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Mapping a multiplexed zoo of mRNA expression

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ABSTRACT

In situ hybridization methods are used across the biological sciences to map mRNA expression within intact specimens. Multiplexed experiments, in which multiple target mRNAs are mapped in a single sample, are essential for studying regulatory interactions, but remain cumbersome in most model organisms. Programmable *in situ* amplifiers based on the mechanism of hybridization chain reaction (HCR) overcome this longstanding challenge by operating independently within a sample, enabling multiplexed experiments to be performed with an experimental timeline independent of the number of target mRNAs. To assist biologists working across a broad spectrum of organisms, we demonstrate multiplexed *in situ* HCR in diverse imaging settings: bacteria, whole-mount nematode larvae, whole-mount fruit fly embryos, whole-mount sea urchin embryos, whole-mount zebrafish larvae, whole-mount chicken embryos, whole-mount mouse embryos and formalin-fixed paraffin-embedded human tissue sections. In addition to straightforward multiplexing, *in situ* HCR enables deep sample penetration, high contrast and subcellular resolution, providing an incisive tool for the study of interlaced and overlapping expression patterns, with implications for research communities across the biological sciences.

KEY WORDS: *In situ* hybridization, *In situ* amplification, Hybridization chain reaction (HCR), Multiplexing, Deep sample penetration, High contrast, Subcellular resolution, Bacteria, Whole-mount embryos and larvae, Tissue sections

INTRODUCTION

The programmable molecular circuits that orchestrate life generate and exploit astonishing spatial complexity. *In situ* hybridization experiments provide biologists with a crucial window into the spatial organization of this circuitry by revealing the expression patterns of target mRNAs within cells, tissues, organs, organisms and ecosystems (Gall and Pardue, 1969; Cox et al., 1984; Tautz and Pfeifle, 1989; Rosen and Beddington, 1993; Wallner et al., 1993; Nieto et al., 1996; Thisse and Thisse, 2008). Because of stochastic variation between specimens, examination of intricate spatial relationships between interacting regulatory elements requires multiplexed experiments in which multiple target mRNAs are mapped with high resolution within a single specimen. However, decades after *in situ* hybridization became an essential research tool, multiplexed studies remain cumbersome or impractical in a variety of model and non-model organisms.

In a multiplexed experiment, the goal is to use N spectrally distinct reporter molecules to map N target mRNAs, yielding an N-channel image that permits detailed comparisons between channels. As spatial complexity increases, so too does the background arising from the sample, increasing the challenge in achieving high signal-to-background in each channel of a multiplexed image. The difficulty arises not from multiplexed target detection, but from multiplexed signal amplification. All N target mRNAs may be detected in parallel using N nucleic acid probe sets (each comprising one or more probes) that hybridize to orthogonal subsequences along the targets. If the background is sufficiently low, probes can be direct-labeled with reporter molecules to enable straightforward multiplexing (Kislauskis et al., 1993; Femino et al., 1998; Levsky et al., 2002; Kosman et al., 2004; Capodieci et al., 2005; Chan et al., 2005; Raj et al., 2008); in many settings, this approach does not yield sufficient contrast, so probes are instead used to mediate *in situ* signal amplification (Tautz and Pfeifle, 1989; Harland, 1991; Lehmann and Tautz, 1994; Kerstens et al., 1995; Nieto et al., 1996; Wiedorn et al., 1999; Player et al., 2001; Pernthaler et al., 2002; Thisse et al., 2004; Denkers et al., 2004; Kosman et al., 2004; Zhou et al., 2004; Larsson et al., 2004, 2010; Clay and Ramakrishnan, 2005; Barroso-Chinea et al., 2007; Acloque et al., 2008; Piette et al., 2008; Thisse and Thisse, 2008; Weiszmann et al., 2009; Wang et al., 2012).

Traditional *in situ* amplification approaches achieve high contrast using enzymes to catalyze reporter deposition (CARD) in the vicinity of probes (Tautz and Pfeifle, 1989; Harland, 1991; Lehmann and Tautz, 1994; Kerstens et al., 1995; Nieto et al., 1996; Pernthaler et al., 2002; Kosman et al., 2004; Thisse et al., 2004; Denkers et al., 2004; Clay and Ramakrishnan, 2005; Barroso-Chinea et al., 2007; Acloque et al., 2008; Piette et al., 2008; Thisse and Thisse, 2008; Weiszmann et al., 2009). A key difficulty is the lack of orthogonal deposition chemistries, necessitating serial amplification for each of N targets (Lehmann and Tautz, 1994; Nieto et al., 1996; Thisse et al., 2004; Denkers et al., 2004; Kosman et al., 2004; Clay and Ramakrishnan, 2005; Barroso-Chinea et al., 2007; Acloque et al., 2008; Piette et al., 2008). The resulting

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lengthy protocols lead to progressive sample degradation and are a significant hindrance to the study of endogenous biological circuitry. For example, it takes 4 days to map two target mRNAs in whole-mount zebrafish embryos (Thisse et al., 2004; Clay and Ramakrishnan, 2005) or 5 days to map three target mRNAs in whole-mount chicken embryos (Denkers et al., 2004; Acloque et al., 2008). A second common difficulty with traditional CARD methods is a loss of resolution resulting from diffusion of reporter molecules prior to deposition, leading to indistinct boundaries in the resulting mRNA expression maps (Tautz and Pfeifle, 1989; Thisse et al., 2004; Thisse and Thisse, 2008; Acloque et al., 2008; Piette et al., 2008; Weiszmann et al., 2009).

Programmable *in situ* amplification based on the mechanism of hybridization chain reaction (HCR) (Dirks and Pierce, 2004) addresses these longstanding challenges (Choi et al., 2010, 2014). Using *in situ* HCR, DNA probes complementary to mRNA targets carry DNA initiators that trigger chain reactions in which metastable fluorophore-labeled DNA hairpins self-assemble into tethered fluorescent amplification polymers (Fig. 1A). Programmability enables multiple orthogonal HCR amplifiers to operate independently in the same sample at the same time; tethering prevents diffusion of the amplified signal away from targets. The same two-stage *in situ* hybridization protocol is used independent of the number of target RNAs: in the detection stage, N orthogonal probe sets are hybridized in parallel; in the amplification stage, N orthogonal HCR amplifiers operate in parallel. We favor a 36 hour protocol with two overnight incubations, enabling researchers to maintain a normal sleep schedule (Fig. 1B).

RESULTS AND DISCUSSION

Building on our technology development in whole-mount zebrafish embryos (Choi et al., 2010, 2014), here, we generalize *in situ* HCR to eight sample types widely studied in the biological sciences (Fig. 2): bacteria, whole-mount nematode larvae, whole-mount fruit fly embryos, whole-mount sea urchin embryos, whole-mount zebrafish larvae [5 dpf compared with the previous 27 hpf embryos (Choi et al., 2014)], whole-mount chicken embryos, whole-mount mouse embryos and formalin-fixed paraffin-embedded (FFPE) human tissue sections. Protocols are provided for each organism in supplementary Materials and Methods, sections S3-S10.

The 24 target mRNAs mapped in Fig. 2 are detected using probe sets containing between 2 and 10 DNA probes (Table S1), each addressing a 50 nt subsequence of a target mRNA. Within each probe set, all probes carry two DNA initiators for the same DNA HCR amplifier. The number of probes in each probe set depends on the expression level of the target, the hybridization yield of the probes, and the level of autofluorescence in the channel corresponding to the target. When mapping the expression pattern for a new target mRNA, we balance brightness, robustness and cost considerations by using a probe set containing five DNA probes.

To characterize signal-to-background for each target mRNA, we compare pixel intensities in representative regions of high and low (or no) expression (Figs S2A-S9A). Indicative of high contrast, pixel intensity histograms for these regions are typically non-overlapping (Figs S2B-S9B) and signal-to-background ratios range from 2 to 110 with a median of 6 (Table S4). All images are presented without background subtraction.

To characterize the resolution achieved using *in situ* HCR, we redundantly detect a target mRNA in the embryonic mouse heart using two probe sets that initiate spectrally distinct HCR amplifiers (Fig. 3A), providing a rigorous test of signal colocalization independent of the expression pattern of the target. Subcellular voxel intensities in the two channels are highly correlated (Pearson correlation coefficient r=0.92 for 0.35×0.35 µm voxels), indicative of subcellular resolution for each channel (Fig. 3B). Putative sites of active transcription (Ruf-Zamojski et al., 2015) appear as two bright dots in some nuclei (Fig. 3A and Fig. S11).

HCR draws on principles from the emerging disciplines of molecular programming and dynamic nucleic acid nanotechnology to provide isothermal enzyme-free signal amplification in diverse technological settings (Zhang et al., 2013; Wang et al., 2014; Jung and Ellington, 2014; Ikbal et al., 2015) and it is particularly well-suited to the demands of *in situ* amplification (Choi et al., 2010, 2014).

First, HCR is programmable, providing the basis for straightforward multiplexing using orthogonal amplifiers that operate independently and carry spectrally distinct fluorophores. Use of a two-stage protocol independent of the number of target mRNAs is convenient for any sample, but essential for delicate samples such as sea urchin embryos that are easily damaged during serial multiplexing protocols. Even in experimental settings where

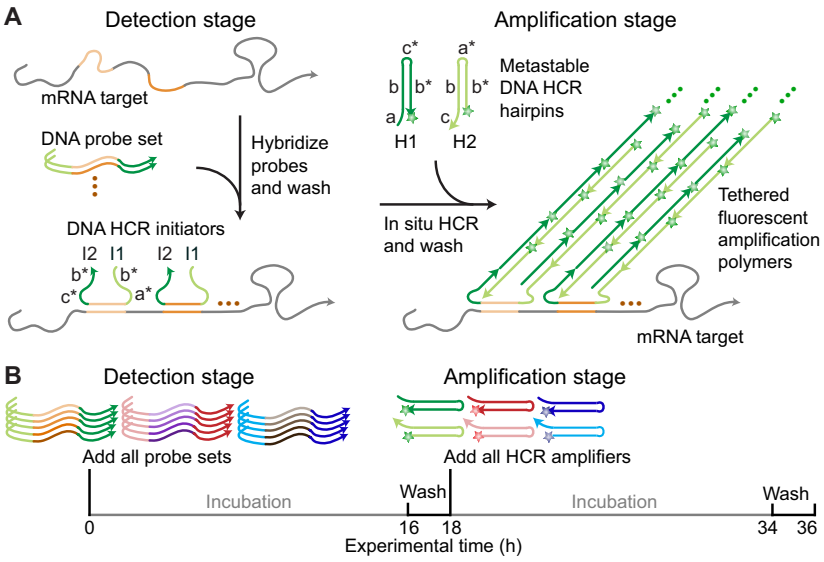

Fig. 1. Multiplexed *in situ* hybridization chain reaction (HCR). (A) Two-stage *in situ* HCR protocol (Choi et al., 2014). Detection stage: DNA probes carrying DNA HCR initiators (I1 and I2) hybridize to mRNA targets and unused probes are washed from the sample. Amplification stage: metastable DNA HCR hairpins (H1 and H2) penetrate the sample, initiators trigger chain reactions in which fluorophore-labeled H1 and H2 hairpins sequentially nucleate and open to assemble into tethered fluorescent amplification polymers, and unused hairpins are washed from the sample. See Fig. S1 for a detailed description of the HCR mechanism. (B) Experimental timeline. The time required to perform an experiment is independent of the number of target mRNAs. Stars denote fluorophores.

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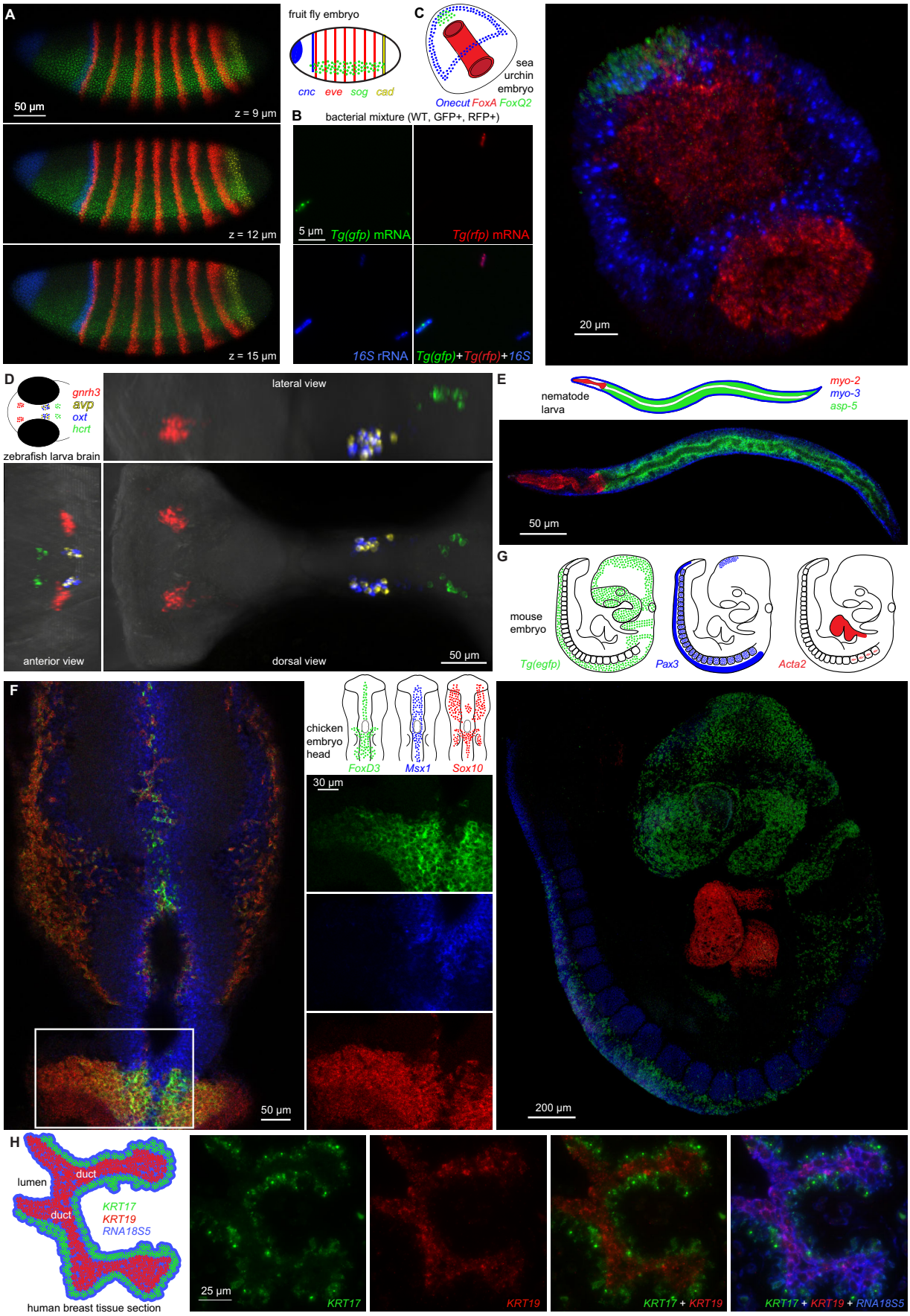

Fig. 2. See next page for legend.

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Fig. 2. Multiplexed mRNA expression maps using *in situ* HCR. (A) Whole-mount fruit fly (*Drosophila melanogaster*) embryo: expression schematic and confocal micrographs for four target mRNAs on three planes. Embryo fixed: stage 4-6. (B) Mixed bacterial populations (*Escherichia coli*: WT, GFP+, RFP+): epifluorescence micrographs (single channels and merge) for three targets (*gfp* and *rfp* mRNAs and 16S rRNA). (C) Whole-mount sea urchin embryo (*Strongylocentrotus purpuratus*): expression schematic and three-dimensional reconstruction from confocal micrographs for three target mRNAs. Embryo fixed: 45 hpf. (D) Whole-mount zebrafish larva (*Danio rerio*): expression schematic and three-dimensional reconstruction from confocal micrographs for four target mRNAs within the brain. Larva fixed: 5 dpf. (E) Whole-mount nematode larva (*Caenorhabditis elegans*): expression schematic and confocal micrograph for three target mRNAs. Larva fixed: L3. (F) Whole-mount chicken embryo (*Gallus gallus domesticus*): expression schematic and confocal micrographs for three target mRNAs in the neural crest (merge and single-channel details). Embryo fixed: stage HH 11-12. (G) Whole-mount mouse embryo [*Mus musculus*: Tg(Wnt1-Cre; R26R-eGFP)]: expression schematic and three-dimensional reconstruction from confocal micrographs for three target mRNAs. Embryo fixed: E9.5. (H) FFPE human breast tissue section (*Homo sapiens sapiens*): expression schematic and epifluorescence micrographs for two target mRNAs and one rRNA (single channels and merges). Thickness: 4 µm. See Figs S2-S10 and Movies 1-5 for additional data.

multiplexing can be achieved by mixing and matching approaches with different sensitivity and resolution to target mRNAs with different abundance and patterning [e.g. simultaneous use of CARD, conjugated secondary antibodies, pre-associated antibody complexes and direct-labeled probes in whole-mount fruit fly embryos (Kosman et al., 2004)], researchers may appreciate the simplicity of *in situ* HCR.

Second, HCR hairpins do not self-assemble until they encounter a probe carrying the cognate initiator, enabling deep sample penetration prior to growth of bright amplification polymers at the site of target molecules (see Movies 1-5). The fact that the amplification reagents are structured hairpins with a duplex stem reduces the potential for non-specific hybridization within the sample and also increases the ease of engineering multiple orthogonal amplifiers. The fact that amplification polymers carry up to hundreds of fluorophores (Choi et al., 2014) makes it possible to achieve high signal-to-background even when autofluorescence is high [e.g. in whole-mount vertebrate embryos (Choi et al., 2014; McLennan et al., 2015; Huss et al., 2015), in thick mouse brain sections (Sylwestrak et al., 2016) or in bacteria contained within environmental samples or other organisms (Rosenthal et al., 2013; Yamaguchi et al., 2015; Nikolakakis et al., 2015)]. The resulting HCR signal is stable for at least 1 week in zebrafish embryos stored in solution (Fig. S12) and for at least 2 years in fruit fly embryos stored in hardset mounting medium (Fig. S13).

Third, HCR amplification polymers remain tethered to their initiating probes, preventing signal from diffusing away from targets and leading to subcellular resolution and sharp cellular boundaries (e.g. note the signal in the zebrafish larva brain of Movie 3). With straightforward modifications, single-molecule resolution can be achieved by using larger probe sets (to better distinguish true dots representing mRNAs bound by multiple probes from false dots representing individual non-specifically-bound probes) and shorter amplification times (to grow shorter amplification polymers and resolve individual mRNAs as diffraction-limited dots) (Shah et al., 2016). So-called single-molecule HCR (smHCR) is compatible with tissue hydrogel clearing and embedding and selective plane illumination microscopy, enabling mapping of single mRNAs in thick samples (e.g. 0.5mm adult mouse brain sections) (Shah et al., 2016) where high background undermines the performance of single-molecule fluorescence *in situ* hybridization (smFISH) using direct-labeled probes (Raj et al., 2008). Used in combination with expansion microscopy (Chen et al., 2015), smHCR enables super-resolution imaging of clustered mRNAs using conventional diffraction-limited microscopes (Chen et al., 2016).

Fourth, because HCR amplifier sequences are independent of mRNA target sequences, previously validated amplifiers (Choi et al., 2014) can be used for new studies without modification. To map a new target mRNA, all that is needed is a new DNA probe set carrying DNA initiators for an existing DNA HCR amplifier. Taken together, the properties of *in situ* HCR lead to straightforward multiplexing, deep sample penetration, high contrast and subcellular resolution in diverse organisms, offering biologists a dramatically improved window into the spatial organization of biological circuitry.

MATERIALS AND METHODS

Probe sets, amplifiers and buffers

Reagents are summarized in supplementary material section S1.1 and Table S1; probe sequences are provided in section S14.

In situ hybridization

In situ HCR was performed in eight organisms using the protocols and recipes detailed in supplementary material sections S3-S10. Frequently asked questions are answered in supplementary material section S2, including questions related to: getting started, sample preparation, optimizing signal-to-background and imaging.

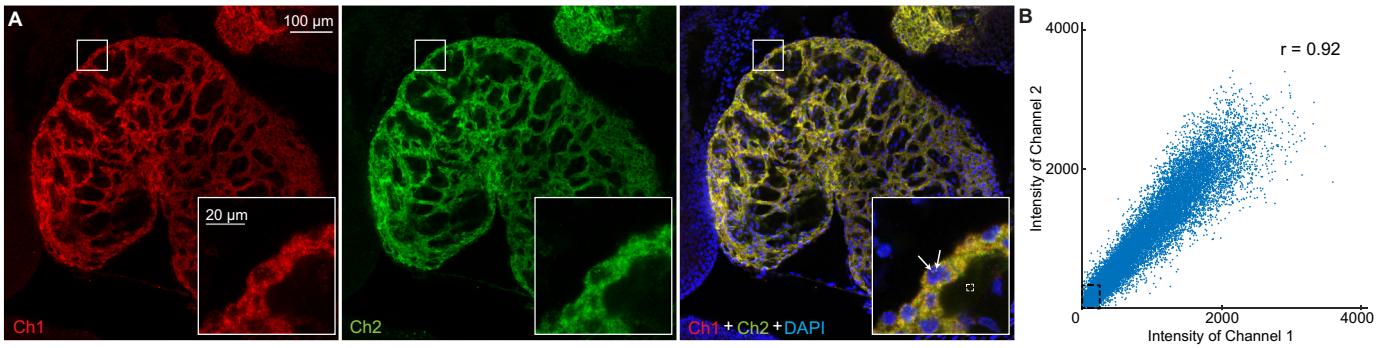

Fig. 3. Subcellular resolution using *in situ* HCR. (A) Redundant two-channel mapping of target mRNA *Acta2* in the heart of a whole-mount mouse embryo. Arrows denote putative sites of active transcription. Probe sets: two probes per channel. Pixel size: 69×69 nm. Embryo fixed: E9.5. (B) Highly correlated intensities for 0.35×0.35 µm voxels in the inset (Pearson correlation coefficient: r=0.92). To avoid inflating the correlation coefficient, we exclude voxels that fall below background thresholds in both channels (excluded voxels lie in the dashed rectangle at the lower left corner of the correlation plot). For each channel, the background threshold is defined as the mean plus two standard deviations for the voxels in the small white square. See Fig. S11 for additional data.

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Microscopy

Thin samples (bacterial populations and human tissue sections) were imaged using epifluorescence microscopy and thick samples (whole-mount embryos and larvae) were imaged using confocal microscopy as detailed in supplementary material section S1.3 and Tables S2 and S3.

Image analysis

Signal-to-background analysis was performed for each target mRNA as detailed in supplementary material section S1.4 based on the data of section S11, yielding the results of Table S4.

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Competing interests

The authors declare competing financial interests in the form of patents and pending patent applications.

Author contributions

Study conceived by N.A.P. in consultation with M.E.B., E.H.D., S.E.F., B.A.H., J.R.L., D.K.N., P.H.P., M.v.d.R., B.W. Experiments designed by H.M.T.C. and N.A.P. Preliminary studies and protocol adaptation performed by S.M.L., R.C.H., A.Z.R., H.M.T.C. (bacteria), C.R.C. (nematode), N.H. (fly), J.C.B., C.R.C. (urchin), H.M.T.C. (zebrafish), T.S.-S., C.R.C. (chicken), A.C.T.A., B.E.D., D.H., H.M.T.C. (mouse), and H.M.T.C., N.H. (human), in consultation with D.K.N., J.R.L. (bacteria), M.K. (nematode), R.L., S.E.F. (chicken), R.L., C.R.C., S.E.F., B.W. (mouse), M.v.d.R. (human), and H.M.T.C., N.A.P. (all organisms). Final protocols optimized and final data collected by H.M.T.C. (bacteria, zebrafish, mouse), C.R.C. (nematode, urchin, chicken) and N.H. (fly, human). Final data analyzed by: S.M.L., S.K.M., R.C.H., D.K.N., A.Z.R., J.R.L. (bacteria), M.K., P.S., C.R.C. (nematode), O.S.A., B.A.H., N.H. (fly), J.C.B., C.R.C. (urchin), D.A.P., S.E.F. (fish), T.S.-S., M.E.B., C.R.C. (chicken), A.C.T.A., B.W., B.E.D., D.H., Y.L., C.R., R.L., S.E.F. (mouse), A.C.M., M.v.d.R., N.H. (human) and H.M.T.C and N.A.P. (all organisms). Paper and supplementary information written by H.M.T.C. and N.A.P. Paper was edited and approved by all coauthors.

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Supplementary information

Supplementary information available online at
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Supplementary Information

Mapping a multiplexed zoo of mRNA expression

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S1 Materials and methods

S1.1 Probe sets, amplifiers, and buffers.

For each target mRNA, a kit containing a DNA probe set, a DNA HCR amplifier, and hybridization, wash, and amplification buffers was purchased from Molecular Instruments (molecularinstruments.org). See Table S1 for a summary of probe set, amplifier, and fluorophore details and Section S14 for probe sequences. Sequences for HCR amplifiers B1, B2, B3, B4, and B5 are given in (Choi *et al.*, 2014). The size of each probe set was based on the expression level of the target, the level of autofluorescence in the channel corresponding to that target, and the hybridization yield of the probes.

Organism	Target	Probes	HCR Amplifier	Fluorophore	Figures
E. coli	*Tg(rfp)*	5	B4	Alexa546	2, S2, S10
	Tg(gfp)	5	B3	Alexa647	2, S2, S10
	16S	1	B1	Alexa488	2, S2, S10
D. melanogaster	*cad*	5	B5	Alexa488	2, S3
	sog	5	B1	Alexa514	2, S3
	cnc	4	B2	Alexa546	2, S3
	eve	5	B3	Alexa647	2, S3, S13
S. purpuratus	*FoxA*	10	B5	Alexa488	2, S4
	Onecut	6	B1	Alexa546	2, S4
	FoxQ2	3	B2	Alexa647	2, S4
C. elegans	*myo-2*	2	B2	Alexa488	2, S5
	myo-3	4	B3	Alexa546	2, S5
	asp-5	3	B1	Alexa647	2, S5
D. rerio	*avp*	5	B5	Alexa488	2, S6
	oxt	5	B2	Alexa514	2, S6
	gnrh3	5	B3	Alexa546	2, S6
	hcrt	6	B1	Alexa647	2, S6
	Tg(egfp)	6	B3	Alexa647	S12
G. gallus domesticus	*FoxD3*	6	B1	Alexa488	2, S7
	Msx1	9	B2	Alexa546	2, S7
	Sox10	5	B3	Alexa647	2, S7
M. musculus	*Tg(egfp)*	6	B3	Alexa488	2, S8
	Acta2	4	B1	Alexa546	2, S8
	Pax3	6	B2	Alexa647	2, S8
	Acta2	2	B2	Alexa546	3, S11
	Acta2	2	B1	Alexa647	3, S11
H. sapiens sapiens	*KRT17*	8	B2	Alexa546	2, S9
	KRT19	3	B1	Alexa647	2, S9
	RNA18S5	1	B5	Alexa488	2

Table S1. Probe set size, HCR amplifier, fluorophore, and figure numbers for each target.

S1.2 In situ hybridization

Procedures for the care and use of zebrafish embryos were approved by the Caltech IACUC and for the care and use of mouse embryos were approved by the USC IACUC. FFPE human tissue sections were purchased from Pantomics (Cat. # BRE01 Block # F091926B08). For each of eight organisms, in situ HCR was performed using the corresponding protocol detailed in Sections S3–S10.

S1.3 Microscopy

Epifluorescence Microscopy

A Zeiss Axio Observer Z1 fluorescent inverted microscope was used to image thin samples (bacterial populations and human tissue sections). Sample slides were illuminated with X-Cite Series 120 (EXFO Photonics) and images were collected with an ImagEM-1K EM-CCD digital camera (Hamamatsu). An Plan-Apochromat 100×/1.4 Oil DIC objective was used to acquire all bacterial images and an Plan-Apochromat 63×/1.4 Oil DIC M27 objective was used to acquire all human tissue images. See Table S2 for a summary of excitation and emission filters for each target. For this epifluorescence microscope, non-negligible instrument Noise was measured in the absence of sample; images are displayed with the mean Noise subtracted from each pixel intensity. However, they are displayed without subtracting background arising from the sample (autofluorescence, non-specific detection, and non-specific amplification; see Section S2.3).

Organism	Target	Fluorophore	Excitation (nm)	Beam Splitter	Emission (nm)	Figures
E. coli	*Tg(rfp)*	Alexa546	BP 550/25	FT 570	BP 605/70	2, S2, S10
	Tg(gfp)	Alexa647	BP 640/30	FT 660	BP 690/50	2, S2, S10
	16S	Alexa488	BP 470/40	FT 495	BP 525/50	2, S10
H. sapiens sapiens	*KRT17*	Alexa546	BP 550/25	FT 570	BP 605/70	2, S9
	KRT19	Alexa647	BP 640/30	FT 660	BP 690/50	2, S9
	RNA18S5	Alexa488	BP 470/40	FT 495	BP 525/50	2
	Nuclear DNA	DAPI	G 365	FT 395	BP 445/50	2

Table S2. Wide-field epifluorescence microscope settings.

Confocal Microscopy

A Zeiss 710 NLO inverted confocal microscope was used to image whole-mount embryos and larvae. An LD LCI Plan-Apochromat 25×/0.8 Imm Korr DIC objective was used to acquire images of fruit fly, chicken, zebrafish, and mouse embryos. An LD C-Apochromat 40×/1.1 W Korr M27 objective was used to acquire images of sea urchin embryos, nematode larvae, zebrafish larvae, and mouse heart. See Table S3 for a summary of excitation laser sources, beam splitters, and tuned emission bandpass filters used for each target. For this confocal microscope, instrument Noise in the absence of sample was negligible; images are displayed without subtracting the mean Noise from each pixel intensity. Furthermore, they are displayed without subtracting background arising from the sample (autofluorescence, non-specific detection, and non-specific amplification; see Section S2.3).

Organism	Target	Fluorophore	Laser (nm)	Beam Splitter	Filter (nm)	Figures
D. melanogaster	*cad*	Alexa488	488	MBS 488/561/633	497–512	2, S3
	sog	Alexa514	514	MBS 458/514	610–670	2, S3
	cnc	Alexa546	561	MBS 458/561	563–612	2, S3
	eve	Alexa647	633	MBS 488/561/633	646–758	2, S3, S13
S. purpuratus	*FoxA*	Alexa488	488	MBS 488/561/633	502–537	2, S4
	Onecut	Alexa546	561	MBS 458/561	563–582	2, S4
	FoxQ2	Alexa647	633	MBS 488/561/633	660–681	2, S4
C. elegans	*myo-2*	Alexa488	488	MBS 488/561/633	502–537	2, S5
	myo-3	Alexa546	561	MBS 458/561	563–582	2, S5
	asp-5	Alexa647	633	MBS 488/561/633	651–690	2, S5
D. rerio	*avp*	Alexa488	488	MBS 488/561/633	488–527	2, S6
	oxt	Alexa514	514	MBS 458/514	537–553	2, S6
	gnrh3	Alexa546	561	MBS 458/561	563–612	2, S6
	hcrt	Alexa647	633	MBS 488/561/633	654–687	2, S6
	Tg(egfp)	Alexa647	633	MBS 488/561/633	654–687	S12
G. gallus domesticus	*FoxD3*	Alexa488	488	MBS 488/561/633	508–538	2, S7
	Msx1	Alexa546	561	MBS 458/561	563–582	2, S7
	Sox10	Alexa647	633	MBS 488/561/633	651–690	2, S7
M. musculus	*Tg(egfp)*	Alexa488	488	MBS 488/561/633	493–556	2, S8
	Acta2	Alexa546	561	MBS 488/561/633	563–582	2, S8
	Pax3	Alexa647	633	MBS 488/561/633	650–755	2, S8
	Acta2	Alexa546	561	MBS 488/561/633	563–592	3, S11
	Acta2	Alexa647	633	MBS 458/561/633	650–689	3, S11
	Nuclear DNA	DAPI	720	MBS 690+	417–505	3, S11

Table S3. Confocal microscope settings.

S1.4 Image analysis

Signal-to-background analysis is performed as described previously (Choi *et al.*, 2014). For each target mRNA, background (BACK) is characterized for pixels in one or more representative rectangular regions of no- or low-expression and the combination of background and signal (BACK+SIG) is characterized for pixels in one or more representative rectangular regions of high expression (Figures S2a–S9a).* For the pixels in these regions, we characterize the distribution by plotting a pixel intensity histogram (Figures S2b–S9b) and characterize typical performance by calculating the mean pixel intensity (x_{BACK} or $x_{\mathrm{BACK+SIG}}$). Performance across experimental specimens ($N = 3$ for each type of organism) is characterized by calculating the sample means, $\bar{x}_{\mathrm{BACK}}$ and $\bar{x}_{\mathrm{BACK+SIG}}$, and sample standard deviations, s_{BACK} and $s_{\mathrm{BACK+SIG}}$. The mean signal is then estimated as

$$\bar{x}_{\mathrm{SIG}} = \bar{x}_{\mathrm{BACK+SIG}} - \bar{x}_{\mathrm{BACK}}$$

with standard deviation estimated via uncertainty propagation as

$$s_{\mathrm{SIG}} \leq \sqrt{(s_{\mathrm{BACK+SIG}})^2 + (s_{\mathrm{BACK}})^2}.$$

The signal-to-background ratio is estimated as

$$\bar{x}_{\mathrm{SIG/BACK}} = \bar{x}_{\mathrm{SIG}} / \bar{x}_{\mathrm{BACK}}$$

with standard deviation estimated via uncertainty propagation as

$$s_{\mathrm{SIG/BACK}} \leq \frac{\bar{x}_{\mathrm{SIG}}}{\bar{x}_{\mathrm{BACK}}} \sqrt{\left(\frac{s_{\mathrm{SIG}}}{\bar{x}_{\mathrm{SIG}}}\right)^2 + \left(\frac{s_{\mathrm{BACK}}}{\bar{x}_{\mathrm{BACK}}}\right)^2}.$$

These upper bounds on estimated standard deviations hold under the assumption that the correlation between SIG and BACK is non-negative. Estimates for background ($\bar{x}_{\mathrm{BACK}} \pm s_{\mathrm{BACK}}$), signal ($\bar{x}_{\mathrm{SIG}} \pm s_{\mathrm{SIG}}$), and signal-to-background ($\bar{x}_{\mathrm{SIG/BACK}} \pm s_{\mathrm{SIG/BACK}}$), are displayed for each target mRNA in Table S4.

*For transgenic target mRNAs in bacteria (*Tg(gfp)* and *Tg(rfp)*), the BACK pixel intensities are obtained from WT organisms lacking the target; BACK+SIG pixel intensities are obtained from transgenic organisms containing the target.

S2 Frequently asked questions

S2.1 Getting started

- **How does the HCR amplification mechanism work?** See Figure S1.

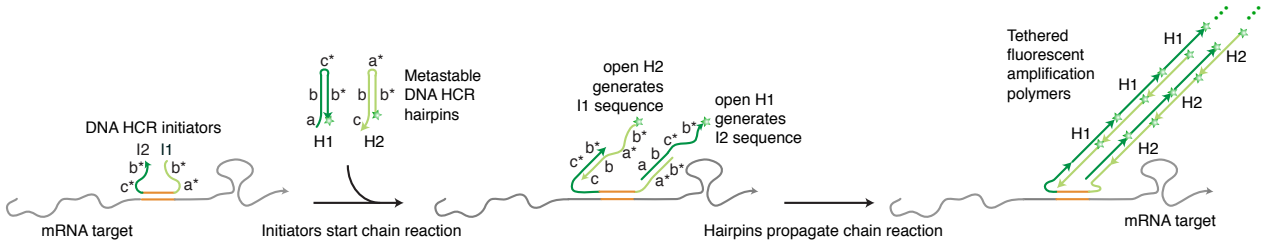


Figure S1. HCR mechanism. Hairpins H1 and H2 are metastable and predominantly do not interact on an experimental time scale in the absence of cognate initiators I1 and I2 (Dirks *et al.*, 2004), enabling them to penetrate deep into the sample prior to self-assembling into tethered fluorescent amplification polymers at the site of probes predominantly bound to cognate target mRNAs (Choi *et al.*, 2010; Choi *et al.*, 2014). Each unstructured probe carries HCR initiator I1 at the 5′ end and HCR initiator I2 at the 3′end (Choi *et al.*, 2014). Initiator I1 nucleates with hairpin H1 via base-pairing to single-stranded toehold 'a', mediating a branch migration (Quartin *et al.*, 1989; Yurke *et al.*, 2000) that opens H1 to expose single-stranded segment 'c*-b*' identical in sequence to initiator I2. Initiator I2 nucleates with hairpin H2 via base-pairing to single-stranded toehold 'c', mediating a branch migration that opens H2 to expose single-stranded segment 'a*-b*' identical in sequence to initiator I1. Thus, addition of each hairpin regenerates an initiator sequence providing the basis for nucleation and opening of a new hairpin, yielding a chain reaction of alternating H1 and H2 polymerization steps. Stars denote fluorophores.

- **What do I need to get started with a multiplexed HCR experiment with N targets?**
  1. N probe sets that initiate different HCR amplifiers.
  2. N orthogonal HCR amplifiers carrying spectrally distinct fluorophores.
  3. Hybridization, wash, and amplification buffers.
- **I've never used in situ HCR – what's the best way to get started?** If possible, start with a probe set that has been previously validated in your model organism (e.g., any of the probe sets in Section S14). Otherwise, start with a high- or moderate-copy target with a known expression pattern. If you have access to transgenic lines that express fluorescent proteins, detection of the transgenic mRNA provides a positive control (e.g., using a validated *Tg(gfp)* probe set), and the corresponding wildtype organism provides a negative control.
- **How many probes do you recommend per probe set?** When mapping the expression pattern for a new target mRNA, we balance brightness, robustness, and cost considerations by using a probe set containing 5 DNA probes, each carrying two DNA HCR initiators (one at each end of the probe).
- **How long does it take to perform in situ hybridization for five target mRNAs?** The same amount of time it takes for one target mRNA. We tend to use 36-hour protocols that include two overnight incubations, enabling the researcher to maintain a normal sleep schedule. Depending on your sample thickness, you may be able to greatly reduce the duration of the protocol to suit your needs.
- **How many HCR amplifiers can I use at once?** We have currently validated five orthogonal DNA HCR amplifiers (B1, B2, B3, B4, B5) that can be mixed and matched with Alexa fluorophores (e.g., Alexa488, Alexa514, Alexa546, Alexa594, Alexa647)(Choi *et al.*, 2014) or custom labels as desired. If you have access to a microscope that can distinguish more than five reporters, additional orthogonal HCR amplifiers can be engineered for simultaneous use.
- **Do you recommend one HCR amplifier over another?** No. All HCR amplifiers have been validated to perform well in a variety of sample types.
- **Do you recommend one fluorophore over another?** Yes. In most sample types, autofluorescence is lowest in the far red, so we recommend using Alexa647 preferentially (e.g., for your first target, your lowest-copy

target, etc).

- **How can I obtain HCR reagents?** HCR kits containing probes, amplifiers, and buffers are available from Molecular Instruments (molecularinstruments.org), a non-profit academic resource within the Beckman Institute at Caltech.
- **What if I need advice or technical support?** Contact Molecular Instruments (support@molecularinstruments.org).

S2.2 Sample preparation

- **Do I need to do anything unusual in preparing my sample?** When starting out in a new model organism, we recommend preparing your sample as you normally would using a traditional in situ hybridization method. Due to the use of relatively short DNA probes and small DNA HCR hairpins, you may find that you can reduce proteinase K treatment.
- **Do I need to use proteinase K to increase target accessibility?** This depends on the model organism and the age of the embryo or larva. In the present work, we use proteinase K for nematode, fly, zebrafish, chicken, mouse, and human samples but not for bacteria and urchin samples. For protocols that use proteinase K, it is *critical* to re-optimize the concentration and duration of treatment due to variability between batches.
- **What precautions should I take to avoid RNA degradation during sample preparation?** Use RNase-free water to make all buffers and use RNaseZap to clean all tools that will be in contact with your samples.
- **How can I minimize autofluorescence?** Use fresh samples. Avoid using calcium chloride and magnesium chloride in PBS.

S2.3 Optimizing signal-to-background

- **What causes background?** For each channel, the fluorescent background (BACK) in a pixel is generated by three sources (Choi *et al.*, 2010; Choi *et al.*, 2014):

  1. Autofluorescence (AF): inherent fluorescence of the fixed sample.
  2. Non-specific detection (NSD): probes that bind non-specifically in the sample and are subsequently amplified.
  3. Non-specific amplification (NSA): HCR hairpins that bind non-specifically in the sample.

  Hence, the total background in a pixel is BACK = AF + NSD + NSA. For each channel, the fluorescent signal (SIG) in a pixel is generated by HCR amplification polymers tethered to probes bound specifically to a target mRNA. For each channel, the total fluorescence in a pixel is BACK + SIG.
- **How do I measure the contributions to background and signal?** The contributions of AF, NSA, NSD, and SIG to the total fluorescence are characterized using the following four types of experiments(Choi *et al.*, 2010; Choi *et al.*, 2014):

  1. **AF:** use the standard in situ protocol omitting probes and hairpins and examine a representative region.
  2. **AF + NSA:** use the standard in situ protocol omitting probes and examine a representative region.
  3. **BACK = AF + NSA + NSD:** use the standard in situ protocol and examine a representative region of no or low expression.[†]
  4. **BACK + SIG = AF + NSA + NSD + SIG:** use the standard in situ protocol and examine a representative region of expression.

  For the pixels in a given representative region, we characterize the distribution by plotting a pixel intensity histogram and characterize typical performance by calculating the mean pixel intensity.[‡]

[†]If the target is transgenic, this experiment can be performed in a wild type organism lacking the target. If the target is endogenous but a knockout strain is available, this experiment can be performed in a knockout organism lacking the target.

[‡]If your microscope generates non-negligible fluorescence intensities in the absence of sample, this instrument noise (NOISE) should be taken into consideration when calculating background and signal contributions, leading to five types of experiment (0. NOISE, 1. NOISE + AF, 2. NOISE + AF + NSA, 3. NOISE + BACK, 4. NOISE + BACK + SIG).

- **What if I don't know what the pattern of non-expression and expression should be?** Perform a redundant detection experiment(Choi *et al.*, 2010; Choi *et al.*, 2014) in which the target is detected with two probe sets that activate orthogonal HCR amplifiers carrying spectrally distinct fluorophores. If the pixel intensities in the two channels are highly correlated, this builds confidence that both probe sets are generating specific staining. To support this interpretation, it is important to check that a third probe set addressing an unrelated target generates staining that is uncorrelated with the first two channels to make sure that the high correlation is sequence-dependent and not simply material-dependent (i.e., not due to general binding of all DNA probes to some feature in the sample).
- **How do I calculate the signal-to-background ratio?** See Section S1.4.
- **How do I increase the signal-to-background ratio?**
  1. If the background is dominated by AF, increase the number of probes in the probe set.
  2. If the background is dominated by NSD, test the probes individually to eliminate any probes that show poor selectivity for the target.
  3. If the background is dominated by NSA, there is a problem with the wash protocol. For an optimized protocol, NSA is typically negligible compared to AF + NSD.
- **What is a good signal-to-background ratio?** As a rule of thumb, we find that a signal-to-background ratio of 5 or more provides excellent contrast when viewed by the human eye, with 2 being a minimum acceptable standard.

S2.4 Imaging

- **What type of microscope should I use?** The choice of microscope is dictated by the sample thickness. Thin samples can be imaged via epifluorescence microscopy (e.g., bacteria and human tissue sections in the current study). For thicker samples, confocal microscopy reduces background by eliminating out-of-plane autofluorescence.
- **How many different target mRNAs can I image in a single sample?** This will depend on the excitation lasers and emission filters available with your microscope setup. Typical fluorescent microscopes can distinguish the spectra of three to five fluorophores.

S3 Protocols for bacteria in suspension (*Escherichia coli*)

S3.1 Sample preparation protocol

1. Grow *E. coli* cells (from frozen glycerol stock) in 2 mL of LB media overnight in a 37 °C shaker.
2. Dilute to make a 5 mL liquid culture with OD_{600} = 0.05.
3. Incubate in a 37 °C shaker until $OD_{600} \approx 0.5$ (exponential phase).
4. Aliquot 1 mL of cells and centrifuge for 10 min.
   NOTE: *Centrifugation should be as gentle as possible to pellet cells. For E. coli, all centrifugation steps are done at 4000 × g.*
5. Remove supernatant and re-suspend cells in 750 μL of 1× phosphate-buffered saline (PBS).
6. Add 250 μL of 4% formaldehyde and incubate overnight at 4 °C.
7. Centrifuge for 10 min and remove supernatant.
8. Re-suspend cells in 150 μL of 1× PBS.
9. Add 850 μL of 100% MeOH and store cells at -20 °C before use.

S3.2 Buffer recipes for sample preparation

LB media
5 g of Novagen LB Broth Miller powder
Fill up to 200 mL with ultrapure H_2O
Autoclave at 121 °C for 20 min

4% formaldehyde
4% formaldehyde
1× PBS

For 10 mL of solution
2.5 mL of 16% formaldehyde
1 mL of 10× PBS
Fill up to 10 mL with ultrapure H_2O

NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the samples.*

S3.3 Multiplexed in situ HCR protocol

Detection stage

1. Transfer 100 μL of cells into a 1.5 mL eppendorf tube.
2. Centrifuge for 5 min and remove supernatant.
3. Wash cells with 500 μL of 1$\times$ PBST and remove the solution by centrifugation.
4. Re-suspend the pellet with 400 μL of probe hybridization buffer and pre-hybridize for 1 h at 65 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *We pre-hybridize for 1h at 65 °C to alleviate signal from fluorescent proteins; we pre-hybridize for 1 h at 45 °C if this is not a consideration.*
5. In the meantime, prepare probe mixture by adding 1 pmol of each probe (1 μL of 1 μM stock per probe) to 100 μL of probe hybridization buffer pre-heated to 45 °C.
6. Add the probe mixture directly to the sample to reach a final probe concentration of 2 nM.
7. Incubate the sample for 2 h at 45 °C.
8. Add 1 mL of probe wash buffer (pre-heated to 45 °C) to the sample to dilute the probes.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
9. Centrifuge for 5 min and remove the wash solution.
10. Re-suspend the cell pellet with 500 μL of probe wash buffer (pre-heated to 45 °C).
11. Incubate for 5 min at 45 °C and remove the wash solution by centrifugation for 5 min.
12. Repeat steps 10 and 11 for two additional times but with 10 min incubation.
13. Proceed to hairpin amplification.

Amplification stage

1. Re-suspend the cell pellet with 250 μL of amplification buffer and pre-amplify for 10 min at room temperature.
2. Prepare 30 pmol of each fluorescently labeled hairpin by snap cooling 10 μL of 3 μM stock in hairpin storage buffer (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
3. Prepare hairpin mixture by adding all snap-cooled hairpins to 250 μL of amplification buffer at room temperature.
4. Add the hairpin mixture directly to the sample to reach a final hairpin concentration of 60 nM.
5. Incubate the sample overnight ($>$12 h) in the dark at room temperature.
6. Add 1 mL of 5$\times$ SSCT at room temperature to the sample to dilute the hairpins.
7. Centrifuge for 5 min and remove the wash solution.
8. Re-suspend the cell pellet with 500 μL of 5$\times$ SSCT.
9. Incubate for 5 min at room temperature and remove the wash solution by centrifugation for 5 min.
10. Repeat steps 8 and 9 for two additional times but with 10 min incubation.
11. Re-suspend the cell pellet in 50 μL of 5$\times$ SSCT.
12. Store the sample at 4 °C before imaging.

S3.4 Sample mounting for microscopy

1. Add 50 μL of SlowFade Gold antifade mountant to each sample.
2. Pipette 50 μL of 0.1% (w/v) poly-L-lysine onto a 22 mm $\times$ 22 mm coverslip.
3. Allow to sit for 10 min and tap off extra solution. Allow it to air dry at room temperature.
4. Add 10 μL of sample to the middle of the coverslip.
5. Place the sample side of the coverslip on top of a 25 mm $\times$ 75 mm glass slide. Lower the coverslip carefully to avoid air bubbles.
6. Sample slide could be imaged on a wide-field fluorescent microscope. In this paper, a Zeiss Axio Observer Z1 fluorescent inverted microscope equipped with an Plan-Apochromat 100$\times$/1.4 Oil DIC objective was used to acquire all bacterial images. Samples were illuminated with X-Cite Series 120 (EXFO Photonics) and images were collected with an ImagEM-1K EM-CCD digital camera (Hamamatsu).

S3.5 Buffer recipes for in situ HCR

Probe hybridization buffer (low MW dextran sulfate)	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
1× Denhardt's solution	800 μL of 50× Denhardt's solution
10% low MW dextran sulfate	8 mL of 50% low MW dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

Probe wash buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
	Fill up to 40 mL with ultrapure H_2O

Amplification buffer (low MW dextran sulfate)	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
10% low MW dextran sulfate	8 mL of 50% low MW dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

Hairpin storage buffer	For 40 mL of solution
10 mM Tris	400 μL of 1 M Tris
1 mM EDTA	80 μL of 0.5 M EDTA
300 mM NaCl	4 mL of 3 M NaCl
	Fill up to 40 mL with ultrapure H_2O

5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
	fill up to 40 mL with ultrapure H_2O

50% dextran sulfate	For 40 mL of solution
50% dextran sulfate	20 g of low MW dextran sulfate powder
	Fill up to 40 mL with ultrapure H_2O

S3.6 Reagents and supplies

LB Broth Miller (Novagen Cat. # 71753-5)
10× PBS (Life Technologies Cat.# AM9625)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Formaldehyde, 16%, methanol free, Ultra Pure (Polyysciences Cat. # 18814-20)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, MW 6,500-10,000 (Sigma Cat. # D4911)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 22 mm No. 1 coverslip (VWR Cat. # 48366-067)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36936)

S4 Protocols for whole-mount fruit fly embryos (*Drosophila melanogaster*)

This protocol has been optimized for embryos at stage 4–6.

S4.1 Sample preparation protocol

1. Collect Drosophila embryos and incubate with yeast paste (food source) until they reach stage 4-6 (approximately 3 h).
2. Rinse embryos into a mesh basket and wash excess yeast paste using DI H_2O.
3. Immerse the mesh basket into 100% bleach and wash for 2 min to dechorinate embryos.
4. Rinse the basket with DI H_2O.
5. Transfer embryos to a scintillation vial containing 8 mL of 4.5% formaldehyde fixation solution.
6. Gently rock embryos in scintillation vial for 25 min on an orbital rocker.
   NOTE: *Rocking does not have to be very vigorous. However, embryos must move continuously within the heptane-fixative interphase to be exposed uniformly to the solvent and fixative.*
7. Remove the bottom liquid phase in the vial.
8. Add 8 mL of methanol (MeOH) and shake the vial hard for 1 min. Devitellinized embryos will sink to the bottom of the vial.
9. Remove all liquid and rinse 2 times in MeOH to remove debris.
10. Store embryos in 1 mL of MeOH at -20 °C before use.

S4.2 Buffer recipes for sample preparation

4.5% formaldehyde fixation solution	For 8 mL of solution
4.5% formaldehyde	975 μL of 37% formaldehyde
0.5× PBS	400 μL of 10× PBS
25 mM EGTA	76 mg of EGTA
50% heptane	4 mL of heptane
	Fill up to 8 mL with ultrapure H_2O

NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the embryos.*

S4.3 Multiplexed in situ HCR protocol

Detection stage

1. For each sample, transfer 50 μL of embryos (using a cut pipet tip) to a 1.5 mL eppendorf tube.
2. Rinse embryos 4 times by quickly adding 1 mL of ethanol (EtOH), inverting the tube, and aspirating the supernatant.
3. Add 500 μL of EtOH and 250 μL of xylene and invert the tube.
4. Add an additional 250 μL of xylene and invert the tube.
5. Add another 250 μL of xylene again and invert the tube. The tube should now contain 500 μL of EtOH and 750 μL of xylene.
6. Rock the tube at room temperature for 45–60 min. Longer rocking time is acceptable.
7. Aspirate the supernatant.
8. Rinse embryos once and wash 3 $\times$ 5 min with EtOH.
   NOTE: *All washes have a volume of 1 mL unless specified. All washes before pre-hybridization (step 16) are done with rocking.*
9. Rinse embryos once and wash 2 $\times$ 5 min with MeOH.
10. Wash with 50% MeOH / 50% PBST for 5 min.
11. Wash 1 $\times$ 10 min and 2 $\times$ 5 min with PBST.
12. Rock embryos in 1 mL of 4 μg/mL proteinase K solution at room temperature for 7 min.
    NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.*
13. Rinse embryos 2 times and wash 2 $\times$ 5 min with PBST.
14. Rock embryos in 4% formaldehyde post-fixation solution at room temperature for 25 min.
15. Rinse embryos and wash 5 $\times$ 5 min with PBST.
16. Pre-hybridize with 100 μL of probe hybridization buffer for 2 h at 65 °C.
    CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
17. Prepare probe solution by adding 0.2 pmol of each probe (0.2 μL of 1 μM stock per probe) to 100 μL of probe hybridization buffer at 45 °C.
18. Remove the pre-hybridization solution and add the probe solution.
19. Incubate embryos overnight (12–16 h) at 45 °C.
20. Remove excess probes by washing with probe wash buffer at 45 °C:
    (a) 2 $\times$ 5 min
    (b) 2 $\times$ 30 min

    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*

Amplification stage

1. Pre-amplify embryos with 1 mL of amplification buffer for 10 min at room temperature.
2. Prepare 6 pmol of each fluorescently labeled hairpin by snap cooling 2 μL of 3 μM stock in hairpin storage buffer (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
3. Prepare hairpin solution by adding all snap-cooled hairpins to 100 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the embryos overnight (12–16 h) in the dark at room temperature.
6. Remove excess hairpins by washing with 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

S4.4 Sample mounting for microscopy

1. Place 25 μL of embryos on a 25 mm $\times$ 75 mm glass slide.
2. Add 1–2 drops of ProLong Gold antifade mountant onto the embryos and stir to mix using a pipette tip.
3. Place a 22 mm $\times$ 22 mm No. 1 coverslip on top and seal using clear nail polish.
4. A Zeiss 710 NLO inverted confocal microscope equipped with an LD LCI Plan-Apochromat 25$\times$/0.8 Imm Corr DIC objective was used to acquire images.

S4.5 Buffer recipes for in situ HCR

Proteinase K solution	For 2 mL of solution
4 μg/mL proteinase K	0.4 μL of 20 mg/mL proteinase K Fill up to 2 mL with PBST
4% formaldehyde post-fixation solution	For 2 mL of solution
4% formaldehyde	216 μL of 37% formaldehyde Fill up to 2 mL with PBST
Probe hybridization buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
1× Denhardt's solution	800 μL of 50× Denhardt's solution
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with ultrapure H_2O
Probe wash buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
	Fill up to 40 mL with ultrapure H_2O
Amplification buffer	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with ultrapure H_2O
5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
	fill up to 40 mL with ultrapure H_2O
50% dextran sulfate	For 40 mL of solution
50% dextran sulfate	20 g of dextran sulfate powder
	Fill up to 40 mL with ultrapure H_2O

S4.6 Reagents and supplies

Baker's yeast (VWR Cat. # IC10140001)
37% formaldehyde (Fisher Scientific Cat. # F79-4)
10× PBS (Life Technologies Cat.# AM9625)
Ethylene glycol tetra acetic acid (EGTA) (Sigma Cat. # E4378)
Heptane, HPLC-grade (EMD Millipore Cat. # HX0080-6)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Ethanol, 200 proof (VWR Cat. # V1001G)
Xylene (Mallinckrodt Chemicals Cat. # 8668-02)
20 mg/mL proteinase K solution (Life Technologies Cat. # 25530-049)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 22 mm No. 1 coverslip (VWR Cat. # 48366-067)
ProLong Gold antifade mountant (Life Technologies Cat. # P36930)

S5 Protocols for whole-mount sea urchin embryos (*Strongylocentrotus purpuratus*)

This protocol has been optimized for embryos 45 hpf.

S5.1 Sample preparation protocol

1. Incubate fertilized embryos with fresh filtered seawater in a 24-well plate at 16 °C until 45 hpf (hours post-feritilization).
2. Transfer embryos from the 24-well plate into a 50 mL falcon conical tube.
3. Centrifuge at 100 × g for 2 min to bring embryos to the bottom of the tube.
4. Remove seawater to reach 20 mL volume and add 2.4 mL of 4.45 M NaCl to deciliate embryos.
5. Incubate on ice until embryos settle to the bottom of the tube.
6. Remove seawater (with NaCl) and add 20 mL of filtered sea water.
7. Incubate on ice until embryos settle to the bottom of the tube.
8. Repeat steps 6 and 7.
9. Aspirate as much sea water as possible without removing embryos.
10. Fix embryos in 40 mL of 4% paraformaldehyde (PFA) overnight at 4 °C on a nutator.
    NOTE: *Use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
11. Remove fixative to reach a volume of ≈ 6 mL.
12. Transfer embryos to six 1.5 mL eppendorf tubes (1 mL each).
13. Centrifuge at 100 × g for 2 min.
14. Wash embryos five times with 1 mL of PBST each. Centrifuge at 100 × g for 2 min to bring embryos to the bottom of a tube in between washes.
15. Wash embryos three times with 1 mL of 70% ethanol each. Centrifuge at 100 × g for 2 min to bring embryos to the bottom of a tube in between ethanol washes.
16. Store embryos in 1 mL of 70 % ethanol at -20 °C before use.

S5.2 Buffer recipes for sample preparation

4% Paraformaldehyde (PFA)	For 40 mL of solution
4% PFA	10 mL of 16% PFA solution
1× PBS	4 mL of 10× PBS
	Fill up to 40 mL with ultrapure H_2O

PBST	For 50 mL of solution
1× PBS	5 mL of 10× PBS
0.1% Tween 20	500 μL of 10% Tween 20
	Fill up to 50 mL with ultrapure H_2O

NOTE: *16% PFA solution is filter sterilized using a 25 mm syringe filter with 0.2 μm membrane before use.*
NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the embryos.*

S5.3 Multiplexed in situ HCR protocol

Detection stage

1. Transfer ≈200 embryos to each well of a 96 well plate.
   NOTE: *Adding and removing solution should be performed under a dissecting scope to avoid losing a large number of embryos.*
2. Rehydrate embryos 3 × 5 min with 250 μL of 5× SSCT.
3. Aspirate with care to reach ≈10 μL of volume.
4. Add 50 μL of probe hybridization buffer and pre-hybridize at 45 °C for 30 min.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
5. Prepare probe solution by adding 0.2 pmol of each probe (0.2 μL of 1 μM stock per probe) to 50 μL of probe hybridization buffer at 45 °C.
6. Add the probe solution to reach a final hybridization volume of 100 μL.
7. Gently stir the solution with a 10 μL pipette tip.
8. Cover plate with Bio-Rad Microseal 'A' film and incubate embryos overnight (12–16 h) at 45 °C.
9. Add 150 μL of probe wash buffer (pre-heated to 45 °C) to each well of embryos.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
10. Incubate at 45 °C for 5 min.
11. Remove excess probes by washing with ≈200 μL of probe wash buffer at 45 °C:
    (a) 2 × 5 min
    (b) 2 × 30 min

    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*
    NOTE: *Fill wash solution to top of each well but do not overfill.*
    NOTE: *It is important to maintain plate temperature at 45 °C during probe washing steps. This can be achieved by placing the 96-well plate containing the embryos on a heat plate while removing solution from each well.*
12. Wash embryos 2 × 5 min with ≈200 μL of 5× SSCT.

Amplification stage

1. Prepare 6 pmol of each fluorescently labeled hairpin by snap cooling 2 μL of 3 μM stock in hairpin storage buffer (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
2. Prepare hairpin solution by adding all snap-cooled hairpins to 100 μL of amplification buffer at room temperature.
3. Aspirate as much 5× SSCT as possible without removing embryos.
4. Add the hairpin solution and incubate embryos overnight (12–16 h) in the dark at room temperature.
5. Add 150 μL of 5× SSCT and incubate for 5 min at room temperature.
6. Remove excess hairpins by washing with ≈200 μL of 5× SSCT at room temperature:
    (a) 2 × 5 min
    (b) 2 × 30 min

S5.4 Sample mounting for microscopy

1. Remove 5× SSCT and add ≈50 μL of SlowFade Gold antifade mountant to each well.
2. A chamber for mounting embryos was made by aligning two stacks of double-sided tape (3 pieces per stack) 1.5 cm apart on a 25 mm × 75 mm glass slide to prevent crushing the embryos.
3. Pipette 50 μL of embryos between the tape stacks on the slide.
4. Place a 22 mm × 30 mm No. 1 coverslip on top of the stacks to close the chamber.
5. A Zeiss 710 NLO inverted confocal microscope equipped with an LD C-Apochromat 40×/1.1 W Korr M27 objective was used to acquire images.

S5.5 Buffer recipes for in situ HCR

Probe hybridization buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
1× Denhardt's solution	800 μL of 50× Denhardt's solution
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

Probe wash buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
	Fill up to 40 mL with ultrapure H_2O

Amplification buffer	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
	Fill up to 40 mL with ultrapure H_2O

50% dextran sulfate	For 40 mL of solution
50% dextran sulfate	20 g of dextran sulfate powder
	Fill up to 40 mL with ultrapure H_2O

S5.6 Reagents and supplies

Paraformaldehyde, 16% w/v aq. soln., methanol free (Alfa Aesar Cat. # 30525-89-4)
Sterile Acrodisc 25 mm syringe filters with 0.2 μm HT Tuffryn membrane (Pall Cat. # PN4192)
10$\times$ PBS (Life Technologies Cat.# AM9625)
Ethanol, 200 proof (VWR Cat. # V1001G)
Formamide (Deionized) (Ambion Cat. # AM9342)
20$\times$ sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50$\times$ Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
Microseal 'A' Film (Bio-Rad Cat. # MSA5001)
25 mm $\times$ 75 mm glass slide (VWR Cat. # 48300-025)
22 mm $\times$ 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36936)

S6 Protocols for whole-mount nematode larvae (*Caenorhabditis elegans*)

This protocol has been optimized for larvae at stages L1–L4.

S6.1 Sample preparation protocol

1. Wash nematode larvae off plate with 1 mL of M9 buffer and transfer to a 1.5 mL eppendorf tube.
2. Centrifuge at 200 × g for 2 min to bring larvae to the bottom of the tube and remove the M9 buffer.
3. Wash larvae 3 times with 1 mL of M9 buffer each. Centrifuge at 200 × g for 2 min between washes.
4. Centrifuge and remove ≈ 800 μL of M9 buffer.
5. Aliquot larvae sufficent for an in situ reaction into 1.5 mL eppendorf tubes.
6. Add 1 mL of 4% paraformaldehyde (PFA).
   NOTE: *Use fresh PFA to avoid increased autofluorescence.*
7. Immediately freeze sample at -80 °C overnight before use. Larvae could stay in -80 °C freezer for long-term storage.

S6.2 Buffer recipes for sample preparation

M9 buffer	For 1 L of solution
22 mM KH_2PO_4	3 g of KH_2PO_4
42 mM Na_2HPO_4	6 g of Na_2HPO_4
20.5 mM NaCl	5 g of NaCl
1 mM $MgSO_4$	1 mL of 1 M $MgSO_4$
	Fill up to 1 L with ultrapure H_2O
	Sterilize by autoclaving
	Store buffer at 4 °C before use

4% Paraformaldehyde (PFA)	For 40 mL of solution
4% PFA	10 mL of 16% PFA solution
1× PBS	4 mL of 10× PBS
	Fill up to 40 mL with ultrapure H_2O

PBST	For 50 mL of solution
1× PBS	5 mL of 10× PBS
0.1% Tween 20	500 μL of 10% Tween 20
	Fill up to 50 mL with ultrapure H_2O

NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the larvae.*

S6.3 Multiplexed in situ HCR protocol

Detection stage

1. Fix larvae by thawing at room temperature for 45 min.
2. Wash larvae 2 times with 1 mL of PBST each. Bring larvae to the bottom of the tube with centrifugation at 200 × g for 2 min in between washes.
3. Treat larvae with 1 mL of proteinase K (100 μg/mL) for 10 min at 37 °C.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K.*
4. Wash larvae 2 times with 1 mL of PBST each.
5. Incubate in 1 mL of 2 mg/mL of glycine solution for 15 min on ice.
6. Wash larvae 2 times with 1 mL of PBST each.
7. Incubate larvae in 1 mL of 50% PBST / 50% probe hybridization buffer for 5 min at room temperature.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
8. Centrifuge at 200 × g for 2 min to remove solution.
9. Pre-hybridize larvae in 300 μL of probe hybridization buffer at 45 °C for 1 h.
10. Prepare probe solution by adding 1 pmol of each probe (1 μL of 1 μM stock per probe) to 200 μL of probe hybridization buffer at 45 °C.
11. Add the probe solution to reach a final hybridization volume of 500 μL.
12. Incubate larvae overnight (12–16 h) at 45 °C.
13. Remove excess probes by washing with 1 mL of probe wash buffer at 45 °C:
    (a) 2 × 5 min
    (b) 2 × 30 min

    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*
    NOTE: *Before each wash, bring larvae to the bottom of the tube with centrifugation at 500 × g for 2 min.*
14. Wash larvae 2 × 5 min with 1 mL of 5× SSCT.

Amplification stage

1. Pre-amplify larvae with 300 μL of amplification buffer for 30 min at room temperature.
2. Prepare 30 pmol of each fluorescently labeled hairpin by snap cooling 10 μL of 3 μM stock in hairpin storage buffer (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
3. Prepare hairpin solution by adding all snap-cooled hairpins to 200 μL of amplification buffer at room temperature.
4. Add the hairpin solution to reach a final amplification volume of 500 μL.
5. Incubate larvae overnight (12–16 h) in the dark at room temperature.
6. Remove excess hairpins by washing with 1 mL of 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

NOTE: *Before each wash, bring larvae to the bottom of the tube with centrifugation at 500 × g for 2 min.*

S6.4 Sample mounting for microscopy

1. Remove 5× SSCT and add ≈50 μL of SlowFade Gold antifade mountant to each tube.
2. Pipette 50 μL of larvae on a 25 mm × 75 mm glass slide.
3. Place a 22 mm × 60 mm No. 1 coverslip on top of the solution to close the chamber.
4. A Zeiss 710 NLO inverted confocal microscope equipped with an LD C-Apochromat 40×/1.1 W Korr M27 objective was used to acquire images.

S6.5 Buffer recipes for in situ HCR

Proteinase K solution	For 1 mL of solution
100 μg/mL proteinase K	5 μL of 20 mg/mL proteinase K Fill up to 1 mL with PBST
Glycine solution	For 50 mL of solution
2 mg/mL glycine PBST	100 mg of glycine Fill up to 50 mL with PBST
Probe hybridization buffer	For 40 mL of solution
50% formamide 5× sodium chloride sodium citrate (SSC) 9 mM citric acid (pH 6.0) 0.1% Tween 20 50 μg/mL heparin 1× Denhardt's solution 10% dextran sulfate	20 mL formamide 10 mL of 20× SSC 360 μL 1 M citric acid, pH 6.0 400 μL of 10% Tween 20 200 μL of 10 mg/mL heparin 800 μL of 50× Denhardt's solution 8 mL of 50% dextran sulfate Fill up to 40 mL with ultrapure H_2O
Probe wash buffer	For 40 mL of solution
50% formamide 5× sodium chloride sodium citrate (SSC) 9 mM citric acid (pH 6.0) 0.1% Tween 20 50 μg/mL heparin	20 mL formamide 10 mL of 20× SSC 360 μL 1 M citric acid, pH 6.0 400 μL of 10% Tween 20 200 μL of 10 mg/mL heparin Fill up to 40 mL with ultrapure H_2O
Amplification buffer	For 40 mL of solution
5× sodium chloride sodium citrate (SSC) 0.1% Tween 20 10% dextran sulfate	10 mL of 20× SSC 400 μL of 10% Tween 20 8 mL of 50% dextran sulfate Fill up to 40 mL with ultrapure H_2O
5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC) 0.1% Tween 20	10 mL of 20× SSC 400 μL of 10% Tween 20 Fill up to 40 mL with ultrapure H_2O
50% dextran sulfate	For 40 mL of solution
50% dextran sulfate	20 g of dextran sulfate powder Fill up to 40 mL with ultrapure H_2O

S6.6 Reagents and supplies

Paraformaldehyde, 16% w/v aq. soln., methanol free (Alfa Aesar Cat. # 30525-89-4)
10× PBS (Life Technologies Cat.# AM9625)
Proteinase K, molecular biology grade (NEB Cat. # P8107S)
Glycine (Sigma Cat. # G7126)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 60 mm No. 1 coverslip (VWR Cat. # 48393-070)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36936)

S7 Protocols for whole-mount zebrafish larvae (*Danio rerio*)

This protocol has been optimized for embryos at 27 hpf and larvae at 5 dpf.

S7.1 Sample preparation protocol

1. Collect zebrafish embryos and incubate at 28 °C in a petri dish with egg H_2O.
2. Exchange egg H_2O with egg H_2O containing 0.003% of 1-phenyl 2-thiourea (PTU) when embryos reach 12 hpf.
   NOTE: *PTU inhibits melanogenesis but can be toxic at high concentrations. PTU treatment must start before the initial pigmentation occurs as PTU does not remove pigment that has already formed. PTU treatment is not necessary for nacre embryos used in this paper.*
3. Replace with fresh egg H_2O containing 0.003% of PTU everyday until the larvae reach 5 dpf (days post-fertilization).
4. Transfer ∼40 larvae (5 dpf) to a 2 mL eppendorf tube and remove excess egg H_2O.
5. Fix larvae in 2 mL of 4% paraformaldehyde (PFA) for 24 h at 4 °C.
   NOTE: *Use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
6. Wash larvae 3 × 5 min with 1 mL of 1× phosphate-buffered saline (PBS) to stop the fixation.
   NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the larvae.*
7. Dehydrate and permeabilize with a series of methanol (MeOH) washes (1 mL each):
   (a) 100% MeOH for 4 × 10 min
   (b) 100% MeOH for 1 × 50 min.
8. Store larvae at -20 °C overnight before use.
   NOTE: *Larvae can be stored for six months at -20 °C.*

S7.2 Buffer recipes for sample preparation

6% PTU stock solution
6% PTU

For 100 mL of solution
6 g of 1-phenyl 2-thiourea powder
Fill up to 100 mL with egg H_2O
Heat solution at 50–60 °C overnight to dissolve powder

0.3% PTU in egg H_2O
0.3% PTU

For 50 mL of solution
2.5 mL of 6% PTU
Fill up to 50 mL with with egg H_2O

4% Paraformaldehyde (PFA)
4% PFA
1× PBS

For 25 mL of solution
1 g of PFA powder
25 mL of 1× PBS
Heat solution at 50–60 °C to dissolve powder

NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the larvae.*

S7.3 Multiplexed in situ HCR protocol

Detection stage

1. Transfer the required number of larvae for an experiment to a 2 mL eppendorf tube.
2. Rehydrate with a series of graded 1 mL MeOH/PBST washes for 5 min each at room temperature:
   (a) 75% MeOH / 25% PBST
   (b) 50% MeOH / 50% PBST
   (c) 25% MeOH / 75% PBST
   (d) 5 × 100% PBST.
3. Treat 5 dpf larvae with 1 mL of proteinase K (30 μg/mL) for 45 min at room temperature.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage. Skip proteinase K treatment and postfixation (steps 3–6) for embryos 30 hpf and younger.*
4. Wash larvae two times with PBST (1 mL each) without incubation.
5. Postfix with 1 mL of 4% PFA for 20 min at room temperature.
6. Wash larvae 5 × 5 min with 1 mL of PBST.
7. For each sample, move 8 larvae to a 1.5 mL eppendorf tube.
8. Pre-hybridize with 350 μL of probe hybridization buffer (with tRNA) for 30 min at 45 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
9. Prepare probe solution by adding 1 pmol of each probe (1 μL of 1 μM stock per probe) to 500 μL of probe hybridization buffer at 45 °C.
10. Remove the pre-hybridization solution and add the probe solution.
11. Incubate larvae overnight (12–16 h) at 45 °C.
12. Remove excess probes by washing at 45 °C with 500 μL of:
    (a) 100% of probe wash buffer for 10 min
    (b) 75% of probe wash buffer / 25% 5× SSCT for 15 min
    (c) 50% of probe wash buffer / 50% 5× SSCT for 15 min
    (d) 25% of probe wash buffer / 75% 5× SSCT for 15 min
    (e) 100% 5× SSCT for 15 min
    (f) 100% 5× SSCT for 30 min.

    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*

Amplification stage

1. Pre-amplify larvae with 350 μL of amplification buffer for 30 min at room temperature.
2. Prepare 30 pmol of each fluorescently labeled hairpin by snap cooling 10 μL of 3 μM stock in hairpin storage buffer (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
3. Prepare hairpin solution by adding all snap-cooled hairpins to 500 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate larvae overnight (12–16 h) in the dark at room temperature.
6. Remove excess hairpins by washing with 500 μL of 5$\times$ SSCT at room temperature:
   (a) 2 $\times$ 5 min
   (b) 2 $\times$ 30 min
   (c) 1 $\times$ 5 min.

S7.4 Sample mounting for microscopy

1. A chamber for mounting the larvae was made by aligning two stacks of Scotch tape (8 pieces per stack) 1 cm apart on a 25 mm $\times$ 75 mm glass slide.
2. Approximately 200 μL of 3% methyl cellulose mounting medium was added between the tape stacks on the slide.
3. Place larvae on the medium and orient for dorsal imaging.
4. Place a 22 mm $\times$ 22 mm No. 1 coverslip on top of the stacks to close the chamber.
5. A Zeiss 710 NLO inverted confocal microscope equipped with an LD C-Apochromat 40$\times$/1.1 W Korr M27 objective was used to acquire images.

S7.5 Buffer recipes for in situ HCR

PBST

1× PBS
0.1% Tween 20

For 50 mL of solution
5 mL of 10× PBS
500 μL of 10% Tween 20
Fill up to 50 mL with ultrapure H_2O

Proteinase K solution

30 μg/mL proteinase K

For 1 mL of solution
1.5 μL of 20 mg/mL proteinase K
Fill up to 1 mL with PBST

Probe hybridization buffer

50% formamide
5× sodium chloride sodium citrate (SSC)
9 mM citric acid (pH 6.0)
0.1% Tween 20
50 μg/mL heparin
1× Denhardt's solution
10% dextran sulfate
500 μg/mL tRNA

For 40 mL of solution
20 mL formamide
10 mL of 20× SSC
360 μL 1 M citric acid, pH 6.0
400 μL of 10% Tween 20
200 μL of 10 mg/mL heparin
800 μL of 50× Denhardt's solution
8 mL of 50% dextran sulfate
200 μL of 100 mg/mL tRNA
Fill up to 40 mL with ultrapure H_2O

Probe wash buffer

50% formamide
5× sodium chloride sodium citrate (SSC)
9 mM citric acid (pH 6.0)
0.1% Tween 20
50 μg/mL heparin

For 40 mL of solution
20 mL formamide
10 mL of 20× SSC
360 μL 1 M citric acid, pH 6.0
400 μL of 10% Tween 20
200 μL of 10 mg/mL heparin
Fill up to 40 mL with ultrapure H_2O

Amplification buffer

5× sodium chloride sodium citrate (SSC)
0.1% Tween 20
10% dextran sulfate

For 40 mL of solution
10 mL of 20× SSC
400 μL of 10% Tween 20
8 mL of 50% dextran sulfate
Fill up to 40 mL with ultrapure H_2O

5× SSCT

5× sodium chloride sodium citrate (SSC)
0.1% Tween 20

For 40 mL of solution
10 mL of 20× SSC
400 μL of 10% Tween 20
Fill up to 40 mL with ultrapure H_2O

50% dextran sulfate

50% dextran sulfate

For 40 mL of solution
20 g of dextran sulfate powder
Fill up to 40 mL with ultrapure H_2O

3% methyl cellulose

3% methyl cellulose

For 100 mL of solution
3 g of methyl cellulose powder
Fill up to 100 mL with ultrapure H_2O
Stir overnight at 4 °C with a stir bar to dissolve powder

S7.6 Reagents and supplies

Paraformaldehyde (PFA) (Sigma Cat. # P6148)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Proteinase K, molecular biology grade (NEB Cat. # P8107S)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
50% Tween 20 (Life Technologies Cat. # 00-3005)
Heparin (Sigma Cat. # H3393)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
tRNA from baker's yeast (Roche Cat. # 10109509001)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 22 mm No. 1 coverslip (VWR Cat. # 48366-067)
Methyl cellulose (Sigma Cat. # M0387)

S8 Protocols for whole-mount chicken embryos (*Gallus gallus domesticus*)

This protocol has been optimized for embryos at stage HH 11–12.

S8.1 Sample preparation protocol

1. Collect stage HH 10–11 chick embryos on 3M paper circles and rinse in 1× DEPC-treated PBS.
2. Immediately transfer into a petri dish containing 4% paraformaldehyde (PFA).
   NOTE: *Use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
3. Transfer all embryos into a new petri dish with fresh 4% PFA after dissection.
   NOTE: *This is to avoid fixing embryos in PFA diluted by egg yolk.*
4. Fix the samples at room temperature for 1 h.
5. Dissect the embryos off the paper, preferably in PFA.
6. Remove the fixed vitelline membrane (opaque) and cut the squares around area pellucida, without leaving excess of extra-embryonic tissue.
7. Transfer embryos into a 2 mL eppendorf tube containing PBST.
8. Nutate for 5 mins with the tube positioned horizontally in a small ice bucket.
9. Dehydrate embryos into methanol (MeOH) with a series of graded 2 mL MeOH/PBST washes, each time nutating for 5 min on ice:
   (a) 25% MeOH / 75% PBST
   (b) 50% MeOH / 50% PBST
   (c) 75% MeOH / 25% PBST
   (d) 100% MeOH
   (e) 100% MeOH.
10. Store embryos at -20 °C overnight before use.
    NOTE: *Embryos can be stored for six months at -20 °C.*

S8.2 Buffer recipes for sample preparation

4% Paraformaldehyde (PFA)	For 25 mL of solution
4% PFA	1 g of PFA powder
1× PBS	25 mL of 1× PBS
	Heat solution at 50–60 °C to dissolve powder

PBST	For 50 mL of solution
1× PBS	5 mL of 10× PBS
0.1% Tween 20	500 μL of 10% Tween 20
	Fill up to 50 mL with ultrapure H_2O

NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the embryos.*

S8.3 Multiplexed in situ HCR protocol

Detection stage

1. Transfer the required number of embryos for an experiment to a 2 mL eppendorf tube.
   NOTE: *Do not place more than 4 embryos in each 2 mL eppendorf tube.*
2. Rehydrate with a series of graded 2 mL MeOH/PBST washes for 5 min each on ice:
   (a) 75% MeOH / 25% PBST
   (b) 50% MeOH / 50% PBST
   (c) 25% MeOH / 75% PBST
   (d) 100% PBST
   (e) 100% PBST.
3. Treat embryos with 2 mL of 20 μg/mL proteinase K solution for 30 min at room temperature.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.*
4. Postfix with 2 mL of 4% PFA for 20 min at room temperature.
5. Wash embryos 2 $\times$ 5 min with 2 mL of PBST on ice.
6. Wash embryos with 2 mL of 50% PBST / 50% 5$\times$ SSCT for 5 min on ice.
7. Wash embryos with 2 mL of 5$\times$ SSCT for 5 min on ice.
8. For each sample, transfer 1–4 embryos to a 2 mL eppendorf tube.
9. Incubate embryos in 1 mL of probe hybridization buffer on ice for 5 min.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
10. Remove the buffer and pre-hybridize with 1 mL of probe hybridization buffer for 30 min at 45 °C.
11. Prepare probe solution by adding 2 pmol of each probe (2 μL of 1 μM stock per probe) to 1 mL of probe hybridization buffer at 45 °C.
12. Remove the pre-hybridization solution and add the probe solution.
13. Incubate embryos overnight (12–16 h) at 45 °C.
14. Remove excess probes by washing with 1 mL of probe wash buffer at 45 °C:
    (a) 2 $\times$ 5 min
    (b) 2 $\times$ 30 min

    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*

Amplification stage

1. Pre-amplify embryos with 1 mL of amplification buffer for 5 min at room temperature.
2. Prepare 60 pmol of each fluorescently labeled hairpin by snap cooling 20 μL of 3 μM stock in hairpin storage buffer (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
3. Prepare hairpin solution by adding all snap-cooled hairpins to 1 mL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the embryos overnight (12–16 h) in the dark at room temperature.
6. Remove excess hairpins by washing with 1 mL of 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

S8.4 Sample mounting for microscopy

1. A chamber for mounting each embryo was made by aligning two stacks of double-sided tape (2 pieces per stack) 1 cm apart on a 25 mm × 75 mm glass slide.
2. Place an embryo between the tape stacks on the slide and remove as much solution as possible.
3. Align the embryo for dorsal imaging and carefully touch the slide with a kimwipe to further dry the area around the embryo.
4. Add two drops of SlowFade Gold antifade mountant on top of the embryo.
5. Place a 22 mm × 30 mm No. 1 coverslip on top of the stacks to close the chamber.
6. A Zeiss 710 NLO inverted confocal microscope equipped with an LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC objective was used to acquire images.

S8.5 Buffer recipes for in situ HCR

Proteinase K solution	For 1 mL of solution
20 μg/mL proteinase K	1 μL of 20 mg/mL proteinase K Fill up to 1 mL with PBST
Probe hybridization buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
1× Denhardt's solution	800 μL of 50× Denhardt's solution
10% dextran sulfate	8 mL of 50% dextran sulfate Fill up to 40 mL with ultrapure H_2O
Probe wash buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin Fill up to 40 mL with ultrapure H_2O
Amplification buffer	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
10% dextran sulfate	8 mL of 50% dextran sulfate Fill up to 40 mL with ultrapure H_2O
5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20 Fill up to 40 mL with ultrapure H_2O
50% dextran sulfate	For 40 mL of solution
50% dextran sulfate	20 g of dextran sulfate powder Fill up to 40 mL with ultrapure H_2O

S8.6 Reagents and supplies

Paraformaldehyde (PFA) (Sigma Cat. # P6148)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Proteinase K, molecular biology grade (NEB Cat. # P8107S)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36937)

S9 Protocols for whole-mount mouse embryos (*Mus musculus*)

This protocol has been optimized for embryos at stage E9.5.

S9.1 Sample preparation protocol

1. Wipe all dissection equipment with RNaseZap.
2. Kill a pregnant female mouse using an IACUC-approved protocol.
3. Immediately remove the uterus and submerge it in 4% paraformaldehyde (PFA) in a fresh RNase-free petri dish.
   NOTE: *Use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
4. Dissect the mouse embryos from the uterus while it is submerged in 4% PFA.
   CAUTION: *Perform this step with active ventilation from a fume hood or a histology slot hood to prevent exposure to the fixative fumes.*
   NOTE: *Each female mouse produces 6–9 embryos. For Steps 5–9, we recommend using ≈1 mL of solution per group of 10 embryos.*
5. Transfer the embryos to a clean vial containing 1 mL of fresh 4% PFA and fix them overnight or longer at 4 °C.
6. Wash 2 × 5 min with 1 mL of PBST on ice.
7. Dehydrate embryos into methanol (MeOH) with a series of graded 1 mL MeOH/PBST washes for 10 min on ice:
   (a) 25% MeOH / 75% PBST
   (b) 50% MeOH / 50% PBST
   (c) 75% MeOH / 25% PBST
   (d) 100% MeOH
   (e) 100% MeOH.
8. Store embryos at -20 °C overnight (> 16 h) or until use.
   NOTE: *Embryos could be stored for six months at -20 °C .*

S9.2 Buffer recipes for sample preparation

4% Paraformaldehyde (PFA)	For 25 mL of solution
4% PFA	1 g of PFA powder
1× PBS	25 mL of 1× PBS
	Heat solution at 50–60 °C to dissolve powder

PBST	For 50 mL of solution
1× PBS	5 mL of 10× PBS
0.1% Tween 20	500 μL of 10% Tween 20
	Fill up to 50 mL with ultrapure H_2O

NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the embryos.*

S9.3 Multiplexed in situ HCR protocol

Detection stage

1. Transfer the desired number of embryos for an experiment to a 1.5 mL eppendorf tube.
   NOTE: *All solutions and washes use a volume of 1 mL unless specified.*
2. Rehydrate with a series of graded MeOH/PBST washes for 10 min each at room temperature:
   (a) 75% MeOH / 25% PBST
   (b) 50% MeOH / 50% PBST
   (c) 25% MeOH / 75% PBST
   (d) 100% PBST
   (e) 100% PBST.
3. Immerse embryos in 10 μg/mL proteinase K solution for 15 min at room temperature.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.*
4. Wash embryos 2 $\times$ 5 min with PBST.
5. Postfix with 4% PFA for 20 min at room temperature.
6. Wash embryos 3 $\times$ 5 min with PBST.
7. For each sample, transfer 1–4 embryos to a 1.5 mL eppendorf tube.
8. Incubate embryos in 1 mL solution containing 50% of hybridization buffer (with tRNA) and 50% of PBST until embryos are settled.
9. Pre-hybridize with probe hybridization buffer (with tRNA) for 30 min at 45 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
10. Prepare probe solution by adding 2 pmol of each probe (2 μL of 1 μM stock per probe) to 500 μL of probe hybridization buffer at 45 °C.
11. Remove the pre-hybridization solution and add the probe solution.
12. Incubate embryos overnight (12–16 h) at 45 °C.
13. Remove excess probes by washing with 500 μL of probe wash buffer at 45 °C:
    (a) 2 $\times$ 5 min
    (b) 2 $\times$ 30 min

    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*
14. Wash with 500 μL of 50% probe wash buffer / 50% 5$\times$ SSCT for 10 min at 45 °C.
15. Wash embryos 3 $\times$ 5 min with 5$\times$ SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos with 1 mL of amplification buffer for 30 min at room temperature.
2. Prepare 30 pmol of each fluorescently labeled hairpin by snap cooling 10 μL of 3 μM stock in hairpin storage buffer (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
3. Prepare hairpin solution by adding all snap-cooled hairpins to 500 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the embryos overnight (12–16 h) in the dark at room temperature.
6. Remove excess hairpins by washing with 500 μL of 5$\times$ SSCT at room temperature:
   (a) 2 $\times$ 5 min
   (b) 2 $\times$ 30 min
   (c) 1 $\times$ 5 min

S9.4 Sample clearing and mounting for microscopy

1. Clear embryos for imaging by incubating them in 500 μL of Scale A2 solution for 1 d in the dark at 4 °C.
2. Clean a 25 mm × 75 mm glass slide and a 22 mm × 30 mm No. 1 coverslip with RNase*Zap*. Add four beads of vaseline to each slide to support a coverslip at the corners.
3. Place and orient an embryo on a slide.
4. Remove excess buffer and add ≈100 μL of SlowFade Gold antifade mountant over the embryo.
5. Place the coverslip over the embryo. Apply enough pressure to push the coverslip onto the embryo without flattening it. Seal the edges of the coverslip with nail polish.
6. Store the slides in the dark at 4 °C until imaging.
7. A Zeiss 710 NLO inverted confocal microscope equipped with an LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC objective was used to acquire images.

S9.5 Buffer recipes for in situ HCR

Proteinase K solution
10 μg/mL proteinase K

For 2 mL of solution
1 μL of 20 mg/mL proteinase K
Fill up to 2 mL with PBST

Probe hybridization buffer
50% formamide
5× sodium chloride sodium citrate (SSC)
9 mM citric acid (pH 6.0)
0.1% Tween 20
50 μg/mL heparin
1× Denhardt's solution
10% dextran sulfate
500 μg/mL tRNA

For 40 mL of solution
20 mL formamide
10 mL of 20× SSC
360 μL 1 M citric acid, pH 6.0
400 μL of 10% Tween 20
200 μL of 10 mg/mL heparin
800 μL of 50× Denhardt's solution
8 mL of 50% dextran sulfate
200 μL of 100 mg/mL tRNA
Fill up to 40 mL with ultrapure H_2O

Probe wash buffer
50% formamide
5× sodium chloride sodium citrate (SSC)
9 mM citric acid (pH 6.0)
0.1% Tween 20
50 μg/mL heparin

For 40 mL of solution
20 mL formamide
10 mL of 20× SSC
360 μL 1 M citric acid, pH 6.0
400 μL of 10% Tween 20
200 μL of 10 mg/mL heparin
Fill up to 40 mL with ultrapure H_2O

Amplification buffer
5× sodium chloride sodium citrate (SSC)
0.1% Tween 20
10% dextran sulfate

For 40 mL of solution
10 mL of 20× SSC
400 μL of 10% Tween 20
8 mL of 50% dextran sulfate
Fill up to 40 mL with ultrapure H_2O

5× SSCT
5× sodium chloride sodium citrate (SSC)
0.1% Tween 20

For 40 mL of solution
10 mL of 20× SSC
400 μL of 10% Tween 20
Fill up to 40 mL with ultrapure H_2O

50% dextran sulfate
50% dextran sulfate

For 40 mL of solution
20 g of dextran sulfate powder
Fill up to 40 mL with ultrapure H_2O

Scale A2
4 M urea
10% glycerol
0.1% Triton X-100

For 50 mL of solution
12.012 g of urea
5 mL of glycerol
50 μL of Triton X-100
Fill up to 50 mL with ultrapure H_2O
Adjust pH to 7.7 with NaOH

S9.6 Reagents and supplies

RNaseZap (Ambion Cat. # AM9780)
Paraformaldehyde (PFA) (Sigma Cat. # P6148)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Proteinase K, molecular biology grade (NEB Cat. # P8107S)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
tRNA from baker's yeast (Roche Cat. # 10109509001)
UltraPure urea (Life Technologies Cat. # 15505-035)
Glycerol, ACS grade (Mallinckrodt Cat. # 5092-16)
Triton X-100 (Sigma Cat. # X100)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36936)
SlowFade Gold antifade mountant with DAPI (Life Technologies Cat. # S36938)

S10 Protocols for FFPE human tissue sections (*Homo sapiens sapiens*)

This protocol has been optimized for 4-μm FFPE sections.

S10.1 Multiplexed in situ HCR protocol

Detection stage

1. Deparaffinize FFPE tissue by immersing slide in Histo-Clear II for 3 × 5 min.
   NOTE: *Each 50 mL falcon tube can fit two outward-facing slides. A volume of 30 mL is sufficient to immerse sections in the tube. If desired, a larger number of slides can be processed together using a Coplin jar.*
2. Rehydrate with a series of graded EtOH washes for 3 min at room temperature:
   (a) 100% EtOH
   (b) 100% EtOH
   (c) 95% EtOH
   (d) 70% EtOH
   (e) RNase-free H_2O
   (f) RNase-free H_2O.
3. Incubate in 1× TBS for 5 min.
4. Immerse slide in 10 μg/mL of proteinase K solution for 40 min at 37 °C.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K.*
   NOTE: *We recommend performing this step in a falcon tube placed inside a 37 °C water bath to minimize temperature fluctuation.*
5. Wash slide 2 × 3 min at room temperature in TBST.
6. Immerse slide in 0.2 N HCl for 20 min at room temperature.
7. Incubate slides in 5× SSCT for 5 min.
8. Place slide in an RNase-free staining glass trough with a stir bar.
9. Add 200 mL of 0.1 M triethanolamine-HCl at pH 8.0.
10. Add 500 μL of acetic anhydride **slowly and carefully** with constant stirring.
11. Turn off stirrer when the acetic anhydride is dispersed and allow the slide to incubate for 10 min.
12. Incubate slide in 5× SSCT for 5 min.
13. Pre-warm two humidified chambers with one at 45 °C and the other one at 65 °C.
14. Dry slide by blotting edges on a Kimwipe.
15. Add 200 μL of probe hybridization buffer on top of the tissue sample.
    CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
16. Pre-hybridize for 10 min inside the 65 °C humidified chamber.
17. Prepare probe solution by adding 0.2 pmol of each probe (0.2 μL of 1 μM stock per probe) to 100 μL of probe hybridization buffer at 45 °C.

18. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
19. Add 50–100 μL of the probe solution on top of the tissue sample.
    NOTE: *Amount of probe solution depends on the size of the coverslip.*
20. Place a coverslip on the tissue sample and incubate overnight (12–16 h) in the 45 °C humidified chamber.
21. Immerse slide in 2× SSC with 0.1% SDS at room temperature to float off coverslip.
22. Remove excess probes by incubating slide at 45 °C in:
    (a) 75% of probe wash buffer / 25% 5× SSCT for 15 min
    (b) 50% of probe wash buffer / 50% 5× SSCT for 15 min
    (c) 25% of probe wash buffer / 75% 5× SSCT for 15 min
    (d) 100% 5× SSCT for 15 min.

    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*
23. Immerse slide in 5× SSCT for 5 min at room temperature.

Amplification stage

1. Dry slide by blotting edges on a Kimwipe.
2. Add 200 μL of amplification buffer on top of the tissue sample and pre-amplify in a humidified chamber for 2 h at room temperature.
3. Prepare 6 pmol of each fluorescently labeled hairpin by snap cooling 2 μL of 3 μM stock in hairpin storage buffer (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
4. Prepare hairpin solution by adding all snap-cooled hairpins to 100 μL of amplification buffer at room temperature.
5. Remove the pre-amplification solution and drain excess buffer on slide by blotting edges on a Kimwipe.
6. Add 50–100 μL of the hairpin solution on top of the tissue sample.
   NOTE: *Amount of hairpin solution depends on the size of the coverslip.*
7. Place a coverslip on the tissue sample and incubate overnight (12–16 h) in a dark humidified chamber at room temperature.
8. Immerse slide in 5× SSCT at room temperature to float off coverslip.
9. Remove excess hairpins by incubating slide in 5× SSCT at room temperature for:
   (a) 2 × 30 min
   (b) 1 × 5 min

S10.2 Sample mounting for microscopy

1. Dry slide by blotting edges on a Kimwipe.
2. Add 50 μL of SlowFade Gold antifade mountant with DAPI on top of human tissue section.
3. Place a 22 mm $\times$ 40 mm No. 1 coverslip on top and seal using clear nail polish.
4. A Zeiss Axio Observer Z1 fluorescent inverted microscope equipped with an Plan-Apochromat 63$\times$/1.4 Oil DIC M27 objective was used to acquire all tissue section images. Samples were illuminated with X-Cite Series 120 (EXFO Photonics) and images were collected with an ImagEM-1K EM-CCD digital camera (Hamamatsu).

S10.3 Buffer recipes for in situ HCR

Proteinase K solution	For 30 mL of solution
10 μg/mL proteinase K	15 μL of 20 mg/mL proteinase K Fill up to 30 mL with 1× TBS
0.2 N HCl	For 30 mL of solution
0.2 N HCl	500 μL of 37% ($\approx$ 12 N) HCl Fill up to 30 mL with ultrapure H_2O
0.1 M Triethanolamine-HCl	For 200 mL of solution
0.1 M triethanolamine Adjust pH to 8.0	2.67 mL of 7.5 mM triethanolamine 350 μL 37% HCl Fill up to 200 mL with ultrapure H_2O
TBST	For 50 mL of solution
1× TBS 0.1% Tween 20	5 mL of 10× TBS 500 μL of 10% Tween 20 Fill up to 50 mL with ultrapure H_2O
Probe hybridization buffer	For 40 mL of solution
50% formamide 5× sodium chloride sodium citrate (SSC) 9 mM citric acid (pH 6.0) 0.1% Tween 20 50 μg/mL heparin 1× Denhardt's solution 10% dextran sulfate	20 mL formamide 10 mL of 20× SSC 360 μL 1 M citric acid, pH 6.0 400 μL of 10% Tween 20 200 μL of 10 mg/mL heparin 800 μL of 50× Denhardt's solution 8 mL of 50% dextran sulfate Fill up to 40 mL with ultrapure H_2O
Probe wash buffer	For 40 mL of solution
50% formamide 5× sodium chloride sodium citrate (SSC) 9 mM citric acid (pH 6.0) 0.1% Tween 20 50 μg/mL heparin	20 mL formamide 10 mL of 20× SSC 360 μL 1 M citric acid, pH 6.0 400 μL of 10% Tween 20 200 μL of 10 mg/mL heparin Fill up to 40 mL with ultrapure H_2O
Amplification buffer	For 40 mL of solution
5× sodium chloride sodium citrate (SSC) 0.1% Tween 20 10% dextran sulfate 100 μg/mL salmon sperm DNA	10 mL of 20× SSC 400 μL of 10% Tween 20 8 mL of 50% dextran sulfate 400 μL of 10 mg/mL salmon sperm DNA Fill up to 40 mL with ultrapure H_2O
5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC) 0.1% Tween 20	10 mL of 20× SSC 400 μL of 10% Tween 20 Fill up to 40 mL with ultrapure H_2O

Development 143: doi:10.1242/dev.140137: Supplementary information

50% dextran sulfate

50% dextran sulfate

For 40 mL of solution

20 g of dextran sulfate powder

Fill up to 40 mL with ultrapure H_2O

S10.4 Reagents and supplies

FFPE human normal breast tissue section (Pantomics Cat. # BRE01 Block # F091926B08)
Histo-Clear II (National Diagnostics Cat. # HS-202)
Ethanol, 200 proof (VWR Cat. # V1001G)
10× Tris-buffered saline solution (TBS) (Research Products International Cat. # T60075)
20 mg/mL Proteinase K (Life Technologies Cat. # AM2546)
10% Tween 20 Solution (Bio-Rad Cat. # 161-0781)
Hydrochloric Acid (HCl) (EMD Millipore Cat. # HX0603-75)
Triethanolamine (Acros Organics Cat. # AC42163-1000)
Acetic anhydride (Mallinckrodt Chemicals Cat. # 2420-04) Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
UltraPure salmon sperm DNA solution (Life Technologies Cat. #15632-011)
22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
SlowFade Gold antifade mountant with DAPI (Life Technologies Cat. # S36938)

S11 Characterization of signal-to-background

The signal-to-background ratio was calculated for each target mRNA following the image analysis approach of Section S1.4 using a subset of the control experiments described in Section S2.3.

Organism	mRNA	Background	Signal	Signal-to-background	Figure
*E. coli**	*Tg(rfp)*	150 ± 20	9000 ± 1000	60 ± 10	S2
	Tg(gfp)	15 ± 3	1600 ± 400	110 ± 30	
D. melanogaster	*cad*	180 ± 30	400 ± 100	2.0 ± 0.7	S3
	sog	170 ± 10	1200 ± 300	7 ± 2	
	cnc	280 ± 20	1200 ± 300	4 ± 1	
	eve	90 ± 20	2000 ± 100	21 ± 5	
S. Purpuratus	*FoxA*	100 ± 20	700 ± 100	7 ± 1	S4
	Onecut	200 ± 50	600 ± 100	3 ± 1	
	FoxQ2	70 ± 20	340 ± 90	5 ± 2	
C. elegans	*myo-2*	191 ± 2	700 ± 100	3.9 ± 0.6	S5
	myo-3	250 ± 40	870 ± 60	3.5 ± 0.6	
	asp-5	78 ± 3	660 ± 10	8.6 ± 0.4	
D. rerio	*gnrh3*	19 ± 6	1230 ± 70	60 ± 20	S6
	avp	22 ± 4	1300 ± 100	60 ± 10	
	hcrt	41 ± 8	1000 ± 200	24 ± 6	
	oxt	17 ± 4	1400 ± 200	80 ± 20	
G. gallus domesticus	*FoxD3*	180 ± 40	1200 ± 100	6 ± 2	S7
	Msx1	160 ± 60	1310 ± 80	8 ± 3	
	Sox10	230 ± 30	1170 ± 90	5.0 ± 0.7	
M. musculus	*Tg(egfp)*	145 ± 5	500 ± 100	3.7 ± 0.8	S8
	Acta2	130 ± 10	790 ± 40	6.0 ± 0.5	
	Pax3	170 ± 30	470 ± 50	2.8 ± 0.5	
*H. sapiens sapiens**	*KRT17*	4000 ± 1000	13,000 ± 2000	3.1 ± 0.9	S9
	KRT19	2200 ± 600	16,000 ± 2000	7 ± 2	

Table S4. Estimated signal-to-background (mean ± standard deviation, $N = 3$ replicates) for target mRNAs based on the images and rectangles depicted in the listed figures.

*The epifluorescence microscope used for bacterial replicates (Figure S2) and FFPE human tissue section replicates (Figure S9) generated non-negligible instrument Noise in the absence of samples. For these two sample types, estimates for Background and Signal in Table S4 incorporate subtraction of Noise with uncertainty propagation (see Sections S1.4 and S2.3).

S11.1 Bacterial replicates

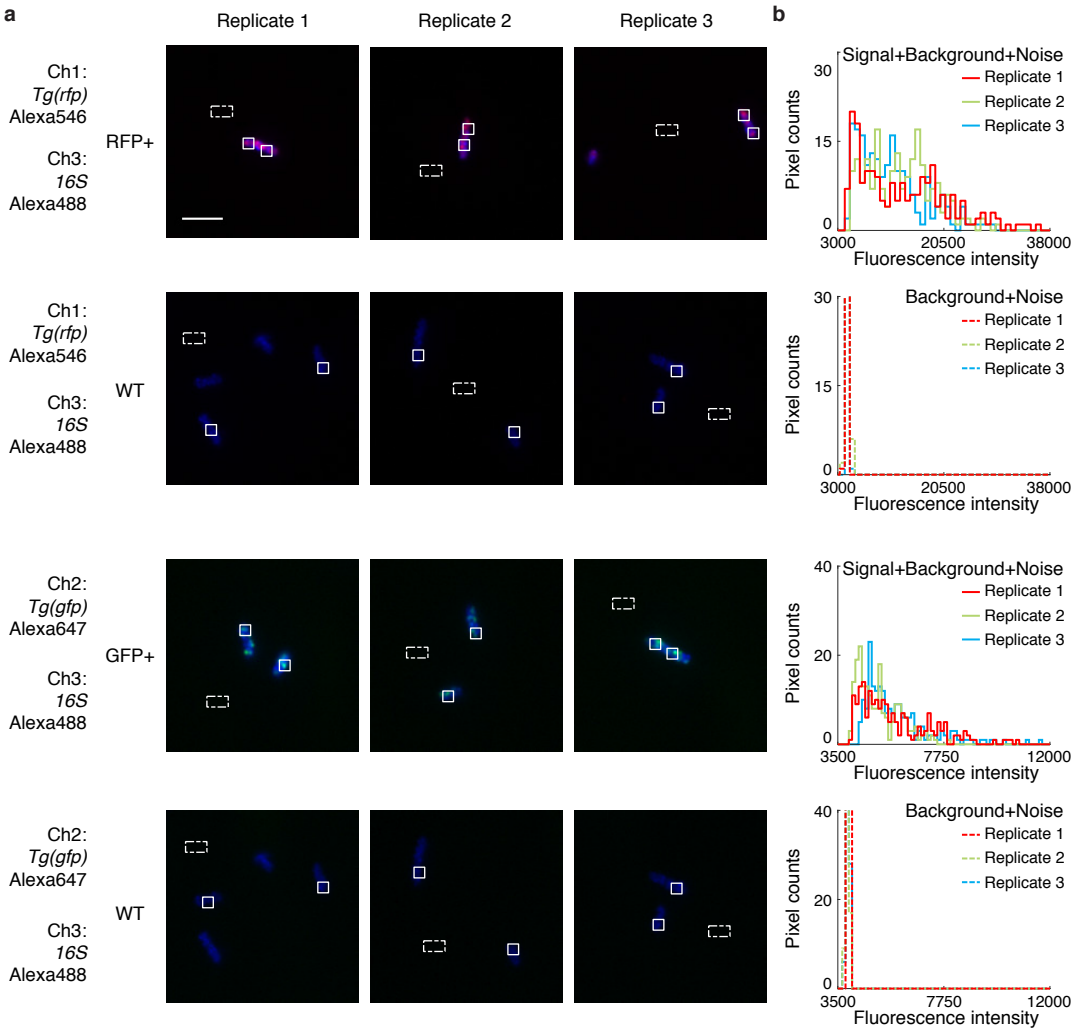


Figure S2. Multiplexed in situ HCR in bacterial populations (*E. coli*: WT, RFP+, or GFP+). (a) Two-channel epifluorescence images: each image contains either Ch1 (for target *Tg(rfp)* mRNA; rows 1 and 2) or Ch2 (for target *Tg(gfp)* or mRNA; rows 3 and 4) plus Ch3 (for target *16S* rRNA solely to help identify the location of cells; rows 1–4). A 1 h pre-hyb at 65 °C is used to eliminate signal from fluorescent proteins (see Section S3.3). Transgenic samples are used to measure Signal + Background + Noise (pixels within solid boundary) and Noise (pixels within dashed boundary) for Ch1 (row 1) and Ch2 (row 3); WT samples (lacking the targets) are used to measure Background + Noise (pixels within solid boundary) and Noise (pixels within dashed boundary) for Ch1 (row 2) and Ch2 (row 4). For each image, the total number of pixels within solid and dashed boundaries is the same. For this epifluorescence microscope, non-negligible instrument Noise was measured in the absence of sample (see Table S5). Images are displayed with the mean Noise subtracted from each pixel intensity for each channel. The estimates for Background and Signal in Table S4 were obtained after subtracting estimates for Noise using uncertainty propagation (see Sections S1.4 and S2.3). (b) Pixel intensity histograms for Signal + Background + Noise (pixels within solid boundary of transgenic samples) and Background + Noise (pixels within solid boundary of WT samples). Scale bar: 5 μm.

Row	*E. coli*	Ch1	Ch2	Ch3
1	RFP+	3870 ± 20	—	3620 ± 10
2	WT	3800 ± 10	—	3570 ± 30
3	GFP+	—	3870 ± 20	3650 ± 10
4	WT	—	3820 ± 20	3570 ± 30

Table S5. Estimated instrument noise for each of the four rows of Figure S2a (mean $\pm$ standard deviation, $N = 3$ replicates, pixels within the dashed boundaries).

S11.2 Whole-mount fruit fly embryo replicates

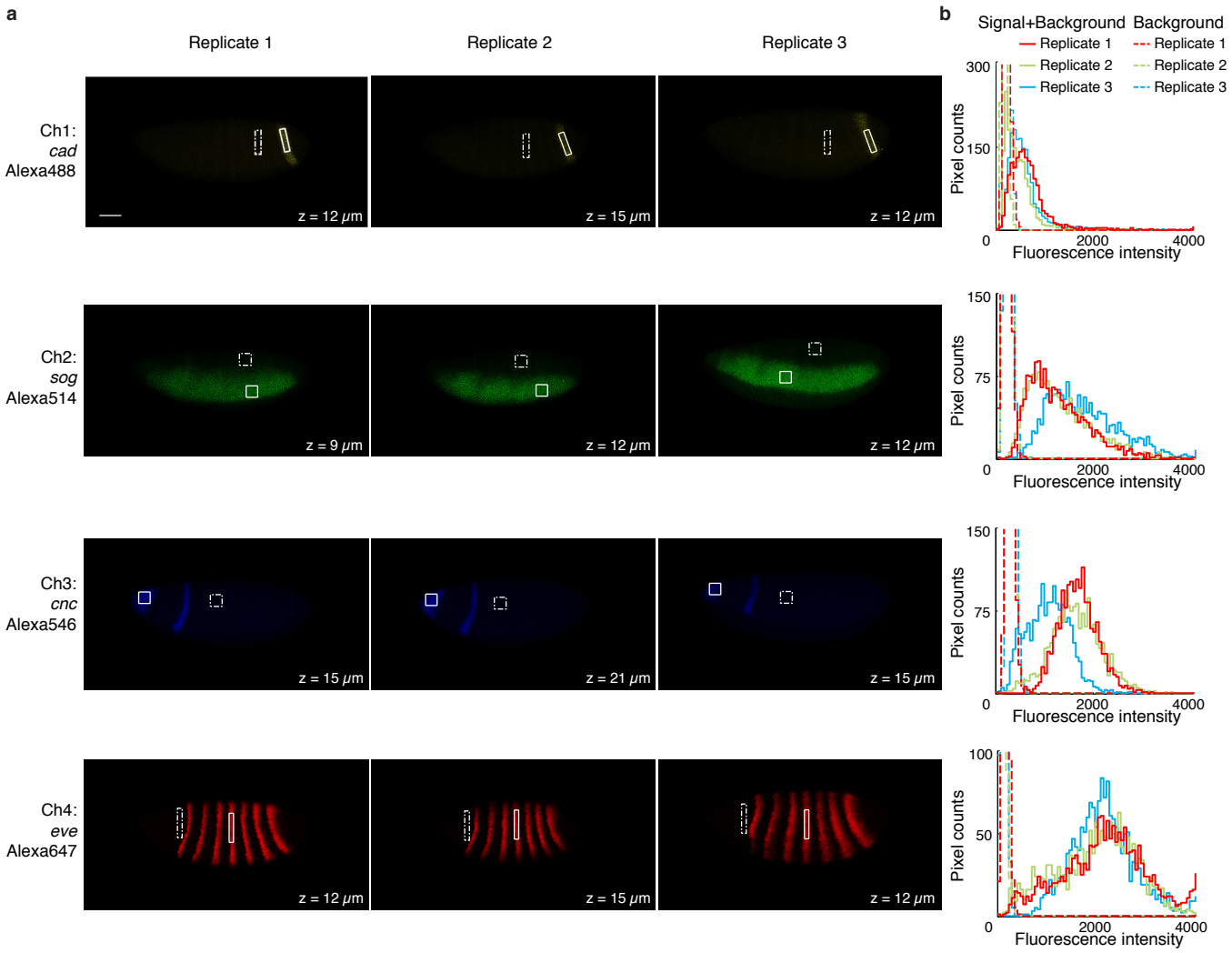


Figure S3. Multiplexed in situ HCR in whole-mount fruit fly embryos (*D. melanogaster*). (a) Individual channels from 4-channel confocal images. For each of three replicate embryos, a representative optical section was selected for each channel based on the expression depth of the corresponding target mRNA. (b) Pixel intensity histograms for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary). For each image, the total number of pixels within solid and dashed boundaries is the same. Note that for speckled expression patterns (*e.g., cad*), there are a substantial number of non-expression pixels even in a rectangle placed in a region of high expression. These non-expression pixels pollute the Signal + Background distribution with some Background pixels and artificially deflate the estimated signal-to-background ratio. Embryos fixed: stage 4-6. Scale bar: 50 μm.

S11.3 Whole-mount sea urchin embryo replicates

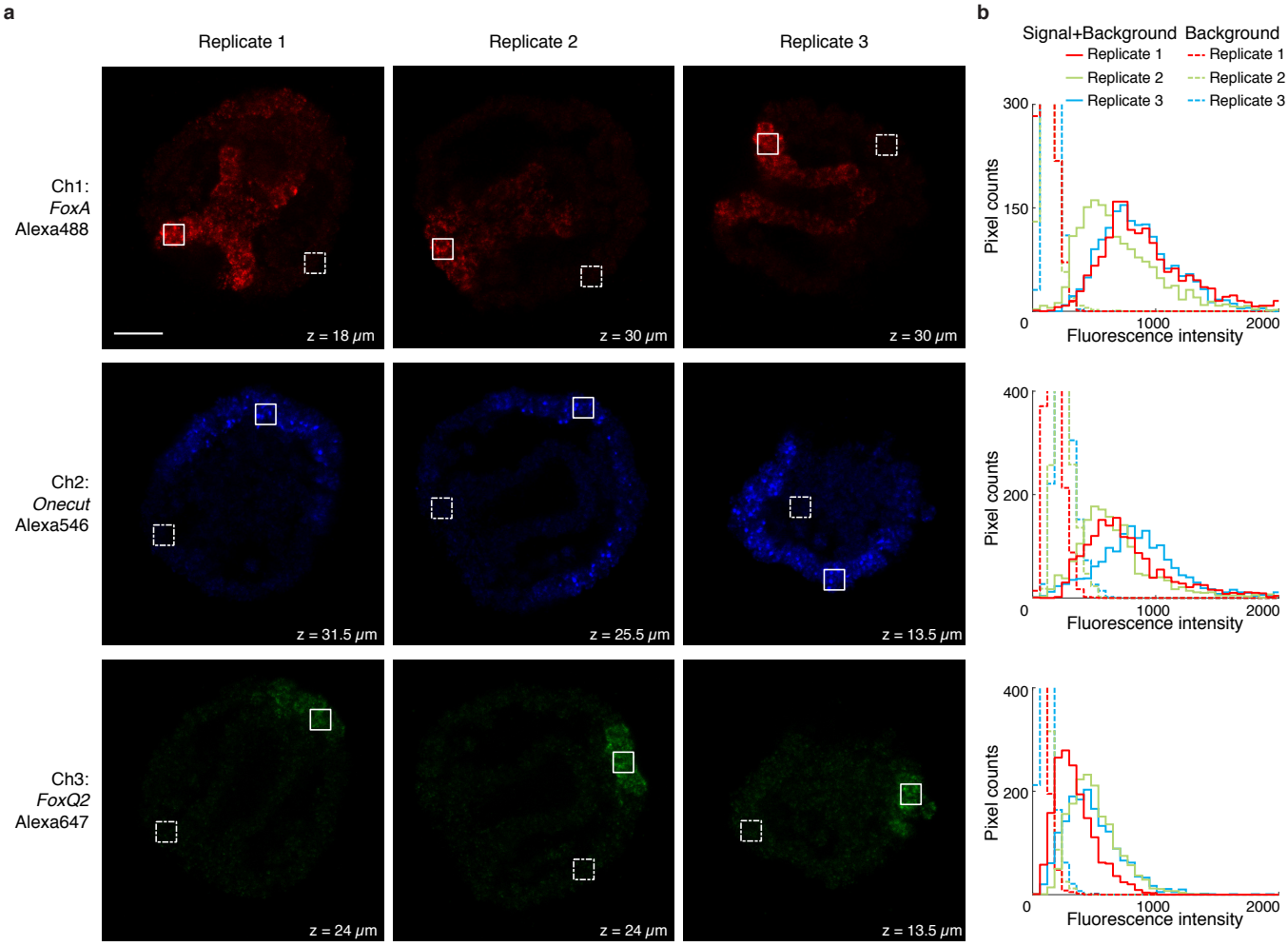


Figure S4. Multiplexed in situ HCR in whole-mount sea urchin embryos (*S. purpuratus*). (a) Individual channels from 3-channel confocal images. For each of three replicate embryos, a representative optical section was selected for each channel based on the expression depth of the corresponding target mRNA. (b) Pixel intensity histograms for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary). For each image, the total number of pixels within solid and dashed boundaries is the same. Note that for speckled expression patterns (*e.g., Onecut* and *FoxQ2*), there are a substantial number of non-expression pixels even in a rectangle placed in a region of high expression. These non-expression pixels pollute the Signal + Background distribution with some Background pixels and artificially deflate the estimated signal-to-background ratio. Embryos fixed: 45 hpf. Scale bar: 20 μm.

S11.4 Whole-mount nematode larva replicates

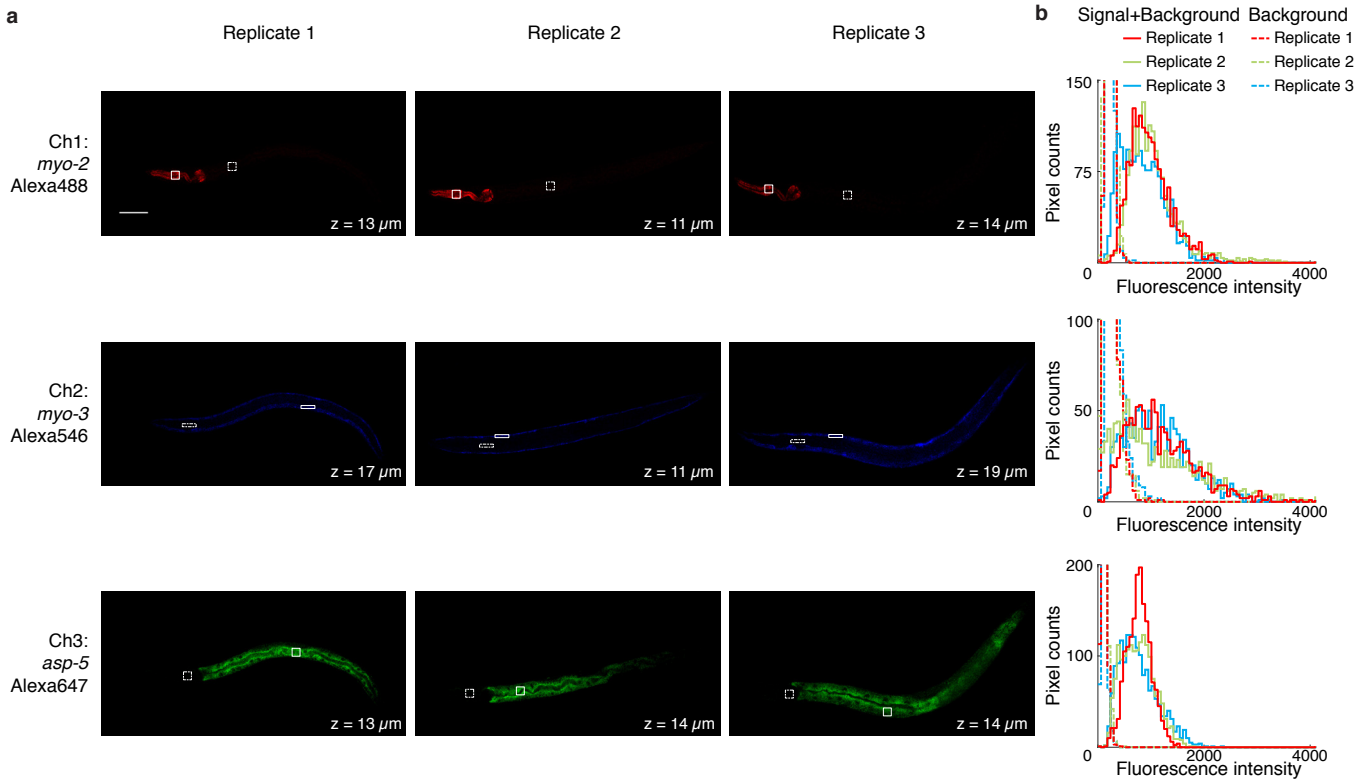


Figure S5. Multiplexed in situ HCR in whole-mount nematode larvae (*C. elegans*). (a) Individual channels from 3-channel confocal images. For each of three replicate larvae, a representative optical section was selected for each channel based on the expression depth of the corresponding target mRNA. (b) Pixel intensity histograms for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary). For each image, the total number of pixels within solid and dashed boundaries is the same. Larvae fixed: L3-L4. Scale bar: 50 μm.

S11.5 Whole-mount zebrafish larva replicates

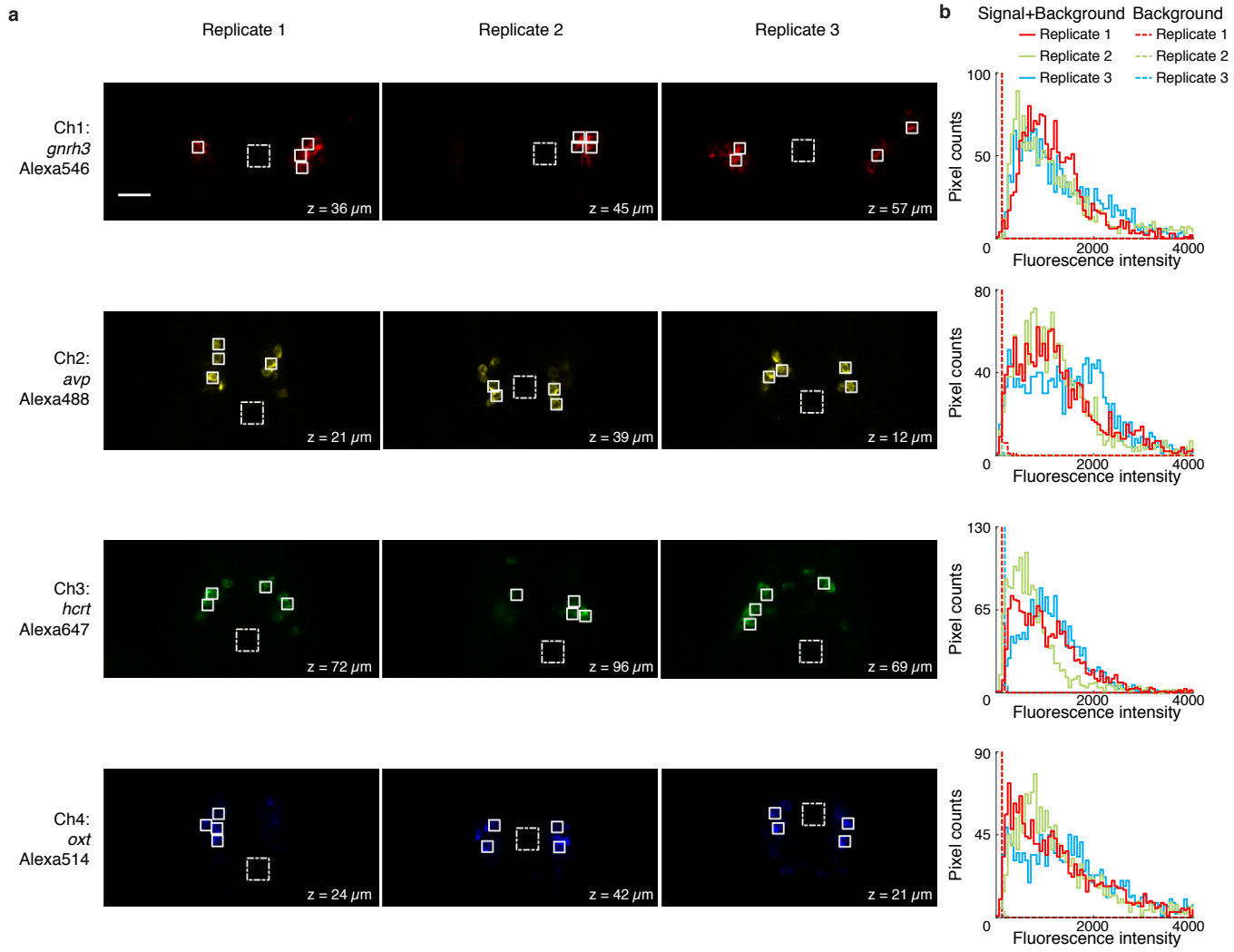


Figure S6. Multiplexed in situ HCR in whole-mount zebrafish larvae (*D. rerio*). (a) Individual channels from 4-channel confocal images. For each of three replicate larvae, a representative optical section was selected for each channel based on the expression depth of the corresponding target mRNA. (b) Pixel intensity histograms for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary). For each image, the total number of pixels within solid and dashed boundaries is the same. Larvae fixed: 5 dpf. Scale bar: 25 μm.

S11.6 Whole-mount chicken embryo replicates

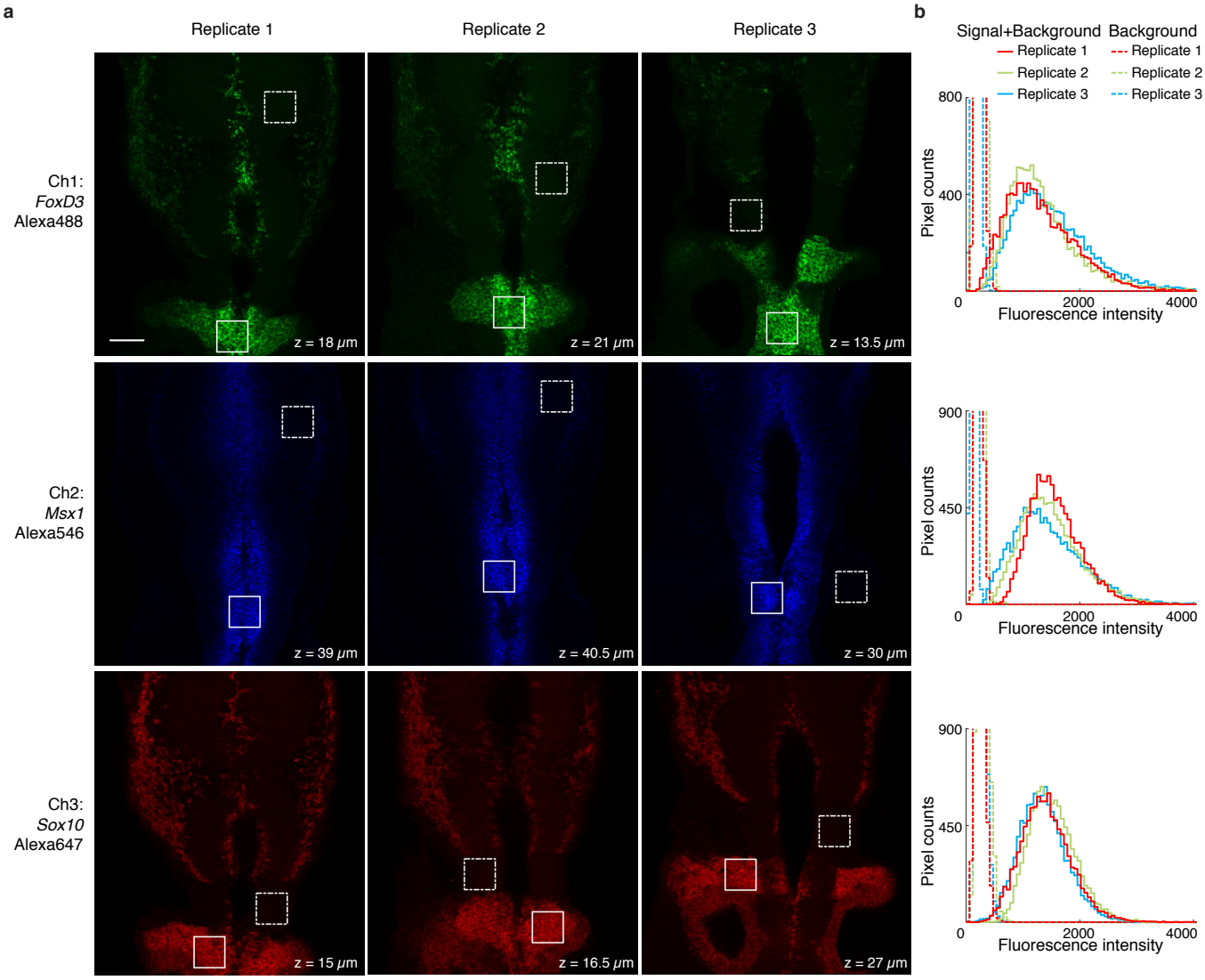


Figure S7. Multiplexed in situ HCR in whole-mount chicken embryos (*G. gallus domesticus*). (a) Individual channels from 3-channel confocal images. For each of three replicate embryos, a representative optical section was selected for each channel based on the expression depth of the corresponding target mRNA. (b) Pixel intensity histograms for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary). For each image, the total number of pixels within solid and dashed boundaries is the same. Embryos fixed: stage HH 11–12. Scale bar: 50 μm.

S11.7 Whole-mount mouse embryo replicates

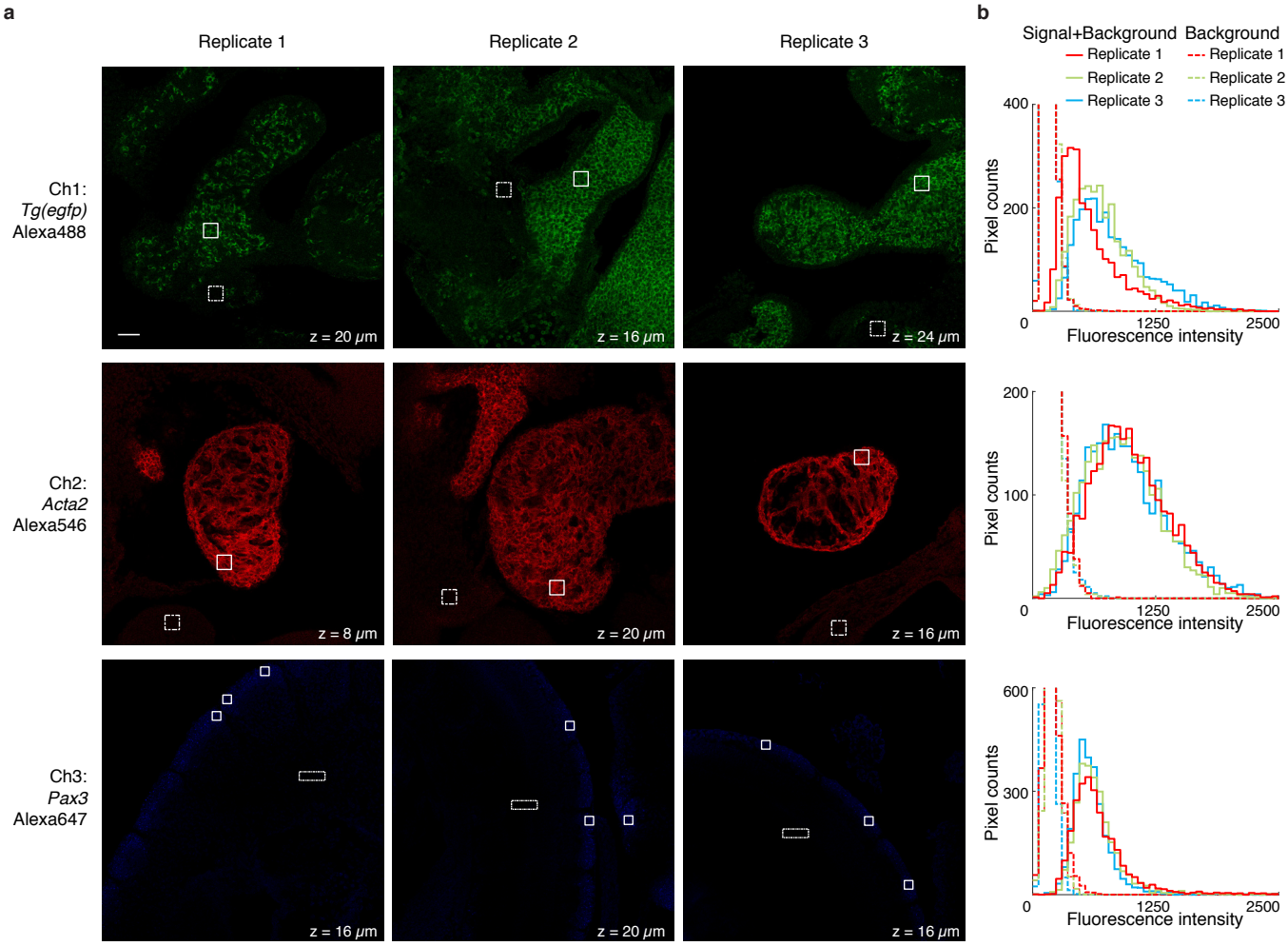


Figure S8. Multiplexed in situ HCR in whole-mount mouse embryos (*M. musculus*: Tg(Wnt1-Cre; R26R-eGFP)). (a) Individual channels from 3-channel confocal images. For each of three replicate embryos, a representative field-of-view and optical section were selected for each channel based on the expression pattern and depth of the corresponding target mRNA. (b) Pixel intensity histograms for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary). For each image, the total number of pixels within solid and dashed boundaries is the same. Embryos fixed: E9.5. Scale bar: 50 μm.

S11.8 FFPE human tissue section replicates

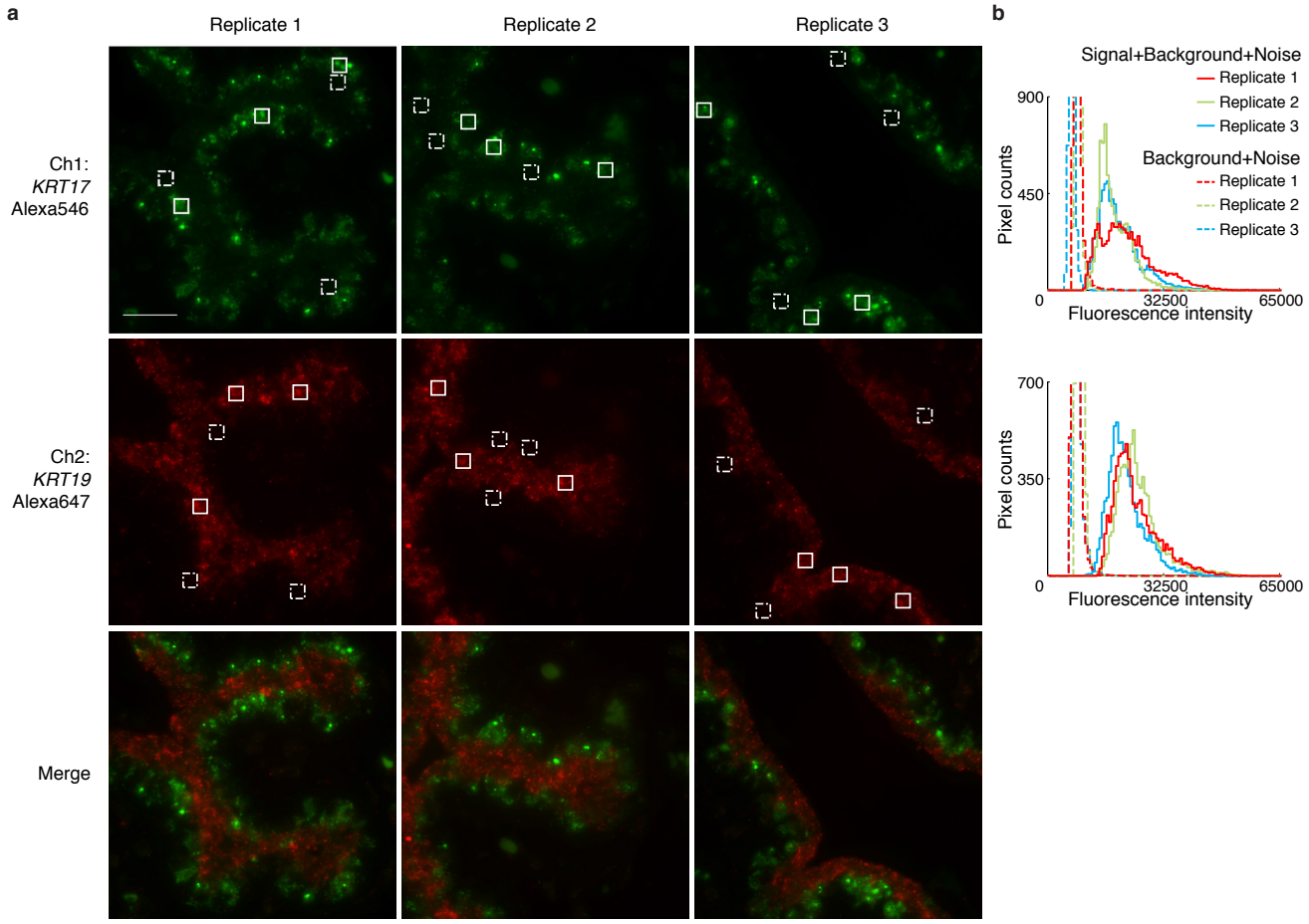
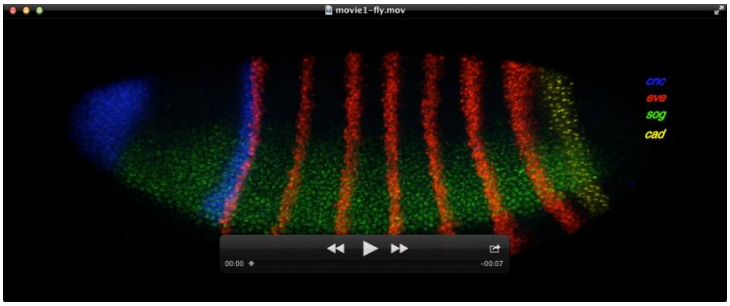


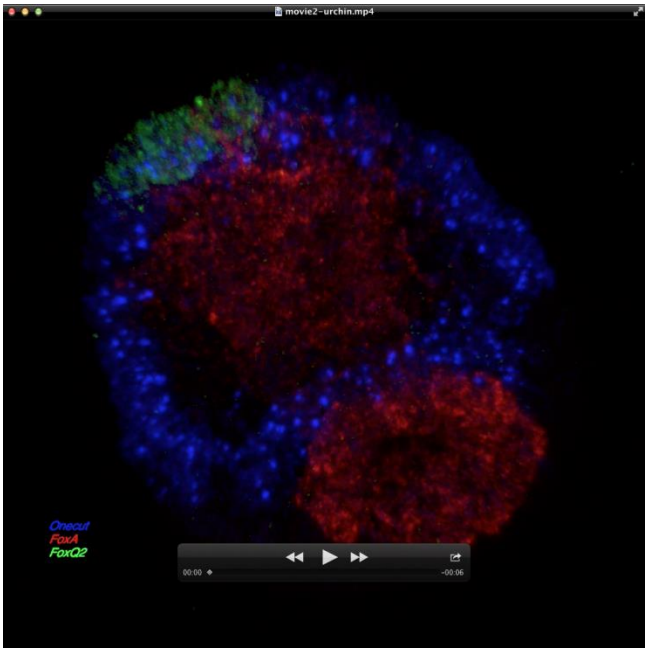
Figure S9. Multiplexed in situ HCR in FFPE human tissue sections (*H. sapiens sapiens*). (a) Individual channels from 2-channel epifluorescence images. For this epifluorescence microscope, non-negligible instrument Noise was measured in the absence of sample (Ch 1: 3500 $\pm$ 6 for *KRT17*; Ch 2: 5950 $\pm$ 30 for *KRT19*; mean $\pm$ standard deviation, $N = 3$ regions of a single slide). Images are displayed with the mean Noise subtracted from each pixel intensity. The estimates for Background and Signal in Table S4 were obtained after subtracting estimates for Noise using uncertainty propagation (see Sections S1.4 and S2.3). (b) Pixel intensity histograms for Signal + Background + Noise (pixels within solid boundary) and Background + Noise (pixels within dashed boundary). For each image, the total number of pixels within solid and dashed boundaries is the same. Thickness: 4 μm. Scale bar: 25 μm.

S12 Movies



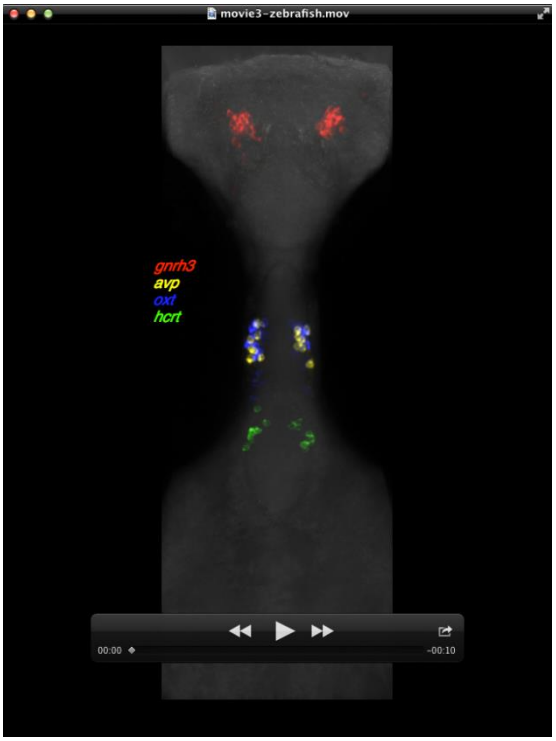
S12.1 Whole-mount fruit fly embryo

Movie 1: movie1-fly.mov
Three-dimensional reconstruction of a whole-mount fruit fly embryo (*D. melanogaster*) for Figure 2A. Target mRNAs: *cnc*, *eve*, *sog*, *cad*. Embryo fixed: stage 4–6.



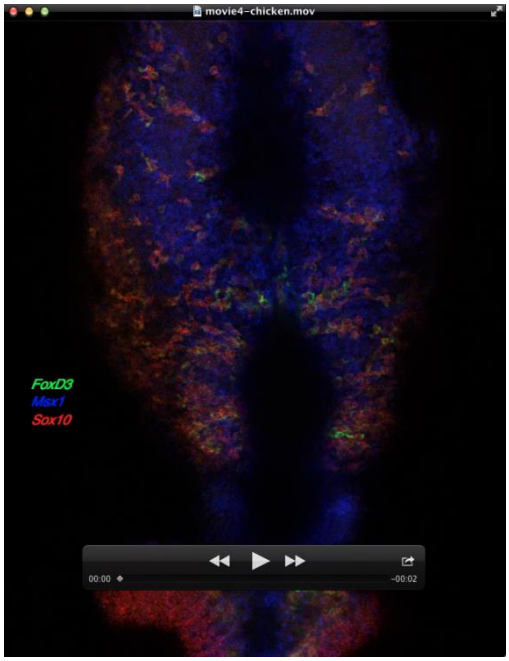
S12.2 Whole-mount sea urchin embyo

Movie 2: movie2-urchin.mov
Three-dimensional reconstruction of a whole-mount sea urchin embryo (*S. purpuratus*) for Figure 2C. Target mRNAs: *Onecut*, *FoxA*, *FoxQ2*. Embryo fixed: 45 hpf.



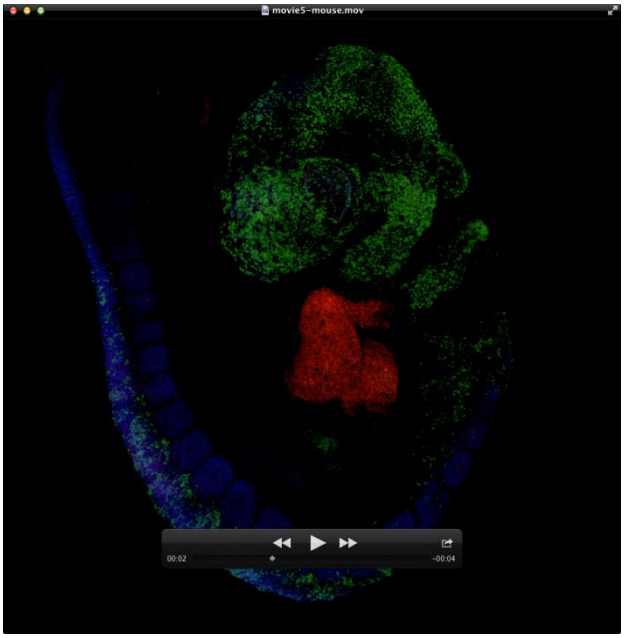
S12.3 Brain of whole-mount zebrafish larva

Movie 3: movie3-zebrafish.mov
Three-dimensional reconstruction of the head of a whole-mount zebrafish larva (*D. rerio*) for Figure 2D. Target mRNAs: *gnrh3*, *avp*, *oxt*, *hcrt*. Larva fixed: 5 dpf.



S12.4 Head of whole-mount chicken embryo

Movie 4: movie4-chicken.mov
Image stack of the head of a whole-mount chicken embryo (*G. gallus domesticus*) for Figure 2F. Target mRNAs: *FoxD3*, *Msx1*, *Sox10*. Embryo fixed: stage HH 11–12.



S12.5 Whole-mount mouse embryo

Movie 5: movie5-mouse.mov
Three-dimensional reconstruction of a whole-mount mouse embryo (*M. musculus*: Tg(Wnt1-Cre; R26R-eGFP)) fo
Figure 2G.
Target mRNAs: *Tg(egfp)*, *Pax3*, *Acta2*. Embryo fixed: E9.5.

S13 Additional data

S13.1 Full matrix of sample types and target channels for bacterial samples of Figure 2

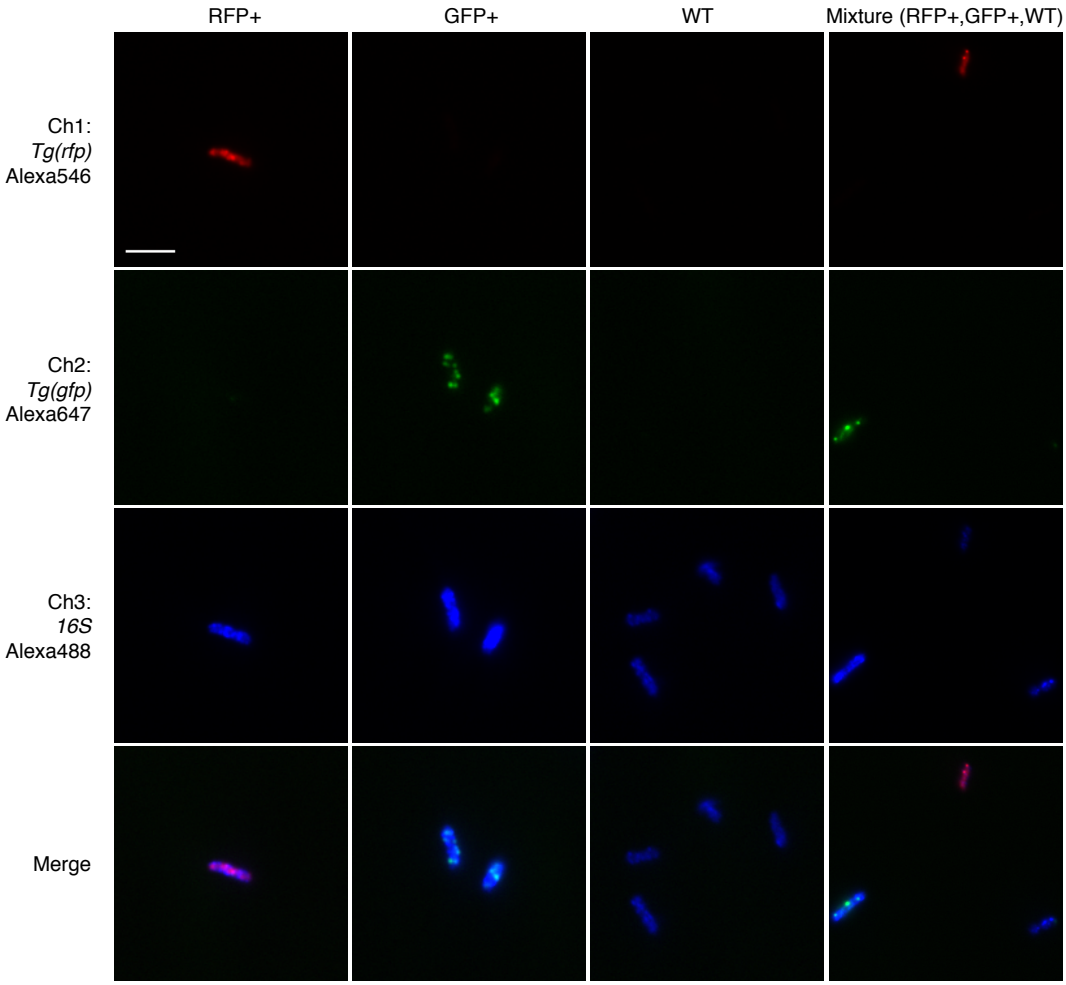


Figure S10. Multiplexed mRNA expression in bacterial populations (*E. coli*: RFP+, GFP+, WT). Epifluorescence microscopy images (single channels and merge) for three targets (*Tg(gfp)* and *Tg(rfp)* mRNAs and *16S* rRNA). Scale bar: 5 μm.

S13.2 Replicates for subcellular resolution study of Figure 3.

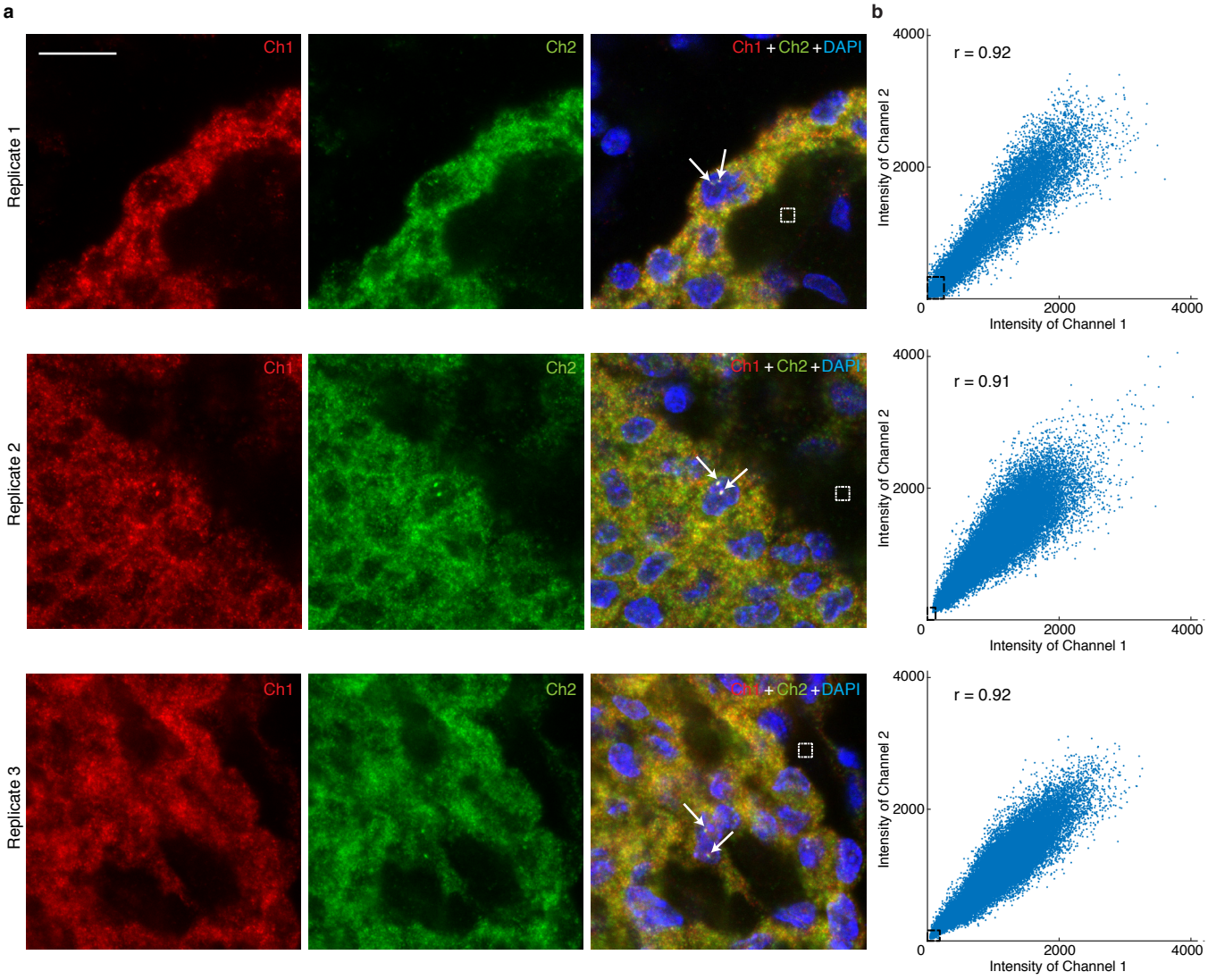
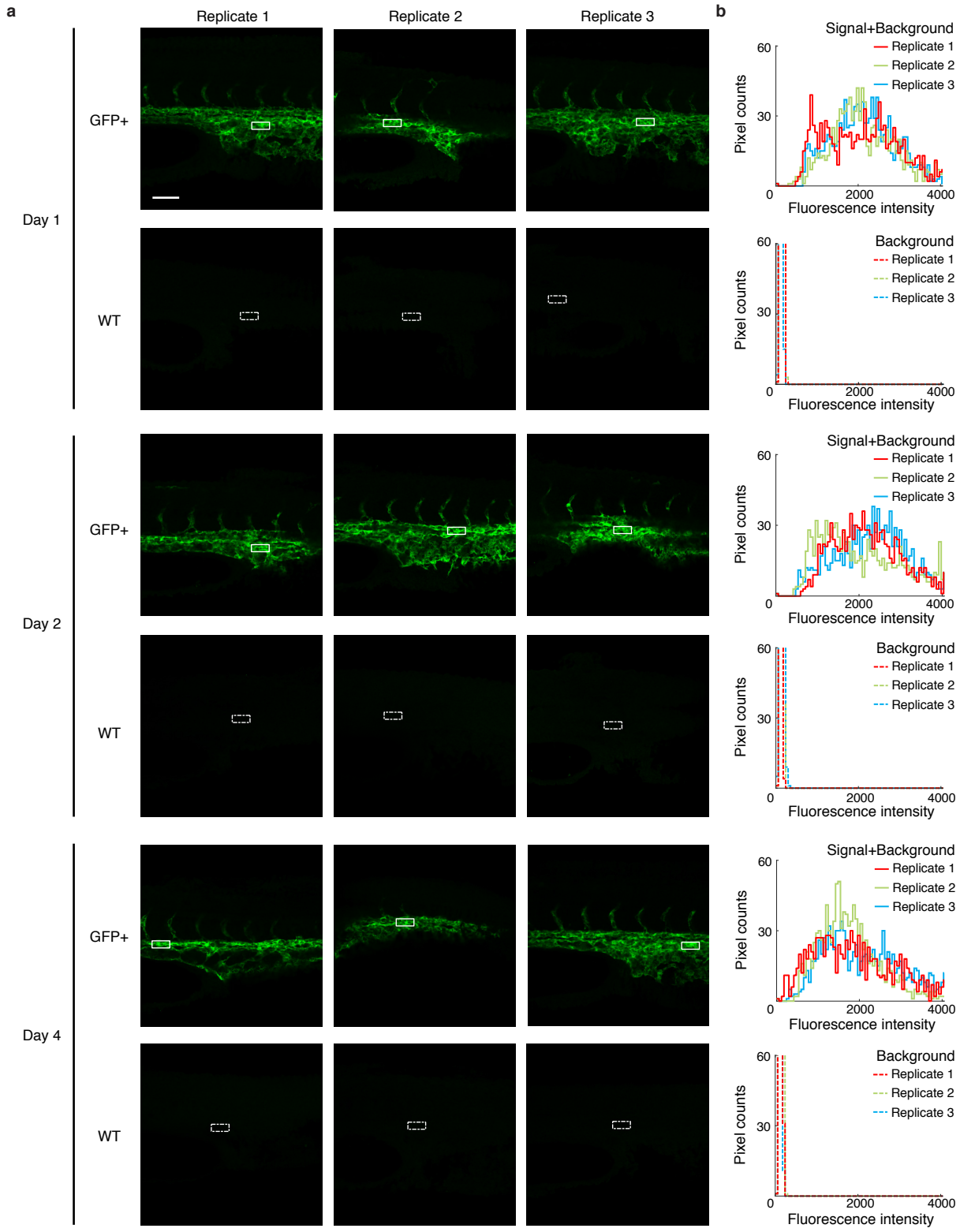


Figure S11. Redundant two-channel mapping of target mRNA *Acta2* in the heart of whole-mount mouse embryos. (a) Individual channels and merged channels with DAPI nuclear stain. Arrows denote putative sites of active transcription. Probe sets: 2 probes per channel. Pixel size: 69 nm $\times$ 69 nm. Embryo fixed: E9.5. Scale bar: 20 μm. (b) Highly correlated intensities for 0.35 μm $\times$ 0.35 μm voxels. To avoid inflating the correlation coefficient, we exclude voxels that fall below background thresholds in both channels (excluded voxels lie in the dashed rectangle at the lower left corner of the correlation plot). For each channel, the background threshold is defined as the mean plus two standard deviations for the voxels in the white dashed square.

S13.3 Stability of HCR signal after in situ amplification



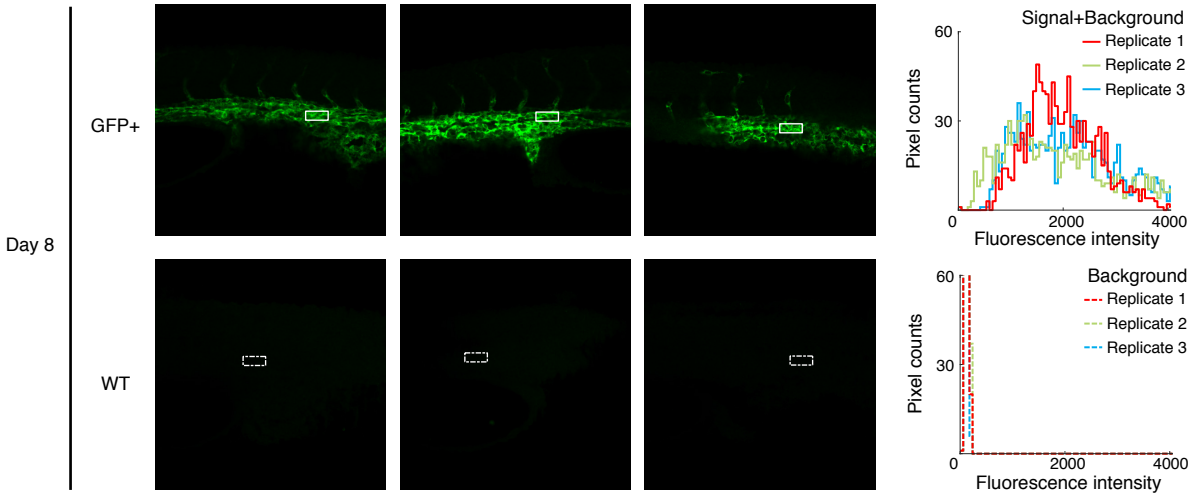


Figure S12. Stability of HCR signal in whole-mount zebrafish embryos (*D. rerio*: Tg(kdrl:egfp) or WT) stored in solution for 1 week. Embryos stored in 5× SSCT at 4 °C for 1, 2, 4, or 8 days before image acquisition. (a) mRNA expression imaged by confocal microscopy with identical microscope settings. Transgenic samples are used to measure Signal + Background (pixels within solid boundary) and WT samples are used to measure Background (pixels within dashed boundary). The total number of pixels within solid and dashed boundaries is the same. (b) Pixel intensity histograms for Signal + Background (pixels within solid boundary of transgenic samples) and Background (pixels within dashed boundary of WT samples). Target mRNA: *Tg(egfp)*. Embryo fixed: 27 hpf. Scale bar: 50 μm.

Day	Background	Signal	Signal-to-background
1	100 ± 20	2050 ± 80	20 ± 4
2	100 ± 20	2140 ± 60	21 ± 3
4	100 ± 10	1900 ± 200	19 ± 3
8	95 ± 6	1940 ± 60	21 ± 2

Table S6. Estimated signal-to-background (mean $\pm$ standard deviation, $N = 3$ replicates) for target mRNA *Tg(egfp)* based on the images and rectangles depicted in Figure S12.

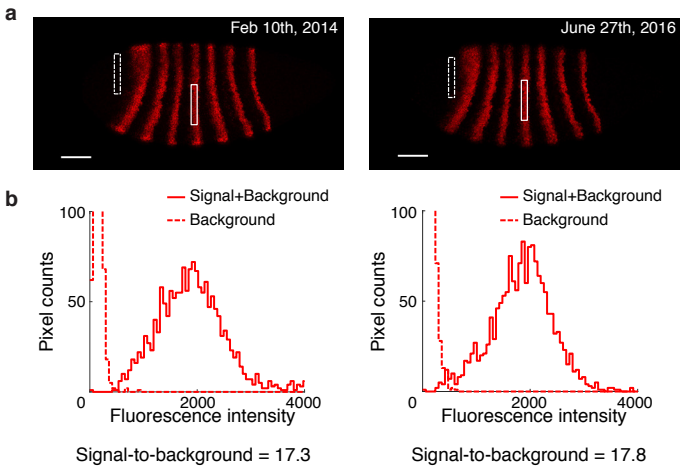


Figure S13. Stability of HCR signal in a whole-mount fruit fly embryo (*D. melanogaster*) stored in hardset mounting media for 2 years. Sample was mounted in ProLong Gold Antifade Mountant and imaged on February 10, 2014, then stored at 4 °C and imaged again on June 27, 2016. (a) mRNA expression imaged by confocal microscopy with the microscope gain adjusted to maximize signal-to-background ratio on different dates. (b) Pixel intensity histogram for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary). For each image, the total number of pixels within solid and dashed boundaries is the same. Target mRNA: *eve*. Embryo fixed: stage 4-6. Scale bar: 50 μm.

S14 Probe sequences

S14.1 *E. coli*

For the bacterial studies of Figures 2, S2, S10, each of three target rRNA or mRNAs is detected with a different probe set. Each probe set contains one to five 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) (McEntyre & Ostell, 2002).

Target mRNA: **16S ribosomal RNA (*16S rRNA*)**
Amplifier: **DNA HCR B1**
Fluorophore: **Alexa Fluor 488**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	TACCgTTTCCAgTAgTTATCCCCCTCCATCAggCAgCTTCCCAgACATTA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

Target mRNA: **green fluorescent protein (*Tg(gfp)*)**
Amplifier: **DNA HCR B3**
Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCgTATgTTgCATCACCTTCACCCTCTCCACTgACAgAAAATTTgTgCCC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TCTCgCAAAgCATTgAAgACCATACgCgAAAgTAgTgACAAgTgTTggCC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ggTCTgCTAgTTgAACgCTTCCATCTTCAATgTTgTgTCTAATTTTgAAg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ggTCTCTCTTTTCgTTgggATCTTTCgAAAgggCAgATTgTgTggACAgg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CATCCATACCATgggTAATCCCAgCAgCTgTTACAAACTCAAgAAggACC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC

Target mRNA: **red fluorescent protein (*Tg(rfp)*)**
Amplifier: **DNA HCR B4**
Fluorophore: **Alexa Fluor 546**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CCTCAACCTACCTCCAACTCTCACCATATTCgCTTC	TAAAA	ATgAACTCCTTgATgATggCCATgTTATCCTCCTCgCCCTTgCTCACCAT	ATTTT	CACATTTACAgACCTCAACCTACCTCCAACTCTCAC
CCTCAACCTACCTCCAACTCTCACCATATTCgCTTC	TAAAA	TCgATCTCgAACTCgTggCCgTTCACggAgCCCTCCATgTgCACCTTgAA	ATTTT	CACATTTACAgACCTCAACCTACCTCCAACTCTCAC
CCTCAACCTACCTCCAACTCTCACCATATTCgCTTC	TAAAA	gTgCTTCACgTAggCCTTggAgCCgTACATgAACTgAggggACAggATgT	ATTTT	CACATTTACAgACCTCAACCTACCTCCAACTCTCAC
CCTCAACCTACCTCCAACTCTCACCATATTCgCTTC	TAAAA	CTCCCACTTgAAgCCCTCggggAAggACAgCTTCAAgTAgTCggggATgT	ATTTT	CACATTTACAgACCTCAACCTACCTCCAACTCTCAC
CCTCAACCTACCTCCAACTCTCACCATATTCgCTTC	TAAAA	ggAggAgTCCTgggTCACggTCACCACgCCgCCgTCCTCgAAgTTCATCA	ATTTT	CACATTTACAgACCTCAACCTACCTCCAACTCTCAC

S14.2 *D. melanogaster*

For the fly studies of Figures 2, S3, S13, each of four target mRNAs is detected with a different probe set. Each probe set contains four to five 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) (McEntyre & Ostell, 2002). Spatial and temporal expression information were obtained from FlyBase (Attrill *et al.*, 2016).

Target mRNA: **caudal (*cad*)**
Amplifier: **DNA HCR B5**
Fluorophore: **Alexa Fluor 488**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	gTTgTTgTTgTTgTTggTTgTTgTTggCgACggCACTCAAATggTgTgCC	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	TgCTgCATCAgCTggTgggCCgAACgTgggCACATTCTgAACgAAgTTAT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	CCAAATTggCgggCACTgTgCTTTTgTgTgTAgggCAgTgTgTTgTAgTA	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	AAgTCCACATCgCCCAggAACTggTggTTCggCggCgTgTggTggTAgTT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	TTggTTgTggTTgTgCTggATAggCgggCTTCTTCATCCAgTCgAAgTAT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC

Target mRNA: **short gastrulation (*sog*)**
Amplifier: **DNA HCR B1**
Fluorophore: **Alexa Fluor 514**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	ATCACggTggAACTCgTCgCCAgACACCgCAgATCTTgCCCgTggCATTC	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	TCCgTCTgCAgggCCgTgTATTTTggCCACCTTTCCggCCAgAgCCAACT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CTggggATCCgTTTTgCCATCgggAAggTggTgCCTCCAgTAgCgAACTg	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gCgACgACAgCTCCAggACATTgATCTCggCAgAgggTTTgCgCACACgT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gCTCgggTCCACgATgTgTCCCTggATgCgCAgATgTgggTACTTCTTgg	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

Target mRNA: **cap-n-collar (*cnc*)**
Amplifier: **DNA HCR B2**
Fluorophore: **Alexa Fluor 546**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	ATCTggTCCAATTTgCgTTTCCTgCCAATTCTgggCAgCgACCTTgTTCT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gTCCATgggCAggTTAATgATgTCCggCACTgAAATgggTATgTTCAAgg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gCgCTTTTCATCgCgTgTCAgATgCTCTTCCTCCAAgTTggCTgCTgTTT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CTgATAggAgTAgCCgggATTAgTgATgCggATACggTgACATATCggAT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

Target mRNA: **even skipped (*eve*)**
Amplifier: **DNA HCR B3**
Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ggCggTCgTgATgggCATggTggCTCTCCATgTTgTAggTTCggTATCCg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTgCCgTACTTgggTggCCAAgAggTCCACAACCAggggCTTCTggTCCA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCgAgCCgCggCTgCCgTTCAAggAgTTATCCggACTTggATAggCATTC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TCCAAgCCgACCCAgCTggTCACgggTgAAggCggTgCgATAgCggCgTA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ggCCAgTTCgCAAgCgACggggACgggACACgTAgTTCTCCTTgTAgAAC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC

S14.3 *S. purpuratus*

For the sea urchin studies of Figures 2 and S4, each of three target mRNAs is detected with a different probe set. Each probe set contains three to ten 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) (McEntyre & Ostell, 2002). Spatial and temporal expression information were obtained from SpBase (Cameron *et al.*, 2009) and (Oliveri *et al.*, 2006; Tu *et al.*, 2006; Barsi & Davidson, 2016).

Target mRNA: **forkhead box A (*FoxA*)**
Amplifier: **DNA HCR B5**
Fluorophore: **Alexa Fluor 488**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	TCATACCCgTCATCgTATTCATgCTgACgggTgATgCTgCCgggTAACCA	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	ATACCCgTggTCgACATCgAgCTgTAgCTgTTCATggACgTCATACTCCC	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	TTCATTCCCATCCCAgCCATACTCgACATgCTCgACATCCCCgTCATACC	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	CATggTgTTCATACTCCCCATCgAgTTCATgTgACCCATgCTTCCCATgC	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	ACATCgCAgCggggTTgTTCATAgCACCCATTgAgTTCATgCCATTgATg	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	TATCAgCACggATACgATCAATggAgTTggCAgCCTgAgCTTgggCgTAT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	TTTCCAggTCgATCgggCgTTCTCgggACTTTgACgAAACAgTCATTgAA	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	CAgATAgCATCCATTCTCgAACATATTACCAgCATCAggATggAgCgTCC	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	TgTgATACAggCgTTgAAgggTCTCTggTATTgggTTCCCCgTTCTCAAT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	ATggATgTTgTCCTACTCCTAgCATACTggCTggATgCATgCCCgTCATT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC

Target mRNA: **ONECUT transcription factor HNF6 beta (*Onecut*)**
Amplifier: **DNA HCR B1**
Fluorophore: **Alexa Fluor 546**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gAgCgAACTCgCCTCggCgAATATCTAgTCCgTATTTAgACAgCTgTAAC	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gCAAAATCgggCCCTgggTAgCgCgAAATCCCTACTCTTCTTTTAAACgA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	TTCTTTgCTACgAAgCTTCTCTCCgCgTCTTggCgACCAATTTCTTCCgA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	ATAATgTggTCTCTCCCgCTTACTCCAAgTTCATggCCTTCTCTAgCgCA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CCAACTAACTCACTTgAAAgCATCTgTACTACTgggCTAggCggTTAgCg	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	AAAgAAATCgCCCgggTTTAAgCCgggTgCTACTgAAgACgAATgggTAT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

Target mRNA: **forkhead box Q2 (*FoxQ2*)**
Amplifier: **DNA HCR B2**
Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CAgTgCTAgATggTCTTTCACTAgTCTgTTCTTCTTgCTgAACgAgAACC	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CATAgATgTCACAgAgCAAAAggTgCTTgTCTTgTgAgTTgATgATAgCC	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gTggCAggAAAACAgTACggATgACTTgATgggATAgTAggTggggTgAA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

S14.4 *C. elegans*

For the nematode studies of Figures 2 and S5, each of three target mRNAs is detected with a different probe set. Each probe set contains two to four 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) (McEntyre & Ostell, 2002). Spatial and temporal expression information were obtained from WormBase (Howe *et al.*, 2016).

Target mRNA: **(*myo-2*)**
Amplifier: **DNA HCR B2**
Fluorophore: **Alexa Fluor 488**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TggCggCAACCAATCggTAgCACTCCATCTTTTCAgTTggggTAAACTTg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CTTCAgCTgTCAAgCggCTgCgAgCATTCTCgAgTgCgACAATCTTggCg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

Target mRNA: **(*myo-3*)**
Amplifier: **DNA HCR B3**
Fluorophore: **Alexa Fluor 546**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CgTTgCTAAgCTTAAgCTTTCCACggAgACTTggATCgTTTCCAgACATA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ACATCAAggAgTTgTCggTAgAgTgCTTgAgAAgAgCgACAgCggTgTCg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCTTggCAAggTAAgAACgAATgCgggATTggAACATggTAACgATTCTA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCAACTCgCgTTCAgCCTCgCTACgggATCTATCAgATTTCTggCgAgAg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC

Target mRNA: **(*asp-5*)**
Amplifier: **DNA HCR B1**
Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gCCgAgCACAggCCCAAAAgggCAACTAgTgCTACgAAAgTggTCTTCAT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CTCAgCggCATCATTCTTgAAgTCggATgAAATgTgTgTggCAATTCCAA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gTTCCgATgTTggTggCgCATggAACATAgTAggAgTCgTCgTTgCTgTg	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

S14.5 *D. rerio*

For the zebrafish studies of Figures 2 and S6, each of four target mRNAs is detected with a different probe set. Each probe set contains five to six 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) (McEntyre & Ostell, 2002). Spatial and temporal expression information were obtained from the Zebrafish Information Network (ZFIN) (Howe *et al.*, 2013).

Target mRNA: **arginine vasopressin (*avp*)**
Amplifier: **DNA HCR B5**
Fluorophore: **Alexa Fluor 488**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	CACACgACATACACTgTCTgATgggCTCCggCTgggATCTCTTgCCTCCT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	ggTCTCACAggggCCCgACAgCTgCTCCTCTTCCATgCAgACCTgCgCCT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	CCTTCAggACAgTCTgggTCTACAgCgCAgCTCTCTgAATCgCAgCAgAT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	ggTTgCCAgATTgAgCAgACgCAgCAgAgTTTCTCCTgAgATgCTCTTAA	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	CAgTgATgTgggggACAgTTTTAggCgATgTgTTCAgAAAggTCTCTgTC	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC

Target mRNA: **oxytocin (*oxt*)**
Amplifier: **DNA HCR B2**
Fluorophore: **Alexa Fluor 514**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TCCAgACATTATTCACCAAggCTgACACCgAgAgCTTgCggAgAAgAAgA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gTTTCTggAgAgCCgACCAAgCAgCCgATgCCTTCACCACAgCAgATACT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CTCATAACCgCAggCCTTTCCAgACATCTCACACggAgAAgggAgAAAAT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	ggCTCCTCCTgAgATgATgTCACAgCTCTgTggCATTTCATTggTggATT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CTAgCgCCTTTACAAAgTTggTTTACAAAAgTgggTggCgAgTCgTgTgg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

Target mRNA: **gonadotropin-releasing hormone 3 (*gnrh3*)**
Amplifier: **DNA HCR B3**
Fluorophore: **Alexa Fluor 546**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ggACCAgCAACCTTCCTTTCCACTCCATgCTAAAACTgCTgTgTTTgTTC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTgCCTCCATTTCACCAACgCTTCTTTTTCCACCgggAAgCCAACCgTAT	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gAATCAgCAggAATAgACAgCACTgTgTCACCAggATCCAACATCCTgAA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TCAgCATCCACCTCATTCACTATgTgTATTggTgAAAgCTgCTCCATTgg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	AATCCATAATTTggAggATATTTCATTAggAgTCCAAAAACATgCTCTCC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC

Target mRNA: **hypocretin (orexin) neuropeptide precursor (*hcrt*)**
Amplifier: **DNA HCR B1**
Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gCAgTCCATgAgTTTAgCTTCTgTCCCCTgTCTTgTAgACAAATgTCATg	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CCAggTgAgCgAgCAgCgCCATgAAgACgAgCACCTggAgCTTCTTAgCT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CAAgAgTgAgAATCCCgACAgCggCgTCgTTgTTgAgATgCACTAAATgT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	AgCAgTTgTTgCAggCggTCgTggACgCggCTTTCgCCCACTTTACgTTT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	TCCAgCCTCTTCCCCATggTgAggATCCCCgCggCTTgATTCCgCgAgTT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	TTCATAACTgTCCACATCCTgTggTACCgTAgggATgAggAATTTggCgg	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

For the HCR signal stability study of Figure S12, *Tg(egfp)* is detected with a probe set containing six 2-initiator DNA probes.

Target mRNA: **enhanced green fluorescent protein (*Tg(egfp)*)**
Amplifier: **DNA HCR B3**
Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gTTCTTCTgCTTgTCggCCATgATATAgACgTTgTggCTgTTgTAgTTgT	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTCAgCTCgATgCggTTCACCAgggTgTCgCCCTCgAACTTCACCTCggC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ACgCTgCCgTCCTCgATgTTgTggCggATCTTgAAgTTCACCTTgATgCC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gCgggTCTTgTAgTTgCCgTCgTCCTTgAAgAAgATggTgCgCTCCTggA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CgTAgCCTTCgggCATggCggACTTgAAgAAgTCgTgCTgCTTCATgTgg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gCggTCACgAACTCCAgCAggACCATgTgATCgCgCTTCTCgTTggggTC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC

S14.6 *G. gallus domesticus*

For the chicken embryo studies of Figures 2 and S7, each of three target mRNAs is detected with a different probe set. Each probe set contains five to nine 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) (McEntyre & Ostell, 2002). Spatial and temporal expression information were obtained from the Gallus Expression in Situ Hybridization Analysis (GEISHA) (Bell *et al.*, 2004; Darnell *et al.*, 2007).

Target mRNA: **Forkhead box D3 (*FoxD3*)**
Amplifier: **DNA HCR B1**
Fluorophore: **Alexa Fluor 488**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	TCgATATCCACgTCCTCggCCgCCAgCgCggTCTggCCggACATATCgCT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	TgCTgAgCTgCAgCTgCAggCTgggCCCgAgCTgCgCgTTgAAggCTTTg	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	TCgATgCTgAACgAggggCggCTgCTgggCTCggATTTCACgATggAgCC	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	ATgATgTTggTAggCACgCTgAggATgggCgCgATggCCgCggTggTCCT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	ATCATgAgCgCCgTCTggTCCCgCAggTgCTCCTgCTggTgCCgCTTgAA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	AAACCCCgAAAgCgACCTCCgCCgTTTCCCAgAgATACgTCCgggggggTC	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

Target mRNA: **Msh homeobox 1 (*Msx1*)**
Amplifier: **DNA HCR B2**
Fluorophore: **Alexa Fluor 546**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TgAAgggCAgCggggAAggggACACCTTgggTTTgTCgCTCTCCTCCTCg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TTCTCggggCTCTCTgCTTTgAgCAgCgCgTCCTCgggCAgCTTgCCCAg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	ggAgCATgggCTTggCTgCCATCTTCAgCTTCTCCAgCTCggCCTCCTgC	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	ACCgTCTCTgCCgCCCggCggCTTCCTgCggTCggCCATgAgCgCTTCCA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	ATgCgCCggCCACTgCTgggCCgCCCAACgggAAggAgATgCCgAATgCA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TgAAggTCTggATgAAgggAgCggAgAAgTCTCTgCCAggggCCgCACgg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CAgCAgggCTgCACCACgggTTCCTTCCTgCCAggCACAgAACAgATCCC	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CggCTCTgCCTCCCACCCTCgCCTTgCAgAAATgTCCTACTgggCTgCCT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	AggAgCTggCggAggAATggCCACAggTTAACAgCTgCCCTgCAgggACg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

Target mRNA: **SRY (sex determining region Y)-box 10 (*Sox10*)**
Amplifier: **DNA HCR B3**
Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CATggACCCgTCACTCCATgTCTTgAgTCTTCCTCATCTAgAAggCCAAT	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCAgCAgggATCAAgATTCATgCATgTgTgAATCTTAggCAggACTgCTg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CgggCTATgAAATgAgAAAggCTAAggCTgACAgTgCAgTTCCTgAATCC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTCACgTTTTCAgCAgACACAgTCAAATgCTggAggAgCAAggACCTggT	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ATTggAACCACATCTgggTgTTggCAAgTgCATggTAgCTTTCTTggTgC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC

S14.7 *M. musculus*

For the mouse studies of Figures 2 and S8, each of three target mRNAs is detected with a different probe set. Each probe set contains four to six 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) (McEntyre & Ostell, 2002). Spatial and temporal expression information were obtained from EMAGE gene expression database (Richardson *et al.*, 2013).

Target mRNA: **enhanced green fluorescent protein (*Tg(egfp)*)**
Amplifier: **DNA HCR B3**
Fluorophore: **Alexa Fluor 488**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gTTCTTCTgCTTgTCggCCATgATATAgACgTTgTggCTgTTgTAgTTgT	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTCAgCTCgATgCggTTCACCAgggTgTCgCCCTCgAACTTCACCTCggC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ACgCTgCCgTCCTCgATgTTgTggCggATCTTgAAgTTCACCTTgATgCC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gCgggTCTTgTAgTTgCCgTCgTCCTTgAAgAAgATggTgCgCTCCTggA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CgTAgCCTTCgggCATggCggACTTgAAgAAgTCgTgCTgCTTCATgTgg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gCggTCACgAACTCCAgCAggACCATgTgATCgCgCTTCTCgTTggggTC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC

Target mRNA: **actin, alpha 2, smooth muscle, aorta (*Acta2*)**
Amplifier: **DNA HCR B1**
Fluorophore: **Alexa Fluor 546**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gCCTTAgggTTCAgTggTgCCTCTgTCAgCAgTgTCggATgCTCTTCAgg	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CAACCATTACTCCCTgATgTCTgggACgTCCCACgATggATgggAAAACA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CggCCTTACAgAgCCCAgAgCCATTgTCgCACACCAgggCTgTgCTgTCT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CTgTTATAggTggTTTCgTggATgCCCgCTgACTCCATCCCAATgAAAgA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

Target mRNA: **paried box 3 (*Pax3*)**
Amplifier: **DNA HCR B2**
Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gTTCCgATCACAgACAgCgTCCTTgAgCAATTTgTCTCTgATTTCCCAgC	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gCTTTCTTCTgCTTCCTTCCTTTCTAgATCCgCCTCCTCCTCTTCTCCTT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	AgCggTAAATCAggTTCAgAgTCAATATCggAgCCTTCATCTgACTgAgg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TCCAgCTgCTCTgCCgTgAAggTggTTCTgCTCCTgCgCTgCTTCCTCTT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	AgTTgATTggCTCCAgCTTgTTTCCTCCATCTTgCACggCggTTgCTAAA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	ggCATggCggTgggAgggAATCCCCCCggAATgAgATggTTgAAAgCCAT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

For the redundant two-color experiment in Figures 3 and S11, *Acta2* is detected with two different probe sets. Each probe set contains two 2-initiator DNA probes.

Target mRNA: **actin, alpha 2, smooth muscle, aorta (*Acta2*)**
Amplifier: **DNA HCR B2**
Fluorophore: **Alexa Fluor 546**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gCCTTAgggTTCAgTggTgCCTCTgTCAgCAgTgTCggATgCTCTTCAgg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CAACCATTACTCCCTgATgTCTgggACgTCCCACgATggATgggAAAACA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

Target mRNA: **actin, alpha 2, smooth muscle, aorta (*Acta2*)**
Amplifier: **DNA HCR B1**
Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CggCCTTACAgAgCCCAgAgCCATTgTCgCACACCAgggCTgTgCTgTCT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CTgTTATAggTggTTTCgTggATgCCCgCTgACTCCATCCCAATgAAAgA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

S14.8 *H. sapiens sapiens*

For the human studies of Figures 2 and S9, each of three target rRNA or mRNAs is detected with a different probe set. Each probe set contains one to eight 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) (McEntyre & Ostell, 2002). Spatial expression information were obtained from literatures (Taylor-Papadimitriou *et al.*, 1989; van de Rijn *et al.*, 2002).

Target mRNA: **18S ribosomal RNA (*RNA18S5*)**
Amplifier: **DNA HCR B5**
Fluorophore: **Alexa Fluor 488**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	CggAATTAACCAgACAAATCgCTCCACCAACTAAgAACggCCATgCACCA	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC

Target mRNA: **keratin 17 (*KRT17*)**
Amplifier: **DNA HCR B2**
Fluorophore: **Alexa Fluor 546**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TgCCggggTTCgCCCgCTTCCTTTATAggCCACCAAgTgggCgTAgCgAT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gCAggCACACAggAgAAgggCTggAgAggAgAggggCCCCAAgTTgTgTA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CCAgCAgATCCCAgCCTgCAggAgCCggCACCCAggCCgCCAgACAgCCg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	AAgCTgTAgCAgCTggAgTAgCTgCTACCCCCgAgggTgCTgCCCAggCC	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TTCCACAATggTACgCACCTgACgggTggTCACCggTTCTTTCTTgTACT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CggCTgCCTCCCTgCCTCCTgggTggCCggCCggggTAgCTgAgTCCTCA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CTgAAgCAgggggCTgAggCTggAgAggCCggAgACTgTggggCAgATgg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gCTgAgTCAACAAgCTTTATTgTCATCAggCAAggAAgCATggggAAggg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

Target mRNA: **keratin 19 (*KRT19*)**
Amplifier: **DNA HCR B1**
Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gAgCACggACggAgCAACCCTggTCTCAgAAgCTgCgATTCgCgggAggA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CCTCCgAAggACgACgTggCCgACgACTggCgATAgCTgTAggAAgTCAT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gTAggCCCCCgAggAggACgAggACACAAAgCgggCggAggACACggATA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

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