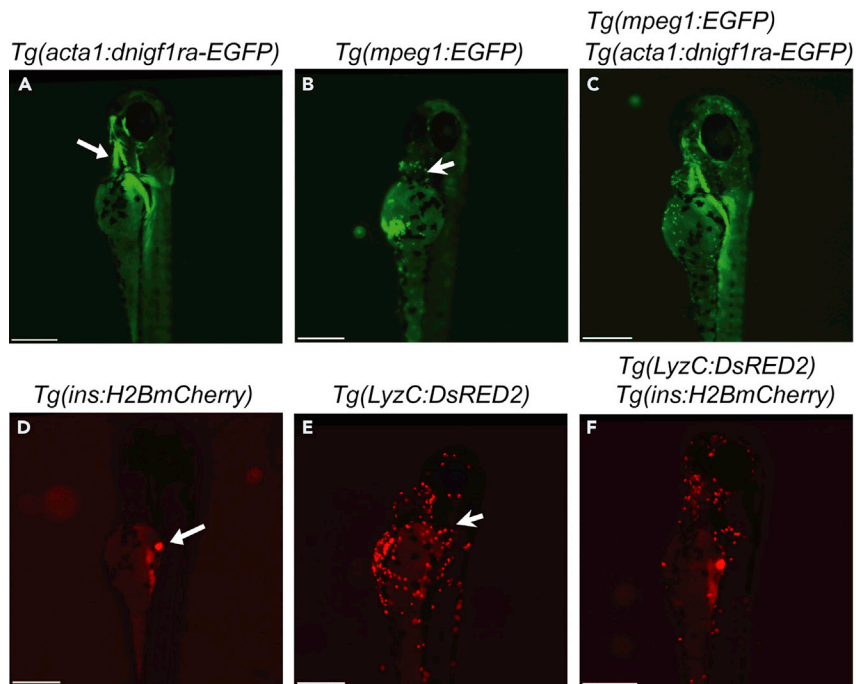
Figure 1. Identify fish with the desired genotypes

(A) Image of *Tg(acta1:dnigf1ra-EGFP)*(zMIR) fish at 4 dpf that expresses a dominant negative Igf1r-EGFP indicated by green immunofluorescence in skeletal muscle, particularly the jaw muscle (White arrow).

(B) Image of *Tg(mpeg1:EGFP)* fish at 4 dpf with its macrophages labelled by EGFP (Green dots). The white arrow points to a macrophage.

(C) Image of a zMIR larva that also has EGFP-labelled macrophages.

(D) Image of a *Tg(ins:H2B-mCherry)* fish at 4 dpf showing red fluorescence in the β cells (White arrow).

(E) Image of a *Tg(lyzC:DsRed2)* fish at 4 dpf showing red fluorescence in neutrophils (Red dot). The white arrow points to a neutrophil.

(F) Image of a larva that has *Tg(ins:H2B-mCherry)* and *Tg(lyzC:DsRed2)*. All scale bars represent 100 μm.

3 cell types, one of the breeder lines should carry 2 cell type markers in addition to the desired mutation(s) or transgene(s). These mutations or transgenes may be obtained de novo, by breeding or from other investigators.

   a. Determine their genotypes using fluorescent marker(s) and/or PCR.
   b. Raise these fish to adulthood.
3. Generate fish of the desired genotypes for the experiment.
   a. Harvest the embryos using a strainer and rinse them with egg water gently and thoroughly to remove fish feces.
   b. Collect them with fresh egg water (0.2 *g*/L Instant Ocean Salt) in 10 cm Petri dishes.
   c. Put the dishes into a 28°C incubator.
   d. Check the embryos daily to remove dead embryos and detached chorions with a transfer pipette.

Note: Do not add methylene blue in egg water. Methylene blue is strongly fluorescent, which will interfere in the screening of fluorescent markers.

⚠ CRITICAL: 70–80 embryos/larvae develop normally in a 10 cm Petri dish. Putting more embryos than this in a dish may impair their development.

4. Identify fish with the desired cell lineage markers at 4 dpf (Figure 1).
   a. Add the 150 µL of Tricaine stock into a Petri dish that contains larvae in 15 mL of egg water. Wait approximately 1 min until all the larvae are anesthetized.
   b. Examine them under a stereo fluorescent microscope (Zeiss Discovery V12) to identify individuals with the desired genotypes.

   Note: Figure 1 shows the expected expression patterns of some of these transgenes.

   c. Use a glass Pasteur pipette to transfer the zMIR+ and zMIR- larvae one by one separately into a cell strainer sitting in a Petri dish with 15 mL egg water.
   d. Transfer each of the cell strainer to another Petri dish containing egg water and gently agitate it to eliminate Tricaine.
   e. Transfer all fish in the strainer to a Petri dish with 25 mL of fresh egg water.

   Note: In our experiments, we have already allocated the RFP channel to islet β cells and assigned the GFP channel to macrophages. Because of their distinct morphology and behavior, neutrophils can use either the GFP or RFP channel.

   ⚠ CRITICAL: Perform the screening process rapidly and gently, avoid damaging the larvae or immersing the larvae in the tricaine solution for too long. Damaged or unhealthy fish will have an abnormal macrophage and neutrophil response in the islet.

5. Put the dishes back into the 28°C incubator until the next step.

   Note: The optimal fish age for this protocol is 6 dpf as they have nearly exhausted the maternal nutrient supply in the yolk and eager to forage. If the desired age of larvae is older than 6 dpf, larvae should be fed with *Paramecium* (400 paramecia per larva) or 1% egg yolk medium (25 mL for 30 min for a group of up to 80 larvae).

Perform the 3-day overnutrition experiment

   ⏱ Timing: 3 days (for steps 6 to 9)

This section provides a detailed description of performing a 3-day 5% egg yolk overnutrition experiment on 6 dpf larvae (Figure 2). The macronutrient composition of chicken egg yolk is 57.7% lipid, 34.5% protein, and 7.8% carbohydrates. It is thus a high fat diet. After culturing in 5% chicken egg yolk emulsion for 8 h, zebrafish larvae have a fully stuffed foregut and a high lipid content in circulation and in the liver.[2] At the end of each session of the 8-h culture in 5% egg yolk emulsion, ER stress can be detected in β cells and compensatory neogenesis occurs,[2] indicating nutrient stress. Therefore, we choose this as an overnutrition regime. We have not tested other means of overnutrition for this stage of larvae, such as overfeeding with paramecia.[7]

6. Formulate the 5% egg yolk medium (Methods video S1).
   a. Isolate egg yolk.
      i. Obtain a fresh egg and break the eggshell.
      ii. Remove the egg white as much as possible.
      iii. Carefully transfer the egg yolk to the other half of the shell or to the palm of the nondominant hand.
      iv. Gently rinse it with 10 mL DI water to remove the egg white (Figure 2A).
      v. Transfer the fresh egg yolk into a 50 mL conical tube. One egg yolk is typically 15 mL in volume (Figure 2B).

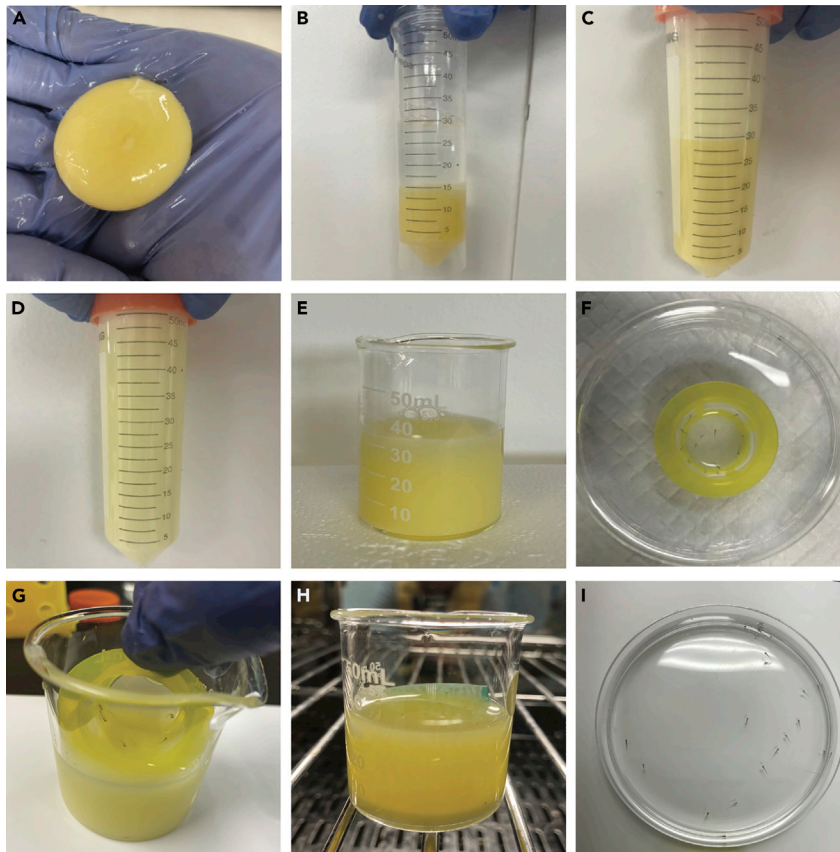   *Note:* Always use fresh eggs before they are expired.

Figure 2. Prepare and use the nutrient-rich medium
(A) Harvest egg yolk.
(B) Add equal amount of 0.3x Danieau's solution to an egg yolk-containing conical tube.
(C) Generate 50% stock by vigorous vortexing.
(D) Prepare working medium by vigorous mixing of 5 mL Stock and 45 mL 0.3x Danieau's solution.
(E) Add 25 mL of the 5% egg yolk medium and < 20 larvae to a clean 50 mL beaker.
(F) Transfer the larvae into the Sterile Cell Strainer in a Petri dish.
(G) Quickly transfer the larvae into a 50 mL clean beaker with 25 mL of the 5% egg yolk medium.
(H) Put the beaker into a 28°C incubator. As sediments accumulate, stir the medium every 2 h
(I) Transfer the larvae clear of egg yolk into a Petri dish with 0.3x Danieau's solution.

⚠ CRITICAL: Before collecting the egg yolk, try to remove as much egg white as possible.
When in contact with salt water (Danieau buffer), the protein from the egg white may dena-
ture and become flocculent, which may stick to the larvae and impede their motility.

b. Make 50% egg yolk emulsion (Figure 2C)
    i. Add equal volume of 0.3x Danieau's solution into the 50 mL conical tube (Figure 2B).
    ii. Close the lid and shake the tube on a tube vortexer at maximum speed for 2 min.
c. Prepare 5% egg yolk medium.
    i. Take 5 mL of the 50% egg yolk emulsion after letting the mixture stand for 5 min and add it
        into a 50 mL centrifuge tube that contains 45 mL of 0.3x Danieau solution.
    ii. Close the lid and shake on a tube vortexer at maximum speed for 2 min (Figure 2D).
    iii. Pour 25 mL of the 5% egg yolk medium into a 50 mL glass beaker.

Note: If the fish need to be treated with an inhibitor during the overnutrition process, dilute
the compound in the appropriate solvent (eg. DMSO) as stock (≥1000x) and dilute to the

desired working concentration(s) in the 5% egg yolk medium. Add the same volume of the solvent (eg. DMSO) into the 5% egg yolk medium for the control group.

7. Setup the overnutrition experiment for larvae (Figure 2E).
    a. Collect larvae for the experiment.
        i.   Put a sterile cell strainer in a petri dish containing 25 mL 0.3x Danieau's solution.
        ii. Transfer all the larvae from the original dish into the sterile cell strainer using a transfer pipette (Figure 2F).
        iii. Take the cell strainer out of the 0.3x Danieau's solution.
        iv. Remove excess liquid by taking the strainer out of the Danieau's solution and tapping it on a clean paper towel as quickly as possible to remove excess liquid.

    Note: Try to remove as much of the excess Danieau's solution on the cell strainer as possible to avoid diluting the egg yolk medium. Gently transfer the larvae into the cell strainer to avoid damaging the larvae.

    b. Submerge the cell strainer in a beaker containing the 5% yolk medium to release the larvae (Figure 2G).

    Note: Put the same number of larvae into the 25 mL 5% yolk medium to create the same condition for both control and experimental groups.

    ⚠ CRITICAL: Do not put more than 20 larvae in a 50 mL beaker that contains 25 mL 5% yolk medium, or 40 larvae in 100 mL beaker containing 50 mL 5% yolk medium as more fish may cause hypoxia and stress.

    c. Put the beaker back into the incubator and stir up the sediments in the egg yolk medium (Figure 2H) with a transfer pipette every 2 h.

    Note: Place the bore of the transfer pipette to the bottom of the beaker and gently pipette the egg yolk medium up and down to break up the sediments. The sediments may bury the larvae and limit their motility and oxygen availability.

    ⚠ CRITICAL: Use a new transfer pipette for each beaker, especially when using a drug or compound within the yolk medium. Some larvae may be stuck onto the transfer pipette and transferred to another beaker.

8. Transfer the larvae to the nutrient-free medium after 8 h in the 5% egg yolk medium.
    a. Gently pour the egg yolk medium into a sterile cell strainer to collect the larvae.
    b. Rinse the residual egg yolk medium off the larvae by gently stirring the cell strainer in a Petri dish with 25 mL 0.3x Danieau's solution.
    c. Move the larvae to a new Petri dish containing 25 mL fresh 0.3x Danieau's solution using a transfer pipette (Figure 2I).

    Note: Gently perform this procedure. Larvae at this stage are fragile and could be harmed or damaged. After transferring the larvae into a Petri dish, remove all the residual egg yolk sediments and replenish the dish with fresh 0.3x Danieau's solution. If an inhibitor is needed during the nutrient free period, then dilute the stock into 0.3x Danieau's solution to the desired working concentration. Add the same volume of solvent (eg. DMSO) into 0.3x Danieau's solution of the control group. We usually put 25 mL solution in a 100 mm × 15 mm Petri dish.

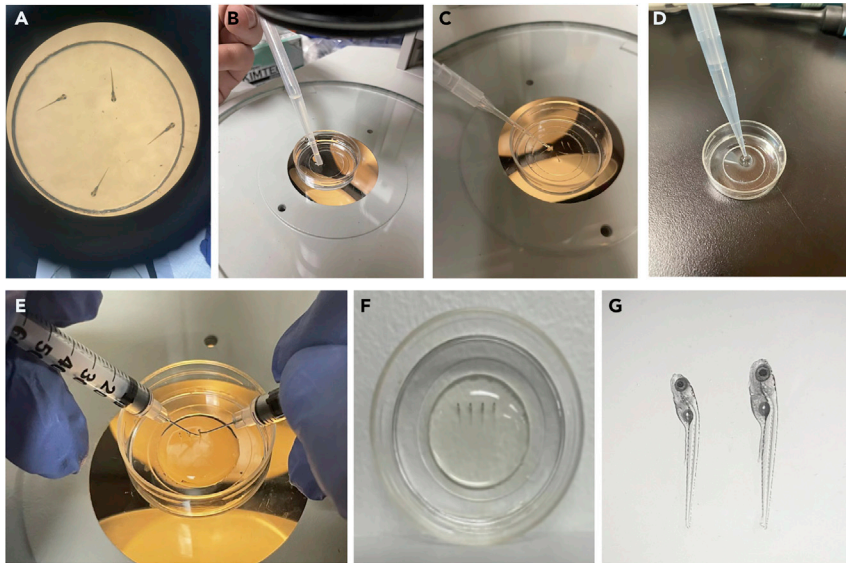   d. Put Petri dish back into the 28°C incubator for 16 h.

Figure 3. Embed larvae in LMA for imaging
(A) Transfer the larvae into a 35 mm Glass Bottom Dish.
(B) After anesthesia, remove most of the medium with a transfer pipette.
(C) Remove the residual medium using a transfer pipette with a 10 μL pipette tip attached.
(D) Add 750 μL of the pre-heated 1.2% LMA at the center of glass bottom using a P1000 pipette.
(E) Position the larvae on their right side using two insulin syringe needles.
(F) Add 750 μL 0.3x Danieau's solution (containing Tricaine) at the periphery of the dish after the LMA solidifies to submerge the gel.
(G) Check the larvae under a stereo microscope.

9. Repeat steps 7 and 8 twice for a complete 3-day overnutrition experiment.

> ⚠ CRITICAL: Follow the same schedule in all 3 days as β cell loss occurs during a 4-h window after a complete 3 day overnutrition (see problem 1 in troubleshooting). We use fresh 5% egg yolk each day because the used 5% egg yolk has a high microbial load after 8 h at 28°C, which not only emits a foul odor but also impacts fish health.

Live confocal imaging of treated zebrafish

> ⏱ Timing: 3–5 h

This section provides a detailed description of performing live imaging on zebrafish to visualize immune cell (macrophages; neutrophils) infiltration into the principal islet after 3 days of overnutrition (Figure 3).

10. Embed larvae in a glass bottom dish (Methods video S2).
    a. Using a transfer pipette, move the larvae from the petri dish into a 35 mm glass bottom dish (uncoated).
    b. Add Tricane stock to a final concentration of 0.16 g/L to anesthetize the larvae (Figure 3A) (see problem 2 in troubleshooting).

> *Note:* Do not transfer more than 4 larvae into a dish. At room temperature (ca. 20°C), we found that only 4 larvae could be embedded in a glass bottom dish before the gelling of LMA. If more fish per dish is necessary, a warm microscope stage may help delay the gelling.

c. Remove liquid.
   i. Use a transfer pipette to aspirate most of the medium in the bottom glass dish (Figure 3B).
   ii. Attach a 10 μL pipette tip to the transfer pipette and aspirate the residual medium as much as possible (Figure 3C).
d. Take one aliquot of LMA out from the 42°C block heater and rapidly add 750 μL of LMA on the bottom glass to cover the larvae (Figure 3D).

Note: Remove the medium out of the bottom glass dish carefully. Avoid contacting the larvae with the transfer pipette or the 10 μL pipette tip.

⚠ CRITICAL: When aspirating the excess medium, slowly release the bulb of the transfer pipette, especially when a 10 μL pipette tip is attached. Larvae will be easily caught by the tip and injured. Macrophages and neutrophils will be attracted to the injury site, which may confound the experiment.

e. Under a stereo microscope, use the needles of two insulin syringes (1 mL) to position the larvae so that their righthand side is close to the glass bottom (Figure 3E). Perform this as quickly as possible.

Note: Beginners may want to start with one larva at a time; it takes around 30–45 s for the LMA to solidify. Once the LMA solidifies, do not move or change the position of the larvae.

⚠ CRITICAL: Carefully position the larvae. Do not touch the larvae with the tip of the needles to avoid injury. Injury may affect the phenotype.

f. Add 750 μL 0.3x Danieau's solution (containing tricaine at a final concentration of 0.16 *g/L*) gently on the periphery of dish to submerge the LMA gel and cover the dish with a lid (Figure 3F).
g. Check the orientation and health of the larvae and put the dish back to the incubator until imaging (Figure 3G).

Note: Wait until the LMA gel completely solidifies before adding the medium to the non-glass portion of the dish to submerge the LMA gel. Do not squirt the medium on the top of the gel as it may loosen up the fish and/or disturb the gel.

⚠ CRITICAL: We immobilize the larvae for about 1.5 h for a 1 h time lapse imaging session. In our model, immobilization for more than 2 h abolishes the phenotypes (macrophage infiltration and β cell loss).

11. Perform live imaging on the principal islet.
   a. Start the LSM 780 Laser Scanning Microscope according to the manufacturer's instructions.

   Note: We usually use a 20x objective (NA 0.5).

   b. Heat up the environment chamber to 30°C.
   c. Transfer the glass bottom dish to the chamber.

   ⚠ CRITICAL: Incubate the larvae at 30°C for at least 20 min before imaging because the expansion of LMA may cause a drift of the samples (see problem 3 in troubleshooting).

   d. Confirm the larvae are laying on the righthand side (Figure 3F) before beginning the imaging session.

e. Setting up the z-stack settings.
f. Take 512 × 512 volumetric time-lapse images at 15-s or 30-s intervals with a z-step size of 1.2 μm and an average total thickness of 35 μm (see Methods videos S3, S4, and S5 for examples).

Note: To catch more details without losing too much image quality, we set the pixel dwell at 3.15 μs. Since macrophages' migration velocity is much slower than that of neutrophils, multiple larvae may be imaged in a session.

⚠ CRITICAL: Maintain the power of all excitation lasers as low as possible (<1.5%) to minimize phototoxicity.

12. Genotype the larvae after live imaging (only necessary if the genotype is unknown).
    a. After live imaging, carefully aspirate the medium with a transfer pipette and use a pair of tweezers to pick up individual larvae from the LMA.
    b. Put each larva into a tube in an 8-strip of PCR tubes to extract DNA.
    c. Add 100 μL 50 mM NaOH to each tube and incubate in a PCR machine at 95°C for 30 min.
    d. Add 20 μL 1 M Tris-HCl (pH 8.0) to neutralize the lysate.
    e. Perform PCR using the Genotyping Primers.

Reagent	Final concentration	Amount
5 X Green GoTaq Flexi Buffer	1 X	4 μL
MgCl$_2$	5 mM	2 μL
dNTP mix	0.2 mM	1 μL
Forwand primer	0.5 μM	1 μL
Reverse Primer	0.5 μM	1 μL
Lysate	N/A	1 μL
GoTaq DNA polymerase (5 U/μL)	1.25 U	0.2 μL
Water	N/A	to 20 μL

f. Run the reaction using the following PCR cycling conditions in a thermocycler.

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 mim	1
Denaturation	95°C	30 s	30 cycles
Annealing	60°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	16°C	1 min	

g. Run the PCR product on a 3% agarose gel to distinguish the mutant, heterozygous and wide type larvae.

Note: The *tnfa* mutation in this study is a 59-basepair deletion.

⏸ Pause Point: Images can be analyzed at a later time.

Analyze images and tracking the immune cells
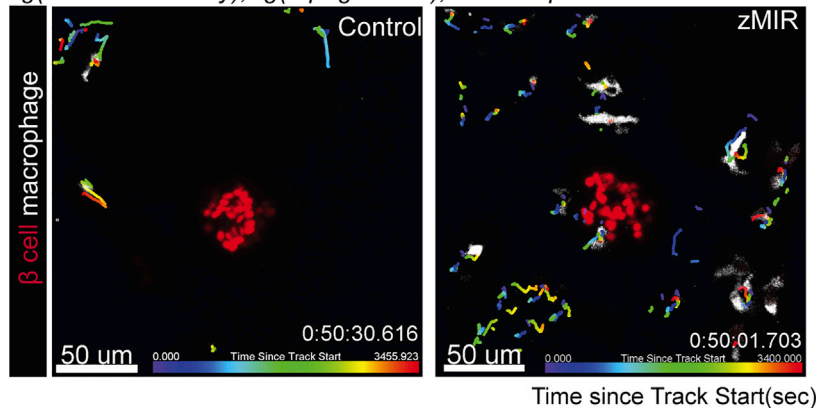
⏱ Timing: 1–2 h

Figure 4. Track macrophages near the principal islet in control and zMIR fish from 64 to 65 h post treatment using Imaris

The islet is labelled by H2B-mCherry (red) and macrophages are labelled by EGFP (white). Each track is color coded to indicate the time during which the macrophage was tracked. The color gradient at the bottom of each image represent the tracking time.

This section provides a detailed description of video analysis and cell tracking using Imaris (Figure 4).

13. Convert the LSM file of images into an Imaris Image File using Imaris File Converter.
14. Open the images with Imaris.
15. To track the movement of macrophages or neutrophils according to the reference manual.
    a. Click on the spot icon. Make sure to unselect "Start Creation with slice view".
    b. Click on the "Start" button.
    c. Slect GFP in the "chanel" pulldown menu. In this protocol, macrophages and neutrophils are labeled with GFP.
    d. Click on "slice view" button, zoom in to measure the largest distance between 2 points within a GFP labelled cell. Go back to "3D view".
    e. In 3D view, key in the XY diameter, which is the distance measured in d.
    f. Click on the next button. Each cell visible in the channel is now automatically labelled with a spot.
    g. Click on the next button until you see the "Algorithm" pulldown menu. Choose "Autoregressive Motion".
    h. Measure the MaxDistance between adjacent frames of a spot. In "Slice View", select a spot and measure the distance it has shifted in the next frame in 4 consecutive frames. Round up the largest distance as Max Distance.
    i. Key in the MaxGapSize. We always use 3.
    j. Click the play button to track all selected images in the video. All cells in the field of view are tracked.
16. Export the tracking images (Figure 4).
17. Quantify the number of macrophages that were in or near (within a defined diameter) the islet or the number of times a neutrophil was detected in the islet within the imaged period in both control and experiment groups. Manually confirm the number frame by frame.
18. Enter the data points into the GraphPad Prism 8.0 and analyze the data through one-way ANOVA.

EXPECTED OUTCOMES

Our workflow provides an optimized protocol for visualizing immune cells that infiltrate a defined target tissue/region *in vivo* through live confocal imaging in zebrafish (Method videos S3 and S4).

With this protocol, we demonstrated that macrophage-derived Tnfa plays a pivotal role in neutrophil recruitment since its deletion results in fewer infiltrating neutrophils in despite of more intra- and peri-islet macrophages (Methods video S5).[1] Understanding the mediators and signaling pathways of these cell-cell communications and interactions between macrophage-β cell-neutrophil may provide insights on how overnutrition induces β cell loss. The protocol may be adapted for imaging macrophage or neutrophil dynamics in other tissues or organs during physiological or pathological processes of interest.

LIMITATIONS

Although this protocol is sufficient to visualize the behavior of infiltrating macrophages and their role in islet inflammation under nutrient stressed condition in zebrafish, it is unable to capture the details of neutrophil behavior as they only stay for a few seconds. Although β cells disappear after multiple encounters with neutrophils, the structural basis, if any, of their interaction leading to the β cell demise is obscure because of the fast neutrophil movement. When acquiring live images, a balance between the acquisition speed and the image quality has to be made. Whether neutrophils cause β cell death through releasing cytotoxic enzymes[8] and/or ROS,[9] or by its phagocytotic function[10] is an opening question. The observed islet inflammation in zMIR fish seems to be transitory and β cell death occurs during a small window of time. Extending the relevance of these findings to adult humans, while necessary, may be even more challenging.

TROUBLESHOOTING

Problem 1
Could not catch macrophages and/or neutrophils in the islet during live imaging (related to step 11).

Potential solution

- Raise the embryos in an incubator with a fixed light/dark cycle. We use 14-h light and 10-h dark, with light on at 8AM.
- Perform the 3-day overnutrition on schedule. We start overnutrition at 9AM each day. Since the infiltration of macrophages and neutrophils occurs sequentially, each in a 2-h window, interrupting or changing the schedule may advance, delay or even abolish the phenotype.

Problem 2
The islet fluctuates during imaging (related to step 11) (Methods video S6).

Potential solution

- This is due to movement of the fish because of insufficient Tricaine in the LMA gel or in the 0.3x Danieau's solution. Always use freshly diluted Tricaine solution. The Tricaine stock should be keep in the dark at −20°C.

Problem 3
The islet keeps shifting during the live imaging (related to step 11) (Methods video S7).

Potential solution

- This may result from expansion of the LMA gel. Heat up the working chamber to 30°C before transfering the glass bottom dish to the chamber. Incubate the larvae in the 30°C chamber for at least 20 min before imaging to equilibrate the LMA temperature.

RESOURCE AVAILABILITY

Lead contact

Materials availability

This study did not generate unique materials.

Data and code availability

No unique datasets or codes were generated in this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.102039.

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AUTHOR CONTRIBUTIONS

Conceptualization, W.C., B.Y.; Methodology, B.Y., Y.Z., L.Y.; Validation, B.Y., Y.Z.; Formal analysis, Y.Z., L.Y.; Investigation, B.Y., Y.Z., L.Y.; Resources, W.C.; Writing – original draft, B.Y., Y.Z.; Writing – review and editing, W.C., B.Y., Y.Z., L.Y.; Visualization, Y.Z., L.Y.; Supervision, W.C.; Funding acquisition, W.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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