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Rapid and Efficient Spatiotemporal Monitoring of Normal and Aberrant Cytosine Methylation within Intact Zebrafish Embryos

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Abstract

Cytosine methylation is highly conserved across vertebrate species and, as a key driver of epigenetic programming and chromatin state, plays a critical role in early embryonic development. Enzymatic modifications drive active methylation and demethylation of cytosine into 5-methylcytosine (5-mC) and subsequent oxidation of 5-mC into 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine. Epigenetic reprogramming is a critical period during *in utero* development, and maternal exposure to chemicals has the potential to reprogram the epigenome within offspring. This can potentially cause adverse outcomes such as immediate phenotypic consequences, long-term effects on adult disease susceptibility, and transgenerational effects of inherited epigenetic marks. Although bisulfite-based sequencing enables investigators to interrogate cytosine methylation at base-pair resolution, sequencing-based approaches are cost-prohibitive and, as such, preclude the ability to monitor cytosine methylation across developmental stages, multiple concentrations per chemical, and replicate embryos per treatment. Due to the ease of automated *in vivo* imaging, genetic manipulations, rapid *ex utero* development time, and husbandry during embryogenesis, zebrafish embryos continue to be used as a physiologically intact model for uncovering xenobiotic-mediated pathways that contribute to adverse outcomes during early embryonic development. Therefore, using commercially available 5-mC-specific antibodies, we describe a cost-effective strategy for rapid and efficient spatiotemporal monitoring of cytosine methylation within individual, intact zebrafish embryos by leveraging whole-mount immunohistochemistry, automated high-content imaging, and efficient data processing using programming language prior to statistical analysis. To current knowledge, this method is the first to successfully detect and quantify 5-mC levels *in situ* within zebrafish embryos during early development. The method enables the detection of DNA methylation within the cell mass and also has the ability to detect cytosine methylation of yolk-localized maternal mRNAs during the maternal-to-zygotic transition. Overall, this method will be useful for the rapid identification of chemicals that have the potential to disrupt cytosine methylation *in situ* during epigenetic reprogramming.

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Disclosures

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Introduction

Enzymatic modifications drive active methylation and demethylation of cytosine into 5-methylcytosine (5-mC) and subsequent oxidation of 5-mC into 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine[1, 2]. Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is a widely used flame retardant in the United States that has been previously demonstrated to alter the trajectory of cytosine methylation following early embryonic exposure from 0.75 hours post-fertilization (hpf) through early gastrulation (6 hpf)[3, 4, 5, 6, 7, 8]. Within vertebrates, 5-mC and its modified derivatives are critical for regulating early embryonic development[9]. Fertilization of an embryo triggers demethylation of parental DNA, followed by maternal mRNA degradation, zygotic genome activation, and remethylation of the zygotic genome[9]. Biologically relevant processes that utilize cytosine methylation include histone modification, recruitment of transcriptional machinery, RNA methylation, epigenetic reprogramming, and determination of chromatin structure[10, 11]. Cytosine methylation is also conserved among vertebrate species, underscoring the importance of understanding and investigating how aberrant cytosine methylation may affect the trajectory of an organism's development[11]. Furthermore, *in utero* development is sensitive to maternal exposure and has the potential to cause adverse outcomes such as immediate phenotypic consequences, long-term effects on adult disease susceptibility, and transgenerational effects of inherited epigenetic marks[12, 13, 14].

Long stretches of cytosine-guanine pairs, or CpG islands, have been the primary foci of investigators that aim to characterize the dynamics of cytosine methylation across the genome[15, 16, 17]. Bisulfite-based strategies such as whole-genome bisulfite sequencing, reduced representation bisulfite sequencing, and bisulfite amplicon sequencing represent the gold standard for interrogating cytosine methylation at basepair resolution. However, sequencing-based approaches are cost-prohibitive and, as such, preclude the ability to monitor cytosine methylation across developmental stages, multiple concentrations per chemical, and replicate embryos per treatment. In addition, sequencing-based approaches do not provide information about spatial localization, which is critical for understanding potentially affected cell types and areas within a developing embryo. Similarly, global DNA methylation assays such as methylation-dependent restriction analysis, 5-mC enzyme-linked immunoassays (ELISAs), and 5-methyl-2′-deoxycytidine (5-mC) liquid chromatography-mass spectrometry (LC-MS) rely on cell or tissue homogenates and, as such, preclude the ability to monitor the localization and magnitude of cytosine methylation over space and time within intact specimens[12, 18].

Due to the ease of automated *in vivo* imaging, genetic manipulations, rapid *ex utero* development time, and husbandry during embryogenesis, zebrafish embryos continue to be widely used as physiologically intact models to uncover xenobiotic-mediated pathways that contribute to adverse outcomes during early embryonic development. Therefore, using commercially available antibodies specific to 5-mC, the protocol below describes a cost-effective strategy for rapid and efficient spatiotemporal monitoring of cytosine methylation within individual, intact zebrafish embryos by leveraging whole-mount immunohistochemistry (IHC), automated high-content imaging, and efficient data processing using programming language prior to statistical analysis.

To current knowledge, this method is the first to monitor 5-mC within intact zebrafish embryos. The method enables the detection of DNA methylation within the cell mass and also has the ability to detect cytosine methylation of yolk-localized maternal mRNAs during the maternal-to-zygotic transition. Overall, this method will be useful for the rapid identification of chemicals that have the potential to disrupt cytosine methylation *in situ* during epigenetic reprogramming.

Protocol

Adult breeders were handled and treated in accordance with an Institutional Animal Care and Use Committee (IACUC)-approved animal use protocol (#20180063) at the University of California, Riverside.

1. Zebrafish embryo collection and chemical exposure

1. Add in-tank breeding traps to tanks containing sexually mature and reproductively viable adult male and female zebrafish. Add at least three traps per 6 L tank at least 12 h prior to collection at ~9:00 AM, which is the approximate time of fertilization and spawning of eggs within tanks.
2. Prepare a fresh exposure solution on the morning of collection. The exposure solution is prepared using a 5 mL pipette and adding 5.0 mL of particulate-free system water to a 60 mm glass Petri dish.
3. Then, use a 10 µL glass microcapillary pipette to transfer 10 µL of vehicle (dimethyl sulfoxide, or DMSO) or chemical stock solution to system water in the glass dish.
4. Swirl the glass Petri dish to ensure the stock solution dissipates. Add another 5.0 mL of system water to the glass Petri dish. Swirl the glass Petri dish to homogenize the exposure solution.
5. Repeat for each treatment group in replicates of four to yield four replicates per treatment.
6. After exposure solutions have been prepared, cap the glass dishes with glass lids to minimize evaporation.
7. While removing the breeding traps from each tank, carefully allow the water within each trap to drain out into the tank before removing from the tanks. Make sure the traps stay right-side up.
8. Transfer the breeding traps to the lab sink and rinse them using a squirt bottle filled with reverse osmosis (RO) water into a fish net previously soaked in 10% bleach and rinsed with water. Rinse the embryos until all debris is cleared and only clean embryos remain.
9. Transfer the embryos from the fish net using an RO water-filled squirt bottle into a 100 mm plastic Petri dish. Randomly sort 50 live embryos at 0.75 h post-fertilization (hpf) and remove excess RO water.

10. Using a microspatula, place sorted embryos into the treatment solution. All embryos must be within vehicle or treatment solution no earlier than 9:45 AM. Swirl the glass dishes to ensure the embryos are evenly coated within the treatment solution.

11. After four replicate dishes (N = 50 per replicate) have been set up for each treatment group, place the glass lid on top of the exposure dishes and transfer all dishes into a temperature-controlled incubator set at 28 °C until 2 hpf, 4 hpf, 6hpf, 8 hpf, or 10 hpf.

12. Prepare fresh 4% paraformaldehyde (PFA) at least 4 h prior to exposure termination and store at 4 °C.

    1. Turn on the hotplate to 115 °C, make 20 mL of 1x phosphate-buffered saline (PBS) (2 mL of 10x PBS + 18 mL of RO water) in a 50 mL centrifuge tube, and weight out 800 mg of paraformaldehyde.

    2. Within a chemical fume hood, transfer 1x PBS solution to a 250 mL Erlenmeyer flask, add 800 mg of paraformaldehyde, and then add 2 μL of 10 N NaOH. Place a thermometer inside the flask, place the flask on the hot plate, and watch the temperature while stirring.

    3. When the temperature reaches 62-65 “Cremove the 4% PFA from the hot plate and allow it to cool to room temperature (RT). Store the 4% PFA at 4 °C.

13. Once the exposure duration has ended, remove the embryos from the incubator and transfer all living embryos to a 1.5 mL microcentrifuge tube. Aspirate residual exposure solution using a 1 mL pipette. Do not aspirate any embryos.

14. Add 500 μL of chilled 4% PFA to a 1.5 mL microcentrifuge tube and mix the embryos in 4% PFA by inverting several times. Allow the embryos to fix in 4% PFA overnight.

    NOTE: It is not recommended to allow embryos to stay in PFA for longer than 12 h.

2. Dechorionation of embryos

1. The following day, aspirate off the 4% PFA, resuspend the embryos in 1x PBS, and gently pipette up and down for 30 s. If needed, stop the protocol at this step and store the embryos in 1x PBS at 4 °C for up to 48 h.

2. Using a plastic transfer pipette, carefully transfer fixed embryos to a RO water-filled glass dish and gently swirl for 30 s. Repeat this step one more time.

3. Use syringe needles under 5.0x magnification using a standard stereomicroscope to manually dechorionate all embryos. Do this by using the needle tip to puncture the chorion and gently peel it away from the yolk and cell mass[4].

4. Once the embryos have been dechorionated, use a glass microcapillary pipette to transfer up to 25 intact embryos into one immunochemistry (IHC) basket and

place the IHC basket into a 96-well plate. Use a 1 mL pipette to aspirate the RO water from each well.

3. Immunohistochemistry using 5-mC-specific antibody

1. Resuspend the embryos in 500 µL of blocking buffer (1x PBST + 2% sheep serum + 2 mg/mL bovine serum albumin) per well, and wrap the plate with parafilm and aluminum foil to protect them from light. Incubate at 4 °C for 4 h on an orbital shaker (100 rpm).
2. Remove the parafilm wrap and aluminum and use a 1 mL pipette to aspirate the blocking buffer from each well. Replace it with 500 µL of a 1:100 dilution of monoclonal mouse anti-5-mC antibody in the blocking buffer. Incubate all embryos with primary antibodies except for a subset of vehicle control embryos which will be incubated with the blocking buffer to account for background noise. Be careful not to disrupt the integrity of embryos, nor to aspirate embryos during this step.
3. Rewrap the plate in parafilm and aluminum foil and allow the plate to incubate at 4 °C overnight on an orbital shaker (100 rpm).
4. The following day, remove the primary antibody solution from each well and replace it with 1x PBS + 0.1% Tween-20 (1x PBST). Wash each well three times with 1x PBST for 15 min per wash on an orbital shaker (100 rpm).
5. Replace 1x PBST with a 1:500 dilution of goat anti-mouse IgG antibody in blocking buffer and incubate all embryos with the secondary antibody. Let the plate incubate at 4 °C overnight on an orbital shaker (100 rpm).
6. The following day, remove the secondary antibody and wash the residual solution three times with 1x PBST for 15 min per wash on an orbital shaker (100 rpm).

   NOTE: The protocol can be stopped here for up to 48 h if the IHC baskets are stored in clean wells filled with 1x PBS.
7. Use a 1 mL pipette to aspirate 1x PBST from each well and place the IHC basket into a glass Petri dish filled with RO water. Under a standard stereomicroscope, randomly sort intact embryos into a 96-well plate, resulting in one embryo per well.
8. Using a 1 mL pipette, remove all RO water from individual wells and replace it with 200 µL of 1x PBS. Centrifuge the plate for 3 min at 1 x *g*.

4. Automated imaging of embryos within 96-well plates

1. Image the embryos with a 2x objective under transmitted light and FITC using a high-content screening system (Table of Materials).

   NOTE: A fluorescent image of each embryo was captured automatically using the above-mentioned magnification and a FITC filter[19].

1. Select **Autofocus** to ensure the focus of the camera is on the bottom of the plate and offset by the bottom thickness.
2. Select a FITC wavelength with an exposure duration of 100 ms, and set the **Autofocus** to laser with a **Z-offset.** Observe and confirm proper image acquisition in several wells prior to commencing plate acquisition.

   NOTE: The instrument automatically acquires images from all selected wells containing embryos. A 96-well plate required approximately 30 min for image acquisition and data storage. For the duration of the acquisition period, the internal temperature within the imaging system was maintained at RT.

2. Following image acquisition, carry out data analysis using the high-content screening system and a custom automated image analysis procedure. Select each embryo on the 96-well plate to be analyzed for total area and integrated intensity of fluorescence.

   NOTE: The analysis included providing images that contained overlays of data points from the analysis.

3. Then, tabulate the data points and export them from the high-content screening system by going under **Measure** and selecting **Open Data Log** to a spreadsheet. The plate acquisition and data analysis portions are depicted in Figure 1.

5. Data analysis

1. Upload the exported spreadsheet into a computer program where the deployer (dplyr) package is used to sort, summate data for each well, and filter by well number. The writexl package is then used to export the summated data into a spreadsheet. The 5mC program code is shown in Supplemental File 1.

Representative Results

The overall aim of this protocol is to determine whether a treatment affects the relative abundance of 5-mC by assessing the total area and relative intensity of fluorescence within fixed and labeled zebrafish embryos. After completing the protocol, a fluorescence stereomicroscope can be used to first determine whether the whole-mount IHC was successful. When labeled embryos are observed under a FITC or GFP filter, a positive result is indicated by a positive FITC signal within the embryo, whereas a negative result is indicated by the absence of fluorescence within control embryos. Using a high-content screening system, these results can also be confirmed during image acquisition using a FITC filter. In addition, during data analysis, the custom module will identify and quantify the total area and integrated intensity of fluorescence. Representative images of successful results are shown in Figure 1, where acquired images were measured successfully by the custom module, and individual data points (shown as blue overlay) were acquired for both total area and integrated intensity.

During data extraction, the threshold stringency within the custom module is an additional variable that needs to be optimized to maximize the signal-to-noise ratio and increase the probability of detecting a significant treatment-specific difference in 5-mC abundance. During optimization, this final threshold provided the most significant separation between medians of control and treatment groups (e.g., the largest signal-to-noise ratio). Representative results at varying custom module thresholds that impact signal-to-noise ratios are presented in Figure 2.

Discussion

During this protocol, there are a few steps that are critical. First, when dechorionating embryos, it is important to point the needle away from the tissue of the embryo/yolk sac/cell mass, as these portions of the developing embryo are very fragile and easy to puncture. Second, when transferring labeled embryos to individual wells, use a glass pipette to transfer embryos as they will adhere to a plastic pipette. Third, when performing whole-mount IHC, ensure that the plate is protected from light. Finally, after completing the whole-mount IHC protocol, allow the plate to incubate in 1x PBS at 4 °C overnight before imaging, as this will minimize autofluorescence that may interfere with imaging.

If there are embryos that have been severed during dechorionation or IHC, exclude these embryos from the remainder of the protocol. If no fluorescence is detected, this may be solved by incubating for longer (up to 16 h). Since this is a 5-mC-specific antibody, both DNA and RNA may be labeled. A general understanding of spatial localization is important.

There are some limitations in this method. First, it is a non-targeted technique, meaning it will not provide the exact quantity of 5-mC, but rather the relative abundance based on the total area and integrated intensity of fluorescence. In addition, this protocol has only been tested on embryos prior to segmentation, so if testing later stages of development, some additional optimization may be needed. Furthermore, since the method is IHC-based, it may be susceptible to non-specific binding; therefore, it is not certain to only be staining 5-mC localized to DNA, but may also be staining 5-mC localized to RNA as well. Lastly, optimization of the data analysis threshold may be needed depending on the chemical and stringency of the conditions that are preferred.

Overall, this method provides quick and cost-efficient detection of 5-mC across multiple stages of development and chemical concentrations[3]. It therefore provides an alternative to cost-prohibitive bisulfite sequencing-based approaches. By offering this protocol, investigators can use this method to quickly screen chemicals and assess how the abundance of 5-mC may be affected during early embryonic development. In addition, this method may be utilized as a prescreening tool to identify the concentration range, period of development, and/or window of sensitivity in which the chemical of interest affects 5-mC abundance. Alternatively, this same method may be utilized for a different biomarker and antibody, albeit further optimization is needed. By utilizing this method, an investigator can quickly, efficiently, and cost-effectively screen and identify a chemical that alters the relative abundance of 5-mC within zebrafish embryos prior to investing in labor-intensive bisulfite sequencing-based approaches. However, this method is zebrafish-specific, and

further research is needed to determine whether 5-mC can be detected *in situ* within early embryos of other model organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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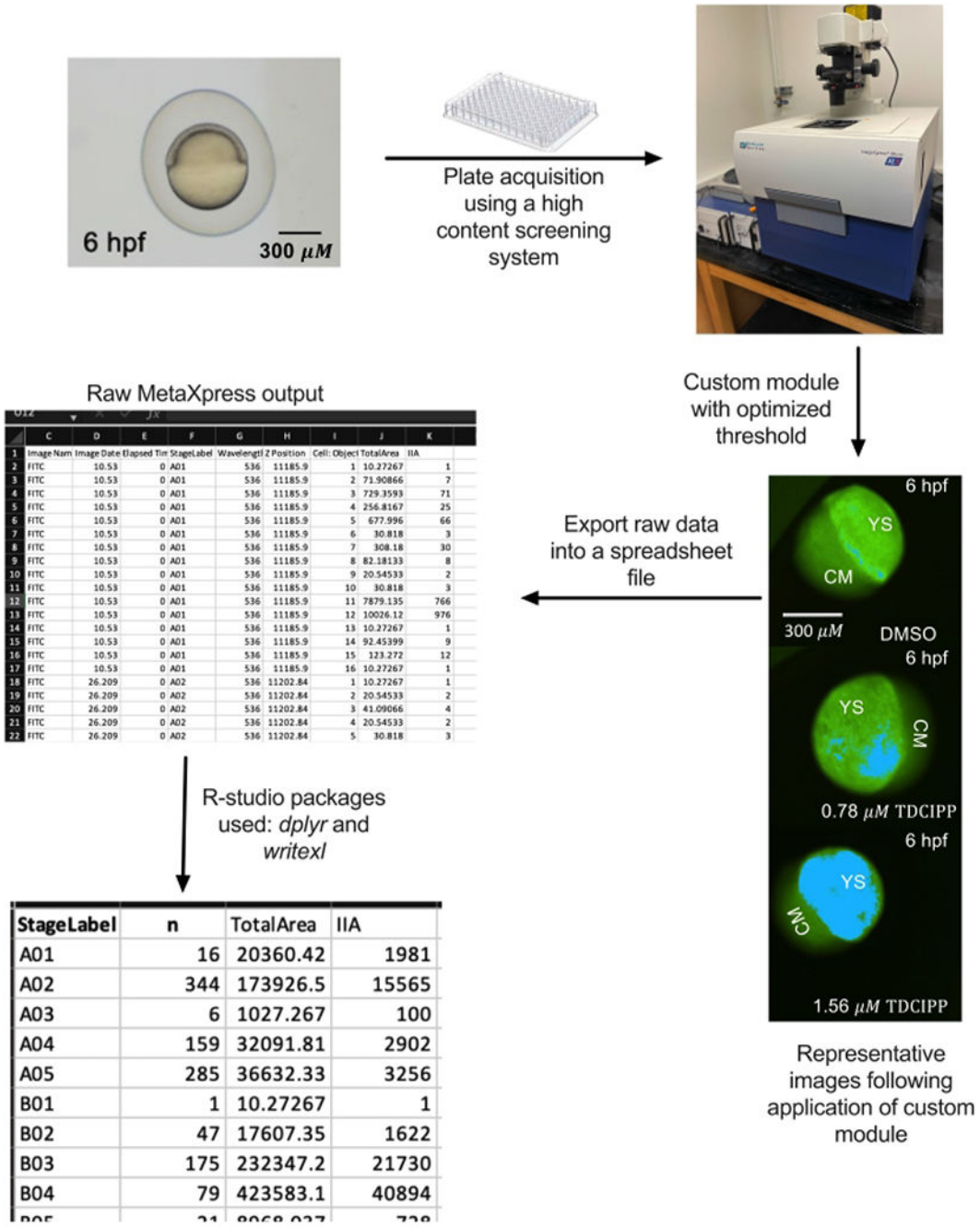

Image Nam	Image Date	Elapsed Tim	StageLabel	Wavelengtl	Z Position	Cell: Object	TotalArea	IIA
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FITC	10.53	0	A01	536	11185.9	3	729.3593	71
FITC	10.53	0	A01	536	11185.9	4	256.8167	25
FITC	10.53	0	A01	536	11185.9	5	677.996	66
FITC	10.53	0	A01	536	11185.9	6	30.818	3
FITC	10.53	0	A01	536	11185.9	7	308.18	30
FITC	10.53	0	A01	536	11185.9	8	82.18133	8
FITC	10.53	0	A01	536	11185.9	9	20.54533	2
FITC	10.53	0	A01	536	11185.9	10	30.818	3
FITC	10.53	0	A01	536	11185.9	11	7879.135	766
FITC	10.53	0	A01	536	11185.9	12	10026.12	976
FITC	10.53	0	A01	536	11185.9	13	10.27267	1
FITC	10.53	0	A01	536	11185.9	14	92.45399	9
FITC	10.53	0	A01	536	11185.9	15	123.272	12
FITC	10.53	0	A01	536	11185.9	16	10.27267	1
FITC	26.209	0	A02	536	11202.84	1	10.27267	1
FITC	26.209	0	A02	536	11202.84	2	20.54533	2
FITC	26.209	0	A02	536	11202.84	3	41.09066	4
FITC	26.209	0	A02	536	11202.84	4	20.54533	2
FITC	26.209	0	A02	536	11202.84	5	30.818	3

StageLabel	n	TotalArea	IIA
A01	16	20360.42	1981
A02	344	173926.5	15565
A03	6	1027.267	100
A04	159	32091.81	2902
A05	285	36632.33	3256
B01	1	10.27267	1
B02	47	17607.35	1622
B03	175	232347.2	21730
B04	79	423583.1	40894

Figure 1: A flow diagram providing a graphical representation of the protocol for exposure and *in situ* detection of 5-methylcytosine for 6 hpf zebrafish embryos.

The direction of the flow diagram is provided with black arrows. Exposures occurred in replicates of four replicate dishes per treatment and 50 embryos per replicate dish. Abbreviations: cm = cell mass; ys = yolk sac. Please click here to view a larger version of this figure.

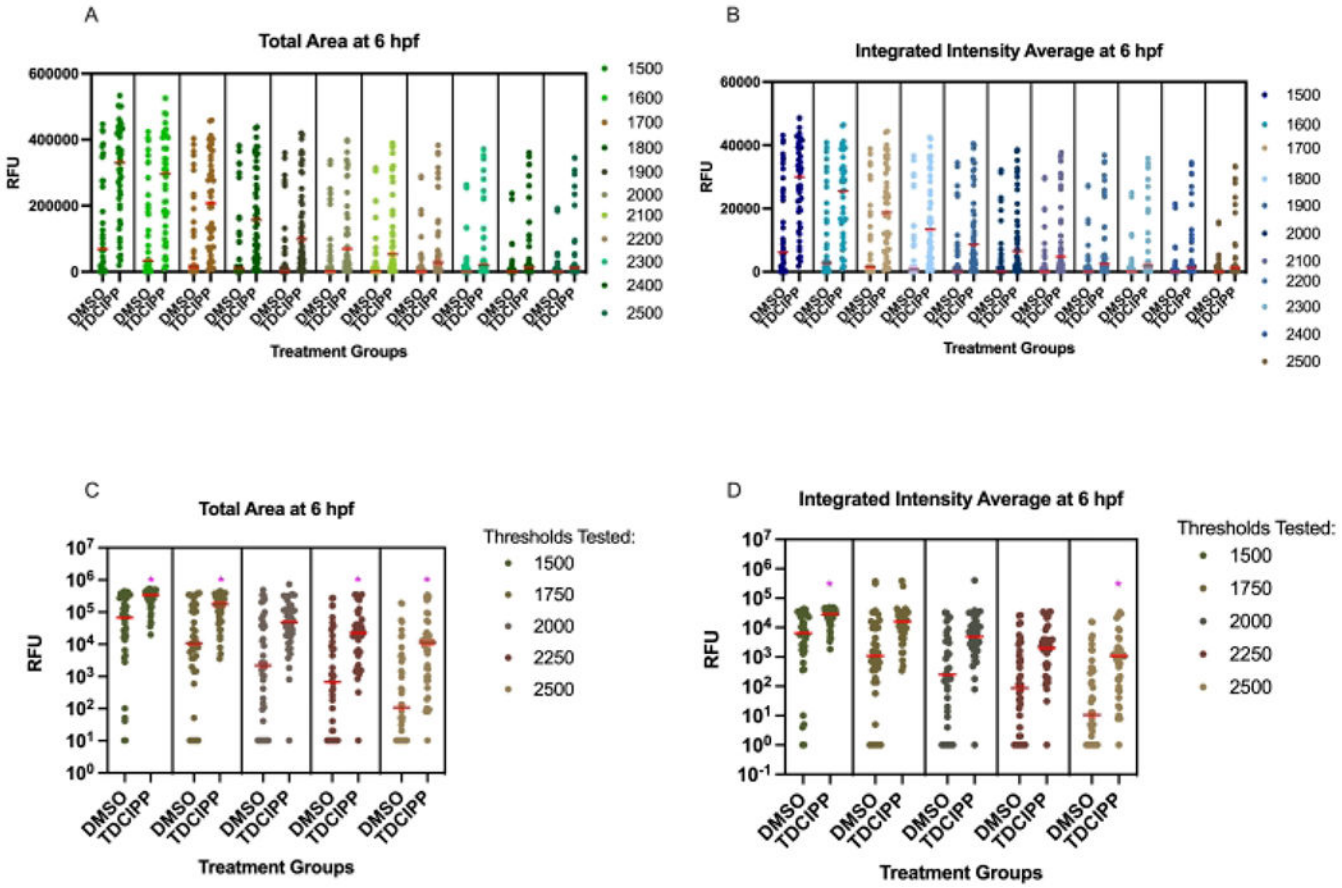

Figure 2: Optimization of the custom module.

Panels **A-D** show optimization of the custom module by assessing the median and distribution of 5-mC-specific total area and integrated intensity within zebrafish embryos at 6 hpf. (**A,B**) Different thresholds tested are listed in the legend to the right (difference of 100 between each threshold) and are ordered by stringency, where 1500 and 2500 represent the least and most stringent thresholds tested, respectively. (**C,D**) Different thresholds tested are listed in the legend to the right (difference of 250 between each threshold) and ordered by stringency, where 1500 and 2500 represent the least and most stringent thresholds tested, respectively. The (*) in **C,D** denotes thresholds that are significantly different from vehicle-treated embryos ($p < 0.05$). For **A,B,** all thresholds tested were significant from the vehicle control. The x-axis denotes exposure to either vehicle (0.1% DMSO) or 0.78 μM TDCIPP (positive control). The y-axis denotes relative fluorescence. Panel **A** displays the total area of 5-mC detected within embryos as a function of treatment, whereas Panel **B** displays the integrated intensity of 5-mC within that same area. One embryo is represented by a single data point for a total of N = 96 for each treatment group. All exposures were performed in replicates of four dishes per treatment with 50 embryos per glass dish. Please click here to view a larger version of this figure.

Table 1.

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
In-tank breeding traps	Aquatic Habitats	N/A	This product is no longer available following acquisition of Aquatic Habitats by Pentair. Investigators can use standard off-system breeding tanks available from multiple vendors.
5-mL pipette	Fisher Scientific	13690033	
60-mm glass petri dishes with lids	Fisher Scientific	08747A	
10-µL glass microcapillary pipette	Fisher Scientific	211762B	
DMSO	Fisher Scientific	BP2311	
100-mm plastic petri dish	Fisher Scientific	08757100D	
Microspatula	Fisher Scientific	2140115	
Temperature-controlled incubator	Fisher Scientific	PR505755L	
Paraformaldehyde	Fisher Scientific	18612139	
Hotplate	Fisher Scientific	1110016SH	
10X phosphate-buffered saline	Fisher Scientific	BP399500	
250-mL Erlenmeyer flask	Fisher Scientific	FB501250	
NaOH	Fisher Scientific	BP359-500	
1.5-mL microcentrifuge tubes	Fisher Scientific	540225	
1-mL pipette	Fisher Scientific	13690032	
Plastic transfer pipette	Fisher Scientific	1368050	
Immunochemistry (IHC) basket	N/A	N/A	Manufactured in-house using microcentrifuge tubes with conical portion removed and bottom fitted with mesh, sized for 24- or 48-well plates.
Parafilm	Fisher Scientific	1337412	
Orbital shaker	Fisher Scientific	50998290	
Monoclonal mouse anti-5-mC antibody	Millipore Sigma	MABE146	
Tween-20	Fisher Scientific	P7949-500ML	
Sheep serum	Millipore Sigma	S3772-5ML	
Bovine serum albumin	Fisher Scientific	BP67110	
AlexaFluor 488-conjugated goat anti-mouse IgG antibody	Fisher Scientific	A21121	
Stereomicroscope	Leica	10450103	
96-well plate	Fisher Scientific	720089	
ImageXpress Micro XLS Widefield High-Content Screening System	Molecular Devices	N/A	Any high-content screening system equipped with transmitted light and FITC filter will be suitable.
MetaXpress 6.0.3.1658	Molecular Devices	N/A	Any software capable of quantifying total area and integrated intensity of fluorescence will be suitable.
Rstudio	RStudio	N/A	RStudio is open-source software and can be downloaded at https://www.rstudio.com.