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TITLE:

Reverse Genetic Approach to Identify Regulators of Pigmentation Using Zebrafish

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SUMMARY:

Regulators of melanocyte functions govern visible differences in the pigmentation outcome. Deciphering the molecular function of the candidate pigmentation gene poses a challenge. Herein, we demonstrate the use of a zebrafish model system to identify candidates and classify them into regulators of melanin content and melanocyte number.

ABSTRACT:

Melanocytes are specialized neural crest-derived cells present in the epidermal skin. These cells synthesize melanin pigment that protects the genome from harmful ultraviolet radiations. Perturbations in melanocyte functioning lead to pigmentary disorders such as piebaldism, albinism, vitiligo, melasma, and melanoma. Zebrafish is an excellent model system to understand melanocyte functions. The presence of conspicuous pigmented melanocytes, ease of genetic manipulation, and availability of transgenic fluorescent lines facilitate the study of pigmentation. This study employs the use of wild-type and transgenic zebrafish lines that drive green fluorescent protein (GFP) expression under *mitfa* and *tyrp1* promoters that mark various stages of melanocytes.

Morpholino-based silencing of candidate genes is achieved to evaluate the phenotypic outcome on larval pigmentation and is applicable to screen for regulators of pigmentation. This protocol demonstrates the method from microinjection to imaging and fluorescence-activated cell sorting

(FACS)-based dissection of phenotypes using two candidate genes, carbonic anhydrase 14 (*Ca14*) and a histone variant (*H2afv*), to comprehensively assess the pigmentation outcome. Further, this protocol demonstrates segregating candidate genes into melanocyte specifiers and differentiators that selectively alter melanocyte numbers and melanin content per cell, respectively.

INTRODUCTION:

While the use of melanin for photoprotection has evolved several times across the animal kingdom, vertebrates have seemingly perfected the process. Dedicated pigment-producing cells with an elaborate machinery to synthesize and contain melanin are conserved from fish to humans[1]. However, the outcome of pigmentation is dramatically varied, ranging in the color to recipience and presenting as vivid patterns on integuments, the skin, and hair[2]. Despite the diversity, the repertoire of genes involved in pigmentation response is strikingly conserved. The core components of the melanin-synthesizing machinery, such as the key melanin-synthesizing enzymes, the components of the melanosomes, and the upstream connectivity to the signaling pathway, remain essentially identical across organisms. Subtle genetic differences bring about dramatic changes in the patterns of pigmentation observed across species[3]. Hence, a reverse genetic approach in a lower vertebrate organism, the zebrafish (*Danio rerio*), offers an excellent opportunity to decipher the involvement of genes in rendering the pigmented state[4].

Zebrafish embryos develop from a single-celled fertilized zygote to a larva within a span of ~24 h post fertilization (hpf)[5]. Strikingly, the melanocyte-equivalent cells—the melanophores—are large cells that are present in the dermis and are conspicuous due to the dark melanin content[6]. These neural crest-derived cells emanate ~11 hpf and begin to pigment ~24 hpf[6,7]. Conserved gene expression modules have enabled the identification of key factors that orchestrate melanocyte functions and led to the development of transgenic fluorescent reporter lines Tg(*Sox10*:GFP), Tg(*Mitfa*:GFP), and Tg(*ftyrp1*:GFP)[8-10] that label selective stages of melanocyte development. Using these transgenic fish lines enables the interrogation of cell biology of melanocytes at the organismal level in the tissue context with appropriate cues according to the developmental timelines. These reporters complement pigment-based quantitation of melanocytes and enable a distinct assessment of melanocyte numbers irrespective of melanin content.

This article provides a detailed protocol for deciphering the biology of melanocytes by assessing two critical parameters, namely melanin content and melanocyte numbers. While the former is a common functional readout emanating from a hypopigmentation response, the latter is associated with a reduction in the specification or survival of melanocytes and is often associated with genetic or acquired depigmentation conditions. The overall strategy of this reverse genetic screen is to silence select genes using a morpholino and investigate the melanocyte-specific outcomes. Melanin content is analyzed using image-based quantitation of mean grey values followed by confirmation using a melanin content assay. The number of melanocytes at various stages of maturation is analyzed using image-based quantitation and further confirmed using FACS analysis. Here, the screening protocol is demonstrated using two candidate genes, namely carbonic anhydrase 14, involved in melanogenesis, and a histone variant H2AFZ.2 involved in the

specification of melanocytes from the neural crest precursor population. While the former alters melanin content and not the melanocyte numbers, the latter alters the number of specified melanocytes and, consequently, the melanin content in the embryo. In all, this method provides a detailed protocol to identify the role of a candidate gene in pigmentation and distinguish its role in controlling melanocyte numbers versus melanin content.

PROTOCOL:

Zebrafish experiments were performed in strict accordance with the institutional animal ethics approval (IAEC) of the CSIR-Institute of Genomics and Integrative Biology (IGIB), India (Proposal No 45a). All efforts were made to minimize animal suffering.

1. Injecting morpholino into zebrafish embryos

1.1. Using a standard needle puller, draw very sharp and closed-tipped pipets.

1.2. Load the solution containing morpholino into the micropipettes using a microloader tip and insert it into the microinjector apparatus. Tighten the screw properly to lock the micropipette.

NOTE: Dosage standardization of morpholinos is necessary. The appropriate dosage has a survival rate of >70% and a specific phenotype at 24 hpf.

1.3. Cut the tip of the micropipette using fine forceps and calibrate it[11].

1.4. To calibrate, inject 1 volume of morpholino solution into the capillary tube from the micropipette, keeping the injecting time at 1 s. Repeat this five times. Using the standard, 1 mm = 30 nL volume, find the volume injected in 5 s by keeping the capillary tube against a measuring scale. Using the above information, calculate the time (in seconds) required to inject 1–3 nL per injection[12].

NOTE: The standard, 1 mm = 30 nL, is specific to the microinjector pressure settings and diameter of the capillary used for calibration. Ideally, the injection must be made into the yolk-cell interface, which is formed within 15–20 min after fertilization. To facilitate multiple injections, development can be slightly delayed by maintaining embryos at a lower temperature (18 °C). However, this should be kept to a minimum and not extended beyond 30 min due to the compounding effect of lower temperature on gene expression changes.

1.5. Mount the embryos on an agarose-cast Petri dish (60 mm). Stack the embryos tightly within the ridges. Using fire-polished glass pipettes, align them in the proper orientation for injection.

1.6. Under a microscope, use manipulators to bring the micropipette closer to the embryo and inject inside the yolk-cell interface by pressing the footswitch.

1.7. Inject all the embryos similarly; collect them in a Petri dish containing fresh embryo water medium and incubate at 28 ˚C.

1.8. Check the injected embryos after 6–8 h. Remove all the dead embryos identifiable due to their high opacity and keep changing the embryo water at least once per day to avoid infection.

2. **Pigmentation analysis**

2.1. Preparation for counting lateral midline melanophores in 3 dpf zebrafish embryos

2.1.1. Treat 48 hpf embryos with 0.016% tricaine to immobilize them.

2.1.2. To mount the embryos for imaging, add a few milliliters of 1.5–2% methylcellulose in the Petri dish (60 mm) so that it forms a thin layer. Using a Pasteur pipette, pick embryos and gently position them in methylcellulose to restrict further movement during imaging.

2.1.3. Adjust their position for optimal lateral (dorsal stripe, ventral stripe, yolk stripe, and mid-line melanophores) or dorsal (melanophores on the dorsal part of the head) imaging. For better resolution of adjacent melanophores, image at higher magnification (>5x)

2.2. Brightfield imaging

NOTE: Brightfield imaging is performed using a stereomicroscope with 8–10x magnification.

2.2.1. Place the Petri dish containing the zebrafish embryos under the microscope. Using a manipulator, adjust the fish in such orientation so that all five melanocyte embryonic stripes are visible (dorsal, ventral, two lateral, yolk) simultaneously (**Figure 1C**). Quickly capture images using the acquisition software. If the fish starts waking up, put it back into tricaine water until immobile and start imaging again.

2.2.2. Repeat this with all the fishes and ensure that magnification remains the same for every image.

2.3. Calculating mean gray value using ImageJ software

2.3.1. Take dorsal and lateral images of 2 dpf zebrafish embryos.

2.3.2. Open the image to be quantified in ImageJ using the **Open** tool. Use the **freehand shape** tool to outline the area for analysis. Go to the **Set measurements** option and select **Mean grey value | area**. Press **M** (or **Analyze | Measure**) to calculate the mean grey value for the selected area.

2.3.3. Keeping the area to be analyzed constant, calculate the mean grey area for every animal separately.

2.3.4. Plot a bar graph with the acquired data (**Figure 2F**).

2.4. Melanin content assay

2.4.1. At 2 dpf, use a glass Pasteur pipette to collect ~25 zebrafish embryos.

2.4.2. Perform manual dechorionation using 1 mL insulin needles and add them to 1.5 mL microcentrifuge tubes.

2.4.3. Discard the embryo medium carefully and add 1 mL of ice-cold lysis buffer (20 mM sodium phosphate (pH 6.8), 1% Triton X-100, 1 mM PMSF, 1 mM EDTA) with protease inhibitor cocktail and prepare protein lysates by sonication.

2.4.4. Dissolve the lysates in 1 mL of 1 N NaOH and incubate the samples at 100 ˚C for 50 min in a water bath. Vortex it intermittently to homogenize the lysates completely.

2.4.5. Take absorbance readings of the samples at 490 nm using a spectrophotometer.

2.4.6. Calculate the melanin content by comparing the sample absorbance to a standard curve of known concentrations of synthetic melanin (**Figure 2G**).

3. Melanophore count

NOTE: Fluorescence analysis in transgenic Zebrafish embryos can be done by two methods: 1) counting GFP-positive cells; 2) measuring fluorescence intensity.

3.1. Preparation for FACS-based counting of GFP-positive cells in early zebrafish embryos

3.1.1. Collect 200–250 *ftyrp:*GFP embryos and wash them in a strainer using plain embryo water. As a negative control, process GFP-negative, stage-matched wild-type (ASWT) embryos[12].

3.1.2. Based on the stage of interest, transfer the embryos to a Petri dish containing 0.6 mg/mL pronase. After 5–10 min, using a Pasteur pipette, transfer the dechorionated embryos to a fresh Petri dish containing plain embryo water. Using a glass Pasteur pipette, collect ~100 embryos and transfer them to a 2 mL microcentrifuge tube.

3.1.3. Discard the medium carefully and add 200 µL of ice-cold Ringer's solution (deyolking buffer). Keeping the tube on ice, pipette the contents up and down ~20 times until the yolk is dissolved. Centrifuge the tubes at 100 × *g* for 1 min in a tabletop centrifuge at 4 °C. Centrifuge again and carefully discard the supernatant.

3.1.4. Using a 1 mL pipette, transfer the deyolked embryos to a Petri dish containing 10 mL of trypsin solution (cell dissociation buffer). Perform it in multiple Petri dishes to avoid overcrowding of the embryos and inefficient trypsinization. Use a 1 mL pipette, mix the solution containing the embryo bodies once or twice to decrease aggregation.

3.1.5. Incubate the Petri dishes at room temperature for 15 min (<24 hpf embryos) or 30 min (24–30 hpf embryos). Aspirate and dispense the suspension occasionally using a 1 mL pipette to aid the disintegration of cells.

3.1.6. During the incubation period, initialize the flow cytometer machine for cell counting.

3.1.7. Place a 70 µm cell strainer above a 50 mL conical tube and pass the trypsinized suspension through the strainer to obtain a single-cell suspension. Wash the Petri dishes with the same suspension a few times to remove cells adhered to the surface.

3.1.8. Spin the samples at 450 × *g*, 4 °C for 5 min in a swinging bucket rotor.

3.1.9. Discard the supernatant and resuspend the pellet in 1mL of ice-cold 1x phosphate-buffered saline (PBS).

3.1.10. Spin again at 450 × *g*, 4 °C for 5 min. Repeat steps 3.1.5–3.1.16.

3.1.11. Finally, resuspend the pellet in PBS + 5% fetal bovine serum and keep it on ice until the FACS run.

3.2. Preparing the flow cytometer

3.2.1. Switch on the flow cytometer and initiate start-up.

NOTE: Before switching on the machine, empty the waste bin and fill the sheath fluid tank.

3.2.2. Normalize the flow of the stream for a 70 µm (or 85 µm) nozzle. Leave it undisturbed for 10–15 min if it is unstable.

3.3. Counting fluorescently labeled cells using a flow cytometer

3.3.1. Initialize a new experiment folder and make a scatter plot of forward scatter versus side scatter and a histogram of fluorescein isothiocyanate (FITC) intensity.

3.3.2. Load the wild-type cells first to set the forward and side scatter gates (to exclude doublets and debris) and the FITC threshold.

3.3.3. After the gates are set, remove the sample and load the cells isolated from Tg(ftyrp1:GFP) embryos to count melanocytes.

NOTE: Here, as *ftyrp1*:GFP specifically labels mature melanocytes, the number of GFP-positive cells under the FITC gate will correspond to the number of melanocytes.

3.3.4. Repeat the above step with morpholino-injected samples to estimate and compare the number of melanocytes with the control.

3.4. Measuring fluorescence intensity in zebrafish embryos

3.4.1. Using a glass Pasteur pipette, collect 100–120 embryos and transfer them to a Petri dish containing plain embryo water.

3.4.2. At ~10–12 hpf, transfer the embryos to 0.003% 1-phenyl-2-thiourea to inhibit pigmentation.

3.4.3. Perform manual dechorionation using insulin needles for imaging <48 hpf embryos.

3.4.4. To immobilize embryos, treat them with 0.016% tricaine.

3.4.5. To mount embryos for imaging, put a few mL of 1.5–2% methylcellulose in the Petri dish (60 mm) so that it forms a thin layer. Add the embryos to methylcellulose to restrict any further movement during imaging. Place the Petri dish containing the samples under the microscope. Using a pipette tip, adjust the animal in the desired orientation.

3.4.6. Acquire images using the acquisition software. For embryos <24 hpf, capture the whole embryo at once under 10x magnification. For >24 hpf stages, acquire multiple scan-fields and subsequently assemble (stitch) them together.

3.4.7. Repeat this with all the fishes and ensure that the magnification remains the same for every image.

3.5. Quantification

3.5.1. Analyze the image further using ImageJ software.

3.5.2. Open the image to be quantified in ImageJ using the **Open** tool.

3.5.3. Use the **freehand shape** tool to outline the area for analysis (**Figure 3E**).

3.5.4. Press **M** (or **Analyze | Measure**) to acquire a **selected area** intensity measurement.

3.5.5. Keeping the area to be analyzed constant, calculate the mean intensity per area for every animal separately.

REPRESENTATIVE RESULTS:

The workflow described in **Figure 1** was used to perform morpholino-based genetic perturbation at the zebrafish one-cell stage. Pigmentation analysis was performed using various methods, as mentioned below. To illustrate the representative results, standardized volumes of antisense morpholino targeting *h2afv* and *ca14* genes were injected in the yolk or one-cell stage of the zebrafish embryo. The initial phenotyping based on brightfield imaging was performed at 48 h postfertilization (hpf), when all the five pigmented melanophore stripes (dorsal, ventral, yolk, two lateral) were evident.

Figure 2A,B represent an approach to analyze pigmentation by calculating the number of lateral melanophores per embryo. Lateral melanophores were manually counted at the 48 hpf stage by zooming in on the lateral region of the tilted zebrafish. An alternative method to quantify the pigmented melanophores (**Supplemental Figure S1**) involves the manual counting of the head melanophores by imaging the dorsal view of the zebrafish.

The melanin content per embryo is calculated by measuring the mean gray value, keeping the region of interest constant with the help of ImageJ software. **Figure 2C–F** show that the mean gray value of ca14 and h2afv morphants was higher than in control morphants. As the mean gray value is inversely proportional to melanin content, knockdown of *ca14* and *h2afv* genes led to a decrease in melanin content per embryo. Another robust method to quantify the melanin content is a NaOH-based spectrophotometric absorption method. As shown in **Figure 2G**, the total melanin content in ca14 morphants was less than in control morphants.

Fluorescent imaging revealed two different stages of zebrafish melanophore development: early specified melanophores (*Mitfa:gfp*) and differentiating melanophores (*ftyrp:gfp*). The morpholino-based alteration was evaluated by fluorescent imaging (**Figure 3A** and **Figure 3C**). To further validate these observations, the frequency of GFP-positive cells was calculated using FACS. The knockdown of *h2afv* but not *ca14* significantly reduced the number of melanophores with respect to control (**Figure 3A–D**). Moreover, a reduction in the mean fluorescence intensity/area can be calculated using ImageJ software, keeping the region of interest constant. H2afv morphants show a significant decrease in the mean fluorescent intensity/area relative to control morphants (**Figure 3E,F**).

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow for a reverse genetic approach to identify regulators of melanocyte biology using zebrafish. (**A**) Flowchart depicting the experimental workflow starting from microinjection to evaluating pigmentation by various methods. (**B**) Microinjection needle holder and micromanipulator, along with a dissecting microscope, is a typical setup for microinjection of one-cell stage zebrafish embryos. (**C**) Embryonic melanocyte patterning in zebrafish at 3 dpf showing all five stripes: dorsolateral, two lateral midlines (pointed by four red arrows), ventral stripe, and yolk stripe. (**D**) To study the pigmentation outcome upon morpholino injection, lateral midline melanophores can be counted under a stereomicroscope. Brightfield image of a dorsal region at 2 dpf; melanin content can be quantified by measuring mean grey value. (**E**) The mean

gray values are inversely proportional to the melanin content of the embryo. Melanin-filled melanophores at 2 dpf in wild-type embryos. (**F**) Melanin content assay can be performed to estimate the melanin production within these melanophores. Transgenic zebrafish labeling the cells expressing TYRP1 protein at 2 dpf. (**G**) Fluorescently labeled cells can be quantified using FACS; scale bar = 100 µm. Transgenic zebrafish labeling the cells expressing Mitfa protein at 1 dpf. Mean fluorescence intensity per animal can be measured using ImageJ software. Scale bars = 100 µm. Abbreviations: dpf = days post fertilization; FACS = fluorescence-activated cell sorting.

Figure 2: Counting lateral midline melanophores, measuring mean gray value, and melanin content assay. Brightfield microscopic images of the lateral view of morphants were used to quantify melanin content using ImageJ software. (**A**) Control and Histone variant h2afv (h2afv) morphants at 3 dpf; on the right are zoomed-in trunk regions of these embryos depicting melanocyte numbers. (**B**) The number of melanophores in h2afv morphants is drastically reduced relative to control, n > 10 each in 3 biological replicates. (**C**) Representative image of control vs carbonic anhydrase 14 morphants at 2 dpf. (**D**) Melanin quantification of ca14 vs control morphants, n > 10 across 3 biological replicates. (**E**) Lateral view of h2afv vs control morphants at 2 dpf. (**F**) Melanin content quantification of h2afv vs control morphants. Red rectangles represent ROI chosen for ImageJ-based analysis. (**G**) Melanin content assay performed on morpholino-injected embryos for quantifying the total melanin content. Mean of three independent experiments ± SEM.; ∗∗P < 0.01, ∗∗∗∗ P < 0.0001. Student's *t*-test; error bars are mean ± standard error of the mean (SEM). Scale bars = 100 µm. Abbreviations: MO = morpholino; dpf = days post fertilization; ROI = region of interest.

Figure 3: Quantifying fluorescently labeled cells using FACS and measuring mean fluorescence intensity. (**A**) Fluorescence images of ca14 and control morphant embryos at 2 dpf from Tg (ftyrp1: GFP) line, where the differentiating melanocytes are marked by GFP expression. (**B**) Number of melanophores in ca14 and control morphant embryos at 2 dpf remain unchanged, n = 3 biological replicates. (**C**) Fluorescence images at 2 dpf of h2afv and control morphants from Tg(*ftyrp1*:GFP) line. (**D**) The number of melanophores in h2afv morphants is significantly reduced compared to the control morphant embryos at 2 dpf, n = 3 biological replicates. (**E**) Fluorescence images of control and h2afv morphant embryos at 36 hpf from Tg(*mitfa*:GFP) with labeled melanocytes. (**F**) MFI per animal is calculated using ImageJ software and represented as a bar graph depicting individual data sets. MFI is significantly reduced in h2afv morphants compared to control. Red rectangle represents ROI chosen for ImageJ software-based analysis. n = 3 biological replicates; ∗∗∗P < 0.001, ∗∗∗∗P < 0.0001. Student's *t*-test; error bars are mean ± standard error of the mean (SEM); scale bars = 100 µm. Abbreviations: MO = morpholino; FACS = fluorescence-activated cell sorting; GFP = green fluorescent protein; dpf = days post fertilization; ROI = region of interest; MFI = mean fluorescence intensity.

Supplemental Figure S1: Quantitation of head melanophores. (**A**) Dorsal view of h2afv and control morphants at 48 hpf showing head melanophores. Red box represents the ROI. Keeping the area of interest constant, the number of head melanophores can be quantified manually. (**B**) Number of head melanophores significantly reduced upon h2afv knockdown. Bars represent geometric mean with 95% CI of the number of head melanophores per embryo with >30 embryos

each. Scale bars = 100 µm. Abbreviations: MO = morpholino; CI = confidence interval; hpf = hours post fertilization; ROI = region of interest.

Supplemental Figure S2: Microarray of human primary melanocytes treated with PTU. Heat map shows the expression of pigmentation genes (*Mitf, tyrosinase, DCT, Mc1r*) upon treatment in human primary melanocytes. Abbreviations: PTU = 1-phenyl-2-thiourea; tyr = tyrosinase.

DISCUSSION:

Pigmentation phenotype is often manifested as alterations in the content of melanin or the number of pigment-bearing melanocytes. The method described herein allows the dissection of this dichotomy and permits qualitative as well as quantitative assessment of melanin content and the number of melanophores per embryo, irrespective of the melanin content. The high fecundity of zebrafish, visible nature of pigmented melanocytes, and lack of melanosome transfer enable the dissection of melanocyte biology using this reverse genetic approach that is amenable to medium-throughput screening.

The choice of morpholino-based silencing emanates from the ease of silencing and the ability to test multiple candidates parallelly. There are certain limitations of using a morpholino-based silencing approach, and guidelines for using and interpreting these valuable tools have been described previously[13]. Essentially, the validation of phenotype upon knockdown using multiple MOs provides a tangible solution to this problem. An alternate strategy is the use of CRISPR-based genetic perturbation. The penetrance of the phenotype is limited due to the inherently lower frequency of genetic events and could be compounded by heterogeneous mutations. That limits its use for reverse genetic screening[14].

Phenotype assessment of melanocyte-selective perturbations often results in decreased pigment content due to altered melanogenic response. However, there are several genes that perturb the specification of melanocytes and hence cause a reduction in pigmentation due to a decrease in overall melanocyte numbers. A systematic dissection of the two scenarios is essential to ascribe the molecular function of the gene in pigmentation. The measurement of melanin content is compounded by the complex nature of melanin polymer. Of the two available methods, namely melanin solubilization by NaOH and HPLC-based detection of stable melanin oxidation products PTCA and PDCA, the former method can be routinely performed in a laboratory setting while the latter requires specific standards and sophisticated instrumentation[15].

Additionally, the number of embryos required by the NaOH method is also higher due to several steps involved in the detection. NaOH-based hydrolysis solubilizes melanin and enables spectrophotometric quantitation. While this method is robust and can be adopted for a medium-throughput screen (10–15 candidates at a time), xanthine present in xanthophores is a potential interfering substance due to its inherent absorption in the 400–450 nm range[16]. Hence, a concordance between image analysis-based mean grey value determination and melanin content assay must be carried out to confirm the reduction in the melanin content.

PTU offers an easy tool to reduce the pigmentation and visualize the otherwise transparent

embryo. The use could possibly result in feedback changes and invoke alterations in melanocytes. However, we have observed minimal changes in the expression of pigmentation genes **(Supplemental Figure S2)**.

The counting of melanocytes is easiest in the lateral mid line and the head region, as the melanocytes are relatively distinctly observable in these regions (**Supplemental Figure S1**). Lateral line melanophores arise from the melanocyte stem cell population that resides close to the dorsal root ganglia. Imaging this population has several advantages such as fixed number per embryo and clear distinction. However, the two contralateral lines need to be imaged by tilting the embryo slightly; else the two merge due to the transparent nature of the embryo, making it difficult to count (**Figure 1C** versus **Figure 2C**).

Zebrafish melanophores are distinct from the pigmented cells of the eye that are derived from neuro-ectoderm. These are distinguishable by their positioning within the embryo and are evident in the eye versus the body pattern. In the FACS-based experiment, the eye-derived cells can be distinguished by their size as they are significantly smaller than the melanophores and can be gated using size or scatter criteria.

Estimates of melanocytes at different maturation stages using transgenic lines are easily carried out using image-based quantitation. Quantitation of the number of lateral-line melanophores is robust as their number varies in a window between 2 dpf and 5 dpf on each side of the embryo[17]. However, the number of lateral-line melanophores is determined by the migration and establishment of a dorsal root ganglion-associated stem cell pool[18]. Hence, any alterations in these processes can lead to a similar observation. To circumvent this confounding factor, FACS-based estimation of all melanophores is a tangible solution.

Alternately, quantitation of head melanophores can be performed as delineated in **Supplemental Figure S1** as a surrogate for melanophore numbers. It is interesting to note that the alterations in steady-state levels of melanocyte numbers could signify two opposite functions for the candidate gene in question. It could either result in decreased specification of melanocytes from neural-crest precursors or cause melanocyte-specific death and need to be addressed using methods such as the tunnel assay. Variations such as microinjections followed by live-cell imaging would enable monitoring other melanocyte-specific phenotypes such as migration and patterning. Of interest to pigment-cell researchers would be the process of melanosome transfer, which is not operative in the fish system. However, concerted melanosome movement in melanophores can be studied with alterations in live-cell imaging described herein with modifications. In all, the detailed protocol presented in this video article would enable pigment cell biology researchers to query candidate genes and assess their role in melanocyte biology.

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DISCLOSURES:

All authors declare no conflict of interest.

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Figure 1

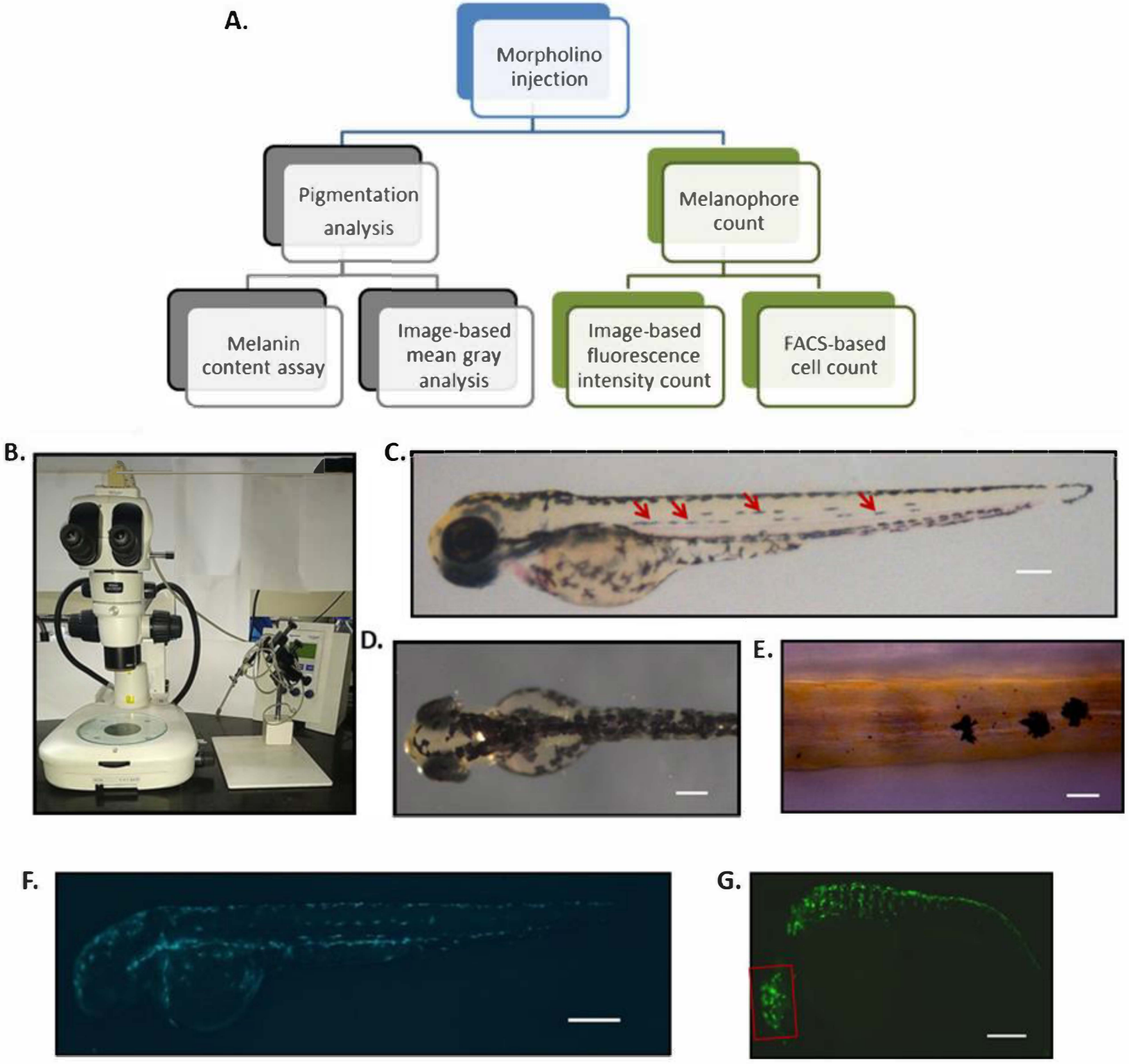
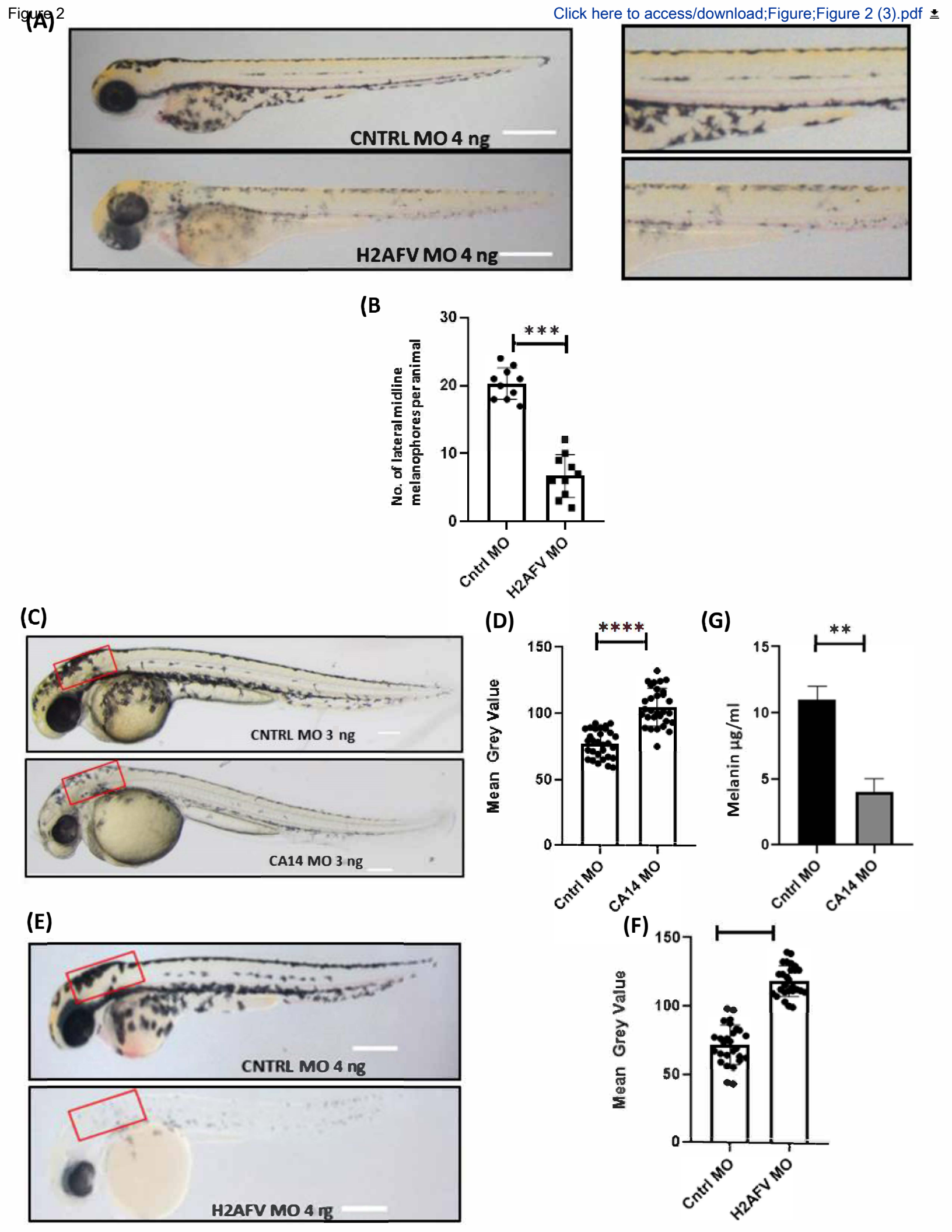


Figure 2



Figure

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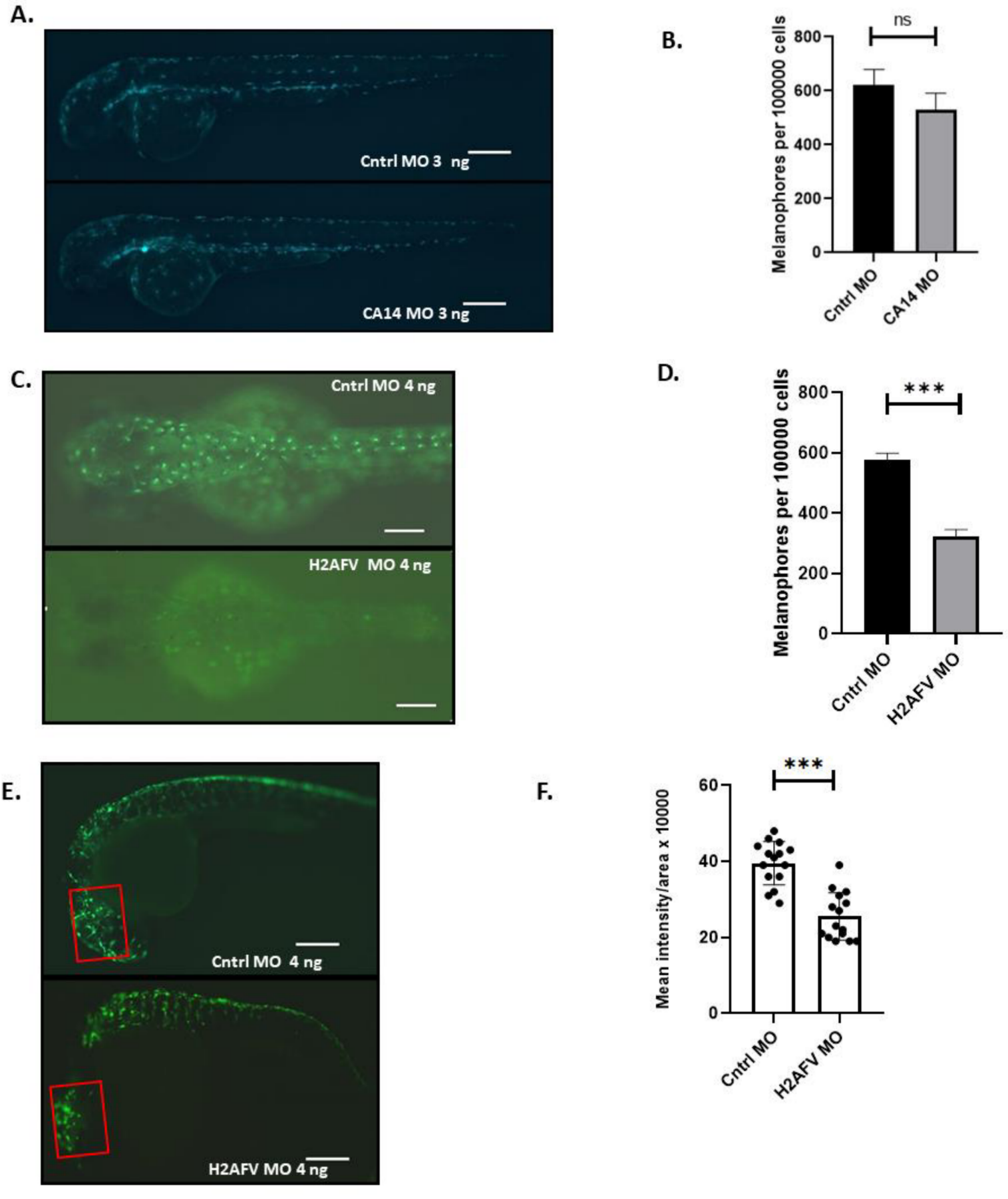


We thank editorial team for reviewing the manuscript thoroughly. We want to address the comments pointwise.

Reviewer #3:

- the morpholinos used are not referenced.

Our response: Thank you for bringing it to our knowledge. We have done that now.

1. Injecting morpholino into zebrafish embryos:
-my assumption is that it is not intended to give an exact protocol for microinjections into zebrafish eggs; otherwise much more detail would be needed. The published protocols should be cited.

-I don't understand the paragraph on calibration (1.4.). It must be specific for the injection setup used; if we inject with our setup for 5 sec the needle will be completely empty.
What does 1mm=30nl mean? If 1 mm is the diameter of a spherical droplet that was produced, then r = 0.5 mm, and therefore the volume would be (v = 4/3 * 3.14 * r[3]) approx. 500 nl. If some specific setup for calibration is used this should be mentioned.

Our response: As microinjection is a very essential part of this protocol, we understand discussing it in the main text is appropriate. Also, we have cited the appropriate published protocols.

Reviewer is correct that the standard stated here, 1mm=30 nl, is specific to microinjector pressure settings and the diameter of the capillary used for calibration. We have mentioned it in the main text as a separate note.

“1mm” is not diameter of the spherical droplet. But this is the length of the capillary tube (Drummond, Catalog Number 1-000-0010), which is used for calibration. It means 1mm of this capillary can hold 30 nl of the solution.

2. Pigmentation analysis

-to visualize all five melanophore stripes simultaneously the larvae must be slightly tilted, otherwise the two lateral stripes will be on top of each other and it will be more difficult to count individual cells. In Fig. 1C and 2A, H2AFV MO 4 ng this is the case; however, not in Fig. 2A, CNTRL MO 4 ng, where, in deed, I find it difficult to distinguish individual cells. This could be mentioned.

Our response: We agree that fish has to be slightly tilted to visualize both the lateral stripes simultaneously. We have incorporated it into the main text.

-morpholino injections frequently lead to developmental delays that might result in reduced melanophore pigmentation, especially when analyzed during very early stages. This could be mentioned in the discussion to caution others who might investigate genes without known mutant phenotypes.

Our response: Morpholino injections often lead to developmental delays especially till 24 hpf. Firstly, control morpholino should account for any developmental delay due to injection. Secondly, in some

cases the experimental morphants show developmental delay whereas control morphant do not. To account such cases the morphological developmental stage of zebrafish should be used as the reference point to compare between experimental and control morphants.

We will mention this in the discussion.

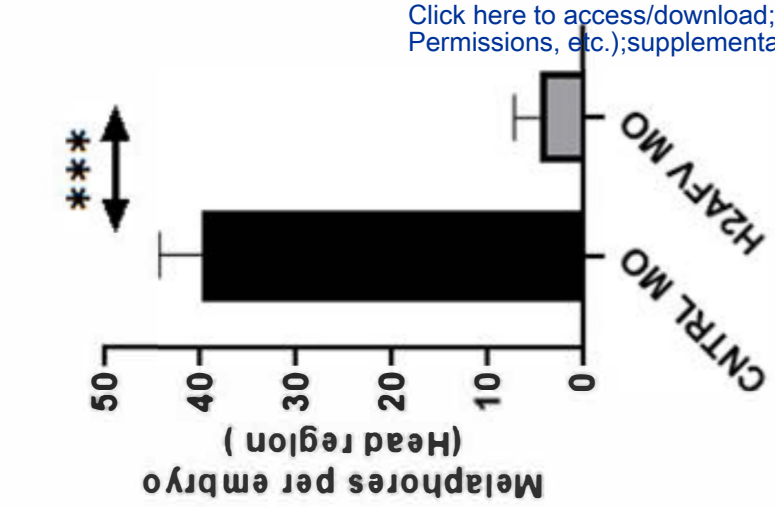
-melanophores in zebrafish are of two different origins: the neural crest derived body melanophores, which are dependent on MitfA; and the melanophores of the retinal pigment epithelium, which are of a neuro-ectodermal origin, and do not depend on MitfA. This could possibly affect the interpretation of the results obtained from melanin quantification of total larvae and should be commented on.
In addition, it seems that in Fig.2A, H2AFV MO 4 ng the larva shows reduced pigmentation in the eyes. If this was indeed the case, and it is not due to the way the picture was taken, not only neural crest derived melanophores would be affected by the knock-down. This could be commented on.

Our response: We agree during NaOH based melanin estimation and fluorescent imaging using Tg(ftyrp:gfp) line, we will be unable to differentiate between RPE and NCDMs. And this will affect the interpretation as mentioned by the reviewer correctly.

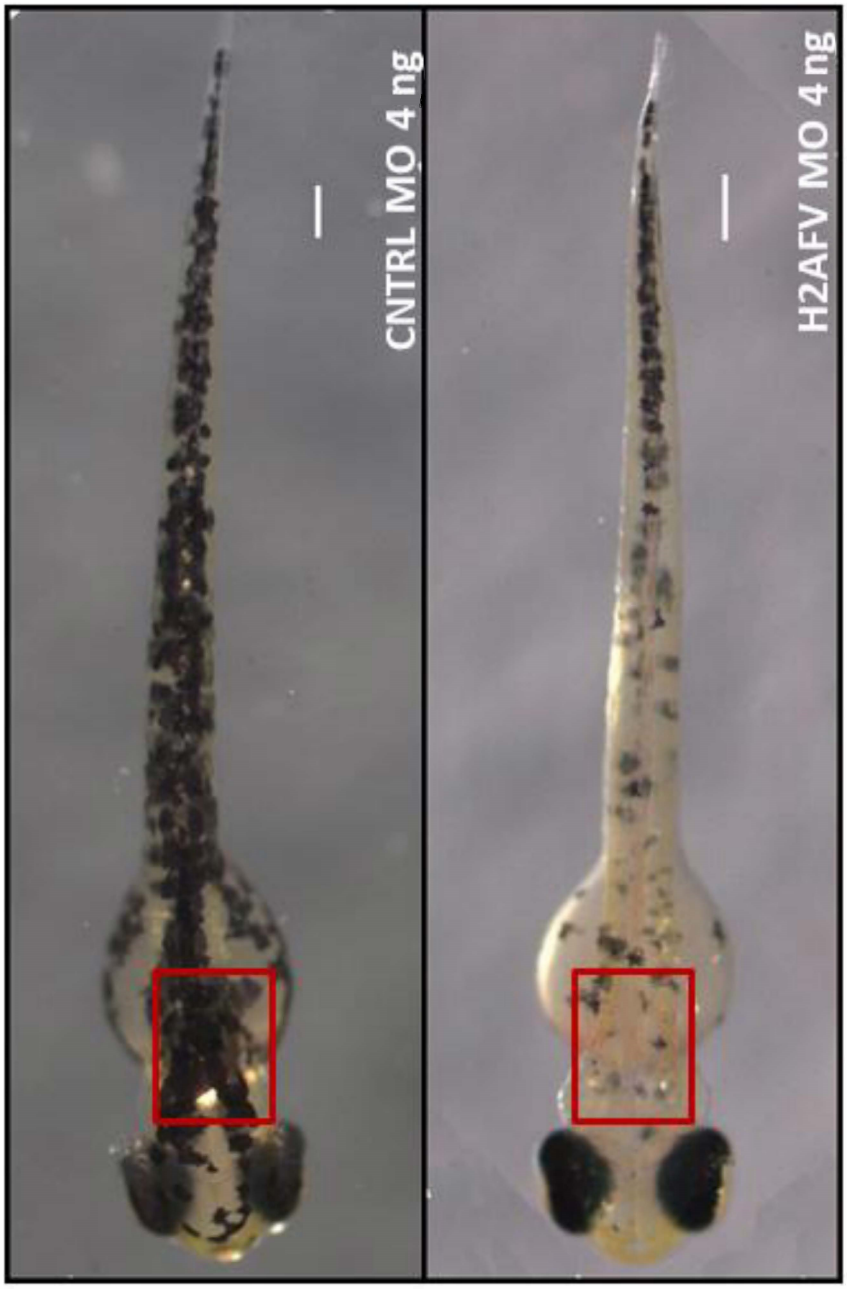
But we do multiple investigations parallelly to appreciate the difference. For example: H2AFV morpholino injection in Tg(mitfa:gfp) that only marks NCDMs not RPE, also mean gray value is calculated from head region to estimate the overall melanin content per embryo. It is necessary to put together all the above obseravations before coming to a justified and valid interpretation.

We will surely comment about this in discussion.

Abbreviatons used: **RPE**= Retinal Pigment Epithelium; **NCDMs**= Neural Crest Derived Melanophores.



B.



A.

Supplemental Figure S2

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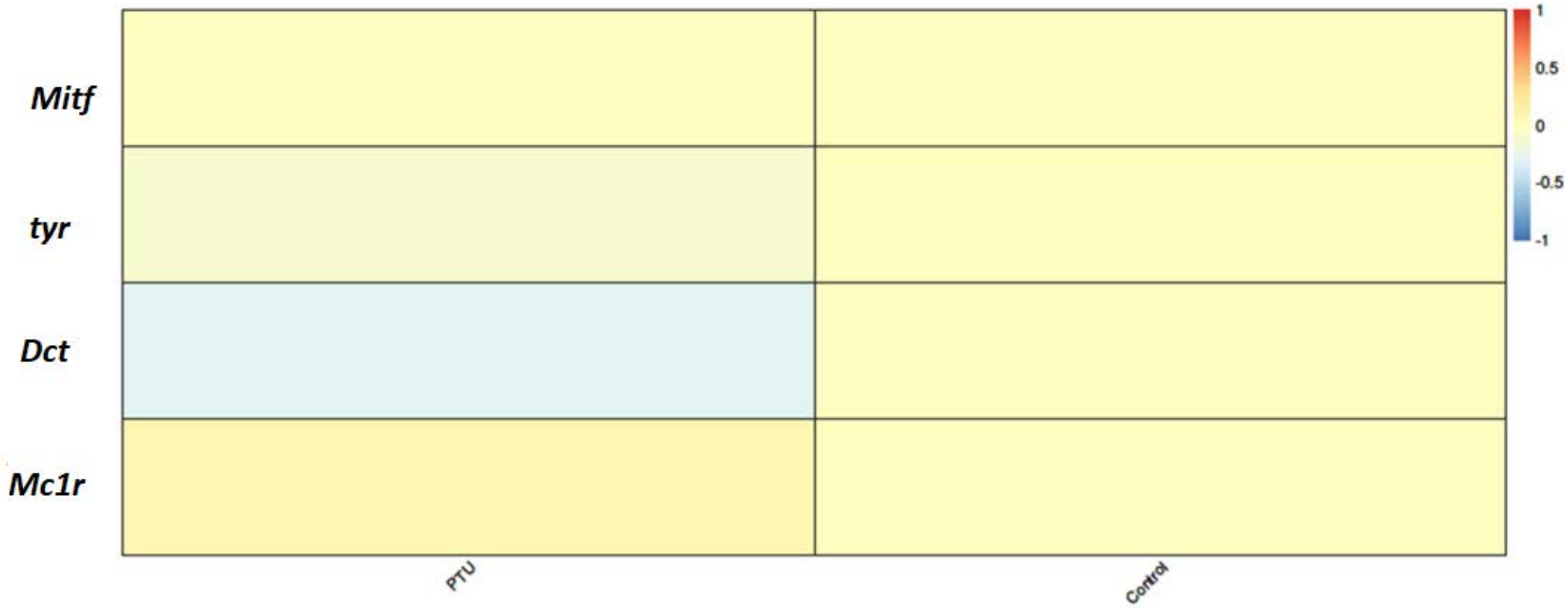

Figure S2