A spatial mRNA profiling workflow using Rapid Amplified Multiplex-FISH (RAM-FISH)

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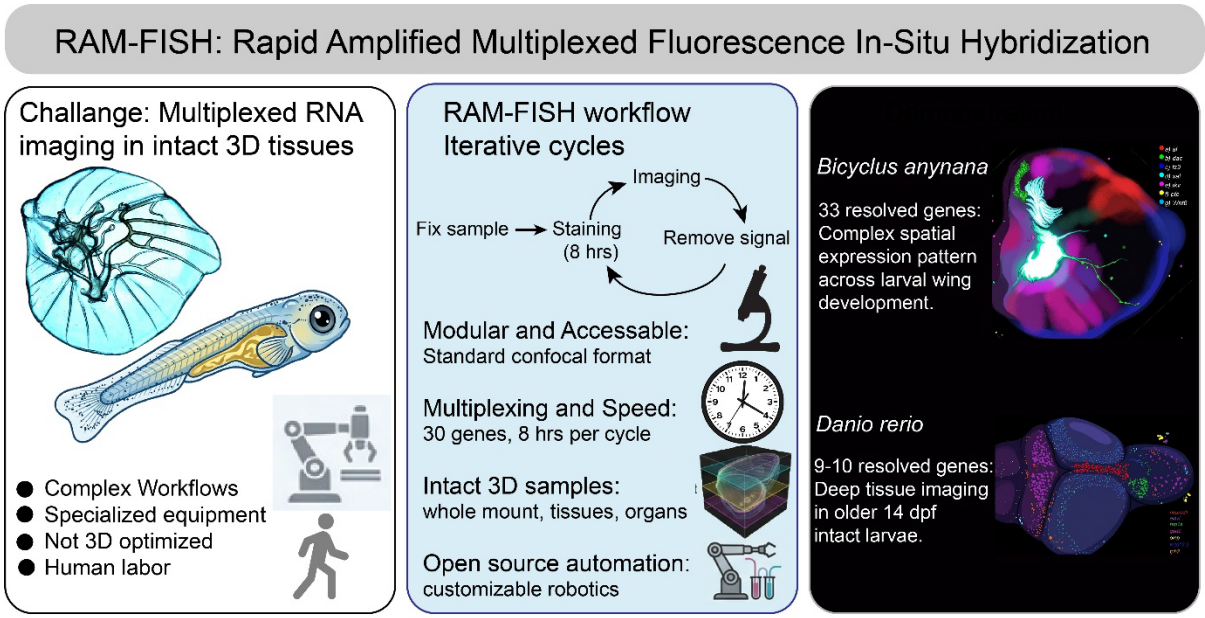
Abstract

Spatial localization of multiple RNA transcripts in intact tissues can reveal gene regulatory interactions that shape development and function. However, multiplex RNA imaging remains challenging to implement in most laboratories because existing techniques often require complex workflows, specialized equipment, or methods that are not optimized for 3D tissue samples. Here, we describe Rapid Amplified Multiplexed Fluorescence In-Situ Hybridization (RAM-FISH), a modular workflow for multiplexed spatial RNA detection in intact tissues, organs, and whole-mount specimens. RAM-FISH achieves multiplexing through iterative cycles of hybridization and imaging, enabling detection of three to four genes per cycle and more than 30 transcripts within the same sample. The workflow is compatible with standard confocal microscopy and performed either manually or using a fluidics automation system. We demonstrate RAM-FISH by visualizing transcription dynamics in developing *Bicyclus anynana* butterfly wings and intact 14-days-post-fertilization zebrafish larvae. In both, RAM-FISH resolved multi-gene spatial expression patterns in intact tissues that were previously inferred through independent experiments as well as genes characterized for the first time. Together, RAM-FISH offers an accessible open source platform for multiplex spatial transcript detection in diverse experimental systems.

Keywords

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

Graphical Abstract



Introduction

Multicellular organisms are organized through dynamic spatial and temporal patterns of gene expression. Visualizing the localization of RNA molecules within intact tissues provides critical insight into developmental processes, tissue organization, disease progression, and the structure of gene-regulatory networks. As a result, there is growing interest in methodologies that enable the simultaneous detection of multiple transcripts while preserving spatial context (Chen et al., 2015; Marx, 2021; Moffitt et al., 2022; Ståhl et al., 2016; Tian et al., 2023). Such approaches are particularly valuable for studying tissue differentiation, mapping regulatory interactions, and linking gene expression patterns to morphological outcomes.

To support these applications, RNA imaging workflows must provide reliable signal detection while remaining practical to implement across diverse sample types. Methods that minimize extensive sample preparation and can be applied to intact tissues, organs, or whole organisms are especially important for extending spatial transcript analysis to a broader range of biological systems.

Recent technological advances now enable spatially resolved transcriptomic profiling at single-cell or subcellular resolution while preserving tissue architecture (Moffitt et al., 2022; Moses and Pachter, 2022; Tian et al., 2023). These approaches broadly fall into two categories: sequencing-based methods such as Visium (Ståhl et al., 2016), Slide-seq (Rodriques et al., 2019), and Stereo-seq (Chen et al., 2022); and imaging-based methods that use fluorescently labelled probes for transcript detection such as MERFISH (Chen et al., 2015), seq-FISH (Lubeck and Cai, 2012), osmFISH (Codeluppi et al., 2018), STARmap (Wang et al., 2018), cycleHCR (Gandin et al., 2024), EASI-FISH (Wang et al., 2021), PRISM (Chang et al., 2024), and FISH&CHIPS (Zhou et al., 2023). While these technologies have greatly expanded the scope of spatial transcriptomics, their implementation can be challenging in certain experimental contexts. Many workflows involve extensive sample preparation, specialized instrumentation or proprietary platforms, or are optimized primarily for thin tissue sections. These requirements can limit routine application in laboratories working with thick tissues, whole-mount specimens, or non-model organisms.

In this study, we apply RAM-FISH workflow to enable iterative spatial detection of mRNA transcripts within intact tissues through repeated cycles of hybridization, imaging, and signal removal. Using this approach, we generated spatial expression maps across multiple developmental stages of *Bicyclus anynana* butterfly wings and in intact larval *Danio rerio*. These datasets reveal reproducible spatial domains for both well-characterized as well as previously unexamined genes, demonstrating the utility of the workflow for investigating complex gene expression patterns in 3D tissues. Together, these results illustrate how iterative multiplex imaging can provide accessible spatial gene expression maps across diverse biological systems.

Workflow Overview

The RAM-FISH workflow consists of tissue collection, fixation, permeabilization, and iterative cycles of probe hybridization, imaging, and signal removal (**Figure 1A**). Depending on experimental requirements and available infrastructure, staining can be performed either manually or using an optional open source automated fluidic system(https://www.biorxiv.org/content/10.64898/2026.04.17.713973v1). Both approaches support repeated hybridization cycles for multiplex transcript detection within the same sample.

A. Manual workflow

In the manual implementation, samples are processed using standard laboratory containers and buffers. Two configurations are supported (**Figure 1A**).

In the free-floating configuration, tissues are maintained in solution throughout hybridization and washing steps. This approach is well suited for single-cycle experiments or applications where precise spatial registration across cycles is not required.

For multi-cycle experiments requiring image registration, samples can be immobilized by embedding them in an acrylamide-based gel within a glass-bottom imaging dish. All hybridization and washing steps are performed directly in the dish, and imaging is carried out between cycles. Immobilization improves spatial stability and facilitates alignment of gene expression patterns across sequential imaging rounds.

B. Automated workflow

For automated hands free experimentation, RAM-FISH can be performed using an optional open source microcontroller-based fluidic system that enables programmable reagent exchange and temperature control (**Figure 1A**). The automation platform supports both free-floating and gel-embedded sample configurations.

In this system, hybridization, washing, and signal removal steps are carried out under controlled fluidic conditions, after which samples are imaged and returned to the system for subsequent cycles. This configuration enables consistent execution of multicycle experiments while reducing manual intervention.

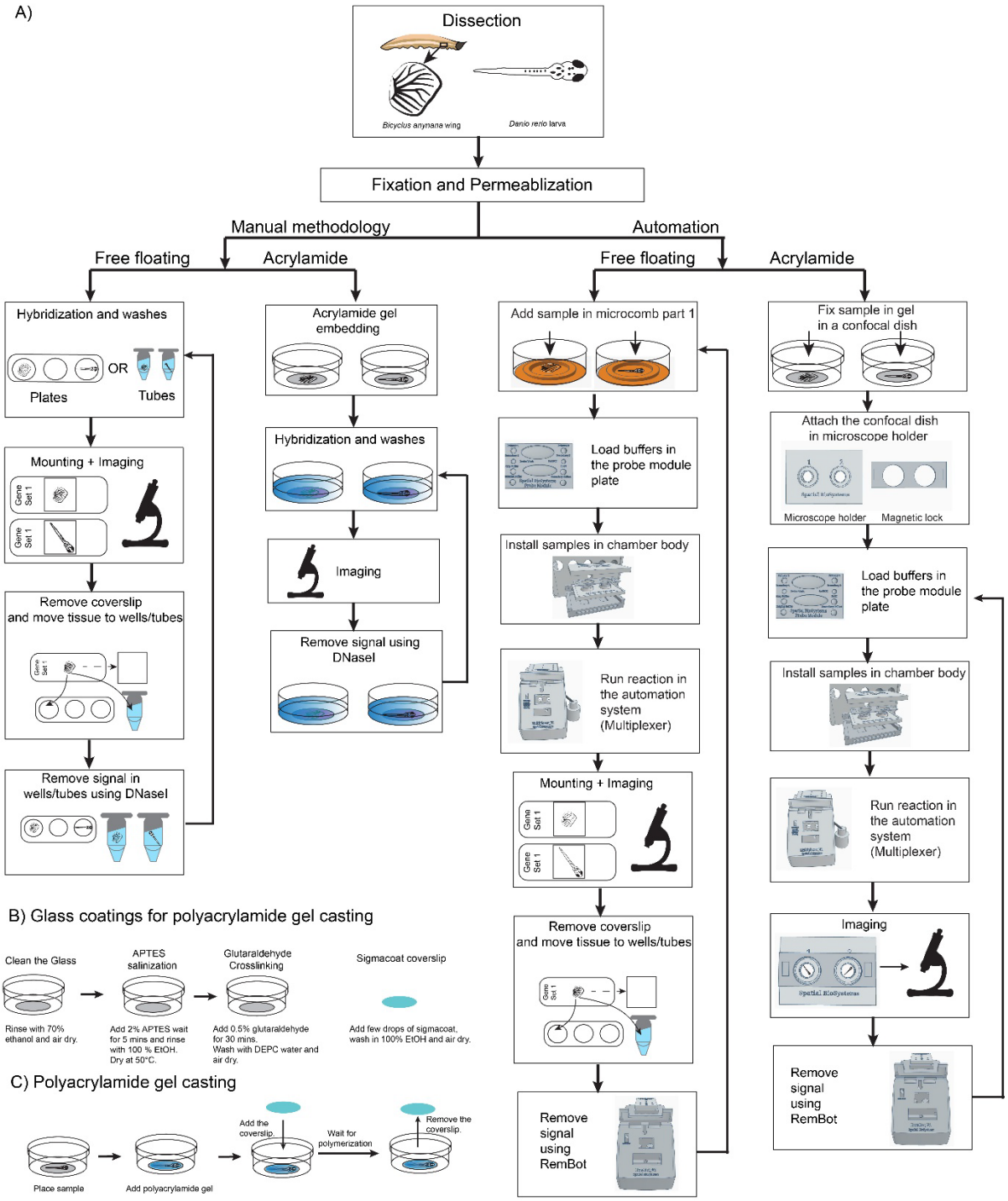


Figure 1: Illustration of the manual and the automation-based multiplexing protocol. (A) Tissues are dissected, fixed, and permeabilized. Afterwards, multiplexing can be performed either manually or using an automation system. (**B**) Simplified method of glass coating and (**C**) method for polyacrylamide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

Visualizing multiple genes on developing butterfly larval wings

We first implemented the RAM-FISH workflow to investigate spatial expression patterns of multiple genes in developing larval wings of *Bicyclus anynana*. A major limitation of traditional mRNA detection protocols in butterfly evolutionary and developmental biology is their restriction to detecting only one to three genes per experiment. This constraint significantly limits the amount of information that can be extracted within a specific developmental context and hampers efforts to dissect gene regulatory networks, particularly in experiments combining CRISPR mediated gene knockout with downstream staining analyses (Matsuoka and Monteiro, 2022; Murugeshan et al., 2022).

Using RAM-FISH, we successfully detected the expression of 21 previously characterized genes and 12 novel genes in *B. anynana* across multiple developmental stages ranging from 0.75 to 3.25 (wing staging as described in (Banerjee and Monteiro, 2020; Reed et al., 2007)). This enabled us to systematically explore gene expression dynamics over 10-14 iterative cycles of hybridization and washing (**Figure 2 and 3; Figure S1 to S6**), demonstrating the robustness and multiplexing capacity of the workflow.

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). The known genes were *Wnt1, Wnt6, 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 and 3; Figure S1 to S6; Video S1**). The 12 new genes for *B. anynana* were *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 S2** 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 S2**.

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 S1, S2, S4, S5**).

2) *homothorax (hth)* expression was newly visualized along two bands along the proximal domain of the developing larval wings (**Figure 2G, Figure 3F, 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 2G, Figure 3J, Figure S1_3, Figure S1_5, Figure S1_6)** and in an anterior proximal domain, with slightly higher expression in the lower posterior compartment on the forewing during wing development (**Figure 2L, Figure S1_2, Figure S1_4**).

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 S1_1 to S1_6**).

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 S1_1 to S1_6**).

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 S1_1 to S1_6**).

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 S1_1 to S1_6**).

8) *eyegone (eyg)* was newly visualized along the wing margin in intervein cells (**Figure 2AC, Figure S1_1 to S1_6**).

9) *bric-a-bac (bab)* was newly visualized in larval wings, expressed in distinct domains in the anterior compartment (**Figure 2AD, Figure 3V, Figure S1_1 to S1_6**). 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 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 S1_1 to S1_6**).

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 S1_1 to S1_6**).

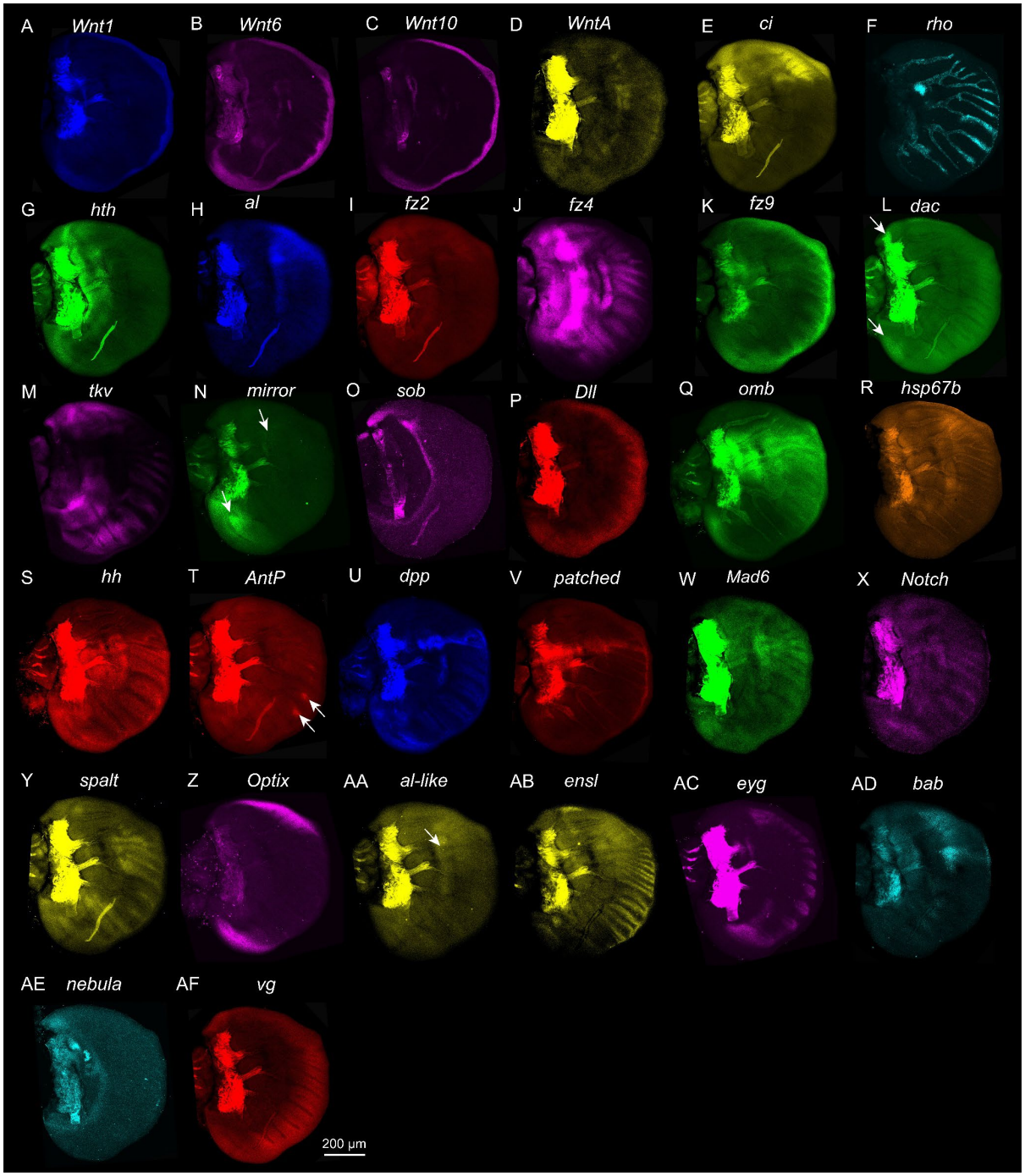


Figure 2: Detection of 32 genes (mRNAs) in a single early larval <u>forewing</u> of *Bicyclus anynana* (stage 1.00) using the manual, free-floating method. (**A-AF**) Expression of multiple target genes (arrows indicate small expression domains). Note: The tracheal tissue along the veins and in the proximal domain are autofluorescent.

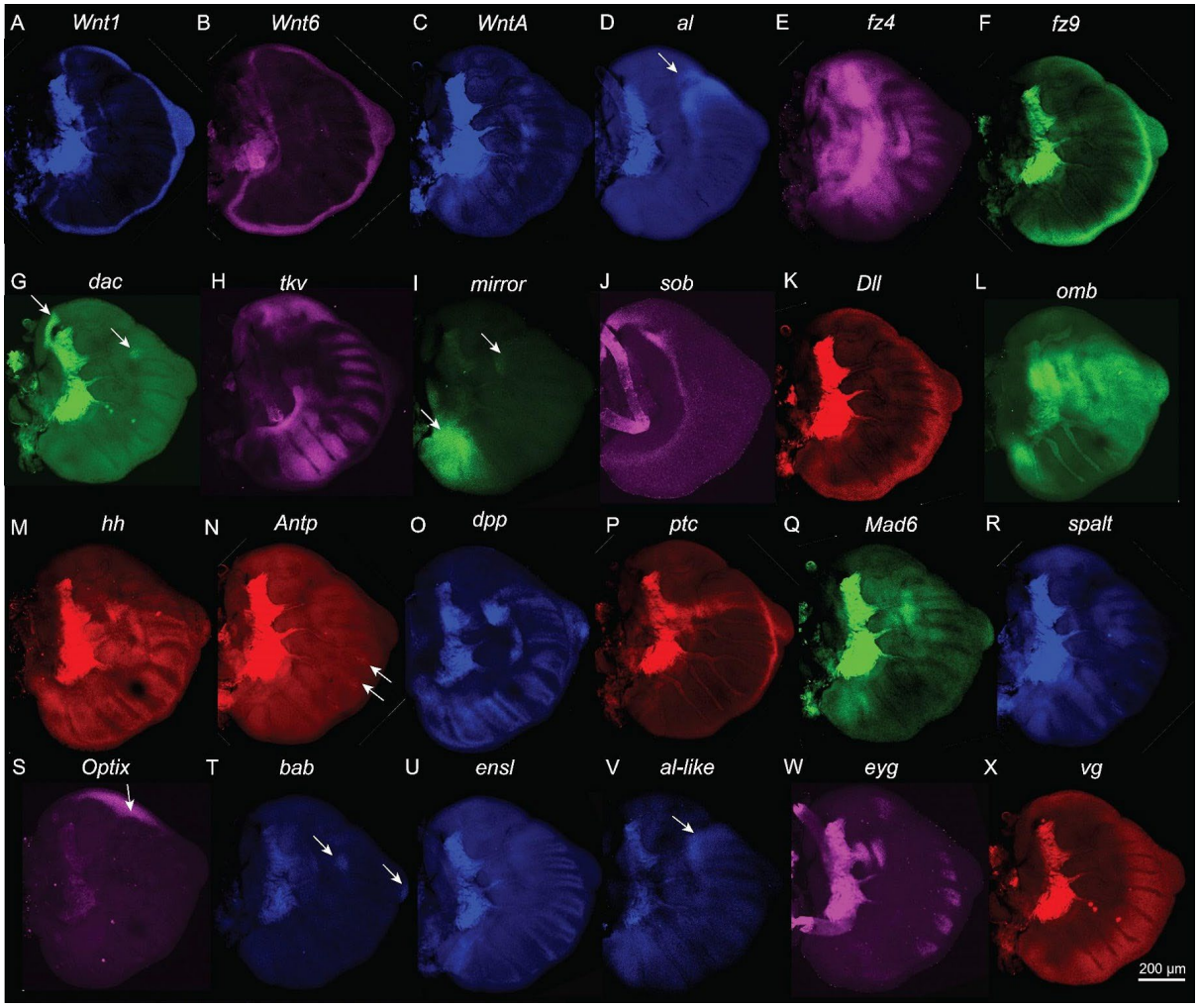


Figure 3. Expression of 24 genes (mRNAs) in a single late larval <u>hindwing</u> of *Bicyclus anynana* (stage 1.00) using the manual, free-floating method. (**A-X**) Expression of multiple target genes (arrows indicate small expression domains). Note: The tracheal tissue along the veins and in the proximal domain are autofluorescent.

Multiplexing in *Danio rerio* (zebrafish) larvae

Zebrafish are commonly used vertebrate laboratory models for fundamental studies including neurobiology (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 of the brain add unique insights into circuit level connections between brain regions that bulk and single cell RNA sequencing alone cannot reveal. This was achieved recently by registering *in situ* results from individual stainings of young larvae (< 6 dpf) to a standard reference brain (Schulze et al., 2023; Shainer et al., 2023). Maps of gene expression in late-stage larvae or juvenile fish brains, however, when complex behaviors including learning and social behaviors emerge, are limited or absent (Gemmer et al., 2022). We spatially profiled 9-10 genes using both the manual and the automation system on 14 days post fertilization (dpf) larvae to examine gene expression in the brain (**Figure 4; Figure S8-S13, S15, and S16; Video 1 and 2**). We examined genes critical to neurotransmitter synthesis such as *tph2 (seratonin), chatb (acetylcholine), oxt (oxytocin),*, and *gad2 (GABA)*, receptor genes like *nrp1a (semaphorin & VEGF)* and *kctd12.2 (GABA),* and neurodevelopmental markers such as *opm* (olfactory bulb), *neurod1,*

and elavl3 (neuronal differentiation)., Overall, the expression patterns observed are consistent with previous reports available at mapZebrain (Shainer et al., 2023), or Zebrafish Information Network (ZFIN; **Table S2**) 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 S14**). The 10 profiled genes were: *glutamate decarboxylase 2 (gad2*), *neuropilin 1a (nrp1a), neuronal differentiation 1 (neurod1), olfactory marker protein b (omp*), *tryptophan hydroxylase 2* (*tph2), oxytocin (oxt*), *potassium channel tetramerisation domain containing 12.2 (kctd12.2), 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 S3**.

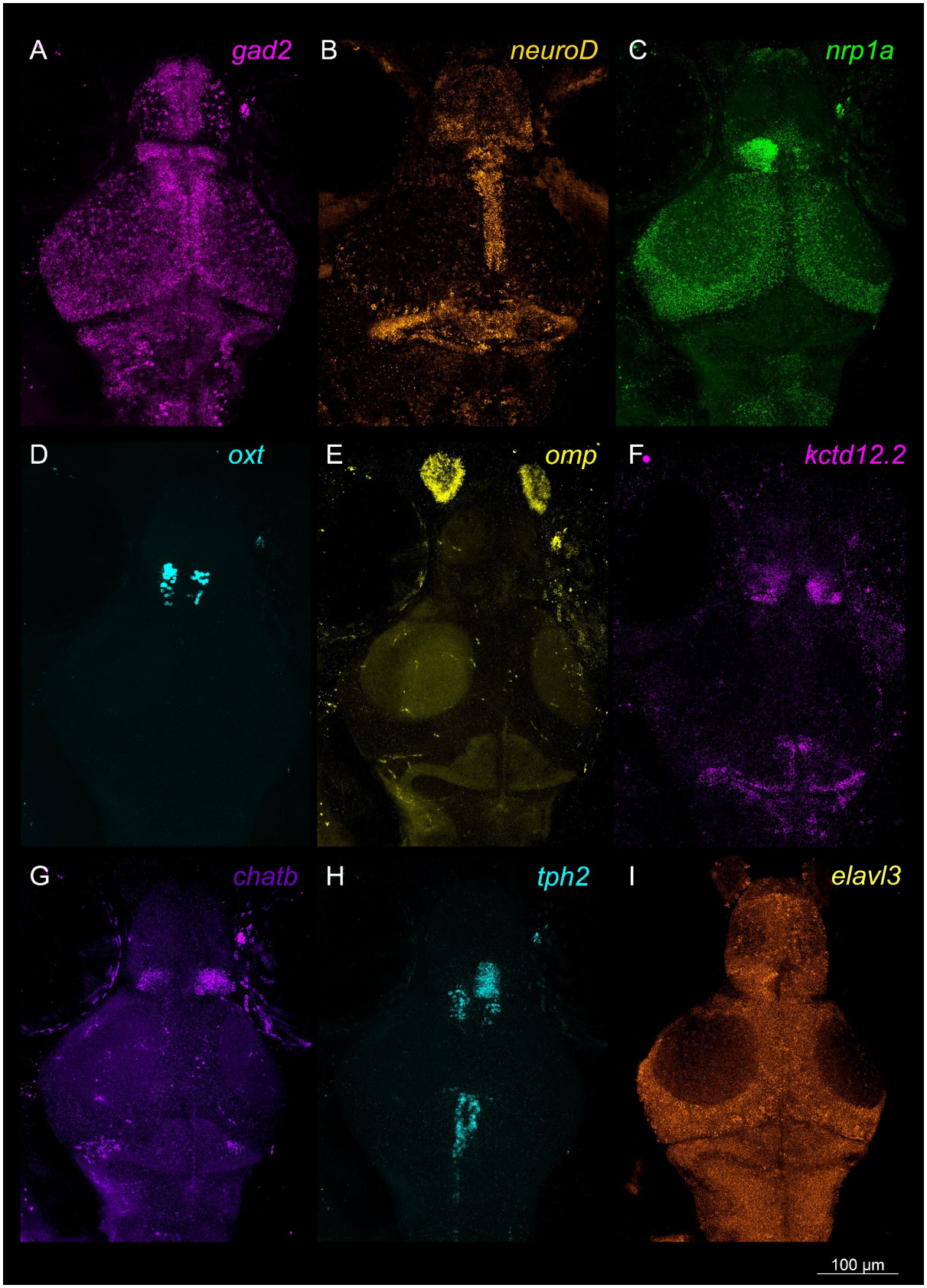


Figure 4. Detection of multiple genes (mRNAs) in a single 14 dpf *Danio rerio* larva across ~190 µm depth (maximum projection of multiple z-stacks). (A-I) Multiplexed expression of *gad2, nrp1a*, *neurod1, oxt, omp*, *kctd12.2, chatb, tph2,* and *elavl3* in a 14 dpf *Danio rerio* larva. Reactions were carried out in the gel embedding format.

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

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

Discussion

Spatial RNA profiling technologies have rapidly advanced, enabling the localization of large numbers of transcripts within tissues and accelerating discoveries across developmental biology, neurobiology, and cancer research (Choi et al., 2023; Coutant et al., 2023; Jung and Kim, 2023; Moffitt et al., 2018; Rao et al., 2021; Zhang et al., 2021). Despite their advantages, many current platforms remain difficult to adopt widely due to high costs, specialized instrumentation, proprietary reagents, lengthy experimental workflows, complex computational requirements, and the need for thin tissue sections. These constraints limit their accessibility and restrict their application to intact organs or whole-mount specimens.

To address these challenges, we developed RAM-FISH, a flexible and accessible spatial profiling workflow designed for use with standard laboratory equipment and commonly available reagents. The method builds on established hybridization chain reaction chemistry (Choi et al., 2018) and integrates known modifications that enable signal removal (Codeluppi et al., 2018; Shah et al., 2016; Sternson et al., 2022; Wang et al., 2021) and new modifications in hybridization and permeabilization buffers for efficient iterative probing, allowing multiplexed detection across multiple cycles within the same sample. Importantly, RAM-FISH is compatible with whole tissues and intact specimens, expanding spatial mRNA detection beyond thin sections.

By combining iterative multiplexing with optional automation and simplified workflows, the method provides a practical balance between experimental scalability and ease of implementation. As demonstrated here in developing butterfly wings and whole zebrafish larvae, RAM-FISH enables spatial analysis of multiple genes in complex three-dimensional specimens and can be applied across diverse biological systems using either manual or automated workflows.

RAM-FISH will be broadly useful to researchers who require routine spatial analysis of multiple genes (<30) within intact tissues, including developmental biologists, developmental geneticists, and disease researchers. The method enables studies of tissue patterning, embryonic development, and the localization of gene-regulatory networks by providing spatial context for the coordinated expression of dozens of transcripts within the same specimen. Importantly, multiplexed spatial readouts generated by RAM-FISH can be integrated with functional perturbation approaches such as CRISPR–Cas9 (Jinek et al., 2012; Matsuoka and Monteiro, 2022; Murugeshan et al., 2022), RNA interference (Fire et al., 1998), or pharmacological treatments to assess how manipulation of individual genes influences downstream expression programs in-situ. This capability enables efficient mapping of regulatory relationships within their native tissue environment.

In addition, RAM-FISH complements existing transcriptomic approaches, including bulk RNA-seq (Pertea et al., 2016), single-cell sequencing (Prakash et al., 2024) and spatial capture-based technologies (such as LCM-Seq (Banerjee et al., 2022)), by providing high-resolution, image-based validation and

contextualization of candidate gene localizations. Together, these features position RAM-FISH as a practical tool for bridging functional genetics and spatial systems-level analysis as demonstrated by atlases of multiple known and newly visualized genes in *B. anynana* larval wings, as well as larval zebrafish brains, which can be used as references in future research.

In butterfly wings, both well-characterized patterning genes and previously unexamined transcripts displayed consistent and reproducible spatial domains across multiple samples. These domains aligned with established developmental axes and signaling territories. For example, compartment-specific regulators such as *hh* and *ci* showed anterior-posterior (A/P) restricted expression consistent with their roles in A/P patterning (Banerjee and Monteiro, 2020; Schwartz et al., 1995), while *mirror* localized to lower posterior compartment cells associated with regional differentiation (Chatterjee et al., 2024). Along the proximal–distal axis, genes including *WntA* and its receptor *fz2* marked territories implicated in the specification of central symmetry system (CSS) bands (Banerjee et al., 2023; Hanly et al., 2023). Margin-associated patterning genes such as *Wnt1, vg*, and *Dll* delineated distal boundary regions (Banerjee et al., 2023), whereas vein-related transcripts including *rho* and *ensl* were enriched in vein or provein territories (Banerjee and Monteiro, 2020; De Celis et al., 1997).

Similarly, in zebrafish larvae, genes associated with neurotransmitter synthesis (*tph2, oxt, gad2, and chatb*), receptors (*nrp1a, and kctd12.2),* and neurodevelopmental markers *(elavl3, neurod1, and omp)* exhibited spatial distributions consistent with known anatomical and functional organization of the brain (Shainer et al., 2023). Signal detection was equally robust regardless of tissue depth or spatial specificity. Genes expressed deep within the brain, such as *oxt* and *tph2* (~100-150 µm)*,* were clearly visible at a cellular level, on par with those localised to the dorsal surface, such as *neurod1* and *nrp1a*, providing confidence that RAM-FISH is well suited to thick tissue and whole mount applications.

The high reproducibility of expression patterns across samples and cycles supports the robustness of the methodology for multi-round imaging in intact tissues. By enabling routine mapping of multiple transcripts within the same specimen, RAM-FISH offers a practical framework for linking functional genetics with spatial systems-level analysis across diverse model organisms. We hope these results will inspire researchers to test the method in their own systems.

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, 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).

Image alignment and composite image creation

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

carried via free-floating method 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. Note: No custom pipeline has been developed for auto image alignment of the free-floating version of samples in this version of the workflow.

For composite image generation (**Figure 5**), we developed a python based script to merge pre-aligned single channel images of individual genes (up to 10 genes) acquired during the separate hybridization cycles (**merge.py**). The format of these images 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 (https://github.com/tdblab/RAMFISH). 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
```

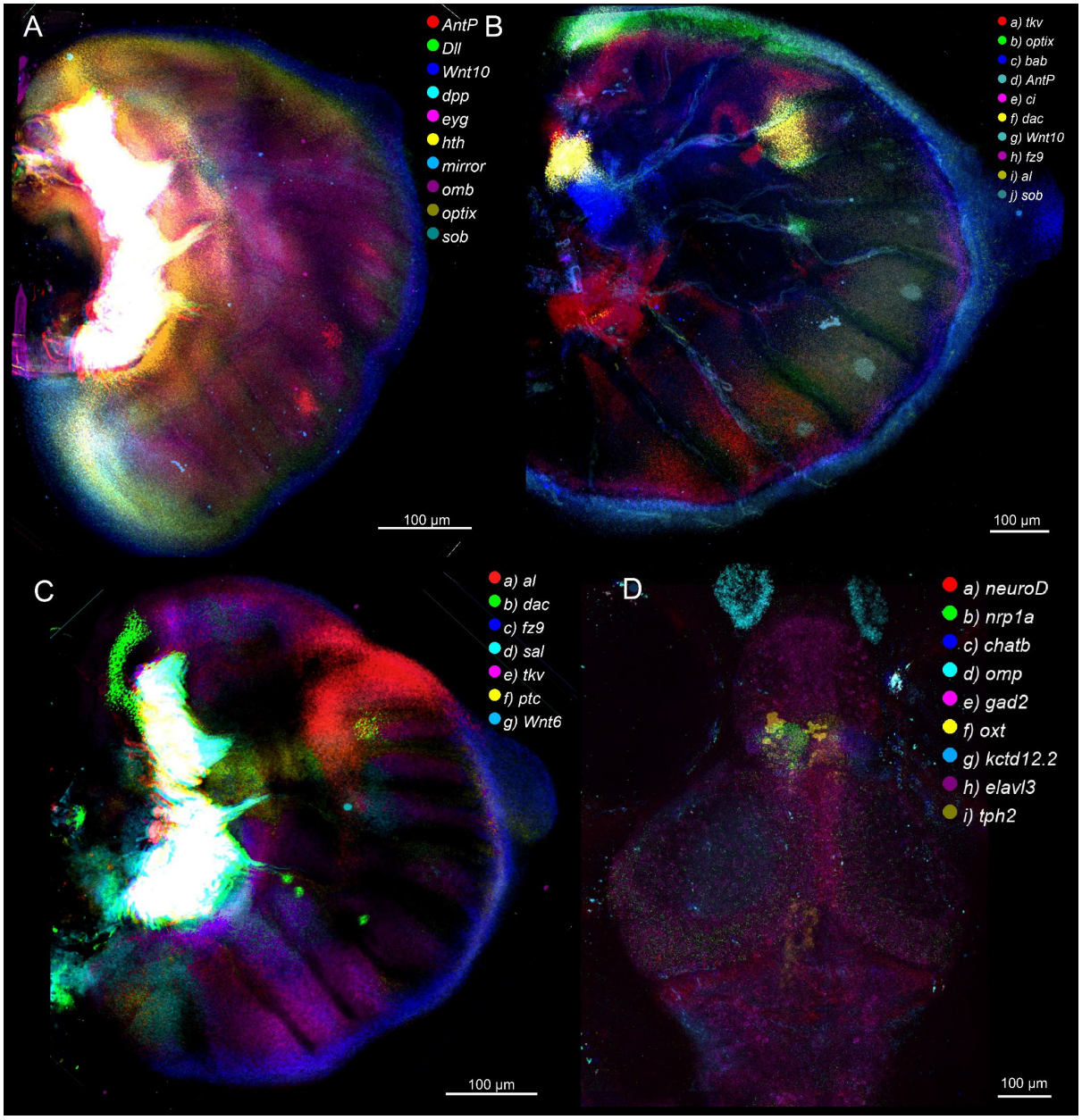


Figure 5: Composite *Bicyclus anynana* and *Danio rerio* RAM-FISH images created using the merge.py script. (A) Merged image of *AntP, Dll, Wnt10, dpp, eyg, hth, mirror, omb, optix,* and *sob* multiplexed expression in a 0.75 stage butterfly larval forewing. **(B)** Merged image of *tkv, optix, bab, 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).

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 S7).** Colocalization plots between the two images were generated using the 'Colocalization finder' plugin **(Figure S7 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 S7D, 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 S7A, B, E, F, I, and J).** Some quantitative reduction due to degradation of RNA levels was observable in the samples **(Figure S7D, H, and L)**.

Materials and labware used

Equipment

1) For the manual multiplexing protocol

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

Pipettes: Micropipette, 10-1000 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 (Multiplexer and RemBot): Custom-built (Construction article available at: https://www.biorxiv.org/content/10.64898/2026.04.17.713973v1).

Pipettes: Micropipette, 10-1000 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. 35 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).

Buffers

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

RAM-FISH buffers

20% EC hybridization buffer:

Composition: 20% v/v 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.

20% EC wash buffer:

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.

Imaging Buffer:

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.

Permeabilization Solution:

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 tissue such as embryos and larvae. Store at RT for up to 6 months.

Mounting Media:

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 incubated 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; optional): 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. Signal 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.

<u>30% probe wash buffer</u>

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.

<u>50% w/v dextran sulfate</u>

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.

<u>Amplification buffer</u>

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.

<u>Detergent Solution</u>

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.

<u>1x PBST (for 50 ml of solution)</u>

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

<u>5× SSCT (for 40 mL of solution)</u>

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 (35 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)
1.	NaCl (2M) (Cat No. S9888-25G)	400 µl	100 µl
2.	Acrylamide/Bisacrylamide 19:1 (A2917-100ML) (100mg/ml)	1200 µl	300 µl
3.	Tris-HCl 1M (Cat No. 10812846001)	120 µl	40 µl
4.	TEMED (Cat No. T9281-25ML)	10 µl	2.5 µl
5.	Ammonium persulfate (1% w/v) (Cat No. 248614-100G)	20 µl	5 µl

Note: A smaller volume can be prepared based on user requirements. If you need the gel to solidify faster use a larger volume of TEMED and APS.

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 S18**), remove the coverslip, and transfer the confocal dish to the 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 µl of each of the 5 µM DNA oligo mixes for each gene for 10 hrs and (Optional: or 20 µl for 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. Longer primary and secondary incubation might be necessary for lowly expressed genes.

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: We use the following combinations: 1) B1: AF546, 2) B2: AF647, 3) B3: AF488. 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 (optional).

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

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 µl of each of the 5 µM DNA oligo mix if running for 8 hrs (Optional: 10 µl of each of the 5 µM DNA oligo mix for 4.5 hrs) reactions, 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, thaw the secondary probes in amplification buffer before use. Longer primary and secondary incubation might be necessary for lowly expressed genes.

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 35 mm 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 in the fluidics robot with thermal control – Multiplexer.

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 (Primaries): 700 µl.

vi) Secondary fluorescence probes in amplification buffer (Secondaries): 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).

Multiplexer instruction:

Button 1: For running single sample FISH cycle: Press the “1 sample” button.

Button 2: For running dual sample FISH cycle: Press the “2 samples” button.

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 (available at: https://github.com/tdblab/RAMFISH).

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 1: 10 ml.

ii) Signal removal solution with DNAse I for first plate (SRS1): 500 µl.

iii) Signal removal solution with DNAse I for second plate (SRS2): 500 µl (optional).

iv) 5x SSCT: 10 ml.

RemBot instruction (buttons):

Button 1: For signal removal in a single sample setup.

Button 2: For signal removal in a dual sample setup.

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 DEPC treated water to clean all the tubing.

Authors contribution

TDB: Conceptualization, hardware design, programming, 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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Availability of resources
All the additional data mentioned in the present method are described in the supplementary files. GitHub link: https://github.com/tdblab/RAMFISH.

Accompanying article
An accompanying article describing the assembly and operation of the Multiplexer and RemBot automation systems is available. Link: https://www.biorxiv.org/content/10.64898/2026.04.17.713973v1

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