Journal of Visualized Experiments

A practical guide for genotyping and quantification of in situ hybridization staining in zebrafish

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59956R1
Full Title:	A practical guide for genotyping and quantification of in situ hybridization staining in zebrafish
Keywords:	Genotyping; in situ hybridization; zebrafish; mutants; Imaging; image quantification.
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Birmingham, United Kingdom

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Phillip Steindel, Ph.D.
JoVE Review Editor

Dear Phillip,

We would like to present our revised manuscript "**A practical guide for genotyping and quantification of in situ hybridization staining in zebrafish**" for publication in JoVE.

We have made modifications to the original manuscript as suggested and added new data to address the reviewer's concerns. The changes are detailed in the accompanying point-by-point response to the editor's and reviewers' comments. All the text revisions are shown as tracked changes in the revised manuscript. We have also added a new Figure (Figure 4) to address some of the reviewers' concerns, in particular those of reviewer #3. We hope that the revised manuscript is now acceptable for publication in JoVE.

Yours sincerely,

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TITLE:

Genotyping and Quantification of In Situ Hybridization Staining in Zebrafish

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KEYWORDS:

Genotyping, in situ hybridization, zebrafish, mutants, bias, image quantification.

SUMMARY:

Gene editing technologies have enabled researchers to generate zebrafish mutants to investigate gene function with relative ease. Here, we provide a guide to perform parallel embryo genotyping and quantification of in situ hybridization signals in zebrafish. This unbiased approach provides greater accuracy in phenotypical analyses based on in situ hybridization.

ABSTRACT:

In situ hybridization (ISH) is an important technique that enables researchers to study mRNA distribution in situ and has been a critical technique in developmental biology for decades. Traditionally, most gene expression studies relied on visual evaluation of the ISH signal, a method that is prone to bias, particularly in cases where sample identities are known a priori. We have previously reported on a method to circumvent this bias and provide a more accurate quantification of ISH signals. Here, we present a simple guide to apply this method to quantify the expression levels of genes of interest in ISH-stained embryos and correlate that with their corresponding genotypes. The method is particularly useful to quantify spatially restricted gene expression signals in samples of mixed genotypes and it provides an unbiased and accurate alternative to the traditional visual scoring methods.

INTRODUCTION:

The introduction of genome editing technologies (ZFN, TALENs and more recently, CRISPR/Cas9) has led to a massive increase in the number of laboratories around the world that make use of these systems to study the function of specific genes in vivo. Zebrafish in particular are amenable to genetic manipulation and many mutants have been generated in the recent past[1,2]. For developmental biologists, one of the most common methods to assess the phenotypical consequences of gene mutations in embryonic development is in situ hybridization (ISH). In the absence of obvious morphological defects that separate homozygous mutants from their wild type or heterozygous siblings, it is essential to be able to correctly identify different genotypes accurately.

Classical ISH relies on qualitative analyses of signal intensities to derive conclusions about regulatory interactions between the gene of interest and selected marker genes. Although useful, these analyses suffer from technical variation and may be biased by researcher expectations. Thus, a method was developed to quantify gene expression after imaging ISH-stained embryos, without prior knowledge of the corresponding genotype. This was followed by an efficient DNA extraction and genotyping that allowed us to quantitatively correlate genotype with gene expression[3]. While the genotyping of embryos post ISH has been used before[4,5], image-based quantification of ISH patterns has not been widely used apart from a couple of studies[6,7]. The most popular alternatives rely on visual scoring or counting of ISH-stained cells[8-10], both prone to poor reproducibility and researcher bias. This method is particularly useful to study changes in genes with expression patterns that are spatially restricted, such as *runx1* or *gata2b*, both expressed in a restricted subset of aortic floor cells called the haemogenic endothelium[11,12].

Here, we aim to provide a practical guide to the implementation of the quantification by image analysis using Fiji[13], as well as the DNA extraction and genotyping protocol. This is meant to illustrate visually our previously published method[3]. Our method allows an accurate representation of the variation in gene expression detected by ISH and an unbiased assignment of gene expression levels to specific genotypes.

PROTOCOL:

Procedures involving animal subjects are regulated by the Animals (Scientific Procedures) Act 1986 and have been approved by the Home Office and the local Animal Welfare and Ethical Review Body.

1. Image ISH-stained embryos

1.1. Prepare a glycerol solution (50%–80% in 1x PBS buffer) and mix to homogenize the solution (e.g., leave in a roller for at least 5 min). This solution can be kept for months at room temperature.

1.2. After in situ hybridization[14-17], transfer embryos to the glycerol solution with a 3 mL Pasteur pipette and leave to settle for at least 5 min. If imaging embryos older than 24 hpf (hours post fertilization), they may be bleached as described[18].

1.2.1. Prepare and label enough PCR tubes to transfer ISH-stained embryos after imaging.

1.3. Add 100% glycerol to the bottom of the well in a glass depression slide with a 3 mL Pasteur pipette.

1.4. Using a 3 mL Pasteur pipette, transfer a single ISH-stained embryo to the glass slide and orient as required under a stereomicroscope equipped with a digital camera and bottom and top illumination.

NOTE: Use gel loading pipette tips to position the embryos for imaging, but other tools (e.g., forceps, dissecting needle) will be equally adequate.

1.5. Using the first embryo, adjust the illumination and exposure time at the desired magnification. Use these conditions for ALL of the embryos in the same experiment (i.e., if imaging 40 embryos from a heterozygous mutant incross, make sure the illumination, exposure time and magnification are the same for all).

1.6. Image as many embryos as required. Label each image with a unique number. After imaging, transfer the embryo to a PCR tube/plate labelled with the same number.

NOTE: Images should be saved as TIF files, but other formats are also adequate.

1.6.1. If required, remove excess glycerol in the PCR tubes/plates.

NOTE: At this point, the embryos can be stored in the PCR tubes for several weeks at room temperature.

2. Extract DNA and genotype the ISH-stained embryos

NOTE: Here, use a reliable and inexpensive method to isolate genomic DNA based on the HoTSHOT method[19] with a DNA extraction efficiency of 95%–100%[3].

2.1. After the imaging is complete, add 40-75 µL of alkaline lysis buffer (e.g., HoTSHOT) to each tube.

2.2. Incubate at 95 °C for approximately 30 min and cool the tubes to 4 °C before adding an equal volume of neutralization buffer. An overnight incubation at 4 °C may improve PCR efficiency.

NOTE: At this point, the genomic DNA can be used for genotyping or stored at -20 °C until it is required.

2.3. Genotype samples with an appropriate method (e.g., HRMA, RFLP)[3,20,21] as required for the mutation of interest.

2.4. Note the genotype corresponding to each sample (e.g., using a spreadsheet software).

3. Quantify the pixel intensity of ISH-stained embryos (image analysis using Fiji software)

3.1. To quantify the in situ hybridization (ISH) staining signal intensity, convert all images to 8-bit grayscale as described[3]. If the images were saved as .TIF files, use a Fiji macro for batch conversion[3]. Alternatively, convert images in other formats (e.g., .JPG) to .TIF using appropriate software and then convert to 8-bit grayscale using Fiji. For convenience, here is the step-by-step procedure that was published previously[3], with some alterations.

3.2. Open images in Fiji and invert the image with **Edit > Invert**. Then change the image type to 8-bit (**Image > Type > 8-bit**).

3.3. Using the polygon selection tool, draw the region of interest (ROI) manually on the image around the region containing the signal.

3.4. Press **t** to open the ROI manager. Use the ROI manager's **Measure** command to measure the intensity of the ROI. Copy the **mean** value from the Results window to a spreadsheet software.

3.5. Move the same ROI, ensuring the same size and shape as the original region, to a region of the zebrafish not containing any staining. Repeat step 3.2 to measure the background.

3.6. To obtain the mean pixel intensity of the ISH signal, subtract the mean intensity value of the background region from that of the stained region for each embryo.

3.7. Assign each intensity value to a genotype (from step 2.3).

4. Analyze the results with appropriate statistical tests

4.1. Plot all the values on one Q-Q plot to identify any deviations from normal distribution.

NOTE: Normal distribution can also be verified with the Kolmogorov-Smirnov test or the Shapiro-Wilk test. However, for large sample sizes there is a high risk of false positives in these tests.

4.2. If there are strong deviations from normal distribution, transform all the values (using ln or sqrt functions) to make sure they are normally distributed before proceeding.

4.3. Analyze the differences between the values (transformed if necessary) assigned to each genotype (wt vs. heterozygote vs. mutant) with 2-tailed ANOVA with 95% confidence levels, accounting for the equality of variances with a Levene's test and Welch correction. For pairwise comparisons between each pair of genotypes, use Tukey's (equal variances) or Games-Howell (unequal variances) post-hoc test.

4.3.1. If the values are not normally distributed despite the transformation, use a non-parametric test (Kruskall-Wallis) to analyze the differences between ranked values and a post-hoc Dunn's multiple comparisons test with Bonferroni correction for pairwise comparisons.

4.4. Plot the untransformed values (from step 3.5) as dot plots for the best representation of the results.

REPRESENTATIVE RESULTS:

Here, we describe the practical application of the pipeline for image quantitation and embryo genotyping as published elsewhere[3]. The workflow for the method is shown in **Figure 1**. To illustrate how to use this method, ISH was performed for *dnmt3bb.1* in 33 hpf embryos from a *runx1*$^{W84X/+}$ [22] incross (**Figure 2**). 130 embryos were imaged using the same illumination conditions as detailed in the protocol and labeling them with a unique number. After imaging, each embryo was transferred to a PCR tube for genotyping. At this point, the image analysis was performed to attribute a pixel intensity value to each image. The genotype was then assigned to its corresponding image and the pixel intensity values grouped according to their genotype for statistical analysis. A decrease in *dnmt3bb.1* expression was detected in *runx1*$^{W84X/W84X}$ mutants (**Figure 2A,B**)[3], in agreement with previous observations[5]. Interestingly, *runx1*$^{W84X/+}$ heterozygous embryos showed no significant differences in *dnmt3bb.1* expression (**Figure 2A,B**) compared to its wild type siblings, suggesting that one copy of Runx1 is sufficient to maintain *dnmt3bb.1* expression at appropriate levels.

Many zebrafish mutants fail to show an embryonic phenotype that can otherwise be detected using other loss of function technologies like morpholino oligonucleotides (MOs). This discrepancy can be attributed to a number of causes including off-target effects, maternal protein compensation, a hypomorphic allele[23] or the recently discovered phenomenon of genetic compensation[24-27]. In this example, we asked whether *runx1* expression was reduced or lost in *lmo4*^{uob100} mutants since previously published data using an *lmo4* MO suggested that *runx1* is decreased in *lmo4* morphants[28]. Here, the analysis revealed no significant differences in *runx1* expression between wild type and *lmo4*^{uob100} homozygous mutants[3] (**Figure 3A,B**). Further analysis by single embryo qPCR showed that there was a small but significant decrease in *runx1* expression in *lmo4*^{uob100} mutants (**Figure 3C**). Thus, it is possible that image quantification might not be able to detect small differences in expression levels. Alternatively, the lack of difference between genotypes that we detected is real and the qPCR experiments are detecting changes in *runx1* expression in other tissues like the telenchephalon where both *lmo4a* and *runx1* are expressed. Researchers should always verify their results with an independent method like qPCR, but ideally enriching for the tissue of interest by flow cytometry, for example.

In rare instances where the ISH has high background (**Figure 3D**), the pixel intensity value of this area is so high that subtraction from the signal value produces a negative number and in such instances those embryos would be excluded from the analysis. In our experience, this occurred in about 0.4% of the *runx1*-probed embryos[3] but may vary between experiments, probes or batches of reagents. Although that might be a limitation of the method, the low frequency of high background is very unlikely to influence the overall results.

To test the effect of selecting different areas for background corrections, we first measured the pixel intensity of the *runx1* ISH signal in 28 hpf embryos, using different regions for background corrections (**Figure 4**). Four different regions were selected: two in the trunk region (R1, and R2), one in the yolk region (unstained, but likely to accumulate background staining) and a smaller area anterior to the ROI (R4, **Figure 4B**). Measuring pixel intensity in these regions showed a relatively stable difference in intensity between ROI and either background area (**Figure 4C**). However, R3 always showed very high values (above those in the ROI). After inversion and conversion to 8-bit, the yolk region appears very bright and thus is not suitable for use as a background correction. R2 was closer to the ROI but contained some ISH signal, and using it for correction decreased the mean pixel intensity when compared with either R1 (located further dorsally, away from the ISH signal) or R4. Thus, either R1 or R4 are appropriate areas that can be used for background correction (despite the area of R4 being smaller than that of R1). Next, we wished to compare how using R1 or R4 affected the outcomes when comparing *runx1* expression. For this, we incrossed dll4$^{+/-}$ heterozygotes[29] and analyzed *runx1* expression in randomly selected wild type and dll4$^{-/-}$ embryos (**Figure 4E**). Although using R1 or R4 for background correction affected individual values, the mean pixel intensities within the same genotype were not significantly different (**Figure 4E**). Moreover, comparing *runx1* expression still yields similar mean intensity values between genotypes using either R1 or R4 areas as background correction (μ_{R1}=16.3 and μ_{R4}=18.2, respectively). Taken together, we concluded that although the choice of background area is important, the main criteria is that it does not include yolk regions (prone to accumulation of background staining) and that it should not contain any (specific) staining that might skew the pixel intensity values of the background.

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow of the parallel image quantitation and genotyping protocol. Embryos collected from an incross of fish heterozygous for a mutant allele are probed for the measured gene with a standard ISH protocol. After imaging, genomic DNA is extracted using the HotSHOT protocol by adding the lysis buffer directly to the embryo in a 0.2 mL PCR tube, followed by a 30 min incubation at 95 °C. This DNA is used for genotyping of the embryos by PCR, PCR and restriction fragment length polymorphism (RFLP), KASP assays or any other appropriate method. In parallel, the images for each embryo are inverted and converted to 8-bit greyscale. ROIs of identical shape and size containing the ISH signal (yellow) and background (blue) are manually selected and measured. The measurements, assigned to corresponding genotypes, are statistically analyzed. Figure adapted from Dobrzycki et al.[3]

Figure 2: Image quantitation in r*unx1* mutants reveals reduced levels of *dnmt3bb.1* expression by ISH. (**A**) Example images of ISH in 33 hpf wild type (blue), *runx1*$^{+/W84X}$ (green) and *runx1*$^{W84X/W84X}$ (orange) embryos, showing *dnmt3bb.1* expression in the dorsal aorta. (**B**) Pixel intensity values of *dnmt3bb.1* mRNA in *runx1*$^{W84X/W84X}$embryos (n=36) are significantly decreased compared to wild types (n=32) and heterozygotes (n=62) (ANOVA, $p < 0.001$). The coefficients of variation are 24%, 22% and 21% for wild type, heterozygote and mutant groups, respectively. Blue, green and orange data point correspond to the example images from panel A. The bars

represent mean ± s.d. ***p<0.001 (Games-Howell post-hoc test). Figure adapted from Dobrzycki et al.[3]

Figure 3: Measuring *runx1* expression levels by ISH in *lmo4a*^{uob100} mutants. (**A**) Representative images of ISH for *runx1* in 28 hpf wild type (blue), heterozygous (green) and *lmo4a*$^{uob100/uob100}$ (orange) embryos, showing the expression in the dorsal aorta. (**B**) Quantification of the *runx1* mRNA signal, detected by ISH, from 28 hpf wild type (n=15), heterozygous *lmo4a*$^{+/-}$ (het) (n=34) and *lmo4a*$^{uob100/uob100}$ mutant (n=18) embryos from one clutch shows no significant difference in *runx1* pixel intensity among the different genotypes (ANOVA,> p 0.6). Blue, green and orange data point correspond to the example images from panel A. The bars represent mean ± s.d. (**C**) Boxplots displaying normalized *runx1* mRNA levels ($2^{-\Delta Ct}$) in single wild type (blue; n=12) and *lmo4a*$^{uob100/uob100}$ (mut, orange; n=12) embryos, measured by qRT-PCR, showing decreased levels of *runx1* in the mutants compared to wild type. *p < 0.05 (t test). (**D**) Example of an ISH experiment on a 28 hpf embryo (stained for *runx1*, yellow arrowheads) showing high background. Figure adapted from Dobrzycki et al.[3]

Figure 4: Effect of background intensity correction on measurement outcomes. (**A**) Representative image of *runx1* ISH staining in a wild type embryo at 28 hpf. (**B**) Same image after inversion and conversion to 8-bit. The region of interest (ROI) is highlighted in green and four different areas used for background correction (R1-R4) are highlighted in yellow. (**C**) Raw pixel intensity measurements in all regions shown in panel B. Note the intensity in R3 (yolk) is consistently higher than the actual ISH signal in the ROI (n=11). (**D**) *Runx1* expression levels in the ROI using R1, R2 and R4 background areas. Areas for ROI, R1, R2 and R3~28500 pixels; R4~8500 pixels. Note that R3 background was not used for this comparison as the background correction (ROI-R3) consistently yielded negative values. (**E**) *Runx1* expression levels in wild type and *dll4*$^{-/-}$ mutants using either R1 or R4 for background correction (n=10 for each sample). Statistical analysis in panels D and E was performed using a non-parametric Kruskal-Wallis test, assuming that the pixel intensity values are not normally distributed.

DISCUSSION:

A few factors should be considered when using this method to quantify gene expression. The imaging conditions must be maintained throughout the experiment (for example illumination, exposure times and embryo positioning) to reduce variability between measurements. A critical point is to avoid overstaining of the samples, as differences in staining between samples may be masked. For example, the decrease in *VegfA* expression in the absence of Eto2 in *Xenopus laevis* embryos[30] could only be detected by carefully monitoring staining over a 24 hour period. Thus, it is good practice to empirically determine adequate staining levels for each gene that best represent its expression, without reaching saturation. Overstaining will also artificially increase the background pixel intensity in the converted 8-bit grayscale images and skew the quantitation results. In extreme cases, the background level in embryonic tissues might be higher than the ISH signal in the selected ROI and these samples should be excluded from the analysis. A similar phenomenon was observed when we tested the suitability of the unstained yolk for background correction (**Figure 4**). After inversion and conversion to 8-bit the darker pixels in the yolk region become brighter than the ISH signal in the embryo and render the background corrected values

negative. Thus, avoid the use of the yolk for background correction. Measuring the background signal in pigmented areas in the embryo (e.g., the eyes or the dorsal part of the trunk from 26/28 hpf onwards) will equally skew the quantitation results and should also be avoided. There are protocols available for bleaching zebrafish embryos, either before or after ISH[18] and bleaching embryos older than 24 hpf before imaging is recommended.

Because this method relies on measuring the pixel intensity in a defined area against a background pixel intensity in an equivalent non-stained area, it is not appropriate for quantitation of ubiquitous or near-ubiquitously expressed genes as is. Instead, it is well suited for measuring expression of genes with a spatially restricted distribution where an area for measuring background pixel intensity can be readily identified. Our additional analysis now suggests that using a smaller area (3–4x smaller) for background correction yields similar outcomes to using an equivalent area to that of the ROI. This extends the applicability of the method to genes expressed in wider spatial domains (and thus requiring larger ROIs for intensity measurements), as long as one can use clearly unstained areas of the embryo for background correction.

Finally, we suggest that the genotyping be performed in parallel or after the image quantitation to minimize experimenter bias. Asking a second experimenter to repeat the quantitation on anonymized samples and compare with the first set of measurements will also help reduce experimenter bias. If the images to be quantified are from a comparison between treatments that does not require genotyping (e.g., wild type vs chemical inhibitor or wild type vs. MO knockdown), the experimenter performing the measurements should be blinded to the identity of the sample.

ACKNOWLEDGMENTS:

We would like to thank the staff in Biomedical Services in Oxford and Birmingham for excellent zebrafish husbandry. T.D. was funded by a Wellcome Trust Chromosome and Developmental Biology PhD Scholarship (#WT102345/Z/13/Z). R.M. and M.K. were funded by the British Heart Foundation (BHF IBSR Fellowship FS/13/50/30436) and are thankful for their generous support. R.M. acknowledges support from the BHF Centre of Research Excellence (RE/13/1/30181), Oxford.

DISCLOSURES:

The authors declare they have no competing financial interests.

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Figure 1

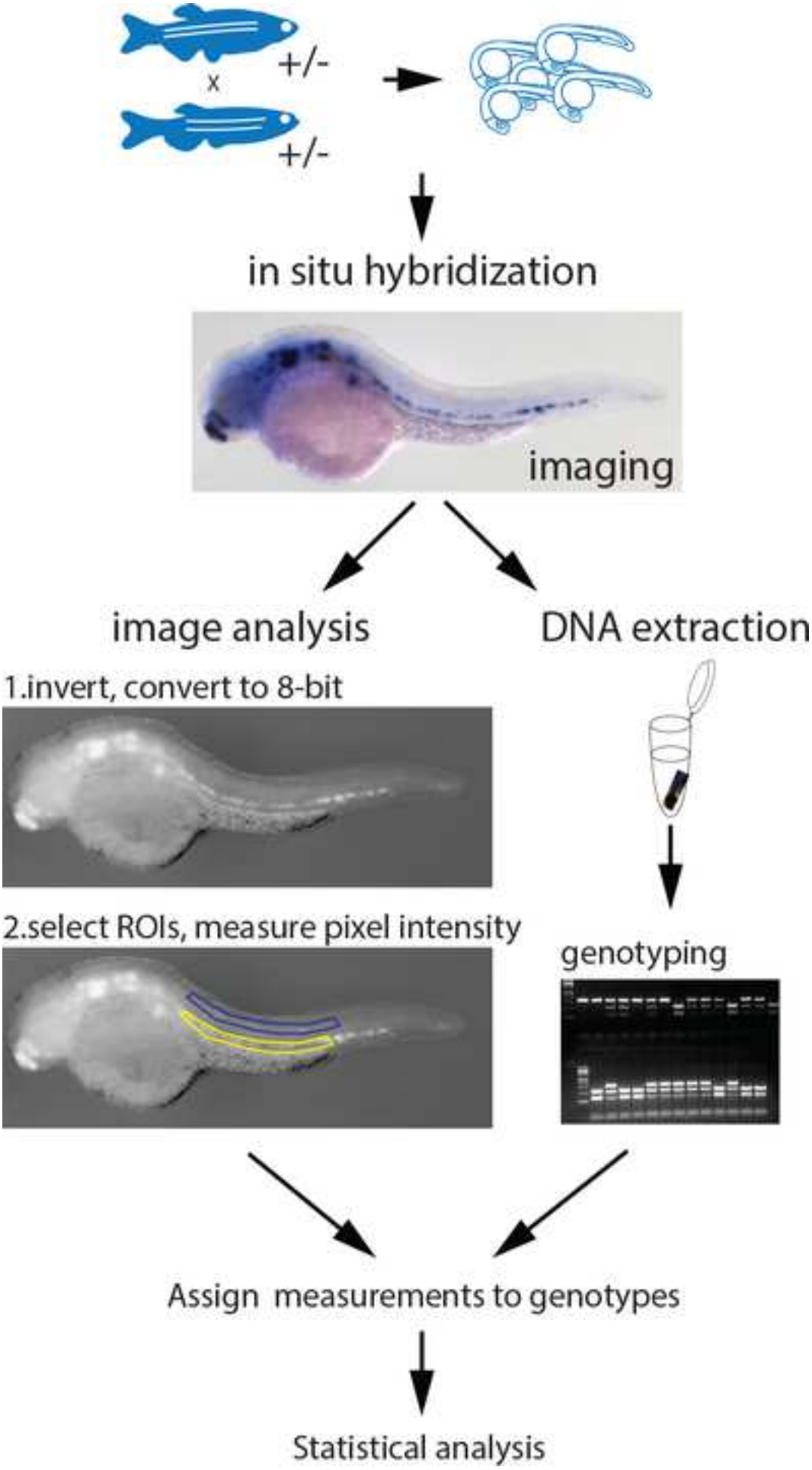


Figure 2

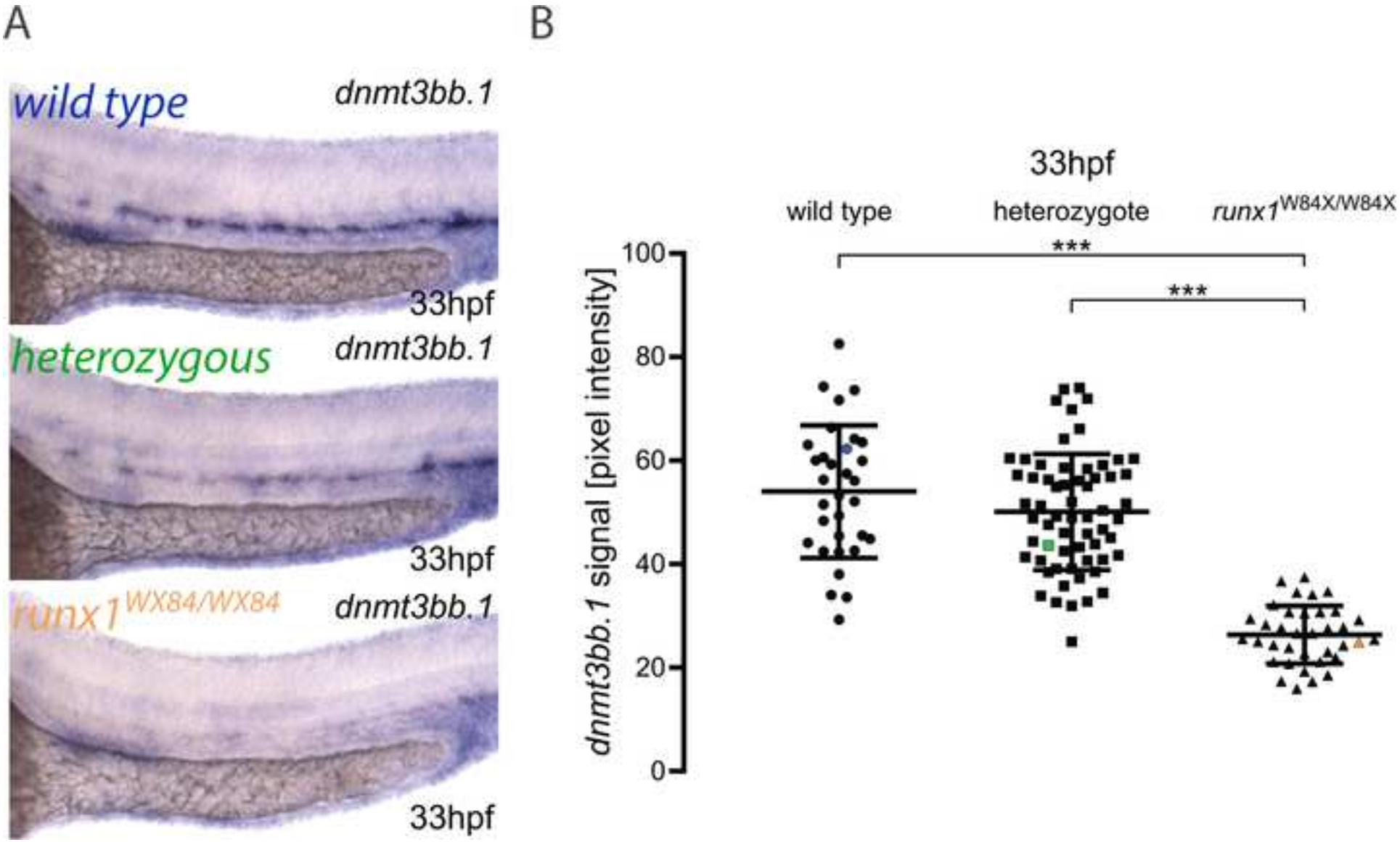


Figure 3

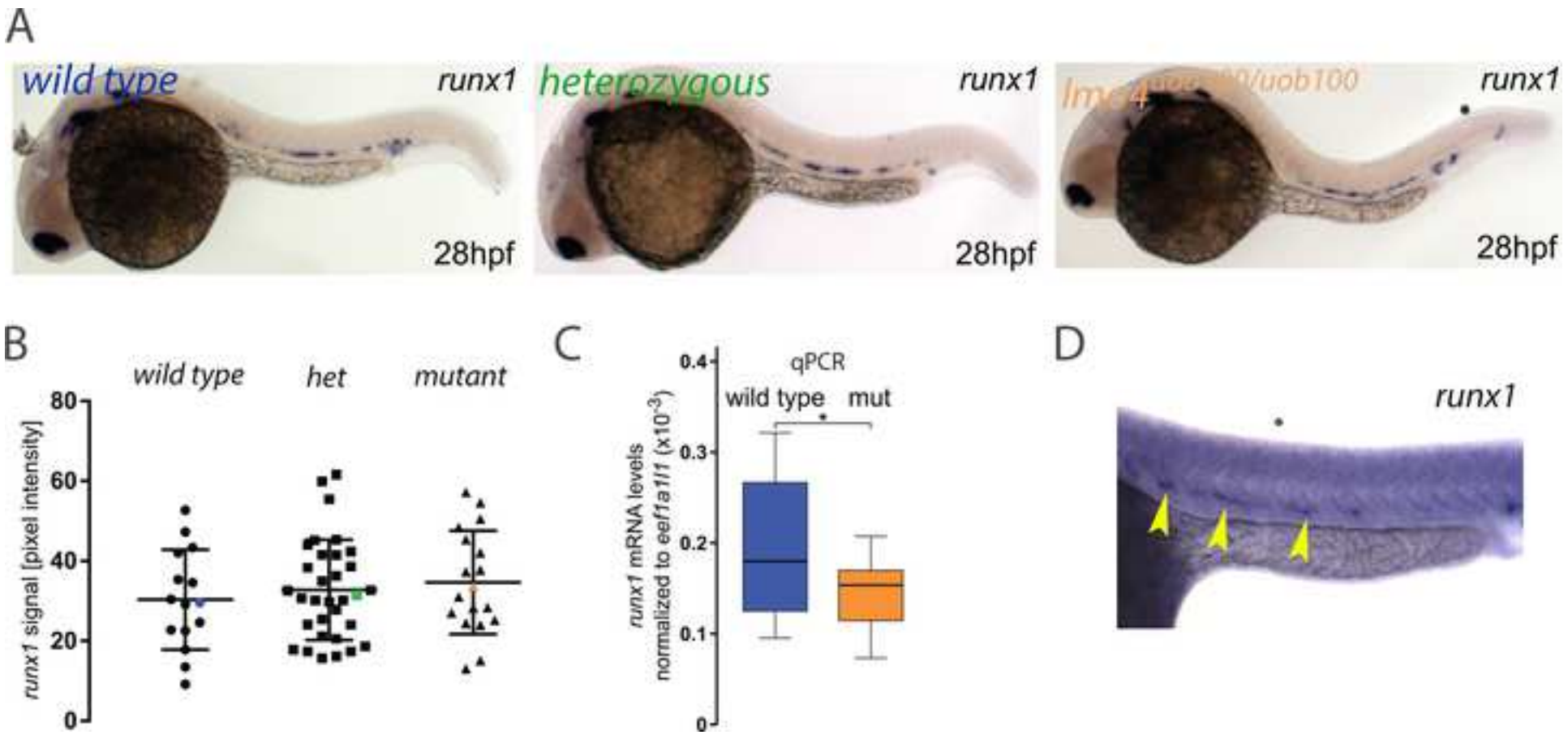
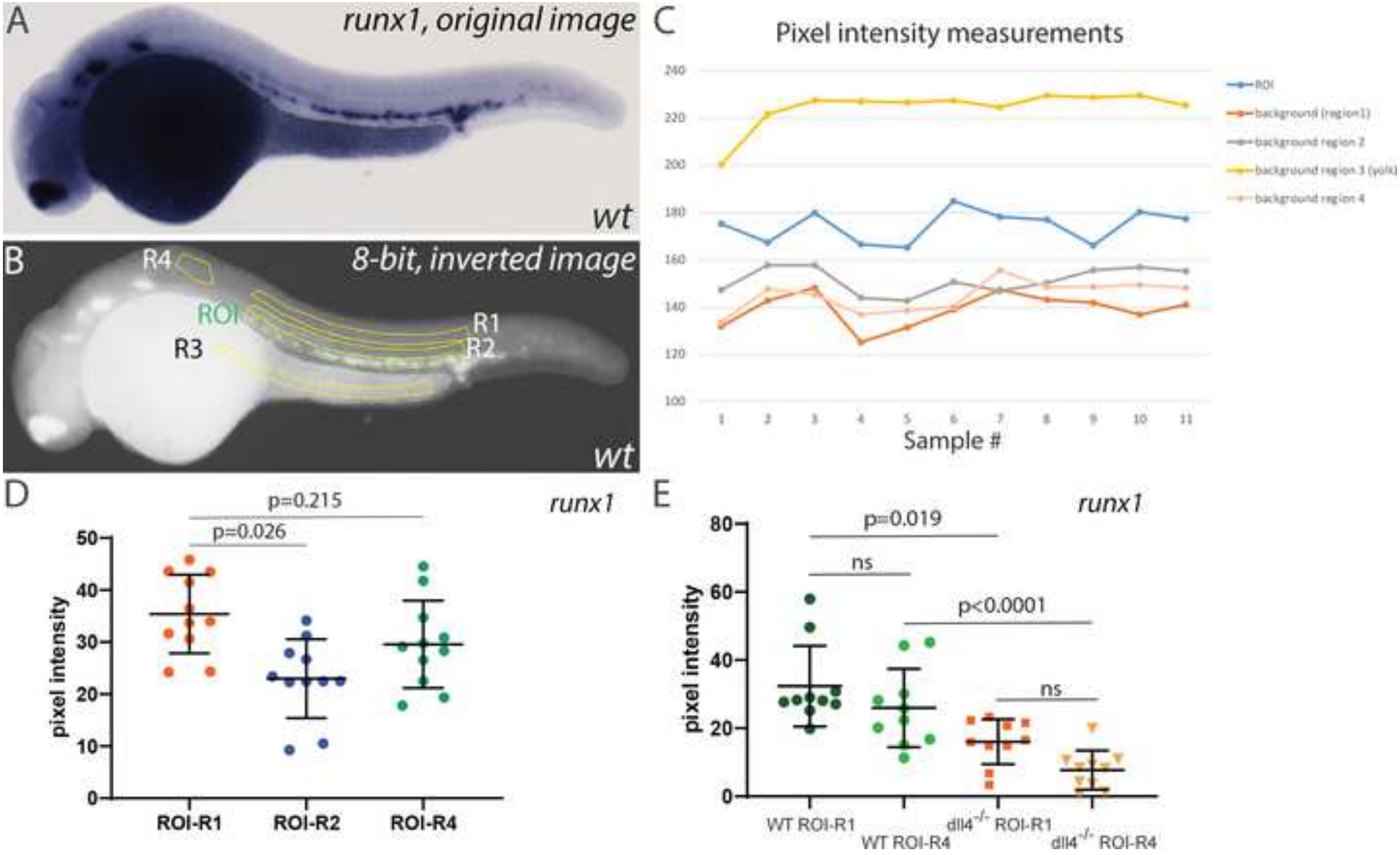


Figure 4



Name of Material/Equipment	Company	Catalog Number	Comments/Description
0.2 mL PCR tubes (8-strips with lids)	StarLab	A1402-3700	96-well plates are equally appropriate for sample handling but beware of cross contamination between samples
3 mL Pasteur pipettes	Alpha Laboratories	LW4114	
Cavity slides	Brand	BR475535-50EA	
Digital Camera (Qimaging Micropublisher 5.0)	Qimaging		
Eppendorf Microloader tips	Eppendorf	10289651	the tips are used to orient the embryos for imaging in glycerol
Excel	Microsoft		
F3000 Fiber Optic Cold Light Source	Photonic		
Fiji			
Glycerol	Sigma	G5516-1L	
Graphpad Prism 8.01	GraphPad Software, Inc.		we prefer to use Graphpad, but other statistics s
HotSHOT alkaline lysis buffer		25 mM NaOH, 0.2 mM disodium EDTA, pH 12	
HotSHOT neutralization buffer		Tris HCl 40 mM, pH 5	
PBS (10X) pH 7.4	Thermofisher	70011044	
Stereomicroscope with illumination stand (Nikon SMZ800N)	Nikon		
Thermocycler	Thermofisher		

oftware packages are also suitable (e.g. SigmaPlot or SPSS)

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Format is as specified.

2. Please check that the access type (standard versus open) is correct. Generally, UK funding requires open access, but there are exceptions.

We will amend the access type upon resubmission.

3. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

4. Please revise lines 138-164 to avoid overlap with previously published material.

We have made some changes to this part of the text. However, the original manuscript is published under a CC-BY license so reproduction of the text is permitted. A copy of Biology Open's publication policy is now attached to the resubmitted version.

5. Please include at least 6 key words or phrases.

We have added two extra key words

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Done; these mentions were removed from the text.

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Done; the protocol is below the 2.75 page limit

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to

perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Done; we have slightly modified some steps and separated others regarding the statistical analysis.

Specific Protocol steps:
1. 1.2: Can you include a reference about preparation of embryos here?

Done; we have slightly changed the text and added a reference as requested

2. 2.3: Please include more information or a reference about how to genotype and annotate.

Done; we have slightly changed the text and added a reference as requested

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2. Please remove 'Figure 1' etc. from the Figures themselves. Please also remove unnecessary whitespace.
Done.

Discussion:
1. Please also include information on the method's significance compared with other methods and future applications in the Discussion.

We have now modified the Discussion to include the new data generated in response to the reviewers and added comments regarding the applicability of the method in the future.

References:
1. Please do not abbreviate journal titles.

We have used the JoVE style as downloaded from the journal's webpage but, unfortunately, we were unable to modify it successfully to prevent it from abbreviating journal titles.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We verified the information and it seems correct.

Reviewers' comments:

Reviewer #1:

In this practical guide, Dobrzycki et al describe a quantification method for in situ hybridization (ISH) in zebrafish, followed by genotyping. The authors provide a detailed protocol for the above-mentioned methods, which could be of broad interest to several developmental (or even other) studies. As the authors explain, ISH stainings are usually evaluated by visual scoring, which is of course a subjective way of analysis and can often bias the results. Instead, they propose a more objective and solid quantification method, by means of Fiji software, which could substantially contribute to avoiding research bias and achieving high reproducibility. Finally, they propose and describe step-by-step a genotyping protocol following quantification, in order to favor blind evaluation and again prevent data interpretation bias stemming from researchers' expectations. This protocol is important since the zebrafish community should become aware that quantification of ISH through imaging must become the standard method in the field.
We thank the reviewer for their comments and their appreciation of the method and agree that it should be more widely used (whenever possible) within the community.

The authors have done a thorough job describing their protocol, its applications, but also its limitations under specific circumstances, except for a few of minor points:
* In lines 131-132 the authors refer to how the images can be converted to 8-bit grayscale and explain that this has been described before (reference is also included). However, it would be more beneficial and easy for the reader to be fully able to apply the instructions based on the specific protocol. Could the authors include a brief explanation of how this conversion can be done?

We have now added a first step to the method (new Step 3.1) explaining the image conversion in line with the reviewer's comment that we hope will help without having to consult the referenced paper.

* The protocol proposed is based on the Fiji software. However, there are more software tools that could in theory be used (e.g. ImageJ). Could the authors explain the advantage of using specifically the Fiji software?

The reviewer is correct that other software would be equally useful/applicable for the quantification. We chose to use Fiji because it is open source, and the latest iteration of Image J (the acronym Fiji means Fiji is just image J). Any software that can measure pixel intensity in an image should be appropriate for this method.

* The resolution of the pictures on the figures is not good. Could the authors provide higher resolution images?
We apologise for this; it is likely due to the PDF conversion process. High resolution images will be provided prior to publication.

Reviewer #2:

Manuscript Summary:
The manuscript by Dobrycki et al describes a detailed protocol for genotyping and quantification of in situ hybridization in zebrafish. The protocol is well written and clearly explains the technique.

Major Concerns:
My major concern is that the same authors have previously published a similar protocol, including the figures, as a methods paper (Dobryzki et al., 2018. Biology Open 7, bio031096), and a previous publication already provided a detailed protocol for quantification and genotyping. Therefore there is little if any novelty in this publication, although I suppose adding video would provide some new angle.

We thank the reviewer for the comment. Indeed, the method we describe is the one we published previously and cite in this manuscript. We felt it was important to more explicitly convey how the method can be applied and thus a video in JoVE describing the method would help in that regard. We hope that adding this extra dimension will increase the reach and hopefully the uptake of quantitative methods to measure ISH straining signals within zebrafish community.

Minor Concerns:
1. It would be useful to provide a reference to the protocol for in situ hybridization that precedes embryo imaging.

The protocol used in our lab is from Jowett and Yan, 1996. However, there are other in situ hybridization protocols available that could be used (e.g. Thisse and Thisse, 2008, *Nature Protocols*, or more recently, Narayanan, R. and Oates, A. C., 2019, *Bio-protocol*) and are equally suitable. The latter (an extended version of Lleras-Forero et al, 2018, *Elife*) discusses in some detail the optimization of conditions for the *in situ* hybridization itself. These references have now been added to the manuscript.

2. It may be worth noting that ISH staining using conventional chromogenic substrates such as NBT / BCIP is non-linear and reaches saturation. Differences in staining intensity may not be apparent in the samples which have been stained long enough to reach the maximum staining intensity (saturation).

We thank the reviewer for their insight. In the majority of cases, the level of saturation for the staining needs to be determined empirically and is different for every probe used. We have now added a cautionary note to the manuscript to alert users to determine those levels of saturation beforehand.

Reviewer #3:

Manuscript Summary:
The authors Dobrzycki, Krecsmarik and Monteiro's manuscript "A practical guide for

genotyping and quantification of in situ hybridisation staining in zebrafish" is a follow-up to their recent Biology Open publication called "An optimised pipeline for parallel image-based quantification of gene expression and genotyping after in situ hybridisation" that appeared in the Techniques and Methods section of this journal.

Major Concerns:
Both the previous publication and the current manuscript present the same data and focus heavily on the description of the author's Fiji-based quantification of gene expression in wild-type, mutant and morphant embryos that have been stained by RNA in situ hybridisation. Unfortunately, having read both papers it is not clear to me what the current manuscript adds that is not already included in the Biology Open publication.
In the current manuscript, the authors could have used examples to highlight pitfalls and strengths of the method. It would have been nice to see the effect of over-staining and the effect of background staining on the outcome of the experiment. The authors stress that the method is less prone to bias, but they do not discuss that the choice of the analysed regions (with and without staining) can easily bias the outcome. Examples could have been given to see how this choice influences the results. I would like to know whether the method can be used if the stained cells are imaged against the background of the yolk and whether the authors recommend its use when staining variably occurs in individual cells or a bulk of cells. I also felt that the authors could have described the statistical methods in much more detail. Here, exemplary data could have been provided to illustrate points 3.6 to 3.8 to distinguish between reliable and unreliable data. Unfortunately, this opportunity was missed. In its current format, the manuscript adds too little to justify publication.

We thank the reviewer for their insight and suggestions to improve aspects of the current manuscript. Paraphrasing reviewer #1, ‘this protocol is important since the zebrafish community should become aware that quantification of ISH through imaging must become the standard method in the field’. Thus, we felt that a video in JoVE describing the method would increase its reach (and hopefully uptake) within the zebrafish community.

To address the reviewer’s concerns, we have tested whether the choice of background affected the outcomes. As we noted on Fig 3D of the manuscript, high background will not allow us to use the method as subtracting the background from the signal leads to negative values. We have now tested what happens when we choose four different regions as background and how that affects the overall pixel intensity values in wildtype embryos stained for runx1. We concluded that the unstained yolk cannot be used as background in our imaging conditions; darker pixels in the yolk region actually become very bright after inversion/conversion to 8-bit so they become brighter than any proper signal in the embryo and render the background corrected values negative. We have also tested two slightly different regions with the same areas as the ROI and a third, smaller area in a different (still unstained) part of the embryo. The choice of background region does affect the intensity values after correction (See new Figure 4A-D), but the difference in mean intensities does not seem to be significant. Notably, using a smaller area for background correction (R4 on Fig 4B-D) yields similar outcomes to using the same area to that of the ROI, as long as there is no *in situ* signal in that area.

Next we compared runx1 levels between 10 wildtype and dll4 mutant embryos stained for runx1, using two very different background correction areas (R1 and R4 in the new Fig 4) and found no significant differences between the mean pixel intensities within the same

genotypes (although individual points do differ to some degree). More importantly, the differences between wildtype and mutant were detected in both cases. Thus, while the choice of background area may affect individual values, overall it does not seem to change the results when comparing conditions (e.g. wildtype vs dll4 mutant expression of runx1). This data and its implications were added to the Results and Discussion Sections and is summarized in the new Figure 4.

We have now also separated the statistical analysis from the rest of the method and added one step to the protocol suggesting how to proceed with the analysis in case the pixel intensity values cannot be transformed so that they fit a normal distribution (new step 4.3.1).

We thank the reviewer for their suggestions and hope the new data and text modifications adequately respond to the concerns regarding background corrections and their effect on the measurement outcomes in this method.

Minor Concerns:

1. a significant downregulation of runx1 expression was measured by quantitative RT-PCR in single lmo4-mutant embryos relative to wt embryos (line 190). This finding has been contrasted with the unaltered level of staining in the ventral wall of the dorsal aorta of the mutant embryos as observed after RNA in situ hybridisation. It might be worth considering whether the different qRT-PCR results are possibly due to differential gene expression in other tissues, e.g. the telencephalon which shows expression of both transcription factors.

We agree that the difference in gene expression found by qPCR could also be due to expression of both factors in other embryonic tissues. We did not expand on this because we wished to focus on the limitations of our method rather than in the limitations of whole embryo qPCR (as opposed to qPCR on FACS-sorted tissue, for example). We have now added this consideration to the manuscript.

2. The authors state that due to genetic compensation, many zebrafish mutants fail to show an embryonic phenotype that can be detected with morpholinos (line 183). This statement is misleading. While compensatory upregulation of functionally redundant proteins has been reported a few times, it is only one of several possible reasons for phenotypic discrepancies between morphants and mutants. Stainier et al (2017, PLOS Genetics 13 (10), e1007000) also list off-target effects, maternal protein compensation and the hypomorphic nature of the mutant allele as possible mechanisms. I would consider all of these explanations more likely than compensatory upregulation of functionally redundant proteins.

The reviewer is correct that, in the absence of further evidence, other mechanisms as stated in the Stainier et al, 2017 publication may also lead to discrepancies between MO- and mutant-induced phenotypes. Thus, we have changed the text in the results section to accommodate the reviewer's comments. Of note, two studies from the Stainier lab and the Chen lab have recently been published showing that mutant strains where mRNAs are still produced induce a nonsense-mediated mRNA decay response that leads to upregulation of related genes and subsequent rescue of the underlying phenotypes (El-Brolosy et al, 2019,Nature and Ma et al, 2019, Nature). This suggests that genetic compensation (or transcriptional adaptation) seems

to be a more common response to buffer against genetic damage than previously appreciated. References to these articles have now been added to the manuscript.

3. Eppendorf Microloader pipette tips are described as cheap alternatives (line 99), but it is unclear to what?

To improve clarity, we have now removed the statement about the costs and added a couple of examples of alternative tools that could be used for embryo manipulation.

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