

Chapter 3

Mast Cell Development and Function in the Zebrafish

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Abstract

The many advantages of the zebrafish model provide a unique opportunity to integrate the tools of developmental embryology, transgenesis, and functional assays to elucidate the molecular pathways underlying hematopoiesis and for modeling human blood diseases. These methodologies have recently been applied to the zebrafish mast cell lineage and have resulted in a better understanding of vertebrate mast cell biology. By employing whole-mount in situ hybridization alone and in combination with co-localization approaches, fluorescence-activated cell sorting (FACS), and morpholino gene knockdown studies, new insights into early mast cell transcriptional regulation and ontogeny have been exposed in vivo. Transgenic strategies have permitted the modeling of human mast cell diseases, like systemic mastocytosis in zebrafish, which can subsequently be exploited for high-throughput chemical screens to identify potential therapies in these conditions. Mast cell functional assays have been adapted to zebrafish providing the opportunity to utilize this model for interrogating the cellular players in innate and adaptive immunity and as a live animal readout for drug responses in allergic and inflammatory reactions. These techniques are detailed in the following chapter.

Key words Zebrafish, Mast cells, Carboxypeptidase A5, Flow cytometry, Morpholino, Cardiac puncture, In situ hybridization, Transgenesis, FcεR, KIT, Tryptase, Mastocytosis

1 Introduction

The zebrafish has emerged as a robust and versatile model system for studying vertebrate hematopoiesis, by virtue of conserved genetics and the accessibility provided by large numbers of transparent embryos that are produced externally [1, 2]. Recently, we have shown that the opportunities afforded by the zebrafish for studying other hematopoietic lineages can be effectively applied to studying mast cell biology [3, 4]. This discovery is timely with the continued growth of this model for better understanding of the complexity of both innate and adaptive immune responses [5–7]. The zebrafish is amenable to a number of genetic manipulations that provide rapid phenotypic in vivo data that have greatly contributed to our understanding of mast cell development and the factors underlying mast cell fate determination. By optimizing whole-mount RNA in situ

hybridization (WISH) and innovatively combining this approach with fluorescence-activated cell sorting (FACS), we identified carboxypeptidase A5 (*cpa5*) as a zebrafish mast cell-specific marker [3, 33]. We subsequently employed this marker as a readout for mast cells in embryos in combination with other analytical techniques including WISH co-localization, morpholino oligonucleotide gene knockdown studies, and chemical inhibition assays. These approaches enabled us to establish *gata2* and *pu.1* as the key transcription factors necessary for early mast cell development, with upstream regulation directed through notch signaling and particularly *notch1b*. Definitive mast cells were also found to arise from the transient erythromyeloid progenitor (EMP) population followed by a gradual transition to hematopoietic stem cells (HSCs), which serve as the cells of mast cell origin for the duration of the life of the zebrafish [8]. These experiments set the stage for using the zebrafish model as a platform for further detailed characterization of the development of the mast cell lineage in vertebrates.

Systemic mastocytosis is a human myeloproliferative neoplasm that can arise from perturbed mast cell development and proliferation [9]. While systemic mastocytosis encompasses a wide spectrum of clinical phenotypes, aggressive forms of the disease include visceral organ involvement with the potential to evolve into mast cell leukemia. These more severe manifestations are currently without curative therapy. As has been demonstrated for other human hematopoietic malignancies, the zebrafish holds tremendous potential to serve as an efficient in vivo tool for use in high-throughput chemical screens [10–13] to identify novel therapies effective in mast cell diseases. We have been modeling systemic mastocytosis in two ways: Given the roles we have identified for the notch signaling pathway in mast cell development, we have employed notch overexpressing transgenic fish lines by breeding *hsp70::GAL4* or *mitfa::GAL4* and *UAS::nicd1a* together and activating notch transcription by heat shock. These fish have been found to have an abundance of *cpa5*-labeled mast cells [8] (and unpublished data). We have also employed the Gateway cloning system (Invitrogen, Carlsbad, CA) to generate stable transgenic lines expressing the human *KIT* D816V mutation, found commonly in human systemic mastocytosis, under the control of the zebrafish ubiquitous β -actin promoter together with an *eGFP* reporter [34]. These transgenic embryos display an apparent block in G2/M phase transition by phosphohistone H3 labeling and decreased expression of DNA methyltransferase 1 (*dnmt1*), as well as reduced expression of epithelial cell adhesion molecule (*epcam*), and decreased neuromast numbers. Adult fish succumb at only several months of age, developing skin and visceral lesions, some containing an abundance of mast cells. Both these models are amenable to targeted (e.g., notch inhibitor) and unbiased small-molecule screens for agents that restore a wild-type embryonic phenotype.

To evaluate phenotypes in adult zebrafish, we have adapted and established the utility of a number of histochemical (hematoxylin and eosin, periodic acid–Schiff, toluidine blue) and immunohistochemical (KIT, tryptase, antihuman FcεRIγ) stains to identify mast cells in zebrafish tissues, such as the gastrointestinal mucosa and gills, which are abundant in mast cells like their mammalian counterparts [3, 4]. The chromogenic tryptase assay is the most reliable technique for measuring mast cell degranulation, and we have used this approach to document response to direct mast cell stimulants such as Compound 48/80, as well as innate immune responses to *Aeromonas salmonicida* infection and adaptive immune responses to passive systemic anaphylaxis employing matched di- and trinitrophenyl-specific antibodies and substrates administered by intraperitoneal injection [4]. Transmission electron microscopy can be employed to examine intracellular structures, including the composition of granules in both resting and activated mast cells [3, 4]. These assays provide the basis for characterizing the phenotype in adult zebrafish models of diseases of perturbed mast cell development and proliferation, as described above. In addition, evidence for conserved components of the mast cell functional “apparatus” in zebrafish, such as an analogous high-affinity immunoglobulin E (IgE)-like receptor and the ability to abrogate responses to irritant and infectious stimuli using clinically relevant agents like ketotifen [4], highlight the feasibility of this model system as a novel *in vivo* tool for elucidating mechanisms underlying the role of mast cells in allergic and inflammatory reactions.

The zebrafish model provides a unique opportunity to integrate the tools of transgenesis, experimental embryology, and functional studies to allow a considerably broad understanding of mast cell biology. Amenable to diverse transgenic approaches, the zebrafish can be exploited in different ways to create fluorescent reporter lines and models of human diseases. In our efforts to use zebrafish as a tool for studying human systemic mastocytosis, the ultimate goal is to employ these transgenic lines for high-throughput small-molecule chemical screens. The zebrafish is inherently suited for these types of screens on account of their small size, large numbers, and robust phenotypic readouts, permitting a more rapid and cost-effective methodology than is possible in mammalian model systems. These screens can easily identify compounds specifically affecting signaling pathways, development, or disease processes. The zebrafish has increasingly been used as this type of platform and has produced promising results in several disease models [10, 11], including hematopoietic malignancies [13].

Reporter lines provide the advantage of labeling individual cell populations and monitoring them during specific activities throughout their life span. These cells can subsequently be sorted by FACS-based approaches, and these lines can be crossed with

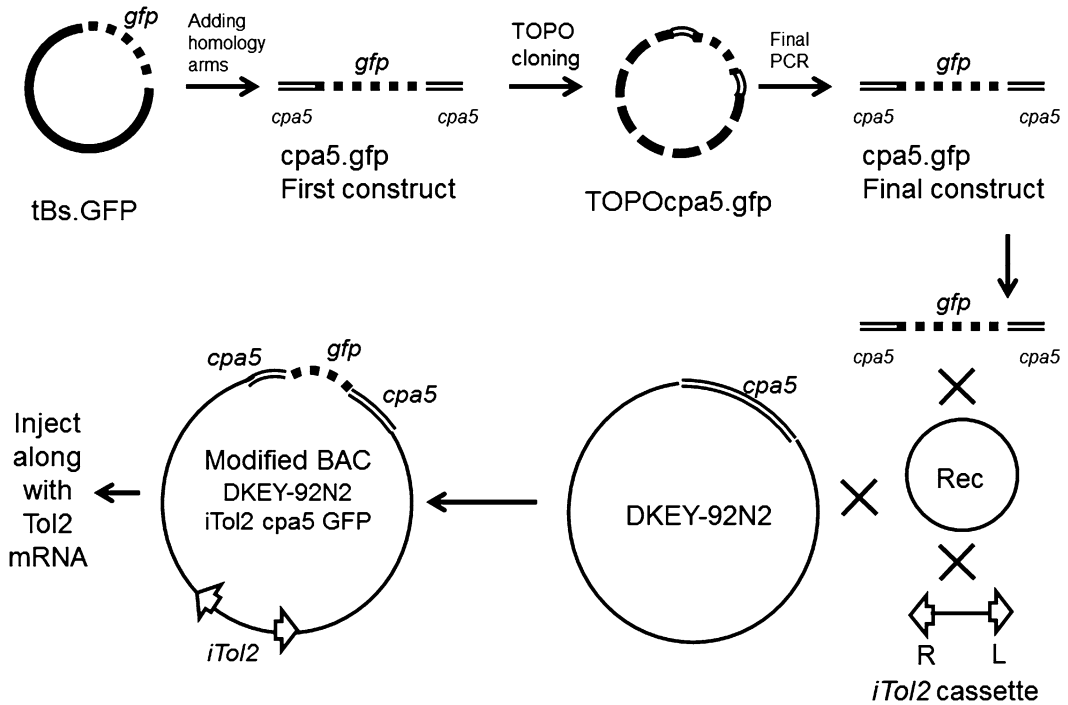


Fig. 1 BAC transgenesis approach for carboxypeptidase A5 promoter (*cpa5*)

other reporter lines to facilitate studies of cellular ontogeny, migration, and interaction [14–17]. With the zebrafish genome sequencing project almost complete and several bacterial artificial chromosome (BAC) libraries available, BAC transgenesis techniques are now being applied to complement more traditional approaches. In order to generate a mast cell reporter line, a BAC-based strategy that has been gaining popularity in the zebrafish field [18, 19] is currently being employed. The technique is based on modifying the BACs using targeting cassettes followed by a recombination step which places *GFP* or *Gal4* reporters under the control of the zebrafish mast cell-specific *cpa5* promoter and BAC regulatory elements (Fig. 1) [19, 20]. More recently, strategies involving the clustered, regularly interspaced, short palindromic repeat (CRISPR) - Cas9 system have facilitated the insertion of fluorescent tags at specific sites in the genome, providing an alternative approach to generating a mast cell reporter lines [35]. A zebrafish mast cell reporter line could eventually be used to specifically show interactions of mast cells with other cell types in immunological or neoplastic processes. Xenotransplantation of human cancer cells into zebrafish has demonstrated the utility of this model for rapid analysis of tumor cell proliferation and response to drugs [36]. In our laboratory, we have established the xenotransplantation of leukemia cell lines and developed a robust cell proliferation assay demonstrating *in vivo* targeted therapeutic inhibition

of these xenografted cells in zebrafish embryos [21]. This method is readily being applied to solid tumors, such as sarcomas, to provide insight into tumor cell invasion and metastatic behavior [22]. A mast cell reporter line has the potential to be incorporated into the xenotransplantation model to reveal the contributions of mast cells during invasion and metastasis, which in turn may shed light on the intricate relations at play in the inflammatory niche of a tumor. In support of this contention, previous studies using reporter lines in zebrafish embryos have already provided new insights into the response of tumor cells to other myeloid cells and drawn parallels between tumor- and wound-mediated inflammation [23]. Additionally, the mast cell reporter line would also make possible the isolation, culturing, and clonal analysis of zebrafish mast cells [24], providing further potential for the exploration of mast cell biology by enabling the application of established mast cell in vitro assays to zebrafish mast cell cultures.

2 Materials

All solutions listed in this section are prepared in distilled and deionized water (ddH₂O) or were purchased as manufacturer-prepared solutions or reagents unless otherwise indicated.

2.1 Equipment

1. 28.5 °C incubator (humidified air).
2. 65 °C incubator.
3. Micropipette.
4. Microcentrifuge.
5. Centrifuge.
6. Hybridization oven (65 °C).
7. Benchtop vortex mixer.
8. Gentle shaker.
9. Humidified chamber.
10. Stereomicroscope.
11. Inverted microscope.
12. Fluorescence-activated cell sorting (FACS) instrument (e.g., FACSaria, BD Biosciences, Canada).
13. 96-well microplate ELISA reader (e.g., BIO-RAD 680 microplate reader).
14. Western blot apparatus.
15. Electron microscope.

2.2 Adult Zebrafish and Embryos

1. Adult wild-type, transgenic, and mutant zebrafish were maintained in tanks with 28.5 °C water (pH 6–8) with a salinity of 1,100–1,300 mS. Fish are exposed to 14 h light per day.

2. Mating tanks with dividers.
3. Egg water: 5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl₂, 0.16 mM MgSO₄.
4. 0.003 % PTU: 1-phenyl-2-thiourea (Sigma-Aldrich, St. Louis, MO).
5. 10 mg/mL pronase (Roche, Indianapolis, IN, USA).
6. Phosphate-buffered saline (PBS).
7. 4 % paraformaldehyde (PFA) prepared in PBS.
8. PBS-T: 0.1 % Tween-20 (v/v) in PBS.
9. Transfer pipettes (wide opening).
10. Injection plate (*see* Subheading 3.19.)
11. Thin-wall glass microinjection capillaries with filaments (dimensions: 100 mm length, 1.0 mm outer diameter, 0.75 mm inner diameter (e.g., TW100F-4 World Precision Instruments, Inc. Sarasota, FL)).
12. 10 mg/mL Proteinase K (Sigma-Aldrich, St. Louis, MO).
13. Fish anesthesia: 4 mg/mL ethyl 3-aminobenzoate methane-sulfonate (Tricaine-MS-222) (Sigma-Aldrich, St. Louis, MO).

2.3 RNA Probe Labeling

1. Plasmid DNA.
2. Restriction enzymes.
3. QIAquick PCR Purification Kit (Qiagen, Toronto, ON, Canada).
4. T7 RNA Polymerase (Roche, Indianapolis, IN, USA).
5. RNase Inhibitor 2,000 U (Roche, Indianapolis, IN, USA).
6. Digoxigenin (DIG) and fluorescein (FITC) RNA labeling mix (Roche, Indianapolis, IN, USA).
7. NucAway spin columns (Ambion, Applied Biosystems, Streetsville, ON, Canada).
8. RNase- and DNase-free sterile distilled water.

2.4 Whole-Mount In Situ Hybridization (WISH)

1. 5× SSC (saline sodium citrate buffer): 43.8 g sodium chloride, 22 g sodium citrate (dihydrate) in ~950 mL ddH₂O. Adjust to pH 7 with concentrated HCl and add ddH₂O to 1 L.
2. SSC-T: 1× SSC, 0.1 % Tween-20.
3. Hyb(−) solution: 50 % formamide, 5× SSC-T. Store at −20 °C.
4. Hyb(+) solution: 50 % formamide, 5× SSC-T, 500 mg/mL torula yeast RNA type IV, 50 µg/mL heparin. Store at −20 °C.
5. Maleic acid buffer-Tween (MAB-T): 100 mM maleic acid, 150 mM NaCl, 10 % Tris base, 0.1 % Tween-20.
6. Blocking solution: 10 % FBS, 2 % blocking reagent for nucleic-acid hybridization (Roche, Indianapolis, IN, USA) prepared in MAB-T.

7. Anti-DIG or anti-FITC Fab fragments from sheep coupled to peroxidase (POD) (Roche, Indianapolis, IN, USA).
8. Fast Red tablets (Roche, Indianapolis, IN, USA).
9. Alkaline phosphate substrate: BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium) (Vector Laboratories, Inc. CA, USA).

2.5 Embryo Dissociation

1. 10 mM dithiothreitol (DTT) in egg water.
2. Embryo dissociation buffer: 0.28 Wünsch U/mL Blendzyme 3 (Roche, Indianapolis, IN, USA) in 1× Hank's buffered saline (HBSS) with 5 mM CaCl₂.
3. 40 µm strainer (BD Falcon, Becton Dickinson, NJ, USA).
4. 0.9× PBS supplemented with 5 % heat-inactivated fetal bovine serum (HI-FBS).

2.6 Morpholinos

1. We use oligonucleotides designed and purchased from Gene Tools, LLC (Philomath, OR).

2.7 Chemical Treatment of Embryos

1. Gamma-secretase inhibitor. Prepare a 1 mM stock of Compound E (Alexis Biochemicals, San Diego, CA) in DMSO. Store at −20 °C.

2.8 Cloning (Gateway®)

1. Multisite Gateway® Pro cloning kit (Invitrogen, Burlington, ON, Canada).

2.9 Histochemical Staining

1. 10 % neutral buffered formalin.
2. 5 µm sections of zebrafish intestine or gills.
3. 1 % (w/v) toluidine blue stock solution: 1 g toluidine blue O dissolved in 100 mL of 70 % EtOH (in water).
4. 1 % sodium chloride (NaCl) solution: 0.5 g NaCl in 50 mL of ddH₂O. Make this solution fresh every time. Adjust pH to 2.0–2.5.
5. Toluidine blue working solution: 5 mL toluidine blue stock solution in 45 mL 1 % NaCl solution (pH 2.0–2.5). Mix well and discard after use.
6. Hematoxylin staining solution: Accustain® Harris' hematoxylin solution.
7. Eosin solution: 2 g of eosin Y disodium salt in 200 mL ddH₂O, 4 mL glacial acetic acid, 600 mL 95 % EtOH.
8. Xylene.
9. 100, 95, and 70 % ethanol (EtOH) diluted in MilliQ water.
10. Gill's hematoxylin (Surgipath Leica, Wetzlar, Germany).
11. Scott's tap water substitute: Add 3.1 g NaHCO₃ and 17.8 g MgSO₄ to 1 L tap water. Add a crystal of thymol (2-isopropyl-5-methylphenol) to prevent the growth of mold and bacteria.

12. 1 % alcoholic eosin (Surgipath, Leica, Wetzlar, Germany).
13. Glycerol Gelatin (Sigma-Aldrich, St. Louis, MO).
14. McManus periodic acid–Schiff (PAS).
 - (a) 1 % periodic acid.
 - (b) *Cold* Schiff's reagent: 1 % (w/v) basic fuchsin, 3.8 % (w/v) sodium metabisulfite in 0.25 N HCl. Shake vigorously and dissolve overnight, add several teaspoons of activated charcoal. Shake well for 2 min, let stand for 5 min, filter, and refrigerate.
15. Fast Red stain: Add one Fast Red tablet to 2 mL of 0.1 M Tris (pH 8.5). Full dissolution requires vigorous vortex mixing.
16. BCIP/NBT stain: Add two drops of reagent 1–5 mL of 0.1 M Tris (pH 9.5), invert to mix. Then add two drops of reagent 2 (invert to mix) followed by two drops of reagent 3 (invert to mix).

2.10 Immunohisto-chemistry

1. 0.01 M sodium citrate buffer (pH 6.1): 2.58 g sodium citrate (dihydrate) in 1 L ddH₂O.
2. 3 % hydrogen peroxide in PBS: 3 mL (30 % H₂O₂) in 27 mL of PBS.
3. 5 % (v/v) normal goat serum in PBS.
4. Antihuman FcεRIγ polyclonal rabbit IgG (Millipore, Billerica, MA) diluted to 1:400 in PBS.
5. Biotinylated goat anti-rabbit IgG diluted in PBS (1:600).
6. Diaminobenzidine (DAB) (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA).
7. Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO).
8. Isotype control consisting of non-immunized rabbit IgG (Cedarlane Laboratories, Hornby, ON, Canada).
9. Cytoseal (Richard-Allan Scientific, Kalamazoo, MI).

2.11 Electron Microscopy

1. 0.1 M sodium cacodylate buffer: 1.78 g sodium cacodylate trihydrate ((CH₃)₂AsO₂Na · 3H₂O) in ddH₂O.
2. 2.5 % glutaraldehyde prepared in 0.1 M sodium cacodylate buffer.
3. 1 % osmium tetroxide.
4. 100 % epon araldite resin.
5. 2 % aqueous uranyl acetate.
6. 0.25 % uranyl acetate.
7. 0.3 % lead citrate.

2.12 Mast Cell Responses to Stimuli

1. 0.07 % bromophenol blue in isotonic saline.
2. Compound 48/80.
3. Live *Aeromonas salmonicida* in a final concentration of 5×10^9 cfu/mL.
4. $1 \times$ PBS.
5. Ketotifen fumarate salt (Sigma-Aldrich, St. Louis, MO).
6. N α -Benzoyl-DL-arginine *p*-nitroanilide (BAPNA) [25].
7. Human LAD2 mast cell line.

2.13 Protein Extraction and Immunoblotting

1. Ringer's solution: 5 mM HEPES (pH 7.2), 116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl₂ in 0.5 L ddH₂O.
2. Lysis buffer #1: 1 % Nonidet P-40, 0.1 % SDS, 100 mM NaCl, 50 mM Tris (pH 7.4–7.7) in 25 mL H₂O. Prepare in advance and store at 4 °C.
3. Lysis buffer #2: 50 mM NaF, 1 mM Na₃VO₄, one tablet complete mini protease inhibitor with EDTA (Roche, Indianapolis, IN, USA). Add lysis buffer #1 to a total volume of 5 mL.

3 Methods

3.1 Embryo Dechoriation

1. Reduce the egg or PTU water to a minimal volume where embryos can still move freely in the dish when swirled.
2. Add 0.5 μ g of pronase (warmed to 28.5 °C) into the Petri dish and incubate for 10–15 min at 28.5 °C. Gentle pronase treatment progressively softens the chorion without damaging the embryos (*see Note 1*). Embryos can be dechorionated by gentle pipetting using 2.5 mL transfer pipette. Alternatively, embryos can be dechorionated by hand using sharpened forceps, but this is a slow process, which can be used only for a small quantity of embryos.
3. After the incubation, gently rinse the embryos at least five times with egg or PTU water.
4. Let the embryos develop at 28.5 °C until they reach the required stage.

3.2 Embryo Fixation

1. Stage the zebrafish embryos according to Westerfield [26].
2. Transfer dechorionated embryos into 1.5 mL microfuge tubes.
3. Wash embryos with PBS-T. Do one quick wash and then wash once for 5 min on shaker. Replace with 1 mL fresh PBS-T.
4. Treat the embryos with Proteinase K (10 mg/mL stock solution) as appropriate for the developmental stage as shown in Table 1.

Table 1
Recommended Proteinase K concentrations and treatment times
appropriate for developmental stage of zebrafish embryos

Embryo stage	Proteinase K treatment
<30 hpf	Not needed or 10 µg/mL, 10 min (1 µL stock in 1 mL PBST)
30 hpf	30 µg/mL, 10 min (3 µL stock in 1 mL PBST)
35–52 hpf	50 µg/mL, 20 min (5 µL stock in 1 mL PBST)
53 hpf+	100 µg/mL, 20 min (10 µL stock in 1 mL PBST)
5 dpf+	100 µg/mL, 30 min (10 µL stock in 1 mL PBST)
7 dpf+	100 µg/mL, 45 min (10 µL stock in 1 mL PBST)

hpf hours postfertilization, *dpf* days postfertilization

5. Wash embryos with PBS-T. Do one quick wash and then wash once for 5 min on shaker. Replace with 1 mL fresh PBS-T.
6. Add 1.5 mL of 4 % PFA and fix on the shaker overnight at 4 °C (embryos can be stored in 4 % PFA up to 6 weeks).
7. Wash embryos by aspirating the PFA.
8. Wash embryos with PBS-T. Do one quick wash and then wash once for 5 min on shaker. Replace with 1 mL fresh PBS-T.
9. Add enough 100 % methanol (MeOH) to cover embryos. Cap and mix gently.
10. Incubate at –20 °C for at least 30 min.
11. Aspirate and replace with fresh 100 % MeOH.
12. Embryos can be stored indefinitely in 100 % MeOH at –20 °C.

3.3 RNA Probe Preparation

The following example methods are for generation of a probe to detect expression of zebrafish mast cell-specific marker, carboxypeptidase A5 (*cpa5*) in situ.

Plasmid Linearization

1. Linearize 2–5 µg plasmid DNA using appropriate restriction enzyme. Mix 2–5 µg of linear DNA (e.g., pBK-CMV-*cpa5* plasmid) with 2 µL 10× restriction enzyme buffer and 2 µL restriction enzyme (e.g., *Bam*HI). Add DEPC-treated or DNase/RNase-free water for a total reaction volume of 20 µL. Let the digestion reaction incubate for 2 h at 37 °C.
2. Clean the linear DNA product using QIAquick PCR Purification Kit according to manufacturers' protocol.
3. Resuspend DNA in 30 µL of DEPC-treated, RNase-free water. Let it sit for 1 min.
4. Run 1–2 µL of digested DNA on agarose gel to make sure the DNA is linear.

3.3.1 Probe Labeling Reaction

1. Prepare a DIG- or FITC-labeled probe using the linearized DNA template. Mix 1–3 μL linearized DNA (2 μg) with 1 μL RNase inhibitor, 2 μL 10 $\times$ DIG or FITC RNA labeling mix (*see Note 2*), 2 μL 10 $\times$ transcription buffer and 2 μL T7 (or SP6) DNA polymerase. Add nuclease free water for a total reaction volume of 20 μL . Incubate at 37 °C for 4 h.
2. Optional: Add 1 μL 10 $\times$ Turbo DNase I and incubate for 15 min at 37 °C.
3. Purify probe using Ambion NucAway spin columns according to manufacturers' protocol.
4. Check 2 μL of the probe on an agarose gel.
5. Add 1 μL of RNase Inhibitor (40 U/ μL) to the completed probe.
6. Store labeled probes at –20 or –80 °C.

3.4 Whole-Mount In Situ Hybridization (WISH) (See Fig. 2)

Day 1: Hybridization

1. Gently wash embryos (**steps 2–7** below) before performing the in situ hybridization (*see Note 3*). During these washes preheat the Hyb(–) and Hyb(+) solutions in the 65 °C incubator.
2. For embryos stored in 100 % MeOH (*see Subheading 3.2*), aspirate MeOH and replace with 75 % MeOH/PBS-T, wash for 5 min at room temperature (RT).
3. Repeat **step 3** with 50 % MeOH/PBS-T and then again with 25 % MeOH/PBS-T.
4. Aspirate and wash twice for 5 min with PBS-T.
5. For embryos stored in paraformaldehyde (4 % PFA) (*see Subheading 3.2*), wash briefly but carefully in PBS-T at RT. Embryos should be kept in PFA at 4 °C at least overnight for best results.
6. Prehybridize in 1 mL of Hyb(–) for at least 15 min in a 65 °C incubator with gentle rocking.
7. Replace Hyb(–) with 300 μL Hyb(+) and incubate in a 65 °C incubator with gentle rocking for 1 h.
8. Add labeled probe to Hyb(+) (4 μL or more if the probe is weak) and incubate overnight in 65 °C incubator with gentle shaking (*see Note 4*).

Day 2: Washing, Blocking, and 2° Antibody

9. Collect the probe solution, and store at –20 °C for a later use (probes in Hyb(+) solution can be used for a total of three times).

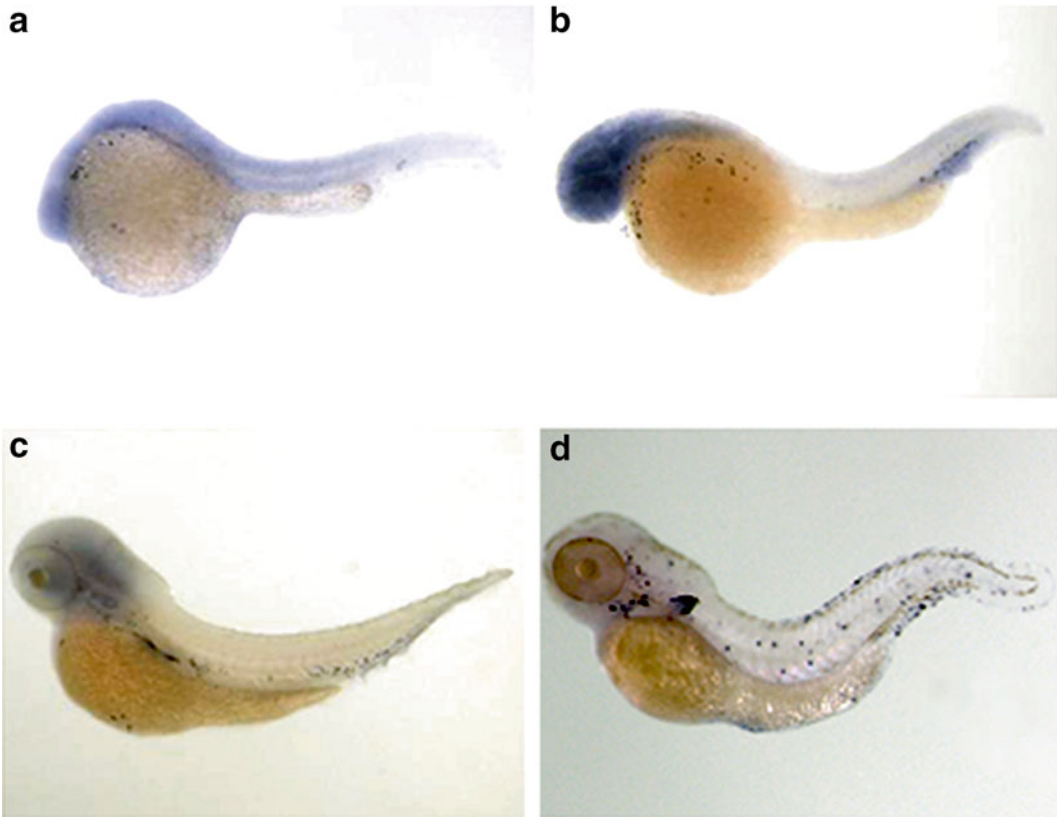


Fig. 2 A time course of *cpa5* expression from 24 hpf to 5 dpf. Expression is observed in white blood cells at the sites of primitive embryonic hematopoiesis (anterior paraxial mesoderm (ALPM) and intermediate cell mass (ICM)) and in circulation. *cpa5* expression begins at 24 hpf (a), peaks at 28 hpf (b) and then remains present through to 72 hpf (c) and 5 dpf (d) (Objective 5×). This research was originally published in *Blood*. Dobson, J.T., Seibert, J., Teh, E.M., Da'as, S., Fraser, R.B., Paw, B.H., Lin, T.J., and Berman, J.N. Carboxypeptidase A5 identifies a novel mast cell lineage in the zebrafish providing new insight into mast cell fate determination. *Blood* 112:2969–2972. © The American Society of Hematology

10. Wash embryos in 65 °C incubator as follows:
 - (a) Wash twice with 1 mL of 2× SSC-T in 50 % formamide (15 min each wash).
 - (b) Wash once with 2× SSC-T (15 min).
 - (c) Wash twice with 0.2× SSC-T (15 min each wash).
11. Wash embryos at RT on a gentle shaker three times with MAB-T (5 min per wash).
12. Prepare the blocking solution: 10 mL of 10 % blocking medium, 5 mL of heat-inactivated FBS, 35 mL of MAB-T. Store any unused blocking solution at 4 °C for washes on day 3.
13. Block in 1 mL of in situ blocking solution. Shake gently at RT for 1 h.

14. Add 0.5 μL 2° antibody (POD-coupled anti-DIG or anti-FITC Fab) to blocking solution and incubate 2° antibody overnight at 4 °C on a gentle shaker.

Day 3: Detection

15. The following steps are performed at RT with gentle shaking (*see Note 5*).
16. Wash once with in situ blocking solution (15 min).
17. Coat transfer pipette in MAB-T to be sure that the embryos will not stick into the transfer pipette. Carefully transfer embryos to new wells in 12-well plate.
18. Wash twice with MAB-T (15 min each).
19. Wash four times (5 min each) with the appropriate buffer. For example, if staining with BCIP/NBT wash with 0.1 M Tris (pH 9.5). If staining with Fast Red, wash with 0.1 M Tris (pH 8.2).
20. Embryo staining
 - (a) BCIP/NBT stain: For two groups of embryos; mix 5 mL of 0.1 M Tris pH 9.5. Add two drops reagent 1, invert to mix. Add two drops reagent 2, invert to mix. Add two drops reagent 3, invert to mix. Develop at RT with gentle shaking in the dark (cover with aluminum foil). Examine after 1.5 h and, if necessary, every 30 min thereafter.
 - (b) Fast Red stain: Add 3 tablets per 6 mL to 0.1 M Tris (pH 8.5) and mix vigorously to dissolve. This is sufficient volume for staining two groups of embryos. Add Fast Red staining solution to the embryos and develop at 37 °C for 2 h then replace with fresh Fast Red and let it shake overnight at 4 °C. Alternatively, incubate at 37 °C for 4 h, then check for staining (this will save you 1 day).
21. Stop the reaction by washing with PBS-T at RT for 5 min (*see Note 6*).
22. Stained embryos (e.g., Fig. 2) are now ready to view under the microscope (*see Note 7*).

**3.5 Co-Localization
Studies Using
Double WISH**

Perform all steps as previously mentioned in Subheading 3.4 except for the following modifications:

Day 1: Hybridization

1. Add both DIG- and FITC-labeled probes to Hyb(+) (4 μL each or more if the probe is weak) and incubate overnight at 65 °C with gentle shaking.

Day 2: Washing, Blocking, and 2° Antibody

2. Add 0.5 μL 2° antibody of the first probe (e.g., anti-DIG or anti-FITC) to the 1 mL blocking solution.

Day 3: Detection of the First Probe

3. Use Fast Red stain as in Subheading 3.4.

Day 4: Washing, Blocking, and 2° Antibody

4. Incubate in 1× MAB with 10 mM EDTA at 60 °C for 10 min (inactivation).
5. Wash three times (5 min each wash) with MAB-T at RT with gentle shaking.
6. Block in 1 mL in situ blocking solution (as in Subheading 3.4, step 20). Shake gently at RT for 1 h.
7. Add 0.5 μL 2° antibody of the second probe, anti-DIG or anti-FITC to the 1 mL blocking solution.

Day 5: Detection of the Second Probe

8. Stain with BCIP/NBT as in Subheading 3.4.

**3.6 Co-localization
Studies Using Double
Fluorescent Whole-
Mount RNA In Situ
Hybridization (WISH)**

Perform all steps as previously mentioned in the section of double WISH except for the following modifications:

Day 2: Washing, Blocking, and 2° Antibody

1. Add 0.5 μL 2° antibody of the first probe, anti-DIG to the 1 mL blocking solution.

Day 3: Detection for the First Probe

2. Use Fast Red stain as in Subheading 3.4.

Day 4: Washing, Blocking, and 2° Antibody

3. Incubate in 1× MAB with 10 mM EDTA in a 60 °C incubator for 10 min (inactivation).
4. Washes at RT on a gentle shaker.
5. Wash three times with MAB-T (5 min each wash).
6. Blocking: Add 0.5 μL 2° antibody of the second probe, anti-FITC to the 1 mL blocking solution.

Day 5: Imaging

7. Wash once with in situ blocking solution for 15 min.
8. Coat transfer pipette in MAB-T to be sure that the embryos will not stick into the transfer pipette. Carefully transfer embryos to net wells in 12-well plate.
9. Wash twice with MAB-T (15 min each wash).

10. Wash once with PBS-T (5 min).
11. View under the microscope for imaging.
12. Embryos can be stored in 4 % PFA at 4 °C.

**3.7 Combination
Fluorescence-
Activated Cell Sorting
(FACS) and WISH Fig. 3**

Perform all steps as previously mentioned in the section of WISH except for the following modifications:

Day 1: Hybridization

1. Add FITC-labeled probe to Hyb(+) (4 µL or more if the probe is weak) and incubate overnight in 65 °C incubator with gentle shaking.

Day 2: Washing, Blocking, and 2° Antibody

2. Add 0.5 µL 2° anti-FITC antibody to the 1 mL blocking solution.

Day 3: Detection for the First Probe

3. Use Fast Red stain as in Subheading 3.4.

Day 4: Dissociation of Embryos for FACS

4. Incubate approximately 100 embryos in 10 mM dithiothreitol (DTT) in egg water for 30 min at RT with gentle shaking.
5. Dissociate in embryo dissociation buffer (Subheading 2.5, **step 2**) for 2–3 h on shaker in 37 °C incubator, vortex every 30 min.
6. Prepare ice-cold 0.9× PBS supplemented with 5 % HI-FBS in pre-coated 50 mL tubes.
7. Pour coating buffer 0.9× PBS with 5 % FBS into two tubes (15 mL): one tube for sorting and one tube (5 mL) for cell suspension.
8. Filter the cell suspension using 40 µm cell strainer into coated 50 mL tube.
9. Centrifuge 10 min at 4,500×*g* speed.
10. Discard supernatant and resuspend pellet in 1,000 µL 0.9× PBS with 5 % FBS.
11. Store at 4 °C wrapped with foil for FACS sorting.
12. Sort and collect desired cell population (Fig. 3) into prelabeled tubes containing 1 mL 0.9× PBS with 5 % FBS (*see Note 8*).

Cytopins of FACS-Sorted Cells

13. Spin down sorted cells in refrigerated centrifuge (5 min, 470×*g*, 4 °C). Resuspend in appropriate volume (~400 µL) of buffer (0.9× PBS with 5 % FBS).

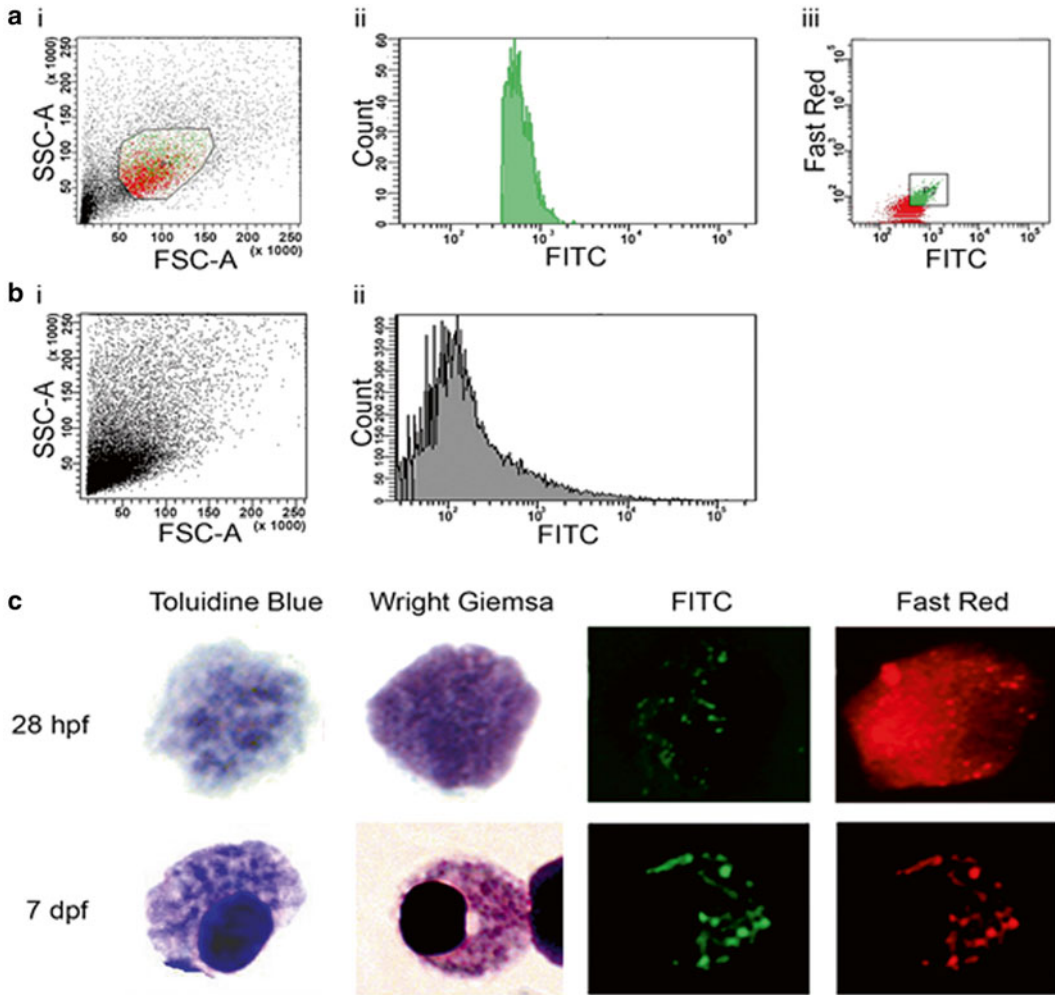


Fig. 3 WISH-FACS isolation technique can identify a specific mast cell population. Cells were isolated based on mRNA expression of *cpa5* labeled by WISH. FACS data for the *cpa5*⁺ cell population isolated from 7 dpf embryos is shown in (a). Cell suspensions were gated based on forward scatter (FSC-A) and side scatter (SSC-A) (a, i) to exclude cellular debris and nonspecific cells and sorted on the basis of FITC fluorescence (a, ii) and subsequently FITC and Fast Red fluorescence together (a, iii). Negative control data from embryos that were subjected to WISH conditions in the absence of an RNA probe is shown in (b). This population was also gated based on FSC-A and SSC-A (b, i), but the resulting population reveals no FITC fluorescence as expected (b, ii). Isolated cell populations were fixed onto slides by cytopsin, stained by Wright Giemsa to examine morphology, or toluidine blue, a mast cell-specific stain. The first row of panel c shows an immature mast cell harvested at 28 hpf. The second row shows a morphologically mature mast cell isolated from embryos at 7 dpf. Toluidine blue staining shows characteristic metachromatic granules. The last two panels in each row demonstrate that the cells shown are both FITC and Fast Red fluorescent, which confirms the presence of *cpa5* mRNA (Objective 100×). This research was originally published in *Blood*. Dobson, J.T., Seibert, J., Teh, E.M., Da'as, S., Fraser, R.B., Paw, B.H., Lin, T.J., and Berman, J.N. Carboxypeptidase A5 identifies a novel mast cell lineage in the zebrafish providing new insight into mast cell fate determination. *Blood* 112:2969–2972. © The American Society of Hematology and in Dobson, J.T., Da'as, S., McBride, E.R., and Berman, J.N. 2009. Fluorescence-activated cell sorting (FACS) of whole-mount in situ hybridization (WISH) labelled haematopoietic cell populations in the zebrafish. *Br J Haematol* 144:732–735

14. Assemble the following parts of the cytospin rig in this order: Bracket (metal, comes with the sealed head), glass microscope slide (frosted side toward outside of rotation), filter card, and then cytofunnel (hole should line up with hole in filter card, funnel points up/out).
15. Pre-wet: Load 100 μL of coating buffer into the funnels and spin at $470\times g$ for 3 min.
16. Cytospin: Load 100 μL of cells into cytofunnel after pre-wetting. Spin at $470\times g$ for 5 min. Carefully dismantle rig (*see Note 9*).

3.8 Morpholino Oligonucleotides

Morpholino oligos are short chains of about 25 nucleotides. Morpholino subunits can inhibit translation, redirect splicing, or inhibit activity, maturation, or target access of a miRNA (*see Note 10*)

3.9 Preparation of Stock Solution

1. Each oligo is delivered as a pre-quantified, sterile, salt-free, lyophilized solid in a glass vial.
2. We recommend making a 2 mM stock solution in distilled water (morpholinos can be damaged by diethyl pyrocarbonate (DEPC)).
3. Store morpholino stock solutions and working stocks at RT, when stored cold or frozen, morpholinos can come out of solution. Before using, heat the solution 10 min at 65 °C and cool to RT before use. If that does not recover full oligo activity, autoclave the solution to dissolve oligo.

3.10 Embryo Collection

1. Set up mating of wild-type or transgenic fish pairs using mating tanks with dividers to separate males and females.
2. Pull the dividers the following morning. One hour later, collect embryos using embryo strainer and place embryos in a Petri dish with PTU water.

3.11 Zebrafish Morpholino Injection

1. Mix 2 % agarose in egg water.
2. Bring to boil in microwave then pour in the Petri dish, insert mold slide, and get rid of bubbles and let it cool down before pulling the mold out.
3. Line the collected embryos in the injection plate.
4. Inject the desired concentration of morpholino with 0.05 % phenol red (a nontoxic injection tracer) into the yolk of 1–4 cell stage embryos (it will be transported into the forming cells).
5. Transfer injected embryos to a clean Petri dish and allow recovering at 28.5 °C incubator until required age before fixation.

3.12 Chemical Inhibitors

Gamma (γ)-Secretase Inhibitor (Compound E) Treatment

1. Dilute Compound E stock (1 mM in DMSO) in egg water and apply to dechorionated zebrafish embryos at 28.5 °C at final concentrations of 50 and 75 μ M from 22 h postfertilization (hpf) to 48 hpf.
2. Fix embryos with 4 % PFA.
3. Treat control embryo groups with (a) egg water and (b) egg water with 0.5 % DMSO.

3.13 Transgenic Mast Cell Disease Models

In our efforts to use the zebrafish as a tool for human systemic mastocytosis (SM), we generated a transgenic line expressing the human KIT gene harboring the specific mutation (D816V) that is seen in SM patients [34]. We used the Multisite Gateway® Pro Technology to express this mutated gene under the ubiquitous zebrafish promoter, beta actin, with a reporter gene. The advantages of the Multisite Gateway system with the flanking Tol2 sites have been well documented in the zebrafish field to provide ease and convenience in generating different constructs to serve various purposes [27–29]:

1. Design primers flanking your gene of interest (in this case, human *KIT D816V*) with attB1 and attB2 sites added to the ends and produce your attB1 and attB4-flanked PCR product.
2. Perform a BP recombination reaction, according to manufacturers' instructions, between the attB1 and attB2-flanked PCR product and pDONR P1-P2 to generate the middle entry clone (pME).
3. Confirm the structure of the cloned DNA by sequencing.
4. Repeat steps for the p5E (5' entry clone, in this case zebrafish β -actin (*actb1*)) and p3E (3' entry clone, in this case enhanced green fluorescent protein (eGFP) with the viral 2A linker) using appropriate att sites.
5. Perform LR reaction and combine the three clones giving rise to pTol2- β -actin-*hKIT D816V*-2AeGFP.
6. Inject along with Tol2 mRNA into the cell of the embryos at 1-cell stage as described above.
7. Screen embryos for ubiquitous expression of GFP and grow them up.
8. When GFP+ fish reach maturity, mate them to wild-type line and screen offspring to identify a founder.
9. Once founders are identified, continue mating and screen for stable reporter expression in the embryos to generate a stable transgenic line.

**3.14 RNA
(Carboxypeptidase A5)
In Situ Hybridization
on Tissue Sections**

Day 1: Hybridization

1. Use 5 μ m sections of paraffin-embedded intestine tissue and mounted on glass slides.
2. Deparaffinize the sections with xylene twice (10 min each). Perform in a fume hood.
3. Rehydrate the tissue sections using serial washes with EtOH as follows:
 - (a) Wash twice with 100 % EtOH (5 min each).
 - (b) Wash once with 95 % EtOH in PBS for 2 min.
 - (c) Wash once with 85 % EtOH in PBS for 2 min.
 - (d) Wash once with 60 % EtOH in PBS for 2 min.
 - (e) Wash once with 30 % EtOH in PBS for 1 min.
 - (f) Wash once with distilled water for 2 min.
 - (g) Wash once with PBS for 5 min.
4. Pre-hybridize sections by immersing in Hyb(+) buffer for 1 h at 37 °C.
5. Add DIG-labeled antisense *cpa5* probe to Hyb(+) 1:150 (enough volume to overlay the sections) and incubate overnight in 37 °C incubator with gentle shaking in a humidified container.

Day 2: Washing, Blocking, and 2° Antibody

6. Collect the probe solution, and store at -20 °C for a later use (solution can be used twice again).
7. Wash tissue section in a 37 °C incubator as follows:
 - (a) Wash twice with 2 $\times$ SSC-T, 50 % formamide (15 min each wash).
 - (b) Wash once with 2 $\times$ SSC-T (15 min).
 - (c) Wash twice with 0.2 $\times$ SSC-T (15 min each).
8. Wash tissue sections at RT on a gentle shaker for 5 min with Buffer A: 100 mM Tris-HCl (pH 7.5), 150 mM NaCl.
9. Prepare the blocking solution: 10 mL of 10 % blocking medium, 5 mL of heat-inactivated FBS, 35 mL MAB-T. Store the blocking solution at 4 °C to use for the washes on day 3.
10. Block in 1 mL in situ blocking solution, shake gently at RT for 1 h.
11. Add 0.5 μ L of the secondary (2°) antibody (anti-DIG or anti-FITC) to the 1 mL blocking solution. Incubate overnight at 4 °C on a gentle shaker.

Day 3: Detection

12. Perform the following detection steps at RT on a gentle shaker.
13. Wash with in situ blocking solution (15 min).
14. Wash twice with Buffer A (5 min each).
15. Wash twice with 0.1 M Tris (pH 9.5) (5 min each wash).
16. BCIP/NBT stain: Add two drops reagent #1 to 5 mL of 0.1 M Tris (pH 9.5) and invert to mix. Then add two drops reagent #2, invert to mix followed by two drops reagent #3, invert to mix. Finally, add 400 µg/mL levamisole.
17. Develop at RT with gentle shaking in the dark (cover with aluminum foil). Examine after 1.5 h and, if necessary, every 30 min thereafter.
18. Counterstain with methyl green for 30 s.
19. Stop the reaction by washing with PBS-T at RT for 5 min.
20. Mount the slide with Glycerol Gelatin (Sigma-Aldrich, St. Louis, MO).
21. View under the microscope.

3.15 Histochemical Stains

Standard protocols were used for staining 5 µm sections of zebrafish intestine. Slides are visualized using Zeiss Z1 microscope and AxioCam Rev 3.0 camera, Wetzlar, Germany (*see Note 11*).

3.15.1 Toluidine Blue Staining (Metachromasia)

This stain is specifically used to visualize mast cells by demonstrating metachromasia (red, pink, or purple) in tissue sections, whereas nuclei and other components will appear as shades of blue (Modified from Carleton's Histological Technique).

1. Deparaffinize and hydrate sections in distilled water.
2. Stain sections in toluidine blue working solution for 3 min.
3. Wash well with three changes of distilled water.
4. Dehydrate rapidly through 95 % and then two changes of 100 % EtOH.
5. Clear in xylene with two changes (3 min each).
6. Mount with Cytoseal (Richard-Allan Scientific) mounting media and a coverslip.

3.15.2 Hematoxylin and Eosin

Reference: Histotechnology: A Self Instructional Text, 3rd Edition, Freida L Carson and Christa Hladik, 2009 [30].

1. Deparaffinize in xylene with three changes (5 min each).
2. Serially hydrate using 100, 95, and 70 % EtOH (2 min each).
3. Rinse 30 s in running tap water.
4. Stain with hematoxylin (2.5 min).

5. Rinse 2 min in running tap water.
6. Blue in Scott's tap water substitute for 1 min.
7. Rinse 1 min in running tap water.
8. Dip in 0.2 % nitric acid to differentiate slide.
9. Rinse 1 min in running tap water.
10. Dip (20 times) in eosin Y solution.
11. Dehydrate through serial washes in 70, 95, and 100 % EtOH (20 dips in each solution).
12. Clear in xylene with three changes (20 dips each).
13. Mount with coverslip using Cytoseal mounting media (Richard-Allan Scientific) (*see* **Note 12**).

3.15.3 *McManus* *Periodic Acid–Schiff (PAS)*

Carleton's Histological Technique

PAS-positive material stains reddish purple or magenta
Nuclei stains blue

1. Deparaffinize sections in xylene and rehydrate through alcohol series as in Subheading "Hematoxylin and Eosin", **steps 1 and 2**.
2. Incubate in 1 % periodic acid for 5–10 min.
3. Wash in tap water for 10 min.
4. Incubate in Schiff reagent 15 min.
5. Wash in tap water 20 min.
6. Incubate in Gill's hematoxylin for 3–5 min.
7. Rinse 1 min in running tap water.
8. Blue in Scott's tap water substitute.
9. Rinse 1 min in running tap water.
10. Dehydrate through serial rinses in 70, 95, and 100 % EtOH.
11. Clear twice in xylene (20 dips each).
12. Cover using Tek® Glas coverslipper.

3.16 *Immunohistochemistry (Fig. 4)*

Day 1: Washing, Blocking, and 1° Antibody

1. Use 5 µm sections of paraffin-embedded intestine tissue and mounted on positive-charged glass slides.
2. Deparaffinize with xylene (three changes, 5 min each).
3. Serial hydrate in EtOH 100, 95, and 70 % (2 min each step).
4. Wash in PBS three times (30 s each).
5. Endogenous peroxidase activity is quenched in 3 % H₂O₂ in PBS for 10 min.
6. Wash in PBS three times (30 s each).
7. Antigen retrieval: 0.01 M sodium citrate buffer (pH 6.1) in a decloaking chamber (Biocare Medical).

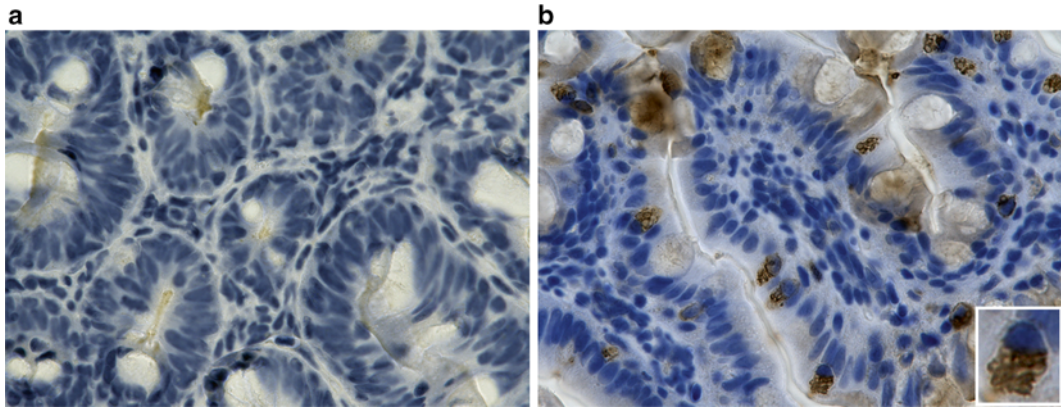


Fig. 4 Zebrafish mast cells express an FcεRI-like receptor and antibody. Immunohistochemistry of wild-type adult zebrafish intestine using antihuman FcεRIγ demonstrates specific staining in intestinal mast cells (**b**, and inset) compared with human IgG isotype control (**a**). This figure is reproduced from Da'as, S., Teh, E.M., Dobson, J.T., Nasrallah, G.K., McBride, E.R., Wang, H., Neuberg, D.S., Marshall, J.S., Lin, T.J., and Berman, J.N. 2011. Zebrafish mast cells possess an FcεRI-like receptor and participate in innate and adaptive immune responses. *Dev Comp Immunol* 35:125–134

8. Wash slides in PBS three times (2 min each).
9. Block by incubating slides in a humidified chamber with 1:20 normal goat or horse serum diluted in PBS for 30 min at RT.
10. Add polyclonal rabbit immunoglobulin G (IgG), antihuman FcεRIγ (Millipore, Billerica, MA) antibody to one slide and add the isotype control consisting of non-immunized rabbit IgG (Cedarlane Laboratories, Hornby, ON, Canada) to the second slide, each diluted at 1:400 in 1× PBS and incubate overnight at 4 °C in a humid chamber.

Day 2: Washing, Blocking, and 2° Antibody

1. Wash slides with PBS (three times, 2 min each).
2. Add secondary antibody, biotinylated goat anti-rabbit IgG diluted in 1:600 in PBS and incubate for 30 min at RT in a humid chamber.
3. Prepare the staining solution. Vectastain ABC kit (Vector Laboratories, Burlingame, CA): To 5 mL of PBS add one drop of solution A. Mix and then add one drop of solution B. Mix and then let sit for 30 min before use.
4. Wash slides with PBS (three times, 2 min).
5. Add the staining solution and incubate for 30 min at RT on a shaker.
6. Wash slides three times with PBS (2 min each wash) (*see Note 13*).

7. Add the DAB (diaminobenzidine) solution (add one drop of DAB and two drops of H_2O_2 to 5 mL of PBS) and incubate for 5 min at RT on a shaker.
8. Rinse slides with running tap water.
9. Counterstain slides with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO).
10. Blue in Scott's tap water substitute.
11. Rinse slides with running tap water.
12. Dehydrate through EtOH series: 70, 95, and 100 % (twice in each solution, 20 dips each).
13. Clear in xylene (three changes, 20 dips).
14. Mount with Cytoseal mounting media (Richard-Allan Scientific).
15. Zebrafish intestine labeled with anti-FcεR1 is provided as sample data in Fig. 4.

3.17 Electron Microscopy (EM)

1. Collect zebrafish intestine immediately following cardiac puncture and fix with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C overnight.
2. Move samples to 1 % osmium tetroxide and 0.25 % uranyl acetate and embed in 100 % epon araldite resin for further fixation.
3. Cut thin sections (100 nm) and place on 300 mesh copper grids.
4. Stain the sections with 2 % aqueous uranyl acetate followed by 0.3 % lead citrate.
5. View the samples using a Transmission Electron Microscope at 80 kV (e.g., JEOL JEM 1230 (Tokyo, Japan)).
6. Capture images using digital camera (e.g., Hamamatsu ORCA-HR (Bridgewater, NJ)).

3.18 Embryo Protein Immunoblotting

The following protocol is modified from Sidi et al. [31]

1. Dechorionate embryos (*see Note 14*).
2. Transfer embryos to ice-cold Ringer's solution to de-yolk if required (*see Note 15*).
3. Transfer embryos to a 2 mL microcentrifuge tube and remove all residual Ringer's solution.
4. Add lysis buffer #2 (1 μL per embryo).
5. Homogenize embryos with a plunger from 1 mL syringe or homogenize for 20–40 s with rotor-stator in 5 mL round bottom tube.

6. Centrifuge $800\times g$, 10 min at 4 °C.
7. Transfer the supernatant to a fresh 1.5 mL microcentrifuge tube and store at -80 °C.

Protein Quantification

8. To quantify protein concentration of embryo lysates, use BIO-RAD DC™ Protein Assay for 96-well plate with the following modifications of the manufacturers' instructions.
9. Dilute 2 µL lysate sample in 20 µL total lysis buffer #2.
10. Use 2 mg/mL BSA to generate standard curve by serial dilution (*see* **Note 16**).
- (a) Add 25 µL of reagent A.
- (b) Add 200 µL of reagent B.
11. Incubate for 15 min at RT.
12. Read on plate reader at 655 nm.
13. Plot standard curve to calculate lysate protein concentration.

Western Blot

14. Extract protein from zebrafish embryos 7 days postfertilization (7 dpf).
15. Treat protein extract with or without 5 % 2-mercaptoethanol.
16. Load a total of 50 µg of protein for Western blot.
17. Add 1:1,000 dilution of the polyclonal rabbit antihuman IgE that reacts with the epsilon-chain of human IgE (A009402, Dako, Denmark) for the detection of the zebrafish Ig heavy chain.
18. Add the secondary antibody.
19. Detect the protein by chemiluminescence detection by using Image Kodak.

3.19 Intraperitoneal Injection and Cardiac Puncture

1. Anesthetize wild-type adult zebrafish in pairs with 100 µg/mL tricaine (MS-222) in a "treatment tank."
2. Inject fish by intraperitoneal (IP) route with 4 µL volume of test treatment or saline control (*see* Subheading 3.17) mixed with 1 µL of 0.07 % bromophenol blue (i.e., a total volume of 5 µL). The bromophenol blue is a marker dye used to ensure correct anatomical location.
3. Allow the zebrafish to recover and monitor for desired postinjection time.
4. At the experimental endpoint, euthanize fish with 2 mg/mL tricaine.
5. Lay the fish on a fitted platform immediately postmortem.
6. Make a small ventral slit using microdissection scissors to reveal the heart.

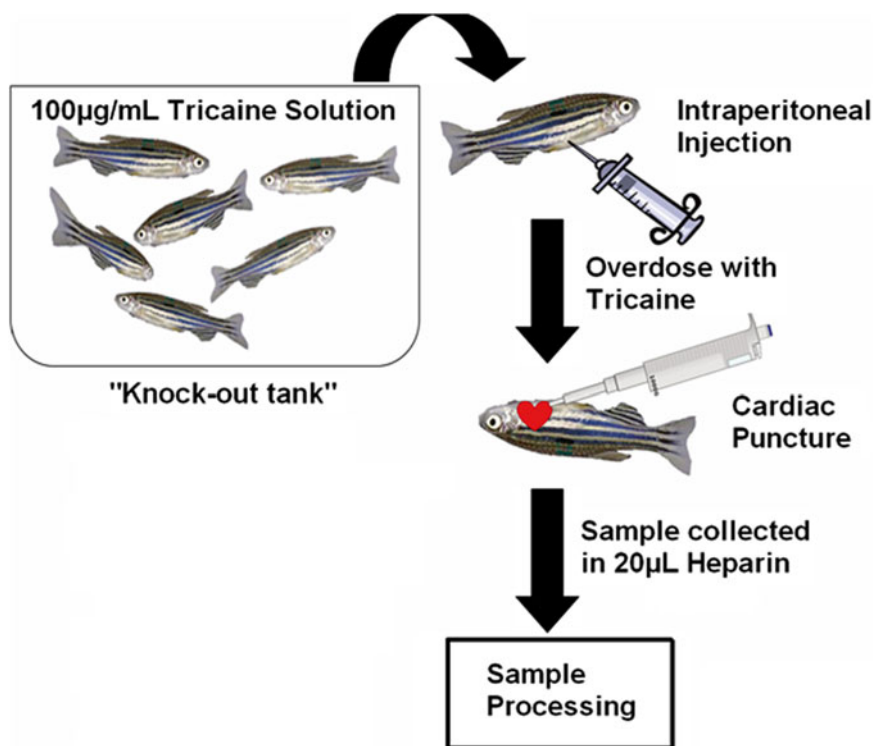


Fig. 5 Method of intraperitoneal injections and blood collection via cardiac puncture from adult zebrafish

7. Use a fine pipette tip (e.g., SDS-PAGE gel-loading tips) preloaded with 10 µL heparin to puncture the heart (*see* procedure illustration in Fig. 5).
8. Dispense the 10 µL heparin around the heart and collect the blood into a 1.5 mL microcentrifuge tube containing 20 µL heparin (*see* **Notes 17** and **18**).
9. Centrifuge collected samples at $855 \times g$ for 5 min at 4 °C.
10. Remove plasma (~20 µL) into a clean microfuge tube.

3.20 Mast Cell Treatments

3.20.1 Activation of Mast Cells

1. Inject (IP) fish with 10 µg of compound 48/80 as described in Subheading 3.19, steps 1–3.
2. Use saline injections for the control group.
3. Allow fish to recover for 5 min prior to blood sampling by cardiac puncture.

3.20.2 Infectious Stimuli

1. Inject fish with live *A. salmonicida* using technique described in Subheading 3.16, steps 1–3. Use a final concentration of 3×10^7 cfu/adult fish.
2. Use saline injections for the control group.
3. Allow fish to recover for 10–60 min prior to blood sampling by cardiac puncture.

3.20.3 Mast Cell Stabilization

1. Add ketotifen fumarate salt (Sigma-Aldrich, St. Louis, MO) directly to the tank water at 400 µg/mL final concentration, 1 h prior to IP injection of compound 48/80 or *A. salmonicida* (or other treatments) (*see* **Notes 19** and **20**).

3.20.4 Antigen-IgE Stimulation

1. Sensitize adult zebrafish by IP injection with 5 µL containing 20 ng of mouse monoclonal anti-DNP (dinitrophenyl) IgE (Sigma-Aldrich, St. Louis, MO) or anti-TNP IgE (trinitrophenyl) (Biosearch Technologies, USA).
2. Saline is used for the control group.
3. Allow fish to recover for 24 h.
4. Challenge fish with IP injections of 20 µg of DNP-BSA (bovine serum albumin; Sigma-Aldrich, St. Louis, MO) or 40 µg of TNP-BSA (Biosearch Technologies, USA) prior to blood sampling by cardiac puncture.

3.21 Tryptase Assay

1. Tryptase is a glycoprotein released from mast cells during degranulation (*see* **Note 21**).
2. Add 20 µL of BAPNA to an equal volume of plasma.
3. Incubate tubes in a humidified incubator at 37 °C for 48 h.
4. Add 60 µL of PBS and mix (by vortex).
5. Centrifuge briefly at high speed.
6. Load 60 µL of the BAPNA/PBS/plasma mixture into a 96-well plate.
7. Read the tryptase activity (proportional to absorbance at $\lambda = 415$ nm) using a 96-well microplate reader (*see* **Note 22**).

4 Notes

1. Once dechorionated, the embryos are very fragile and should be manipulated gently.
2. Generally, DIG-labeled probes are much stronger than FITC-labeled probes.
3. Use 15–20 embryos per in situ labeling reaction and be gentle with the embryos because they are fragile.
4. Label the tube with the probe whether it is DIG- or FITC-labeled probe.
5. Detection at RT on a gentle shaker.
6. To eliminate diffuse background stain, wash embryos with 100 % MeOH at RT for 5–10 min then wash with PBS-T.
7. Embryos can be stored in 4 % PFA at 4 °C.
8. We perform cell sorting (FACS) using a FACSaria machine (BD Biosciences). Cells were gated by forward and side scatter

and subsequently by FITC fluorescence at 515–545 nm. The population with the highest fluorescent signal was then gated using FITC and Fast Red (600–620 nm) to provide a more intense fluorescent signal before sorting.

9. Take apart rig, making sure to lift the filter card off the slide rather than sliding it off. If cytopspins are to be stained later, store them in 10 % buffered formalin.
10. Types of morpholinos:
 - (a) *Translation Blocking*: By blocking the translation initiation complex, morpholinos can knockdown protein expression sufficiently such that the band corresponding to that protein is eliminated on Western blot. Inhibition of translation by a morpholino should be assayed by immunoblotting as the mRNA is not rapidly degraded. RT-PCR will not provide an accurate readout.
 - (b) *Splice Blocking*: Used to block sites involved in splicing pre-mRNA, morpholinos can modify splicing, usually causing targeted exon deletions or intron insertions. This activity can be assayed by RT-PCR, with successful splice-modification visualized on electrophoretic gels as either shifts in the size of cDNA or disappearance of the RT-PCR product.
 - (c) *miRNA Blocking*: A morpholino bound to the guide strand of a miRNA can inhibit its activity (Modified from Gene Tools, LLC, Philomath, OR, USA).
11. Use 5 μm sections of paraffin-embedded intestine tissue and mounted on glass slides.
12. Look at slides regularly under the microscope to be sure that nuclear detail is sufficient and that eosinophilic staining is well differentiated—not too pale or pale darkly stained.
13. The avidin–biotin complex (ABC) method is used, and the peroxidase reaction with diaminobenzidine (DAB) will be employed for visualization (Vectastain ABC Kit).
14. To obtain sufficient yields of protein, use at least 50 embryos chilled on ice for 60 min in egg water; if younger than 48 hpf we recommend using 100 embryos.
15. If younger than 36 hpf, embryos should be de-yolked by mechanical shearing using gel-loading tips.
16. Use serial dilutions of 0, 1, 2, 5, 7, and 10 μg .
17. Heparin is required for proper RBC processing to prevent sample clotting.
18. All tubes should be kept on ice through the collection and processing.

19. Ketotifen fumarate is an H1 histamine receptor antagonist and mast cell-stabilizing agent used in the treatment of asthma and other allergic diseases [32]. It is used here to prevent mast cell degranulation.
20. *A. salmonicida* can be used as an infectious agent to show accumulation and activation of mast cells.
21. Tryptase activity is measured by the release of *p*-nitroanilide from *N* α -Benzoyl-DL-arginine *p*-nitroanilide (BAPNA), a tryptase substrate [25].
22. Relative levels of tryptase activity in zebrafish samples were normalized by using a standard curve generated from a human LAD2 mast cell line.

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