Spatial mRNA profiling using Rapid Amplified Multiplexed-FISH (RAM-FISH)

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Abstract

Localizing multiple RNA molecules simultaneously in intact tissues and organs is valuable for gaining insights into gene-regulatory interactions underlying biological function. Existing technologies for multiplexed RNA localization are expensive, computationally and experimentally complex, have elaborate sample preparation steps, have thin-slice limitations, and require weeks of processing time. This limits the routine use of such techniques in most labs. Here, we describe an easy-to-use methodology, Rapid Amplified Multiplexed-FISH (or RAM-FISH), for localizing dozens of transcripts in the same sample. This methodology achieves multiplexing by localizing 3-4 genes per cycle to detect 30 or more genes. All steps can be done manually or with the help of automation. The method can be applied to fixed tissue sections, entire organs, or whole organisms such as larval *Danio rerio*, without extensive sample preparation steps. Here, we demonstrate its utility, flexibility, and versatility for gene expression analysis in two very different types of samples, *Bicyclus anynana* butterfly larval wings and intact 14-days post fertilization zebrafish larvae.

Keywords

Multiplexing, HCR3.0, *Bicyclus anynana*, zebrafish, RAM-FISH.

Introduction

Multicellular lifeforms are complex systems controlled by dynamic spatial and temporal patterns of gene expression. These gene expression dynamics can be visualized via the detection of RNA molecules in cells. Many biologists are therefore interested in simple, robust methodologies for the simultaneous detection of multiple RNA molecules in intact tissues. This detection can be used to describe natural tissue differentiation, initiation of disease pathologies, or, when combined with CRISPR-Cas9, used to describe the architecture of gene-regulatory networks. These investigations demand a robust and expedient method combining high signal specificity and sensitivity while reducing human labor and errors. In addition, reduced requirements for extensive sample preparation

and increased applicability to diverse sample types are also desirable features for RNA localization methodologies.

Recent technological advancements enable single-cell RNA expression profiling while preserving tissue architecture. These methods can be categorized into two primary types: sequencing-based and imaging-based, using fluorescently-tagged probes for RNA signal quantification. Sequencing-based methods include platforms such as Visium (Ståhl et al., 2016), Stereo-seq (Chen et al., 2022), and Slide-seq (Rodriques et al., 2019). Imaging-based technologies include MERFISH (Chen et al., 2015), osmFISH (Codeluppi et al., 2018), CosMx SMI (He et al., 2021), STARmap (Wang et al., 2018), seq-FISH (Lubeck and Cai, 2012), PRISM (Chang et al., 2024), and FISH&CHIPS (Zhou et al., 2023a). While these methods are valuable, challenges such as complex tissue preparation steps, the need for thin sections, slow reaction speeds, high costs, proprietary setups and buffer composition, and the potential for false positives and background noise can prevent researchers from using them.

To address false positives and background noise, other technologies provide signal amplification and background suppression but still face some limitations. Methods such as HCR3.0 (Choi et al., 2018; Schulte et al., 2024), SABER-FISH (Kishi et al., 2019), split-FISH (Goh et al., 2020), and RNAScope (Wang et al., 2012) provide signal amplification and use split probes to reduce noise. Yet, these technologies still have 1) limited multiplexing capacity of 3-10 genes, 2) lengthy experimental timelines of over three days, and 3) labor-intensive protocols requiring training and manual sample handling, all of which are error prone. HCR3.0 based technologies, such as cycleHCR (Gandin et al., 2024) and EASI-FISH (Wang et al., 2021), have overcome the limited multiplexing capacity by colocalizing a few dozen to hundreds of genes in around 300 µm tissues, but they still rely heavily on specialised experimental setups or equipment, including dedicated confocal systems, robot-handling solutions, and computationally challenging data processing steps that may not be accessible to an average lab for routine experimentation.

Here, we present RAM-FISH, a Hybridization Chain Reaction (HCR3.0) (Choi et al., 2018) based method, coupled with multiplexing and optional automation, to efficiently detect 30 or more target genes through multiple cycles on the same sample. We have previously employed this automation system with a single HCR cycle to characterize gene expression (micro RNAs, long non-coding RNAs, and mRNAs) in *Bicyclus anynana* developing wings and intact *Danio rerio* larval brains (Goel et al., 2025; Prakash et al., 2024; Raine et al., 2025; Tian et al., 2024).

Several features make RAM-FISH unique and an improvement over currently available methods. This includes the flexibility to use a fully manual methodology reliant on standard lab equipment and buffers, or a completely automated, controlled fluidics with a cyclic thermal gradient system for faster, consistent, and hands free experiments (**Supplementary file S2**). Automation reduces human errors and utilizes lower volumes of primary probes for comparable signal capture. Both protocols are independent of dedicated imaging system requirements, allowing researchers to use the microscopy available to them. The protocol also replaces the conventional toxic formamide-based buffers with safer (for the user and the environment) ethylene carbonate-based buffers, and uses a milder permeabilization buffer. The protocol allows the user to pause and store samples at multiple stages for up to a few months without noticeable signal loss. This affords greater control and flexibility for researchers. Finally, a user-friendly probe designer web-app with customizable features and a composite image generator app that can merge up to 10 single FISH channel images provides an end-to-end solution for multiplexed spatial gene expression analyses.

Overview of the Methodology

The workflow involves the dissection or collection of tissues, followed by fixing and permeabilization. Afterwards, the tissue samples are either stained manually or using the automation system (**Figure 1A**).

A) Manual protocol

1) Tissue free-floating in buffer: The hybridization, washes, and signal removal steps are carried out in glass spot plates or in 1.5 ml centrifuge tubes with samples immersed in the buffer (**Figure 1A**). This method is ideal for a single cycle because overlaying aligned images from multiple cycles is difficult.

2) Tissue immobilized via gel-embedding: The samples are embedded in an acrylamide-based gel in a 35 mm glass-bottom dish. First, the gel is cast with samples embedded and positioned appropriately for microscopy. All hybridization and washes are conducted on this dish directly. The dish is moved to a microscope for imaging between each cycle of probe hybridization and signal removal (**Figure 1A**). This option is ideally suited for overlaying the expression of multiple genes from RAM-FISH cycles as the samples are immobilized. However, fewer samples can be processed at a time depending on the imaging area available on the glass-bottom dish. Overlaying images after each cycle can be improved by using a custom 3D-printed holder for the stage that constrains the x-y coordinates of samples (CAD files of the confocal holder for Olympus FV3000 are provided in the GitHub link for this manuscript).

B) Automation-based

The automation protocol requires the use of a microcontroller based on custom fluidics and thermal gradient modules (as described in **Supplementary file 2; Figure S2_1 – S2_7)**. In the system, reactions can be carried out via:

1) Tissue free-floating in buffer: In the free-floating protocol, tissue samples are placed in a microcomb holder, followed by the installation arc reaction chamber (**Figure 1A)**. The reactions are performed in a controlled fluidic and thermal environment with timed delivery and removal of buffers. Afterwards, the samples are mounted in a slide with mounting media and imaged. Samples are taken out of the mounting media and returned to the microcomb for signal removal and repetition of the cycle.

2) Tissue immobilized via gel-embedding: In the gel-based environment, samples are embedded in the confocal dish and placed in the custom 3D-printed holder. All the remaining processes follow as described above in the arc reaction chamber in a controlled thermal and fluidic environment. Microscopy is conducted directly on the dish in the stage holder, following which the signal is removed, and the samples are returned to the automation system for the next cycle (**Figure 1A**).

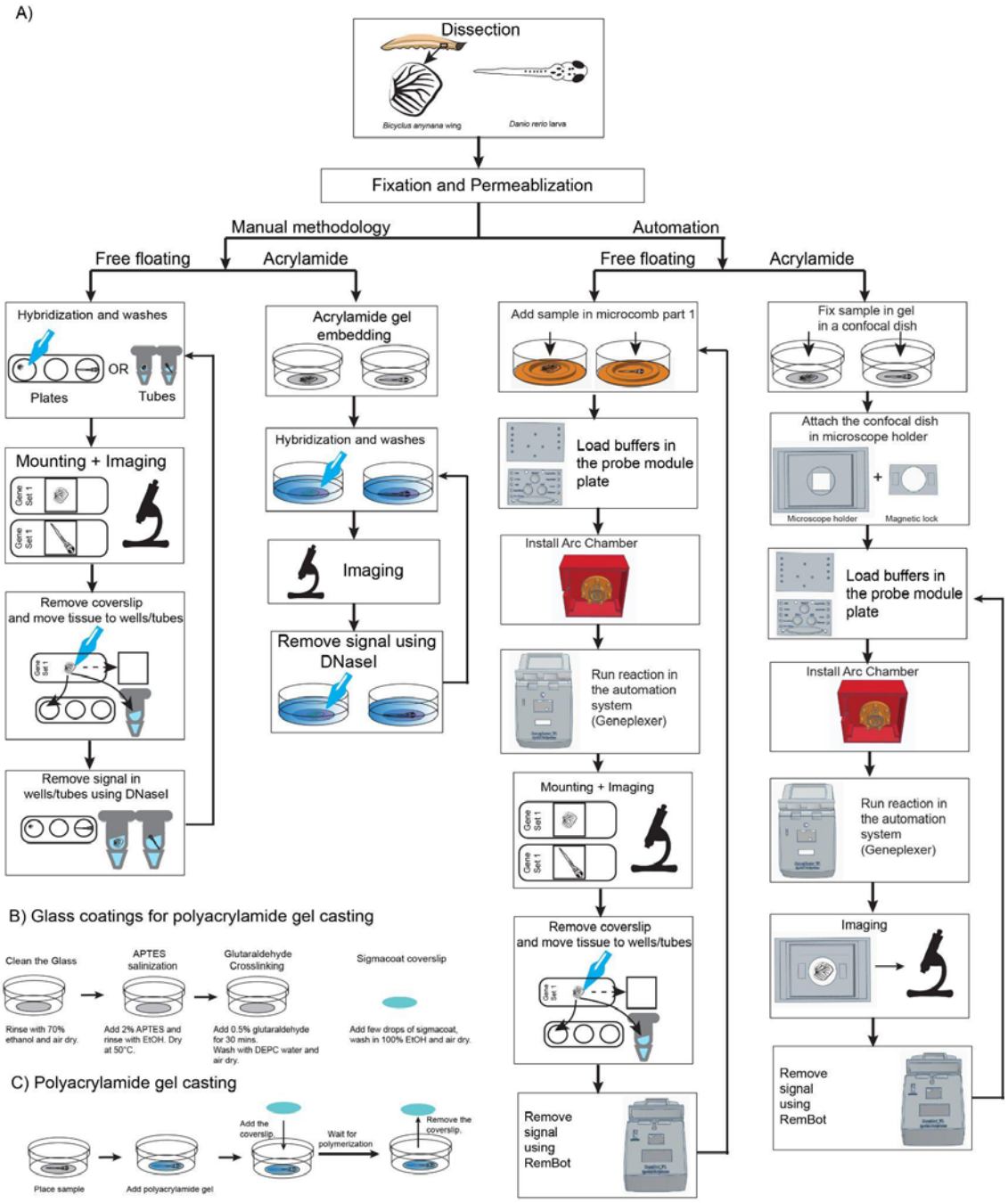

Figure 1: Illustration of the manual and the automation-based multiplexing protocol. (A) Tissues are dissected, fixed, and permeabilized. Afterwards, multiplexing can be performed either manually or using an automation system. (**B**) Simplified method of glass coating and (**C**) method for polyacrylamide gel casting.

Probes preparation

To aid the probe design process, we developed an online web-based probe designer that can generate HCR3.0 style probes. Link: https://tdblab.github.io/hcrprobedesigner/hcr_22.1.html

Input from the user requires: gene name, gene sequence, selection of amplifier color, selection of GC concentration threshold, and the gap in between the probe pairs. Clicking 'Design Probe' and 'Download Excel File' exports the probe sequence formatted for orders from Integrated DNA Technologies (IDT) as oligos (100 µM) or as Pools (oPool at 50 pmol/oligo).

Alternatively, two macro-based MS Excel sheets (Supplementary material: CDS_gene_extractor.xml and amplifier_adder.xml). The first sheet (CDS_gene_extractor.xml) can be used to extract 25 bps nucleotides for both the forward and reverse strands of the chosen HCR primary probe binding sites and reverse-complement them. The second step on the next sheet (Amplifier_adder.xml) adds the amplifiers specific to the selected pairs of complementary sites. Briefly, a candidate gene coding sequence file from a repository such as NCBI (https://ncbi.nlm.nih.gov/) is downloaded and copied and pasted into the first column of the CDS_gene_extractor file. A reverse complement macro (https://github.com/LJI-Bioinformatics/Excel-Reverse-Complement) is loaded into the MS Excel sheet. This produces 25 bp pairs of nucleotides, which are copied and pasted to the amplifier_adder.xml file that contains the secondary fluorescent-specific amplifier sequence.

Imaging multiple genes on the developing butterfly wing (Stages 0.75-3.25)

The manual multiplexing methodology was used to investigate the expression patterns of multiple genes on the developing larval wings of *Bicyclus anynana* butterflies. Many of these genes play important functions in the development of distinct color patterns observed in the adults. During the larval stages, gene expression patterns are highly complex and dynamic (Banerjee et al., 2023; Connahs et al., 2019). We tested the expression of 21 known and 12 novel genes for *B. anynana* during different developmental stages from 0.75-3.25 (staging of wings is described in (Banerjee and Monteiro, 2020a; Reed et al., 2007)) to explore expression dynamics, over 10-14 cycles of hybridization and washes (**Figure 2-4; Figure S1_1 to S1_5**). The known genes are *Wnt1, Wnt 6, Wnt10, WntA, cubitus interruptus (ci), frizzled2 (fz2), frizzled4 (fz4), frizzled9/frizzled3 (fz9), thickvein (tkv), Distal-less (Dll), optomotor-blind (omb), hedgehog (hh), engrailed* (*en*), *Antennapedia* (*Antp*), *decapentaplegic (dpp), patched (ptc), Mothers against dpp 6 (Mad6), Notch, spalt, Optix,* and *vestigial* (*vg*) (**Figure 2-4; Figure S1_1-S1_5; Video S1_1**). The 12 new genes for *B. anynana* are *rhomboid (rho,* at the mRNA level), *homothorax (hth), aristaless (al/al1), dachshund (dac), mirror, sister of odd and bowel (sob), heat shock promoter 67b (hsp67b), aristaless-like (al-like /al2), enhancer of split mbeta (ensl), eyegone (eyg), bric-a-bac (bab),* and *nebula*. **Table S1_2** contains a description of the expression domains of the 21 known genes, whether it is consistent with previous findings in other butterfly species, as well as their function, if known. The coding sequences and the probes used for the detection of the genes are provided in the **Supplementary file S3**.

Below, we provide the description of the novel genes described for the first time in *B. anynana* butterflies.

1) *rhomboid (rho)* mRNA expression was visualized in the veins of butterflies, consistent with previous protein expression results from butterflies (Banerjee and Monteiro, 2020a) and mRNA results from *Drosophila* (Guichard et al., 1999) (**Figure 2F, Figure 3F, Figure S1_1 to S1_5**).

2) *homothorax (hth)* expression was newly visualized along two bands along the proximal domain of the developing larval wings (**Figure 2G, Figure 3G, Figure 4F, Figure S1_1 to S1_5**). A previous study has looked at the expression of *hth* in the pupal wing (Hanly et al., 2019).

3) *dachshund (dac)* was newly visualized in three distinct domains on the anterior compartment of the hindwing (stage 1.00 and 2.50) (**Figure 3G, Figure 4J, Figure S1_2, Figure S1_4, Figure S1_5)** and in an anterior proximal domain, with slightly higher expression in the lower posterior compartment on the forewing during wing development (**Figure 2L, Figure 3L, Figure S1_1, Figure S1_3**).

4) *mirror* was expressed in the lower posterior compartment of the developing larval wings, consistent with a previous study (Chatterjee et al., 2024). Slightly higher expression of *mirror* was also observed in the proximal domain in the anterior compartment of the developing wings (**Figure 2N, Figure 3N, Figure S1_1 to S1_5**).

5) *sister of odd and bowel (sob)* was newly visualized in a strong proximal band as well as at lower levels in a broad distal band (**Figure 2O, Figure 3O, Figure S1_1 to S1_5**).

6) *heat shock promoter 67b (hsp67b)* was newly visualized in complex and dynamic patterns in larval wings. Major expression domains were observed along the anterior compartment and in the wing margin (**Figure 2R, Figure 3R, Figure S1_1 to S1_5**).

7) *enhancer of split mbeta (ensl)* was newly visualized in provein cells adjacent to the veins, from early to late stages of wing development (**Figure 2AB, Figure 3AB, Figure S1_1 to S1_5**).

8) *eyegone (eyg)* was newly visualized along the wing margin in intervein cells (**Figure 2AC, Figure 3AC, Figure S1_1 to S1_5**).

9) *bric-a-bac (bab)* was newly visualized in larval wings, expressed in distinct domains in the anterior compartment (**Figure 2AD, Figure 3AD, Figure 4V, Figure S1_1 to S1_5**). A previous study has looked at the expression of *bab* in pupal *Colias eurytheme* butterflies (Ficarrotta et al., 2022).

10) *nebula* (uncharacterized gene: XP_023941093.2) was a newly visualized gene expressed strongly along a proximal band, and at lower levels throughout the wing tissue (**Figure 2AE, Figure 3AE, Figure S1_1 to S1_5**).

11) *aristaless (al/al1)* was expressed in the anterior compartment along a band running the proximal AP domain of the larval wings, consistent with previous protein and mRNA expression patterns in different butterfly species (Martin and Reed, 2010) (**Figure 2H, Figure 3H, Figure S1_1 to S1_5**).

12) *aristaless-like (al-like /al2)* was expressed in a band along the proximal axis consistent with a previous study in butterflies (Martin and Reed, 2010) (**Figure 2AA, Figure 3AA, Figure S1_1 to S1_5**).

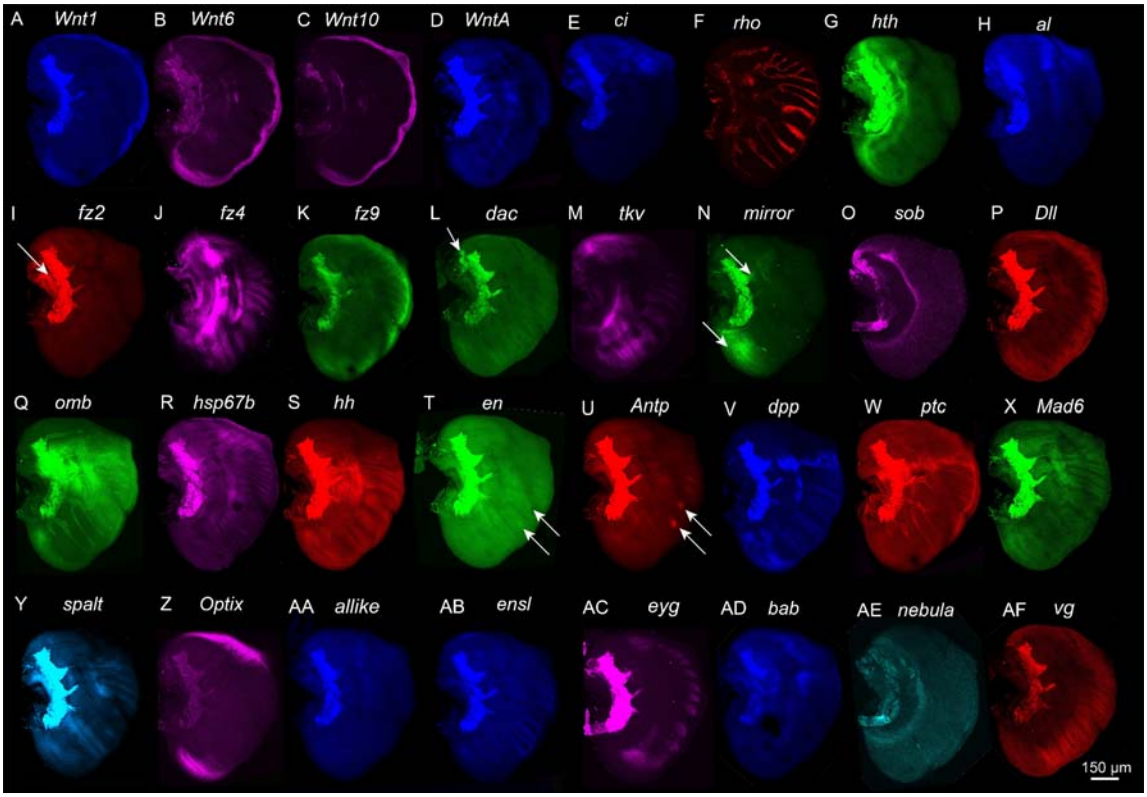

Figure 2. Multiple genes (32 genes) are visualized in a single larval forewing of *Bicyclus anynana* (Stage 0.75). (**A-AF**) Expression of individual target genes (arrows indicate small expression domains). Note: The tracheal tissue along the veins and in the proximal domain are autofluorescent.

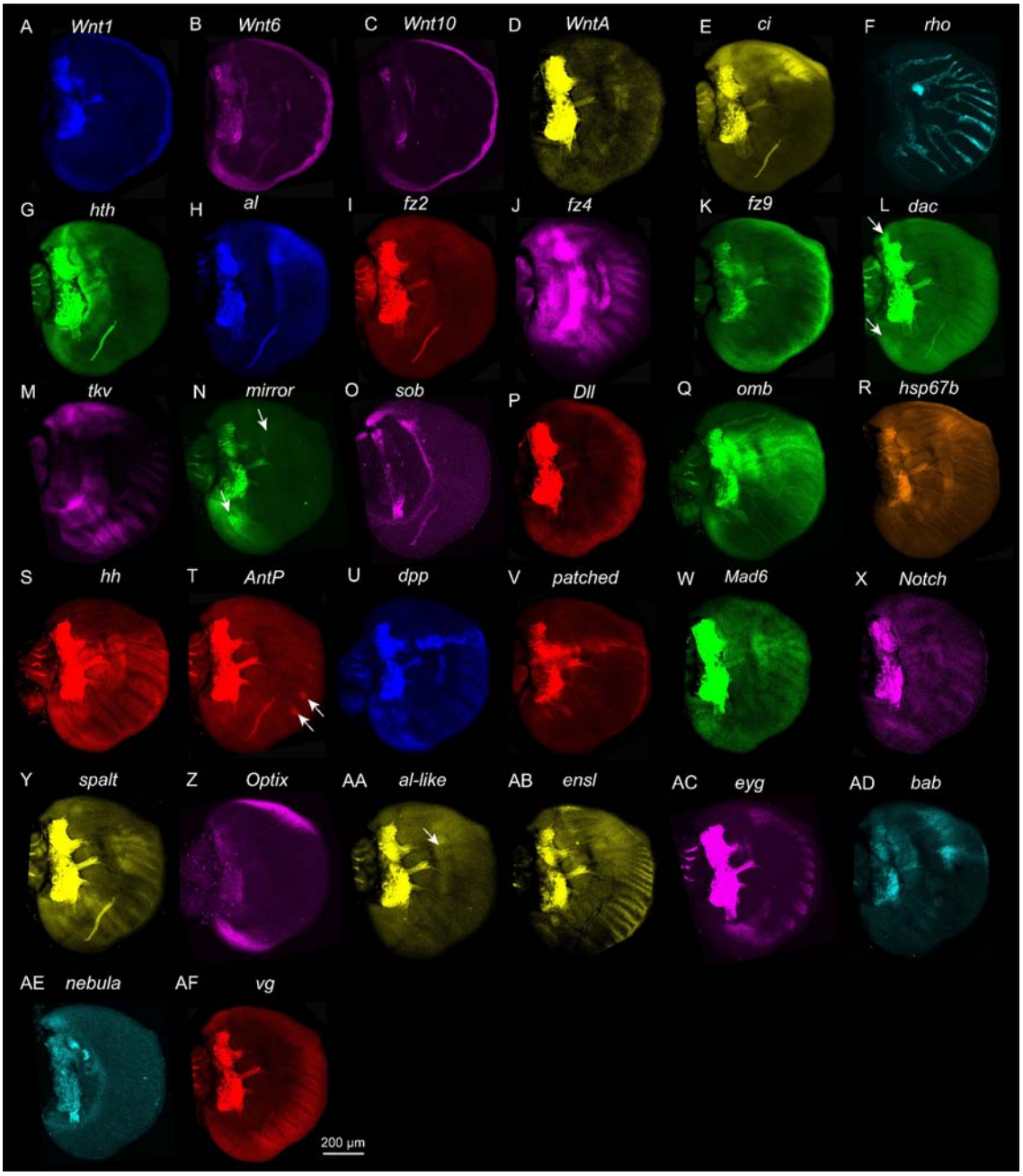

Figure 3: Detection of 32 genes in an early larval forewing of *Bicyclus anynana* (stage 1.00). (**A-AF**) Expression of multiple target genes (arrows indicate small expression domains). Note: The tracheal tissue along the veins and in the proximal domain are autofluorescent.

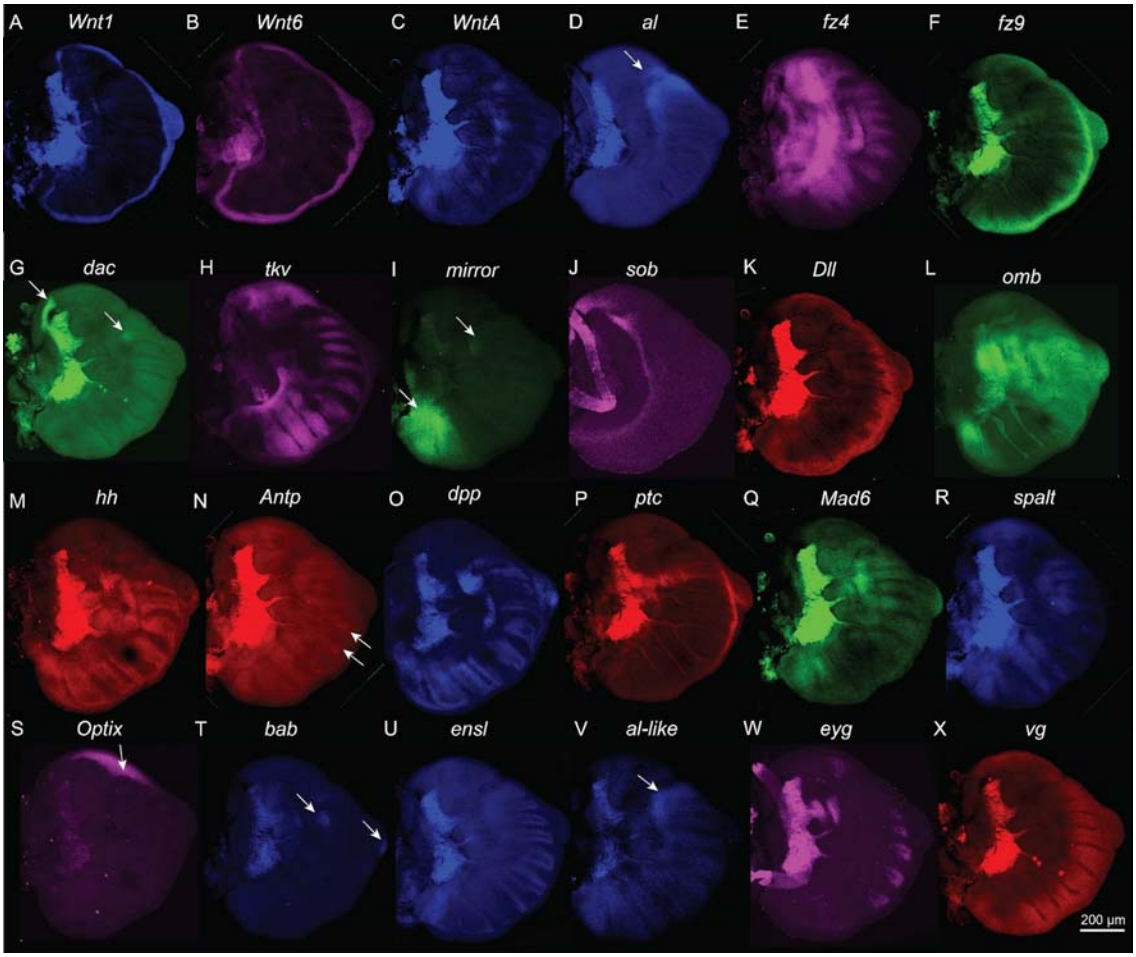

Figure 4. Expression of 24 genes in a late larval hindwing of *Bicyclus anynana* (stage 1.00). (**A-X**) Expression of multiple target genes (arrows indicate small expression domains). Note: The tracheal tissue along the veins and in the proximal domain are autofluorescent.

Robustness analysis: Qualitative and quantitative measurement of signal quality after multiple cycles

We performed a robustness analysis to quantify the signal degradation due to multiplexing. To achieve this, we examined the expression of the gene *optomotor-blind* (*omb*) after multiple cycles, at cycle 1 and cycle 12. The aligned wings from the experiment were imported in FIJI **(Figure S1_6).** Colocalization plots between the two images were generated using the 'Colocalization finder' plugin **(Figure S1_6 C, G, and K).** Pearson correlation coefficient (R) values were generated by selecting Analyse -> Colocalization -> Colocalization test. To calculate the area of expression, color thresholding with a brightness filter of 100 was applied. The selected regions were measured and imported into an Excel sheet to generate the RNA-level graphs **(Figure S1_6D, H, and L).** We confirmed that *omb* expression was qualitatively similar between cycles with clear expression domains spanning the Anterior-Posterior boundary and in the eyespot centers as expected **(Figure S1_6 A, B, E, F, I, and J).** Some quantitative reduction due to degradation of RNA levels was observable in the samples **(Figure S1_6D, H, and L)**. **Note:** Fluorescent signals were stable till 10 cycles of probing and stripping.

Multiplexing in *Danio rerio* (zebrafish) larvae

Zebrafish are commonly used vertebrate laboratory models for fundamental discovery science as preclinical models, and for neurobehavioral studies (Lieschke and Currie, 2007). Larval zebrafish, particularly pigment free mutants (Antinucci and Hindges, 2016), are ideal for high throughput spatial gene expression techniques because they are abundant, transparent, require no special preparation before the procedure, and are small enough to process dozens of animals in parallel. Spatial transcriptomic profiles add unique insights into the brain functionality and circuit level connections between brain regions that bulk and single cell RNA sequencing alone cannot reveal. One way in which this was achieved recently was by registering *in situ* results from individual staining to a standard reference brain (Schulze et al., 2023). However, these existing datasets focus on young larvae (< 6 dpf). Maps of gene expression in late-stage larvae or juvenile fish brains when complex behaviors including learning and social behaviors, emerge are limited or absent (Gemmer et al., 2022). We performed the multiplexing experiment using both the manual and the automation system on 14 days post fertilization (dpf) larvae to examine gene expression in the brain (**Figure 5; Figure S1_7-9; Video 1 and 2; Video S1_2**). Here, we spatially profiled 9-11 genes to illustrate the use of RAM-FISH. We examined neurotransmitter associated genes that have broad brain-wide expression (such as *tph2, neurod1,* and *gad2*), neurotransmitter producing enzyme genes (such as *chatb*), and neuropeptide genes that are expressed in a few neurons in deep seated brain regions (such as *oxt* expressing neurons in the hypothalamus). Overall, the expression patterns observed are consistent with previous reports available at mapZebrain (Shainer et al., 2023), or Zebrafish Information Network (ZFIN; **Table S1_3**) for younger larvae. For example, as seen in the mapZebrain atlas for *tph2,* the HCR technique revealed a broader mRNA expression profile compared to promoter-GAL4 fusion transgenic lines (**Figure S1_10**). The expression of the following 11 genes was carried out by RAM-FISH as a representative example: *glutamate decarboxylase 2 (gad2*), *neuropilin 1a (nrp1a), neuronal differentiation 1 (neuroD), olfactory marker protein b (omp*), *tryptophan hydroxylase 2* (*tph2), oxytocin (oxt*), *potassium channel tetramerisation domain containing 12.2 (kctd12.2), cholinergic receptor, muscarinic 3a (chrm3a), cholinergic receptor, nicotinic, alpha 3 (chrna3), ELAV like neuron-specific RNA binding protein 3 (elavl3),* and *choline O-acetyltransferase b (chatb).* The details of the expression domains are described in **Table S1_3**.

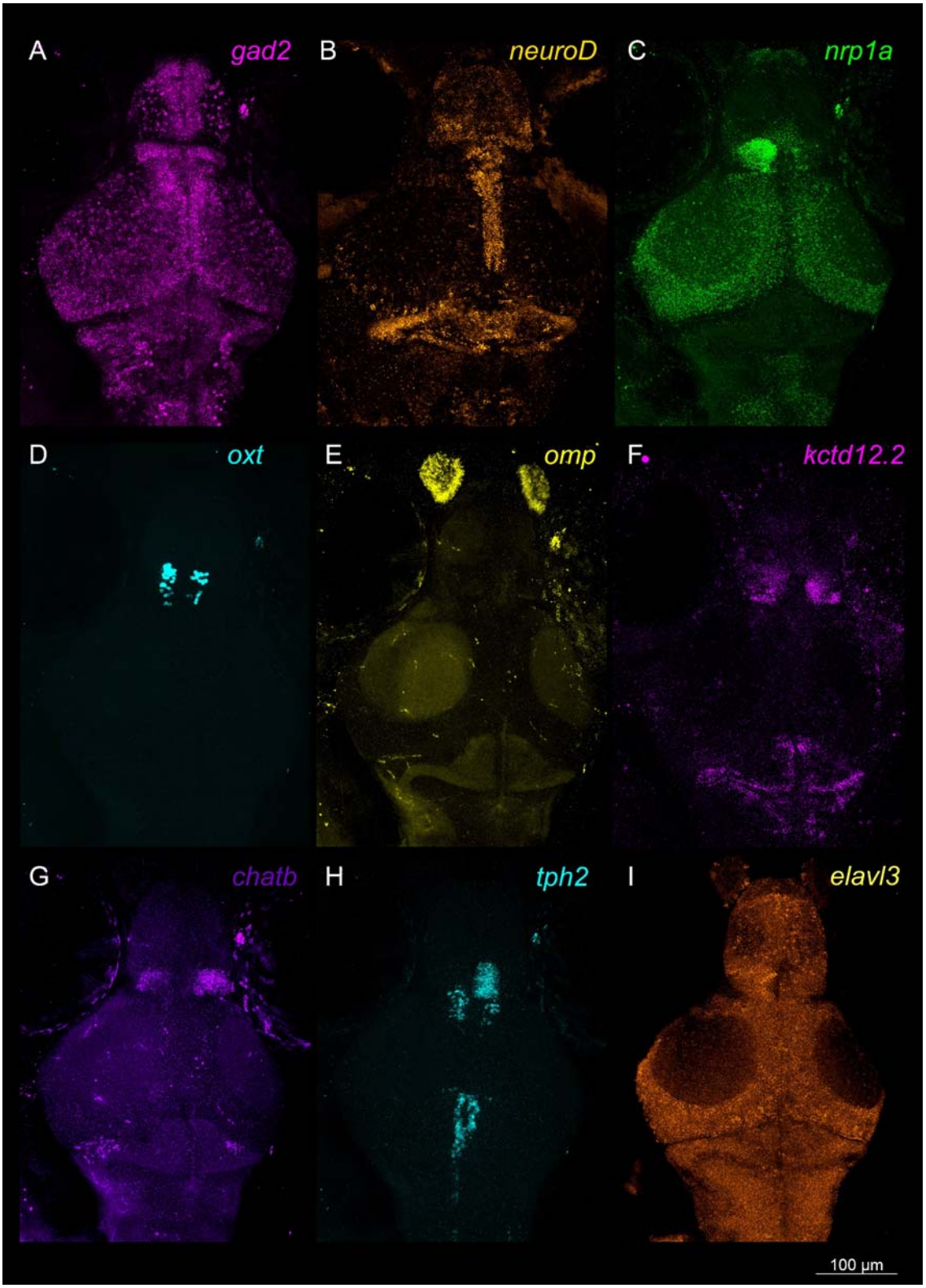

Figure 5. Detection of multiple gene targets in a 14 dpf *Danio rerio* larva across ~190 µm depth (maximum projection of multiple z-stacks). (A-I) Multiplexed expression of *gad2, nrp1a, neuroD, oxt, omp, kctd12.1, chatb, tph2,* and *elavl3* in a 14 dpf *Danio rerio* larva. Reaction carried out using the long reaction protocol.

Video 1. A volume rendered 14 dpf zebrafish larva stained against multiple gene targets across ~190 µm depth (Images acquired as mirror images).

Video 2. Detection of multiple genes in a 14 dpf zebrafish larva across ~190 µm depth (Images acquired as mirror images).

Benchmarking

To benchmark the buffers and thermal gradients for a single cycle, we compared the results obtained using two time parameters: Long (8-10 hrs) and short (4.5 hrs). In the table below, we summarize the results:

Table 1: Benchmarking of different reaction parameters on longer and shorter reaction times.

Reaction Parameters	**Long reaction (8-10 hrs)** **Figure 6**			**Short Reaction (4.5 hrs)** **Figure 7**		
Timing	Primary hybridization: 4-5 hrs Secondary hybridization: 3 hrs Washes: 1-2 hrs			Primary hybridization: 2 hrs Secondary hybridization: 1.5 hrs Washes: 1 hr		
Reaction type	Manual		Automation	Manual		Automation
	RT	37°C	Thermal gradient	RT	37°C	Thermal gradient
Primary Probe volume in µL/mL of hybridization buffer (5 µM stock concentration)	10	10	5	20	20	10
Reaction with 20% Ethylene Carbonate (Signal intensity)	Very weak	Strong	Strong	Not detected	Medium	Medium
Reaction with 30% Probe Hybridization	Not detected	Strong	Strong	Not detected	Weak	Medium

Buffer (Signal intensity)						
Reaction with 20% Ethylene Carbonate (Signal-to-noise)	NA	Medium	High	NA	Medium	Medium
Reaction with 30% Probe Hybridization Buffer (Signal-to-noise)	NA	Medium	High	NA	Low	Medium

Overall, the 8-10 hrs protocol produced a stronger signal and a higher signal-to-noise ratio compared to the 4.5 hrs protocol (**Figures 6 and 7**). Some regions, such as the habenula, however, produced a saturated signal in the 8 hrs protocol using automation, likely due to overamplification of the secondary amplifiers, and is not ideal for single-molecule detection (**Figure 6 AG, AH**).

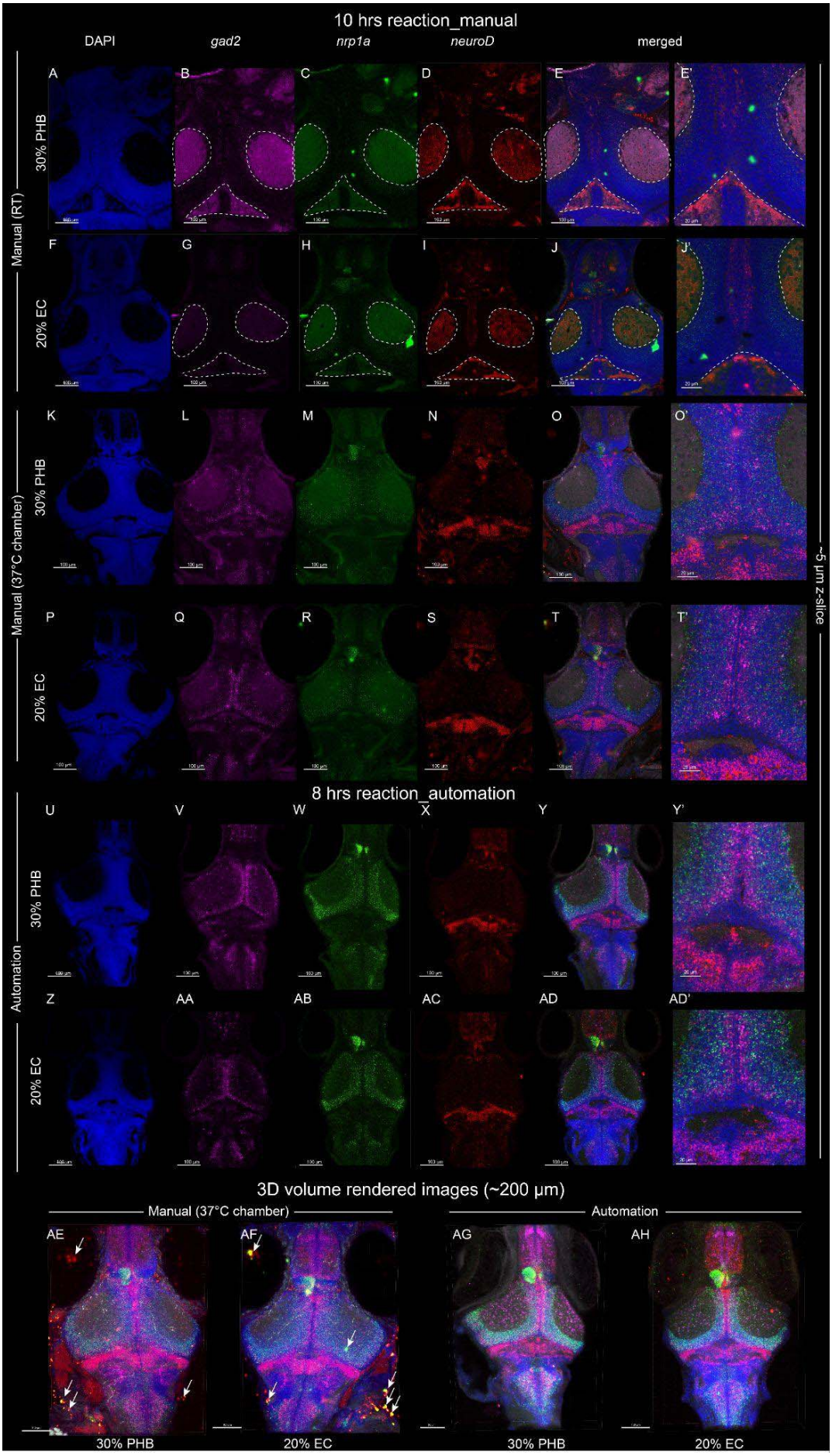
10 hrs reaction_manual
DAPI
gad2
nrp1a
neuroD
merged
Manual (RT)
30% PHB
20% EC
Manual (37°C chamber)
30% PHB
20% EC
~5 µm z-slice
8 hrs reaction_automation
Automation
30% PHB
20% EC
3D volume rendered images (~200 µm)
Manual (37°C chamber)
Automation
30% PHB
20% EC
30% PHB
20% EC

Figure 6. Multiplexed-FISH reaction on *gad2*, *nrp1a*, and *neuroD* was carried out over 10 and 8 hrs of total reaction time in 14-dpf zebrafish larvae. (A-J) Reaction done at room temperature didn't show a stronger specific signal. (**K-T**) Reactions done in a 37°C chamber showed a stronger signal-to-noise for all three genes. (**U-AD**) Reactions done using the automation systems showed specific signals for all three genes. Signals were slightly stronger in images processed by the automation system. (**AE-AH**) 3D volume rendered images. Automation-based experiments showed fewer non-specific signals (arrows). **Note:** In the automation-based methodology, 50% less primary probes have been used to avoid signal saturation. Three biological replicates were imaged for each reaction type. The dashed line from panel B-J represents the autofluorescent domains of the larvae, and the white arrows in panel AE and AF are non-specific signals. Z-depth of the volume rendered images is ~200 µm.

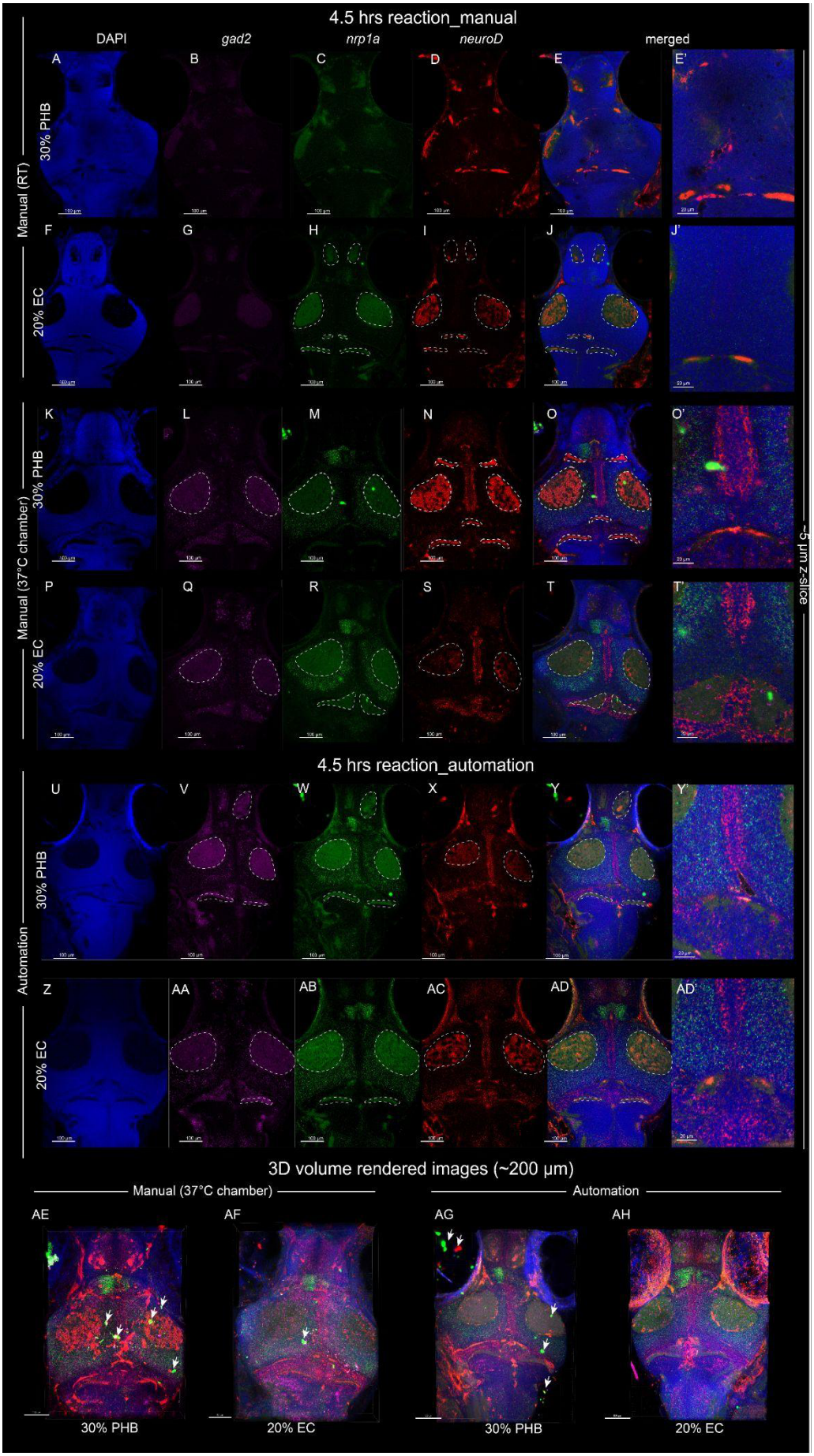
4.5 hrs reaction_manual
DAPI
gad2
nrp1a
neuroD
merged
Manual (RT)
30% PHB
20% EC
Manual (37°C chamber)
~5 µm z-slice
4.5 hrs reaction_automation
Automation
3D volume rendered images (~200 µm)
Manual (37°C chamber)
Automation
30% PHB
20% EC
30% PHB
20% EC

Figure 7. Multiplexed-FISH reaction on *gad2*, *nrp1a*, and *neuroD* carried out over 4.5 hrs of total reaction time in 14dpf zebrafish larvae. (A-J) Reactions done at room temperature didn't show stronger specific signals. (K-T) Reactions done in a 37°C chamber showed specific signals for all three genes. (**U-AD**) Reactions done using the automation systems showed specific signals for all three genes. The stainings from panel A-AD are 5 µm z-slices. (**AE-AH**) 3D volume rendered images. High noise was observed in 30% PHB samples. **Note:** In the automation based methodology 50% less primaries have been used to avoid signal saturation. Three biological replicates were imaged for each reaction type. The dashed line from panel H-AD represents the autofluorescent domains of the larvae, and white arrows in panel AE, AF, and AG mark non-specific staining. Z-depth of the volume rendered images is ~200 µm.

Composite image creation

For composite image generation, we developed a python based program (**merge.py**). It takes pre-aligned single channel images of individual genes (up to 10 genes). The image format can be jpg, bmp, png, and tiff. The name of the image files should be gene_name.format. For example, spalt.jpg, optix.jpg, omb.jpg, etc. The algorithm will automatically place the name of the genes and the associated color in the top-right of the composite image.

For execution, create a folder and copy the merge.py file (GitHub link). Create a subfolder named multiplex_fish_images and place all your images there. Make sure images are of the same dimensions. Install the latest version of Python. Open the command prompt (or terminal on Mac) and move to the location where merge.py is copied. To install the dependencies, type:

```
pip install opencv-python numpy
```

To run the file type:

```
python merge.py
```

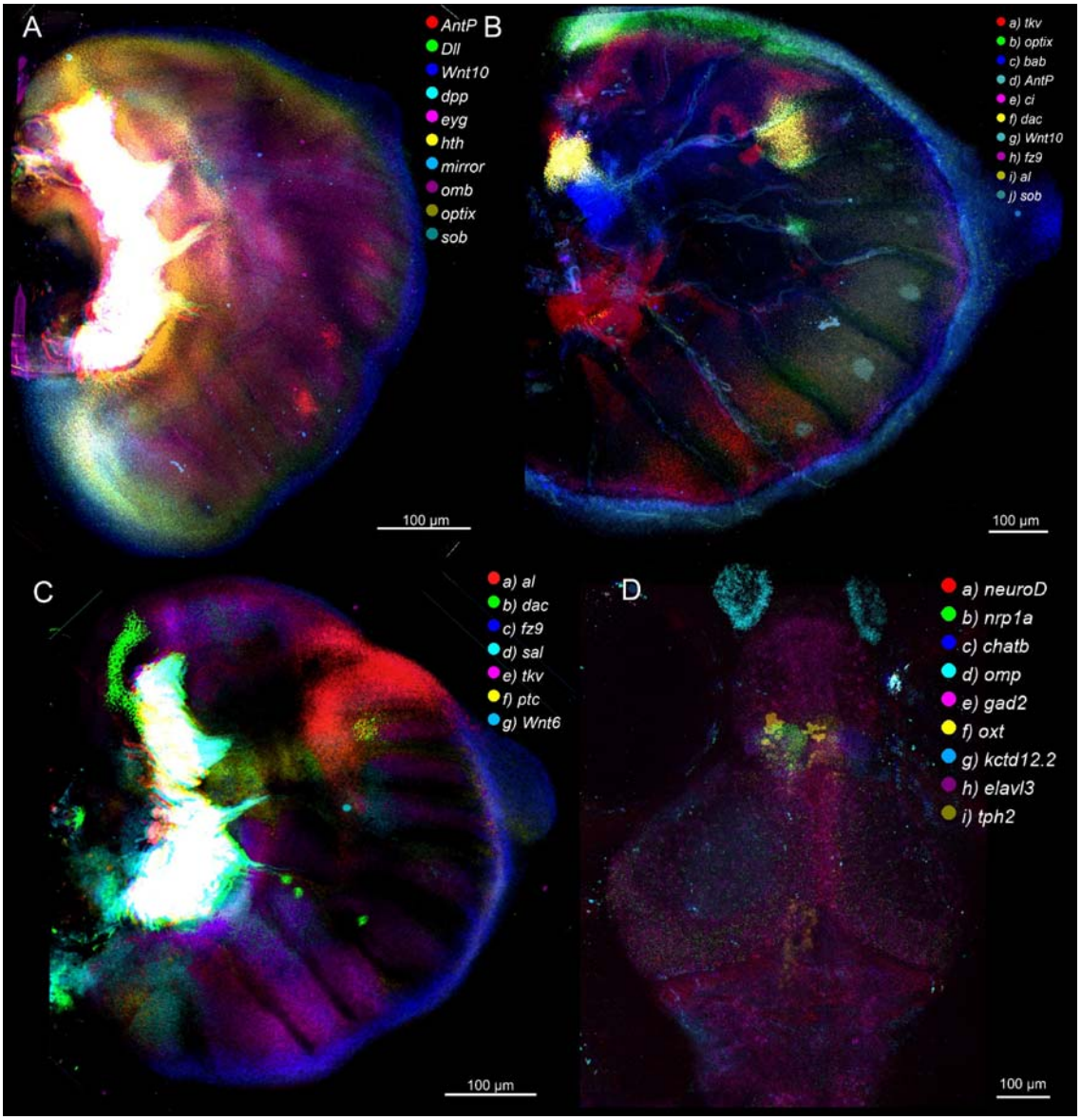

Figure 8: Composite *Bicyclus anynana* and *Danio rerio* RAM-FISH images created using the merge.py script. (A) Merged image of *AntP, Dll, Wnt10, dpp, eyg, hth, mirror, omb, optix,* and *sob* multiplexed expression in a 0.75 stage butterfly larval forewing. **(B)** Merged image of *tkv, optix, bab, Antp, ci, dac, Wnt10, fz9, al,* and *sob* multiplexed expression in a 2.50 stage butterfly larval hindwing. **(C)** Merged image of *al, dac, fz9, sal, tkv, ptc,* and *Wnt6* multiplexed expression in a 0.75 stage butterfly larval hindwing. **(D)** Merged, z-stacked (maximum projection) image of *neuroD, nrp1a, chatb, omp, gad2, oxt, kctd12.2, elavl3,* and *tph2* in a 14 dpf zebrafish larva.

Alternatively, for merging up to 7 channels, the open source platform FIJI (Schindelin et al., 2012) can be used. For creating a merged image, the aligned image files (with the same width and height, alignment method described below) can be imported into FIJI. Image > Color > Merge Channels with all channels selected (C1 to C7 from the drop-down menu) and 'create composite' box selected will produce the desired output. A control protein marker, nuclear marker, or cell membrane marker is required to acquire aligned images after each cycle of RAM-FISH. Here, we used DAPI nuclear staining for cell registration to align 3D images (Sternson et al., 2022).

For registration of multiple 3D frames, cell boundaries based on DAPI stainings were aligned in Imaris 10.2, followed by alignment by the "Image Alignment" tool (Image Processing → Image Alignment → Align Images). 3D image rendering and video capture were carried out using Imaris 10.2 and Imaris Viewer 10.2. For 2D images (single z-slice) from *Bicyclus anynana* wings from different FISH cycles were aligned in different Photoshop layers by aligning the cell boundaries, eyespot center cells, and the autofluorescent signal along the tracheal tissue. The 2D images (max projection files from multiple z-stacks) of *Danio rerio* larvae from different cycles were aligned using the cell boundaries as observed via the DAPI channel in each multiplexed cycle.

Discussion

Spatial RNA profiling technologies, which involve the localization of multiple transcripts, are rapidly evolving and are capable of profiling thousands of mRNA species. The technologies are accelerating basic and applied research, including in the fields embryology and organ development (Choi et al., 2023; Rao et al., 2021; Zhang et al., 2021), neurobiology (Jung and Kim, 2023; Moffitt et al., 2018), and cancer research (Coutant et al., 2023; De Zuani et al., 2024; Valdeolivas et al., 2024; Wu et al., 2021a; Wu et al., 2021b; Wu et al., 2023; Zhang et al., 2022; Zhou et al., 2023b). Yet they face major limitations such as 1) complex and costly setups for dedicated sequencing, microfluidics, and imaging; 2) extensive time consuming steps to perform each experiment; 3) proprietary reagents; 4) complex and computationally intensive analyses pipelines for RNA localization; 5) special sample preparations which are often limited to thin (5-10 µm) tissue slices; and 6) mRNA detection errors.

Our goal was to solve these limitations by deploying a cost-effective, less complex, and faster spatial profiling methodology that can be applied to whole tissues and can be adapted quickly by laboratories using common reagents and labware. To achieve this, we modified previously described methods. We modified the polyacrylamide gel casting methodology and the widely used HCR3.0 hybridization and amplification reaction (Choi et al., 2018) by deploying thermal gradients inside the reaction chamber (**Figure S2_1 – S2_7**) and by modifying the buffers. We used a DNaseI based signal removal strategy (Codeluppi et al., 2018; Kishi et al., 2019; Shah et al., 2016; Sternson et al., 2022; Wang et al., 2021), followed by reprobing and imaging, to achieve multiplexing. Finally, the methodology is not restricted to thin slices of tissue, allowing multiplexing experiments on intact organs and animals. As demonstrated here for butterfly wings and whole fish larvae, this methodology can be applied to diverse species with or without automation.

This methodology will be useful to developmental biologists, developmental geneticists, and pathologists, who require high-quality spatial information on dozens of genes (rather than hundreds of genes) in tissues on a regular basis. This technology will aid in basic studies of pattern formation, embryological development, and in dissecting the architecture of gene-regulatory networks. For instance, the multiplexed *in-situ* images, when combined with functional tools such as CRISPR-Cas9, drug inhibitors, and RNAi, can be used to test how perturbations to single genes affect the expression of multiple possible targets in a single sample. The current method can also complement other technologies such as single-cell and laser-based spatial transcriptomics (Banerjee et al., 2022; Datta et al., 2015; Liu et al., 2019; Nichterwitz et al., 2016; Nichterwitz et al., 2020; Saliba et al., 2014; Shalek et al., 2014).

In the present work, we provided atlases of multiple known and newly visualized genes during different stages of larval wing development of *Bicyclus anynana* butterflies (~80-100 µm thick), as well as larval zebrafish brains, which can be used as references in future research. Many classic genes, such as *WntA, Optix, spalt, dpp, Dll, Antp, vg, hh, notch,* and *ptc*, and newly visualized genes, such as *hth, dac, hsp67b, nebula, bab,* and *eyg,* showed highly consistent expression patterns in the multiple

wing samples tested, supporting the reliability of the current methodology. Similarly, *tph2, neurod1, gad2, oxt, nrp1a, chrna3, kctd12.2, elavl2,* and *omp* expression patterns matched expected profiles in zebrafish. We hope these results will inspire researchers to test the method in their own systems.

Materials and labware used

Equipment

1) For the manual multiplexing protocol

37°C incubator: Incubating Shaker Rocking, Brand: Ohaus, United States.

Pipettes: Micropipette, 0.5–10 µL (Eppendorf, Hamburg, Germany; Cat. no.: 3123000020, 3123000055, 3123000063)

Confocal microscope: Olympus FLUOVIEW FV3000 confocal LSM (Olympus Life Science, Waltham, MA, USA; Product ID: FV3000)

2) For the automation system

Automation systems (Geneplexer and RemBot): Custom-built.

Pipettes: Micropipette, 0.5–10 µL (Eppendorf, Hamburg, Germany; Cat. no.: 3123000020, 3123000055, 3123000063).

Confocal microscope: Olympus FLUOVIEW FV3000 confocal LSM (Olympus Life Science, Waltham, MA, USA; Product ID: FV3000).

Consumables (common)

Pipette tips: Filter pipette tips, 10 µL, 300 µL, 1250 µL. Biotix, San Diego, CA, USA; (Cat. No.: 63300041, 63300045, 63300047).

Confocal dish: Confocal dish. 3.5 mm, SPL (Cat No. 101350).

Coverslips: Cover Glasses 18 mm Ø, Thickness No.1 Circular (Cat No. 0111580). Corning® cover glasses square, No. 1, W x L 18 mm x 18 mm (Cat No. CLS284518-2000EA).

Glass-slides: Slides, microscope plain, size 25 mm × 75 mm (Cat No. S8902-1PAK)

Common Buffers

All the chemicals, unless mentioned, were ordered from Sigma-Aldrich (Merck).

RAM-FISH buffers

20% EC hybridization buffer:

Composition: 20% ethylene carbonate (Cat No. E26258-3KG), 5× sodium chloride sodium citrate (SSC), 12 mM citric acid (pH 6.0) (Cat No. 251275-100G), 0.5% Tween 20 (Cat No. P1379-100ML), 100 µg/mL heparin (Cat No. H3393-50KU), 1.2× Denhardt's solution (Cat No. D6001-50G), 2.5% dextran sulfate (Cat No. D6001-50G), 1.0mM EDTA (pH 8.0) (Cat No. E9884-100G).

For 50 mL of solution:

Mix in a 50 ml tube 10 mL ethylene carbonate (Cat No. E26258-3KG), 12 mL of 20× SSC, 400 µL 1 M citric acid (pH 6.0 (Cat No. 251275-100G), 200 µL of Tween 20 (Cat No. P1379-100ML), 400 µL of 10 mg/mL heparin (Cat No. H3393-50KU), 1000 µL of 50× Denhardt's solution (Cat No. D6001-50G), 5 mL of 25% dextran sulfate (Cat No. D6001-50G), 100 µl of 500mM EDTA (pH8.0) (Cat No. E9884-100G). Fill up to 50 mL with DEPC (Cat No. D5758-25ML) H_2O.

<u>20% EC wash buffer:</u>

Composition: 20% ethylene carbonate (Cat No. E26258-3KG), 5× sodium chloride sodium citrate (SSC), 12 mM citric acid (pH 6.0) (Cat No., 0.5% Tween 20 (Cat No. P1379-100ML), and 100 µg/mL heparin (Cat No. H3393-50KU).

For 50 mL of EC wash solution:

Mix in a 50 ml tube 10 mL ethylene carbonate (Cat No. E26258-3KG), 10 mL of 20× SSC, 400 µL 1 M citric acid (Cat No.), pH 6.0, 200 µL of Tween 20 (Cat No. P1379-100ML), 400 µL of 10 mg/mL heparin (Cat No. H3393-50KU). Fill up to 50 mL with DEPC H_2O.

Note: The use of ethylene carbonate accelerates the overall FISH hybridization time. Previous reports have suggested the use of 15-20% ethylene carbonate as an alternative to accelerate DNA-FISH experiments (Kalinka et al., 2020; Matthiesen and Hansen, 2012). 10% ethylene carbonate has also been used in hybridization mixture and wash buffer in MERFISH experiments (Fang et al., 2023; Moffitt et al., 2016). The buffer presented in this study will freeze at 4°C. Kindly thaw before starting the experiment or prepare fresh.

<u>Imaging Buffer:</u>

Composition: 70% glycerol (Cat No. G7893-500ML) with 1.0mM EDTA (pH 8.0) (Cat No. E9884-100G). For a 50 ml solution in a 50ml tube, add 35ml 100% glycerol and 100 µl of 500 mM EDTA. Top up to 50 ml using DEPC water, and store at room temperature for up to 6 months.

<u>Permeabilization Solution:</u>

Composition: 4.0% Ammonium lauryl sulfate (Cat No. 09887-250ML), 5.0% Tween 20 (Cat No. P1379-100ML), Tris-HCl pH 7.5 (Cat No. 10812846001), 1.0 mM EDTA pH 8.0 (Cat No. E9884-100G), and 200.0 mM NaCl (Cat No. S9888-25G).

For 50 mL of Solution:

Mix in a 50 ml tube 10.00 mL 20% Ammonium lauryl sulfate (Cat No. 09887-250ML) (filtered), 2.50 mL Tween 20 (Cat No. P1379-100ML), 2.50 mL 1M Tris-HCl (Cat No. 10812846001), pH 7.5, 0.10 mL 0.5 M EDTA (Cat No. E9884-100G) pH 8.0, and 2.00 mL 5 M NaCl (Cat No. S9888-25G). Fill up to 50 mL with DEPC H_2O.

Note: The use of ammonium lauryl sulfate provides an alternative to the harsh sodium dodecyl sulfate (SDS) for permeabilization of the probes. Store at RT for up to 6 months.

<u>Mounting Media:</u>

Composition: 70% glycerol (Cat No. G7893-500ML) with 1.0mM EDTA (pH 8.0) (Cat No. E9884-100G). For a 50 mL solution in a 50 mL tube, add 35 mL 100% glycerol and 100 µl of 500 mM EDTA. Top up to 50 ml using DEPC water, and store at room temperature for up to 2 months.

Signal removal and wash solutions

Signal removal solution (1ml): In a 1.5 ml tube add Tris pH7.5 (Cat No. 10812846001 (1M) – 100 µl, $MgCl_2$ (Cat No. M8266-100G) (0.5M) – 50 µl, $CaCl_2$ (Cat No. C4901-100G) (0.5M) – 5 µl, DnaseI (ThermoFisher-Cat No.: EN0521) – 15 µl. Add dH_20 to fill till 1ml. Prepare fresh.

Note: Signal removal solution can remove the signal when kept at 37°C for 30-60 mins when performed manually and as fast as 15 mins in the robotic system in samples up to 50-100 µm thick.

Signal wash buffer 1 (10ml): In a 15 ml tube, add Tris pH7.5 (Cat No. 10812846001) (1M) – 1000 µl, $MgCl_2$ (Cat No. M8266-100G) (0.5M) – 500 µl, $CaCl_2$ (Cat No. C4901-100G) (0.5M) – 50 µl. Add dH_20 to fill till 10ml.

Signal wash buffer 2 (1ml): In a 1.5 ml tube, add Tris pH7.5 (Cat No. 10812846001 (1M) – 100 µl, $MgCl_2$ (Cat No. M8266-100G) (0.5M) – 50 µl, $CaCl_2$ (Cat No. C4901-100G)(0.5M) – 5 µl, 500mM EDTA pH 8.0 (Cat No. E9884-100G) – 5 µl, 10% SDS (Cat No L3771-100G) - 150 µl. Add dH_20 to fill till 1ml.

Note: Prepare the signal removal fresh. Singal Wash buffer 1 and 2 can be stored at room temperature for up to 2 months.

Robot cleaning solution

Composition: 2% SDS (Cat No. 436143-25G), 1% Sodium dichloroisocyanurate (Cat No. 218928-25G), and 1% NaOH (Cat No. 221465-25G).

Alternatively, prepare 20ml of solution by mixing 5ml RNaseZap (ThermoFisher, Cat No. AM9780) and 15ml DEPC water.

HCR3.0 Buffers (from Choi et al., 2018; Bruce et al., 2021)

30% probe hybridization buffer

Composition: 30% formamide (Cat No. F7503-1L), 5× sodium chloride sodium citrate (SSC), 9 mM citric acid (Cat No. 251275-100G) (pH 6.0), 0.1% Tween 20 (Cat No. P1379-100ML), 50 µg/mL heparin (Cat No. H3393-50KU), 1× Denhardt's solution (Cat No. D2532-5X5ML), and 5% dextran sulfate (Cat No. D6001-50G).

For 40 mL of solution:

Mix in a 50 ml conical tube (Cat No. BS-500-MJL-S) 12 mL formamide (Cat No. F7503-1L), 10 mL of 20× SSC, 360 µL 1 M citric acid (Cat No. 251275-100G), pH 6.0, 40 µL of Tween 20 (Cat No. P1379-100ML), 200 µL of 10 mg/mL heparin (Cat No. H3393-50KU), 800 µL of 50× Denhardt's solution (Cat No. D2532-5X5ML), and 4 mL of 50% dextran sulfate (Cat No. D6001-50G). Fill up to 40 mL with DEPC (Cat No. D5758-25ML) H_2O.

30% probe wash buffer

Composition: 30% formamide (Cat No. F7503-1L), 5× sodium chloride sodium citrate (SSC), 9 mM citric acid (Cat No. 251275-100G) (pH 6.0), 0.1% Tween 20 (Cat No. P1379-100ML), and 50 µg/mL heparin (Cat No. H3393-50KU).

For 40 mL of solution

Mix in a 50 ml tube 12 mL formamide (Cat No. F7503-1L), 10 mL of 20× SSC, 360 µL 1 M citric acid (Cat No. 251275-100G), pH 6.0, 40 µL of Tween 20 (Cat No. P1379-100ML), and 200 µL of 10 mg/mL heparin (Cat No. H3393-50KU). Fill up to 40 mL with DEPC H_2O.

50% dextran sulfate

For 40 mL of solution

Mix in a 50 ml tube 20 g of dextran sulfate powder (Cat No. D6001-50G). Fill up to 40 mL with DEPC H_2O.

Amplification buffer

5× sodium chloride sodium citrate (SSC), 0.1% Tween20, and 5% dextran sulfate (Cat No. D6001-50G).

For 40 mL of solution

Mix in a 50 ml tube 10 mL of 20× SSC, 40 µL of Tween 20 (Cat No. P1379-100ML), 4 mL of 50% dextran sulfate (Cat No. D6001-50G). Fill up to 40 mL with DEPC H_2O.

Detergent Solution
Composition: 1.0% SDS (Cat No. 436143-25G), 2.5% Tween (Cat No. P1379-100ML), Tris-HCl (Cat No. 10812846001) (pH 7.5), 1.0 mM EDTA (Cat No. E9884-100G) (pH 8.0), and 150.0 mM NaCl (Cat No. S9888-25G).

For 50 mL of Solution
Mix in a 50 ml tube 5.00 mL 10% SDS (Cat No. 436143-25G) (filtered), 1.25 mL Tween 20 (Cat No. P1379-100ML), 2.50 mL 1M Tris-HCl (Cat No. 10812846001), pH 7.5, 0.10 mL 0.5 M EDTA (Cat No. E9884-100G), pH 8.0, 1.50 mL 5 M NaCl (Cat No. S9888-25G). Fill up to 50 mL with DEPC H_2O.

1x PBST (for 50 ml of solution)

5ml 10x PBS, 50 µL Tween 20 (Cat No. P1379-100ML). Fill up to 50mL with water.

5× SSCT (for 40 mL of solution)

10 mL of 20× SSC, 40 µL of Tween 20 (Cat No. P1379-100ML). Fill up to 40 mL with DEPC H_2O.

DEPC H_2O (1000 ml)

Add 1ml of DEPC to 1000 mL of MilliQ water. Mix well and store in the dark overnight. The next day, autoclave.

10x PBS

For 1 liter of solution, add 81.8 g of NaCl (Cat No. S9888-25G), 5.28 g of KH_2PO_4 (Cat No. P0662-25G), and 10.68 g of K_2HPO_4. (Cat No. P3786-100G) in a glass bottle. Fill till 1000ml using dH_2O and autoclave. Store at RT for up to 6 months.

20x SSC

For 1000 ml of solution, add 175.3 g of NaCl (Cat No. S9888-25G) and 88.2 g of trisodium citrate (Cat No. S1804-500G). Add dH_2O to 1000 ml and autoclave. Store at RT for up to 6 months.

Alternatively prepare, 2 mM Trolox (Cat No. 238813-5G), 10% w/v glucose (Cat No. G7021-100G), 0.1mg/ml glucose oxidase (Cat No. 345386-10KU), 50 µg/ml catalase (Cat No. C9322-5G), 5x SSC, Tris-HCl (Cat No. 10812846001), 50 mM, 60% Glycerol (Cat No. G5516-100ML). Modified from ref (Zhang et al., 2021). If possible, prepare fresh. Otherwise, store at 4°C and use within 1-2 weeks after preparation.

DAPI buffer:

For 1 mL of solution, add 5 µL of stock DAPI (Cat No. D9542-5MG) (1 mg/mL in DMSO) in 995 µL of 5x SSCT in a 1.5 mL tube. Prepare fresh before use.

Sample embedding in a confocal dish:

Glass coatings (**Figure 1B**)

Confocal dish (3.5 mm with glass cover slip) (SPL, Cat No. 101350). Steps:

1) On a glass confocal dish, add 200 µL of 2% APTES (Cat No. 440140-100ML) in 100% ethanol for 5 mins, followed by two washes with 100% ethanol. Dry the dish at 50°C for 30 mins. This can also be done on a regular microscope glass slide.

2) Afterwards, add 0.5% glutaraldehyde (Cat No. G7776-10ML) in DEPC water for 30 mins and wash with DEPC treated water. Air dry the dish for 30 mins.

3) Coat coverslips (18 mm; Cat No. 0111580) with Sigmacoat (Cat No. SL2-25ML) by adding a few drops of Sigmacoat in a fume hood for 1 min, washing twice with 100% ethanol, and letting the coverslips dry.

Finally, add the fixed and permeabilized tissues and embed them in a polyacrylamide gel (see gel composition below).

Hydrogel casting (**Figure 1C**)

A polyacrylamide gel (**Table 1**) is necessary to keep thick tissues in place during the multiple cycles of hybridization, washing, imaging, and alignment. The gel will slightly expand after buffers are added, so a gel can be cast that is narrower than the coverslip diameter of the confocal dish. The expansion factor, i.e., the diameter of the gel (Dgel) divided by the diameter of coverslips (Dcp) for different buffers, is provided in Fang et al. (2023).

Table 1. Composition of polyacrylamide gel.

S.N.	Reagent	Volume	Volume (small)
2.	NaCl (2M) (Cat No. S9888-25G)	400 µl	100 µl
3.	Acrylamide (Cat No. A9099-100G) (100mg/ml)	1200 µl	300 µl

4.	Tris-HCl 1M (Cat No. 10812846001)	120 µl	40 µl
5.	TEMED (Cat No. T9281-25ML)	10 µl	2 µl
6	Ammonium persulfate (2% w/v) (Cat No. 248614-100G)	20 µl	5 µl

Note: A smaller volume can be prepared based on user requirements.

Add around 70-100 µl of the above solution on top of the glass with the tissue samples. Add the Sigmacoated coverslip on top of the gel (**Figure 1C**). After gel solidification (**Figure S1_11**), remove the coverslip, and transfer the confocal dish to the arc chamber for automation or for manual experimentation (**Figure 1A**).

Pause step: The samples embedded in the gel can be stored either dehydrated or in 30% PHB buffer for over 2 months before starting the reaction.

Note: For clearing tissues, these can be left in the gel in 2% SDS, 0.5% Triton-X 100 in 2x SSC at 37°C overnight (Liu et al., 2022). Alternatively, the gel can also be left in 5% SDS+2% boric acid at 37°C overnight.

Procedure

Manual multiplexing protocol

A) Probe preparation

<u>Preparing the primary stock solution (10 pairs of probes: 5µM final volume)</u>

Up to 10 pairs of oligonucleotides were used, each at a concentration of 100 µM. 100 µl of oligos from each tube were mixed to form a master mix with a final probe concentration of 5 µM in a 2 ml microcentrifuge tube (Labselect, Cat No. MCT-001-200). Three to four genes with different hairpin amplifiers (Molecular Instruments) can be designed and tested in a single cycle of HCR. The number of genes tested in each cycle depends on the number of laser lines in the confocal being used. The stock mix of primary probes can be stored at -20°C.

<u>Prepare the 30% probe hybridization buffer or 20% EC hybridization buffer with probes complementary to the target RNA.</u>

Preparation of DNA oligo mix: Add 10 or 20 µl of each of the 5 µM DNA oligo mixes for each gene for 10 hrs and 4.5 hrs manual methodology respectively (e.g., add 10+10+10 µl or 20+20+20 µl for 3 genes) in a 1.5 ml tube and adjust the volume to 1000 µl in 30% probe hybridization buffer or 20% EC hybridization buffer in a 1.5ml microcentrifuge tube (Labselect, Cat No. MCT-001-150).

Note: If you are targeting a lowly expressed gene, you might need to either increase the concentration of the probes for that gene or the number of probe pairs, in the DNA oligo mix.

<u>Prepare the secondary probes with fluorescent tags.</u>

Add 5 µl of each H1 and H2 hairpins (Molecular Instruments) separately in 150+150 µl of Amplification buffer in two 200 µl PCR tubes.

Heat at 95°C for 90 secs in a thermocycler and cool down at room temperature in a dark environment for 30 mins. Mix the two tubes together in a 1.5 ml microcentrifuge tube (Labselect, Cat No. MCT-001-150) and use.

Note: We use the following combinations: 1) B1: AF546, 2) B2: AF647, 3) B3: AF 488. We have used B4 with an AF514 tag, which sometimes cross-talks with the AF546 fluorophore.

The secondaries can be used multiple times. After using the probes, collect them from the sample and store at -20°C. For repeated use, heat the secondary probes in the dark in amplification buffer at 37-42°C for 30 mins before use.

B) Steps

1) Dissect the tissue in 1x PBS at Room Temperature (RT ~ 22°C). For the dissection of butterfly larval wings, follow the protocol described in Banerjee and Monteiro, 2020b.

2) Transfer the tissue to either a 1.5ml tube or glass spot plate containing 500 µL 1x PBST supplemented with 4% formaldehyde and fix for 30-60 mins at room temperature (RT) with shaking. A smaller fixation time is required for thinner tissues, while a longer time is required for thicker tissues.

4) Wash the tissue 3 times with 500 µl 1x PBST (3mins each) at RT.

5) Add 500 µl of permeabilization solution and leave the tissue for 30 mins at 37°C.

Note: If clearing tissue (for transparency), the tissue can also be left in 500 µL of 10% SDS (Cat No. L3771-100G) + 2% boric acid (Cat No. B0394-100G) at 37°C for clearing for up to 10 hrs.

6) Wash 3 times with 500 µl 1x PBST (3 mins each) at RT.

7) Wash 2 times with 500 µl 5x SSCT (3 mins each) at RT.

Note: At this step you can either embed the sample in the acrylamide gel mentioned above or perform the experiment with the tissue free-floating in the buffer. For the free-floating experiments, the reactions are carried out on glass spot plates (PYREX™; Corning, Corning, NY, USA; Cat. No.: 722085) or in 1.5 ml microcentrifuge tubes (Labselect, Cat No. MCT-001-150).

8) Transfer the tissues to 500 µl 30% probe hybridization buffer or 20% EC hybridization buffer and incubate at 37°C for 30 mins.

Pause step: You can store the tissue in 30% probe hybridization buffer at 4°C for over 8 weeks without any significant reduction in signal quality. Do not store samples in the 20% EC hybridization buffer, as the solution will crystallize at 4°C.

9) Prepare 1000 µl 30% probe hybridization buffer or 20% EC hybridization buffer supplemented with DNA oligos complementary to the target RNA (**described above**).

10) Remove the 30% probe hybridization buffer or 20% EC hybridization buffer added before and replace it with 500µl of the solution mentioned in step 9 supplemented with DNA oligos at 37°C.

12) Incubate the samples at 37°C for 4 hrs (for the detection of lowly expressed genes, the samples can be left overnight). For faster detection, primary incubation can be as short as 2 hrs.

13) Wash 6 times with 500 µl of 30% probe wash buffer or 20% EC wash buffer (5-10 mins each) at 37°C.

15) Wash 2 times with 500 µl 5x SSCT (3 mins each) at 37°C.

16) Incubate the tissue in 500 µl Amplification buffer for 30 mins at 37°C.

Pause step: You can store the tissue in Amplification buffer at 4°C for over 8 weeks without any significant reduction in signal quality.

17) Replace the Amplification buffer with 200 µl secondary probes (**described above**) at 37°C.

Note: It is critical to keep the sample away from light as much as possible from this step onwards.

18) Incubate the samples in the dark for 3 hrs at 37°C. For faster detection, secondary incubation can be as short as 1.5 hrs incubated at 37°C.

19) Wash the tissue 5 times with 500 µl 5x SSCT (in a dark environment) for 5 mins each at 37°C.

Note: If you want to stain nuclei, add 5 µL of DAPI to 500 µL 5x SSCT and incubate for 5 mins. Afterwards, wash 2 times with 500 µL 5x SSCT for 3 mins each.

Pause step: You can store the tissue in 5x SSCT at 4°C for over 8 weeks without any significant reduction in signal quality prior to imaging.

20) Replace the 5x SSCT with fluorescent mounting buffer or media and proceed for confocal imaging.

Imaging: Imaging was carried out using an Olympus FV3000 confocal microscope with 405 nm, 488 nm, 555 nm, and 647 nm lasers. Images were captured at 2k resolution using 10x and 20x objective lenses.

Note: If you are performing the experiment with free floating tissue in buffer, mount the sample on a slide, add mounting media, place the coverslip, and proceed for imaging. Do not seal the coverslip. After imaging, remove the coverslip and wash with 5x SSCT to remove the sample from the glass slide. For experiments on microtome sectioned tissues (10-50 µm sections) on coverslips, all the reactions and washes can be directly performed on the coverslips. If you are performing the experiment in the acrylamide gel, add imaging buffer and proceed for imaging.

Subsequent cycles

21) For the second cycle of hybridization with a new set of probes, you need to remove the first set of probes. To remove the signal, first wash the tissue two times with 500 µl 5xSSCT for 3 mins at RT.

22) Wash 3 times with 500 µl signal wash buffer 1 at 37°C for 3 mins each.

23) Add 500 µl of signal removal solution and incubate at 37°C for 15-90 mins (depending on the thickness of the tissue). For wings, signals were removed within 15 mins, and for the zebrafish embedded in acrylamide gel, signal removal was carried out for 60-90 mins.

24) Wash 2 times with 500 µl signal wash buffer 2 at 37°C for 2 mins each.

25) Wash 3 times with 500 µl signal wash buffer 1 at 37°C for 3 mins each.

26) Wash 2 times with 500 µl 5x SSCT 1 at 37°C for 3 mins each.

Pause step: You can store the tissue in 5x SSCT at 4°C for over 8 weeks without any significant reduction in signal quality.

Note: Image the tissue after signal removal to estimate the time required for signal removal. If additional time is necessary, repeat from step 16. The acrylamide gel-based method usually requires a longer time compared to the free floating tissues. After signal removal, repeat from step 7.

Protocol on automation platform: Total time 4.5 hrs or 8 hrs.

A) Probe preparation

Preparing the primary stock solution (10 pairs of probes: 5µM final volume)

Up to 10 pairs of oligonucleotides were ordered from IDT for one gene, each at a concentration of 100 µM. 100 µl of oligos from each tube were mixed to form a master mix with a final probe concentration of 5 µM in a 2ml microcentrifuge tube (Labselect, Cat No. MCT-001-200). Three to four genes with different hairpin amplifiers (Molecular Instruments) can be designed and tested in a single cycle of HCR (Choi et al., 2018). The number of genes tested in each cycle depends on the number of laser lines in the confocal being used. The stock mix of primary probes can be stored at -20°C.

Prepare the 20% EC hybridization buffer with probes complementary to the target RNA

Preparation of DNA oligo mix: Add 5 or 10 µl of each of the 5 µM DNA oligo mix if running for 8 hrs or 4.5 hrs reactions respectively, and adjust the volume to 1000 µl in 20% EC or 30% PHB buffer in an 1.5 mL microcentrifuge tube (Labselect, Cat No. MCT-001-150).

Prepare the secondary probes with fluorescent tags

Add 5 µl of each H1 and H2 hairpins (Molecular Instruments) separately in 150+150 µl of Amplification buffer in two 200 µl PCR tubes.

Heat at 95°C for 90 secs in a thermocycler and cool down at room temperature in a dark environment for 30 mins. Mix the two tubes together in a 1.5 ml microcentrifuge tube (Labselect, Cat No. MCT-001-150) and use.

Note: You can also prepare a larger volume of H1 and H2 hairpins and store at -20°C for future use. The secondaries can be used multiple times. After the reaction, collect them from the secondary collection tube and store at -20°C. For repeated use, heat the secondary probes in amplification buffer at 42°C for 30 mins before use.

B) Steps

Fixation and permeabilization steps (done manually):

1) Dissect the tissue in 1x PBS at room temperature.

2) Transfer the tissue in 500 µl 1x PBST supplemented with 4% formaldehyde and fix for 30-60 mins at room temperature (RT) with shaking. A smaller fixation time is required for thinner tissues, while a longer time is required for thicker tissues.

4) Wash the tissue 3 times with 500 µl 1x PBST (3mins each) at RT.

5) Add 500 µl of detergent solution or alternative permeabilization buffer and leave the tissue for 30 mins at 37°C.

6) Wash 3 times with 500 µl 1x PBST (3 mins each) at RT.

7) Wash 2 times with 500 µl 5x SSCT (3mins each) at RT.

8) Transfer the sample to a 3.5mm confocal dish (SPL, Cat No. 240202783).

Note: At this step you can either embed the sample in the acrylamide gel mentioned above or perform the experiment with the tissue free-floating in the buffer.

9) Transfer the tissue to 500 µl 30% probe hybridization buffer or 20% EC hybridization buffer at RT.

Pause step: Tissues can be stored in 30% probe hybridization buffer at 4°C for 8 weeks without any significant loss of signal. Do not store samples in 20% EC hybridization buffer as the solution will crystallize at 4°C.

10) Add all the buffers to the separate wells of the Probe module (**Figure S2_1F**) in the thermo-fluidics robot – Geneplexer.

i) 5x SSCT: 15ml.

ii) 20% EC Wash buffer: 10 ml.

iii) Amplification buffer: 700 µl.

iv) 20% EC hybridization buffer: 700 µl.

v) 20% EC hybridization buffer with probes: 1000 µl.

vi) Secondary fluorescence probes in amplification buffer: 300 µl.

vii) DAPI buffer: 700 µl.

viii) Imaging buffer: 700 µl.

11) Transfer the confocal dish with the sample to the arc chamber and make sure the microcomb chip fits perfectly on top of the confocal dish.

12) Turn on the fluidics cycle by pressing the appropriate button (see below).

<u>Geneplexer instruction:</u>

Button 1: For running 4.5 hrs FISH cycle: Press the Cycle (4.5 hrs) button. This runs a script that performs 4.5 hrs of RAM-FISH.

Button 2: For running 8 hrs FISH cycle: Press the Cycle (8 hrs) button. This runs a script that performs 8 hrs of RAM-FISH.

Button 3: For calibration: This will execute a script that is used for pump calibration.

Button 4: For cleaning: Press the Clean button. This will execute a script to flush any remaining buffer/probes in all the tubes. After each reaction, load 1 ml of the robot cleaning solution to clean all the tubing.

Note: The system is designed to collect the secondary fluorescent probes in a separate container. The collected probes can be reused 2-3 times (optional). The script is fully customizable based on the user's needs.

12) **Imaging:** Take the microscope plate with the confocal dish under a confocal microscope and perform the imaging. In the present work, imaging was carried out using an Olympus FV3000 confocal microscope with 405 nm, 488 nm, 555 nm, and 647 nm lasers. Images were captured at 2k resolution using 10x and 20x objective lenses. **Note:** In case of drying, rehydrate in 5x SSCT and wash 2 times in 5x SSCT, followed by the addition of imaging buffer before confocal imaging.

Pause step: You can store the tissue in 5x SSCT at 4°C after the reaction for over 8 weeks without any significant reduction in signal quality.

13) Remove the signal using RemBot. Load the following buffers in the RemBot buffer plate.

i) Signal wash buffer 2: 10 ml.

ii) Signal removal buffer: 500 µl.

iii) Signal wash buffer 1: 500 µl.

iv) 5x SSCT: 10 ml.

<u>RemBot instruction (buttons):</u>

Button 1: For Signal removal. This will remove the signal from the sample.

Button 2: For calibration: This will execute a script that is used for pump calibration.

Button 3: For cleaning: Press the Clean button. This will execute a script to flush any remaining buffer/probes in all the tubes. After each reaction, load 1 ml of DEPC treated water to clean all the tubing.

Authors contribution

TDB: Conceptualization, hardware design, programming, simulations, methodology (butterfly wings, zebrafish), writing - original draft, writing -review and editing. JR: Methodology (zebrafish), writing – review and editing. JLCH: Methodology (butterfly wing, zebrafish). KHC: Supervision, writing – review and editing, funding. ASM: Supervision, writing – review and editing, funding. AM: Conceptualization, supervision, funding, writing – review and editing.

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Conflict of Interest

A provisional patent involving the arc chamber and the automation system mentioned in the present methodology has been filed.

Availability of resources

All the data mentioned in the present method are described in the supplementary information. GitHub link:

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