ONLINE METHODS

Probe synthesis. RNA probes are 81-nt long (26-nt initiator, 5-nt spacer, 50-nt mRNA recognition sequence). mRNAs are addressed by probe sets containing one or more probes that hybridize adjacently at 50-nt binding sites. Probe sequences are displayed in Supplementary Information S10. RNA probes were synthesized by in vitro transcription. The coding strand for each probe contained three random nucleotides and a 19-nt SP6 promoter sequence upstream of the 81-nt initiator-linker-probe sequence. Complementary DNA coding and template strands were ordered (unpurified) from Integrated DNA Technologies (IDT). Strands were resuspended in ultrapure water (resistance of 18 MΩ cm) and concentrations were determined by measuring absorption at 260 nm. The double-stranded template was formed by annealing the two strands (heat at 95 °C for 5 min, cool 1 °C/min to room temperature) in 1× SPSC buffer (0.4 M NaCl, 50 mM Na_2HPO_4, pH 7.5). RNA probes were transcribed overnight at 37 °C using an AmpliScribe SP6 high yield transcription kit (Epicentre Biotechnologies) with four unmodified ribonucleotide triphosphates. Probes were purified using an RNeasy mini kit (Qiagen) and concentrations were determined by measuring absorbance at 260 nm.

HCR hairpin design. RNA HCR hairpins are 52-nt long (10-nt toehold, 16-bp stem, 10-nt loop). Hairpin dimensioning was performed based on in vitro and in situ binding studies performed in 40% hybridization buffer[26]. HCR hairpin sequences were designed by considering a set of target secondary structures involving different subsets of the strands (I, H1, H2, I•H1 and I•H1•H2, each as depicted in Fig. 1a). For a given target secondary structure, the ensemble defect represents the average number of incorrectly paired nucleotides at equilibrium, calculated over the ensemble of unpseudoknotted secondary structures [31,32]. Sequence design was performed by mutating the hairpin sequences so as to reduce the sum of the calculated ensemble defects over the set of target structures (J.N. Zadeh, personal communication). Multiple HCR amplifiers were designed independently and then sequence orthogonality was checked using NUPACK (www.nupack.org)[33] to simulate the equilibrium species concentrations and base-pairing properties for a test tube[34] containing different subsets of strands. This approach was used to check for off-target interactions between each of the five initiators and the other four hairpin sets, as well as between the 10-nt toehold and loop segments of each hairpin set and the 10-nt toehold and loop segments of the other four hairpin sets. The sequences are shown in Supplementary Information S11.

HCR hairpin synthesis. Each HCR hairpin was synthesized by IDT as two segments with one segment end-labeled with an amine (3'-end for H1 and 5'-end for H2) to permit subsequent coupling to a fluorophore. The strand with a 5' end at the ligation site was ordered with a 5' phosphate to permit ligation. Ligation of the two segments produced the full 52-nt hairpin. The ligation was performed using T4 RNA ligase 2 (New England Biolabs) at 16 °C for a minimum of 8 hr. The ligated strands were purified using a 15% denaturing polyacrylamide gel. The bands corresponding to the expected sizes of the ligated products were visualized by UV shadowing and excised from the gel. The RNA strands were then eluted by soaking in 0.3 M NaCl overnight and recovered by ethanol precipitation. The pellet was dried and resuspended in ultrapure water and quantified by measuring absorbance at 260 nm. The dye coupling reaction was performed by mixing an amine-labeled hairpin with an Alexa Fluor succinimidyl ester (Invitrogen) and incubating in the dark for 3 hr. Alexa-labeled hairpins were separated from unincorporated dyes by repeating the denaturing PAGE purification described above. To ensure that H1 and H2 form hairpin monomers, the strands were snap-cooled in 1× SPSC buffer before use (heat at 95 °C for 90 sec, cool to room temperature on the benchtop for 30 min).

Multiplexed gel electrophoresis. Reactions for Figure 1b were performed in 40% hybridization buffer without blocking agents (40% formamide, 2× SSC, 9 mM citric acid (pH 6.0), 0.1% Tween 20) with 0.1 μg/μL of total RNA extracted from zebrafish using TRIzol (Invitrogen). Each of the eight hairpin species (two for each of the four HCR amplifiers) was snap cooled at 3 μM in 1× SPSC buffer. The RNA initiator for each HCR system was diluted to 0.3 μM in ultrapure water. Each lane was prepared by mixing 12 μL of formamide, 6 μL of 5× HB supplements without blocking agents (10× SSC, 45 mM citric acid (pH 6.0), 0.5% Tween 20), 1.76 μL of 1.7 μg/μL extracted zebrafish total RNA, and 1 μL of each of the eight hairpins. When an initiator was absent (lane 1), 2.24 μL of ultrapure water was added to bring the reaction volume to 30 μL. For lanes 2 to 5, 1 μL of 0.3 μM initiator for one HCR amplifier and 1.24 μL of ultrapure water were added. The reactions were incubated at 45 °C for 1.5 hr. The samples were supplemented with 7.5 μL of 50% glycerol and loaded into a native 2% agarose gel, prepared with 1× LB buffer (Faster Better Media). The gel was run at 150 V for 90 min at room temperature and imaged using an FLA-5100 fluorescent scanner (Fujifilm Life Science). The excitation laser sources and emission filters were as follows: a 473 nm laser and a 530 ± 10 nm bandpass filter (amplifier HCR3, Alexa 488), a 532 nm laser and a 570 ± 10 nm bandpass filter (amplifier HCR5, Alexa 546), a 635 nm laser and a 665 longpass filter (amplifier HCR1, Alexa 647), and a 670 nm laser and a 705 nm longpass filter (amplifier HCR4, Alexa 700).

In situ hybridization studies. Procedures for the care and use of zebrafish embryos were approved by the Caltech IACUC. Embryos were fixed and permeablized using the protocol of Supplementary Information S1.1. For the transgenic samples, GFP+ embryos were identified using a Leica MZ16 FA fluorescence stereomicroscope. In situ hybridization experiments for Figures 2-4 were performed using the protocol of Supplementary Information S1.2. Overnight incubations were performed for 16 hr. For Figures 2a-i, probe solution was prepared by introducing 6 pmol of each probe (1-3 μL depending on the stock solution) into 300 μL of 50% HB at 55 °C. Hairpin solution was prepared by introducing 10 pmol of each hairpin (snap-cooled in 5 μL) into 300 μL of 40% HB at 45 °C. For Figures 2j-m, experiments were performed using WT embryos. A probe set with three probes (1 pmol of each probe) was used for Figures 2j-l; probe sets with 1, 3, or 9 probes (1 pmol of each probe) were used for Figure 2m. The standard in situ protocol was used for both the (AF + NSA) sample (with probes excluded) and for the AF sample (with probes and hairpins excluded). For the (AF + NSA + NSD) sample, *desm* probes were replaced with *egfp* probes carrying the same initiator sequence as the *desm* probes. For the ex situ HCR study of Figures 2k and 2l, snap-cooled hairpins (30 pmol of each hairpin) and probes (1 pmol of each probe) were added to 300 μL of 40% HB and incubated at 45 °C for 16 hr while the embryos were incubated without probes in 50% HB at 55 °C. For consistency, these embryos were subjected to the standard probe washes and the standard amplification protocol (substituting the pre-assembled polymer solution for the hairpin solution). Experiments for Figures 3 and 4 were performed with *Tg(flk1:egfp)* embryos using probe and hairpin solutions prepared following the protocol in Supplementary Information S1.2.

Vibratome sectioning. After completion of the standard in situ protocol (Supplementary Information S1.2), embryos were post-fixed with 4% PFA at room temperature for 20 min. Fixation was stopped by washing the embryos 3 times with 1× PBST. Embryos were then embedded in 4% low-melting agarose (Cambrex) in 1× PBST and sectioned into 200 μm slices with a Vibratome Series 1000 tissue sectioning system (Vibratome).

Confocal microscopy. A chamber for mounting the embryo was made by aligning 2 stacks of Scotch tape (8 pieces per stack) 1 cm apart on a 25 mm × 75 mm glass slide (VWR). Approximately 200 μL of 3% methyl cellulose mounting medium was added between the tape stacks on the slide and embryos were placed on the medium oriented for lateral imaging. A 22

mm × 22 mm No. 1 coverslip (VWR) was placed on top of the stacks to close the chamber. The sectioned sample of Figure 3d was mounted using a SlowFade Gold antifade reagent (Molecular Probes). A Zeiss 510 upright confocal microscope with an LD LCI Plan-Apochromat 25× / 0.8 Imm Corr DIC objective was used to acquire the images for Figure 2. The excitation laser sources and emissions filters were: 488 nm Ar laser excitation source and a 520 ± 10 nm bandpass filter (gray; autofluorescence), 633 nm HeNe laser and a 650 nm long pass filter (green; Alexa 647). A Leica TCS SP5 inverted confocal microscope with an HCX PL APO 20× / 0.7 Imm objective was used to acquire the 5-color images of Figures 3b and 3d. Excitation laser sources and tuned emissions bandpass filters were as follows: 488 nm/500-540 nm (Alexa 488), 514 nm/550-565 nm (Alexa 514), 543 nm/550-605 nm (Alexa 546), 594 nm/605-640 nm (Alexa 594), 633 nm/655-720 nm (Alexa 647). Cluster analysis (Leica) was performed to enhance dye separation. A Zeiss 510 META NLO inverted confocal microscope with an LD C-Apochromat 40× / 1.1 W Corr objective was used to acquire the images for Figure 4. Excitation laser sources and emission filters were: 488 nm/tunable 500-522 nm (Alexa 488), 514 nm/tunable 543-586 nm (Alexa514), 561 nm/575-630 nm (Alexa 594), 633 nm/650-710 nm (Alexa 647). For the images of Figure 4, image registration (rigid body translation and rotation) was performed to correct for possible misalignment between the two channels (TurboReg plugin for ImageJ). All images are presented without background subtraction.

Note: Supplementary information is available on the Nature Biotechnology website.

Competing Financial Interests

The authors declare competing financial interests in the form of US patents and pending US and EU patents.