Supplementary Information

Programmable in situ amplification for multiplexed bioimaging

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S1 Protocols

S1.1 Preparation of fixed whole-mount zebrafish embryos

1. Collect embryos and incubate at 28 °C in a petri dish with egg H_2O until they reach 20 hr post-fertilization (20 hpf).
2. Dechorinate using two pairs of sharp tweezers under a dissecting scope.
3. Transfer ∼80 embryos (25 hpf) to a 2 mL eppendorf tube and remove excess egg H_2O.
4. Fix embryos in 1 mL of 4% paraformaldehyde (PFA)[1] for 24 hr at 4 °C .
5. Wash embryos 3 × 5 min with 1 mL of phosphate-buffered saline (PBS) to stop the fixation. Fixed embryos can be stored at 4 °C at this point.
6. 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.
7. Rehydrate with a series of graded 1 mL MeOH/PBST washes for 5 min each:
   (a) 75% MeOH / 25% PBST
   (b) 50% MeOH / 50% PBST
   (c) 25% MeOH / 75% PBST
   (d) 5 × 100% PBST.
8. Store embryos at 4 °C before use.[2]

[1] Use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.
[2] Prepare embryos every two weeks to avoid increased autofluorescence.

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S1.2 Two-stage multiplexed in situ hybridization using HCR

Detection stage

1. For each sample, move 8 embryos to a 1.5 mL eppendorf tube.
2. Pre-hybridize with 300 μL of 50% hybridization buffer (50% HB) for 30 min at 55 °C.
3. Prepare probe solution by adding 6 pmol of each probe (1-3 μL per probe depending on the stock) to HB reagents at 55 °C to yield probes in 500 μL of 50% HB.
4. Remove the pre-hybridization solution and add the 500 μL of probe solution.
5. Incubate the embryos overnight (12-16 hr) at 55 °C.
6. Remove excess probes by washing at 55 °C with 500 μL of:
   (a) 75% of 50% HB / 25% 2× SSC for 15 min
   (b) 50% of 50% HB / 50% 2× SSC for 15 min
   (c) 25% of 50% HB / 75% 2× SSC for 15 min
   (d) 100% 2× SSC for 15 min
   (e) 100% 2× SSC for 30 min.

   Wash solutions should be pre-heated to 55 °C before use.
7. Wash at room temperature for 10 min each with 500 μL of:
   (a) 75% 2× SSC / 25% PBST
   (b) 50% 2× SSC / 50% PBST
   (c) 25% 2× SSC / 75% PBST
   (d) 100% PBST.

Amplification stage

1. Prepare 30 pmol of each fluorescently labeled hairpin by snap cooling in 10 μL of 1× SPSC buffer (heat at 95 °C for 90 seconds and cool to room temperature on the benchtop for 30 min).
2. Pre-hybridize embryos with 300 μL of 40% HB for 30 min at 45 °C.
3. Prepare hairpin solution by adding all snap-cooled hairpins to HB reagents at 45 °C to yield hairpins in 500 μL of 40% HB.
4. Remove the pre-hybridization solution and add the 500 μL of hairpin solution.
5. Incubate the embryos overnight (12-16 hr) at 45 °C.
6. Repeat step 6 above using 40% HB at 45 °C (instead of 50% HB at 55 °C).
7. Repeat step 7 above.

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S1.3 Buffer recipes

50% Hybridization Buffer (50% HB)	For 40 mL of solution
50% Formamide	20 mL formamide
2× Sodium Chloride Sodium Citrate (SSC)	4 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
500 μg/mL tRNA	200 μL of 100 mg/mL tRNA
50 μg/mL Heparin	200 μL of 10 mg/mL Heparin
	fill up to 40 mL with ultrapure H_2O

40% Hybridization Buffer (40% HB)	For 40 mL of solution
40% Formamide	16 mL formamide
2× Sodium Chloride Sodium Citrate (SSC)	4 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
500 μg/mL tRNA	200 μL of 100 mg/mL tRNA
50 μg/mL Heparin	200 μL of 10 mg/mL Heparin
	fill up to 40 mL with ultrapure H_2O

5× HB Supplements	For 40 mL of solution
10× Sodium Chloride Sodium Citrate (SSC)	20 mL of 20× SSC
45 mM Citric Acid (pH 6.0)	1.8 mL 1 M Citric Acid, pH 6.0
0.5% Tween 20	2 mL of 10% Tween 20
2.5 mg/mL tRNA	1 mL of 100 mg/mL tRNA
250 μg/mL Heparin	1 mL of 10 mg/mL Heparin
	fill up to 40 mL with ultrapure H_2O

5× HB Supplements without Blocking Agents	For 40 mL of solution
10× Sodium Chloride Sodium Citrate (SSC)	20 mL of 20× SSC
45 mM Citric Acid (pH 6.0)	1.8 mL 1 M Citric Acid, pH 6.0
0.5% Tween 20	2 mL of 10% Tween 20
	fill up to 40 mL with ultrapure H_2O

10× PBS[3]	For 1 L of solution
1.37 M NaCl	80 g NaCl
27 mM KCl	2 g KCl
100 mM Na_2HPO_4	14.2 g Na_2HPO_4 anhydrous
20 mM KH_2PO_4	2.7 g KH_2PO_4 anhydrous
pH 7.4	Adjust pH to 7.4 with HCl
	fill up to 1 L 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

5× Sodium Phosphate Sodium Chloride (SPSC)	For 50 mL of solution
2 M NaCl	25 mL of 4 M NaCl
250 mM Na_2HPO_4	12.5 mL of 1 M Na_2HPO_4
	12.5 mL of ultrapure H_2O

[3] Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the embryos.

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S1.4 Reagents and supplies

SP6 Transcription Kit (Epicentre Cat. # AS3106)
RNeasy Mini Kit (Qiagen Cat. # 74104)
T4 RNA Ligase II (NEB Cat. # M0239L)
Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetraFluorophenyl ester (Molecular Probes Cat. # A30005)
Alexa Fluor 514 carboxylic acid, succinimidyl ester (Molecular Probes Cat. # A30002)
Alexa Fluor 546 carboxylic acid, succinimidyl ester (Molecular Probes Cat. # A20002)
Alexa Fluor 594 carboxylic acid, succinimidyl ester (Molecular Probes Cat. # A20004)
Alexa Fluor 647 carboxylic acid, succinimidyl ester (Molecular Probes Cat. # A20006)
Alexa Fluor 700 carboxylic acid, succinimidyl ester (Molecular Probes Cat. # A20010)
Dimethyl Sulfoxide (DMSO) (Sigma Cat. # 276855)
Paraformaldehyde (PFA) (Sigma Cat. # P6148)
Formamide (EMD Cat. # FX0420-6)
20$\times$ Sodium Chloride Sodium Citrate (SSC) (Invitrogen Cat. # 15557044)
Tween 20 (Sigma Cat. # P1379)
tRNA from baker's yeast (Roche Cat. # 109495)
Heparin (Sigma Cat. # 3393)
TRIzol (Invitrogen Cat. # 15596-026)
SYBR Gold Nucleic Acid Gel Stain (Invitrogen Cat. # S-11494)
25 mm $\times$ 75 mm glass slide (VWR Cat. # 48300-025)
22 mm $\times$ 22 mm No. 1 coverslip (VWR Cat. # 48366-067)
SlowFade Gold Antifade Reagents (Molecular Probes Cat. # S36936)

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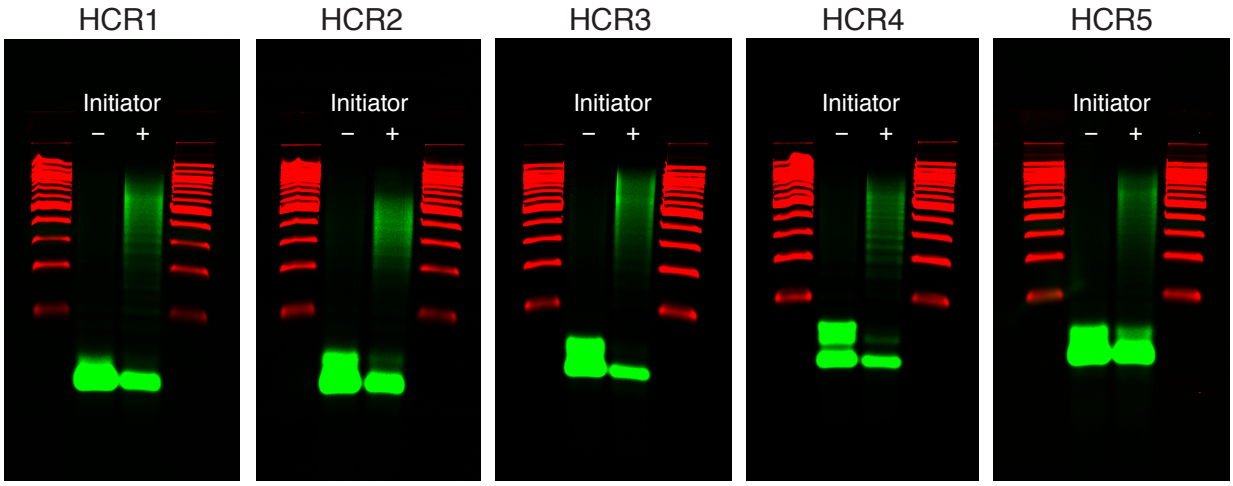
S2 Gels for in vitro validation of HCR amplifiers

Supplementary Figure 1 demonstrates the triggered polymerization properties of each of the five HCR amplifiers used in Figure 3. The hairpins for each HCR amplifier exhibit metastability in the absence of initiator and undergo triggered polymerization upon the introduction of initiator. Previous control experiments (data not shown) show that the H1 and H2 hairpins migrate as separate bands. The hairpins for amplifier HCR4 exist metastably as both monomers and as putative dimers; introduction of initiators triggers polymerization from either metastable state.



Supplementary Figure 1. Agarose gel electrophoresis for five HCR amplifiers. The reaction conditions were the same as for Figure 1b. Each gel tests the hairpins for one HCR amplifier. All hairpins were labeled with Alexa 647. Native 2% agarose gels were run at 150 V for 90 min and imaged with a 635 nm laser and a 665 nm longpass filter. The 100 bp DNA ladders (red) were pre-stained with SYBR Gold (Invitrogen) and imaged using a 488 nm laser and a 575 nm longpass filter.

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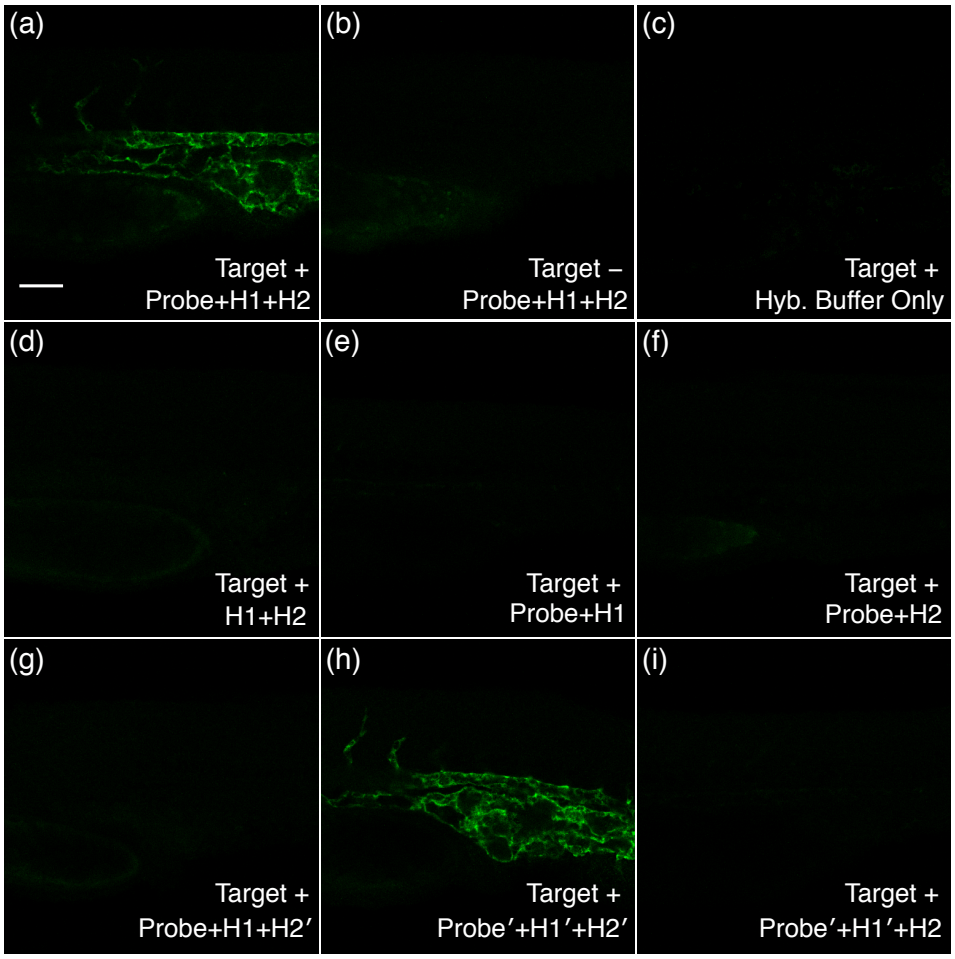
S3 Single-channel images for in situ validation of HCR amplifiers



Supplementary Figure 2. Single-channel version of Figures 2a-i. Turning off the gray autofluorescence channel emphasizes the minimal degree of background staining. Scale bar: 50 μm.

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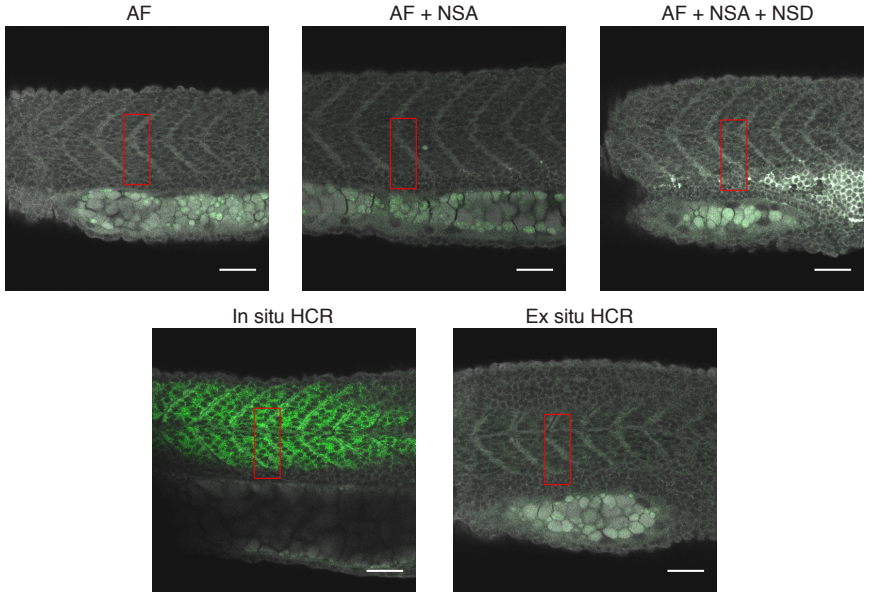
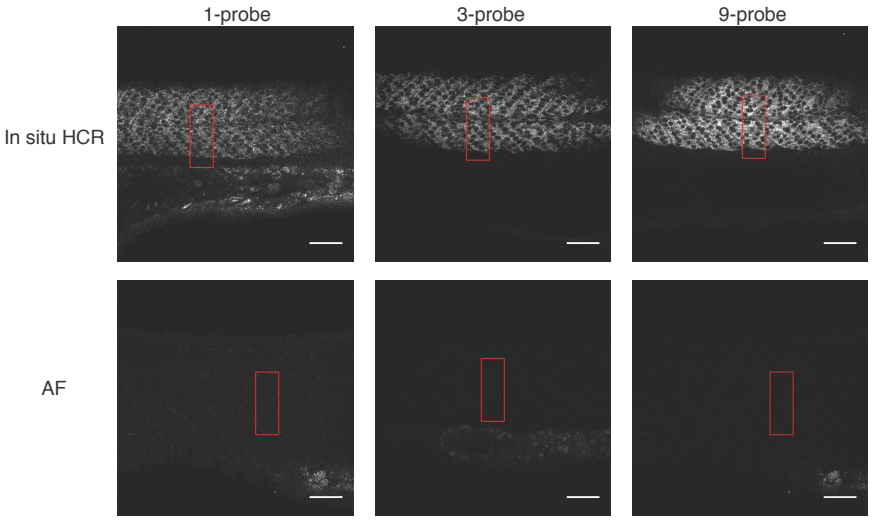
S4 Images for signal-to-background studies

The pixel intensity histograms of Figures 2l and 2m are calculated within the rectangles depicted in Supplementary Figures 3 and 4. These rectangles are positioned so that they encompass both a region with high target expression (to characterize signal) and a region with no target expression (to characterize background). The reproducibility of this type of study is illustrated in Supplementary Figure 5.



Supplementary Figure 3. Images and rectangle placements for the pixel intensity histograms of Figure 2l. Scale bars: 50 μm.



Supplementary Figure 4. Images and rectangle placements for the pixel intensity histograms of Figure 2m. The microscope PMT gain was optimized for each probe set (1, 3, or 9 probes) to avoid saturating pixels using HCR amplification. The two images in each column were obtained using the same microscope settings. Scale bars: 50 μm.

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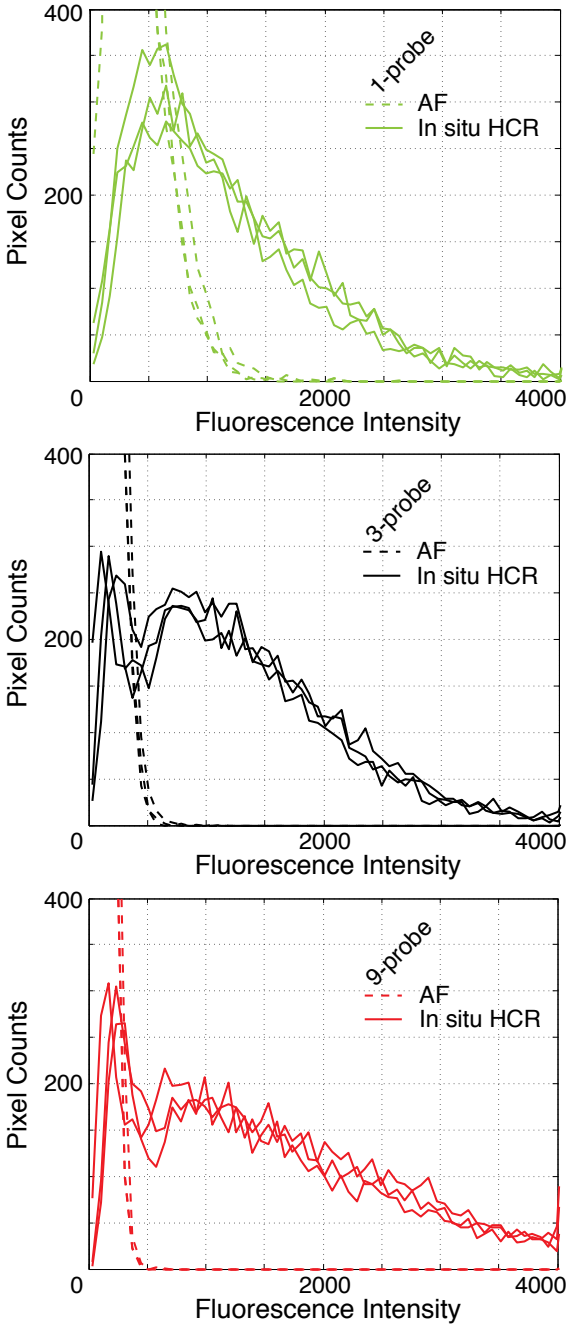
S5 Reproducibility of signal-to-background studies

To illustrate the reproducibility of signal-to-background characterizations, Supplementary Figure 5 displays pixel intensity histograms for probe sets with 1, 3, or 9 probes in three fish for each probe set. Images and rectangle placements for the pixel intensity histograms are shown in Supplementary Figure 6. These experiments were performed using standard probe and hairpin concentrations (see the protocol of Section S1.2).



Supplementary Figure 5. Reproducibility of pixel intensity histograms. Comparison of autofluorescence (AF) and in situ HCR in three fish for each of three probe sets (1, 3, or 9 probes). Target mRNA: *desm*.

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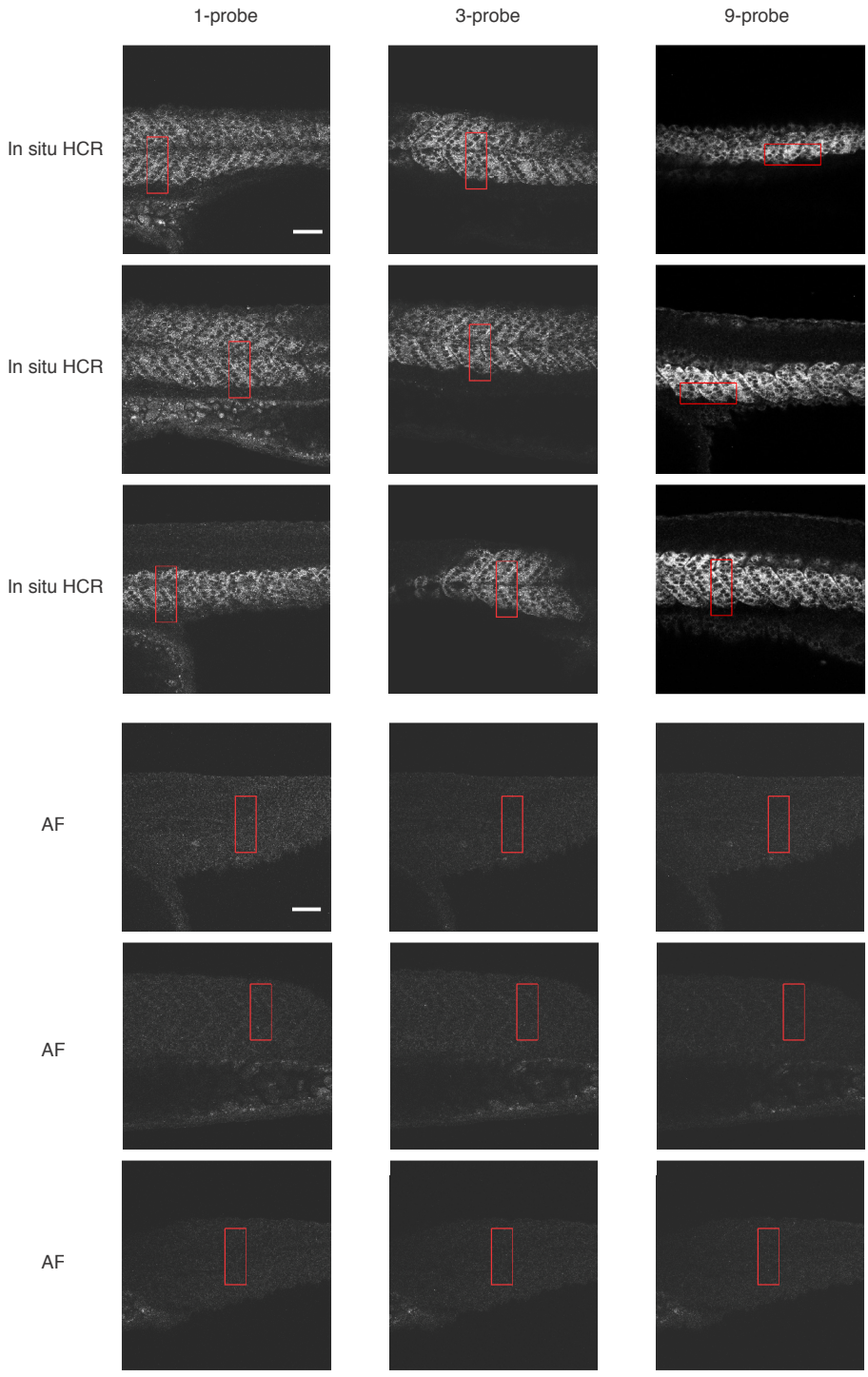

Supplementary Figure 6. Images and rectangle placements for the pixel intensity histograms of Supplementary Figure 5. The microscope PMT gain was optimized for each probe set (1, 3, or 9 probes) to avoid saturating pixels using HCR amplification. The six images in each column were obtained using the same microscope settings. Target mRNA: *desm*. Embryos fixed at 25 hpf. Scale bars: 50 μm.

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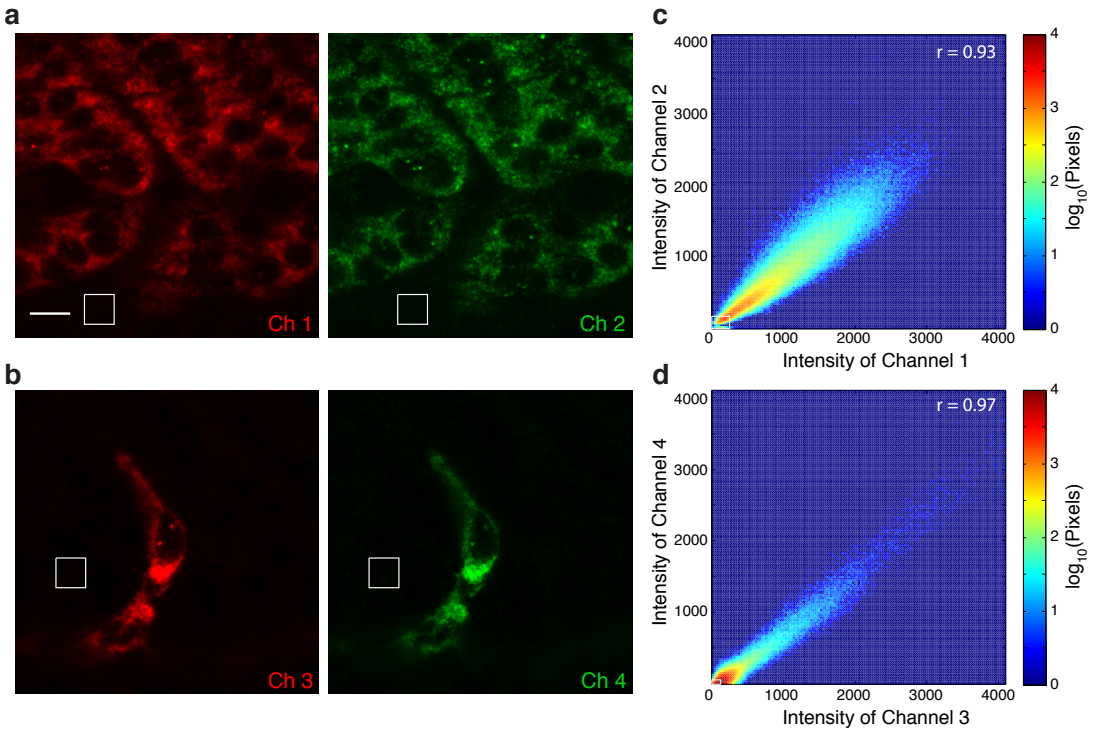
S6 Image stacks for five-color whole-mount and sectioned zebrafish embryos

The full image stack for the embryo depicted in Figure 3b is available as Supplementary Movie 1. Each plane in the stack is separated by 4 μm. The full image stack for the embryo depicted in Figure 3d is available as Supplementary Movie 2. Each plane in the stack is separated by 5 μm. For each frame in either movie, a 3×3 median filter was applied to each channel and the dimensions were reduced by a factor of two.

S7 Signal correlation analysis for co-localization studies

We use the Pearson correlation coefficient, $r \in [-1, 1]$, to quantify the correlation between pixel intensities[1] for channels 1 and 2 of Figure 4a and channels 3 and 4 of Figure 4b. To ensure that pixels representing background in both images do not inflate the correlation coefficient, we exclude pixels that fall below a background threshold in *both* channels. For each image, the threshold was calculated based on pixel intensities within a region of background signal (white boxes of Supplementary Figs 7a,b). We define the threshold to be the mean plus two standard deviations.



Supplementary Figure 7. Signal correlation for channels 1 and 2 of Figure 4a and channels 3 and 4 of Figure 4b. (a,b) Images. For each image, the region used to define the background threshold is depicted by a white square. Scale bar: 10 μm. (c,d) 2D histograms of pixel intensity. Each 2D bin within a 2D histogram represents a range of pixel intensities along each axis. A bin is shaded based on the number of pixels falling into that bin for a given pair of images. Pixels falling into bins within the white square at the lower left corner are excluded from calculation of the correlation coefficient. For target *desm* (panels a and c), the Pearson correlation coefficient is $r = 0.93$. For target *Tg(flk1:egfp)* (panels b and d), the Pearson correlation coefficient is $r = 0.97$. Embryos fixed at 27 hpf.

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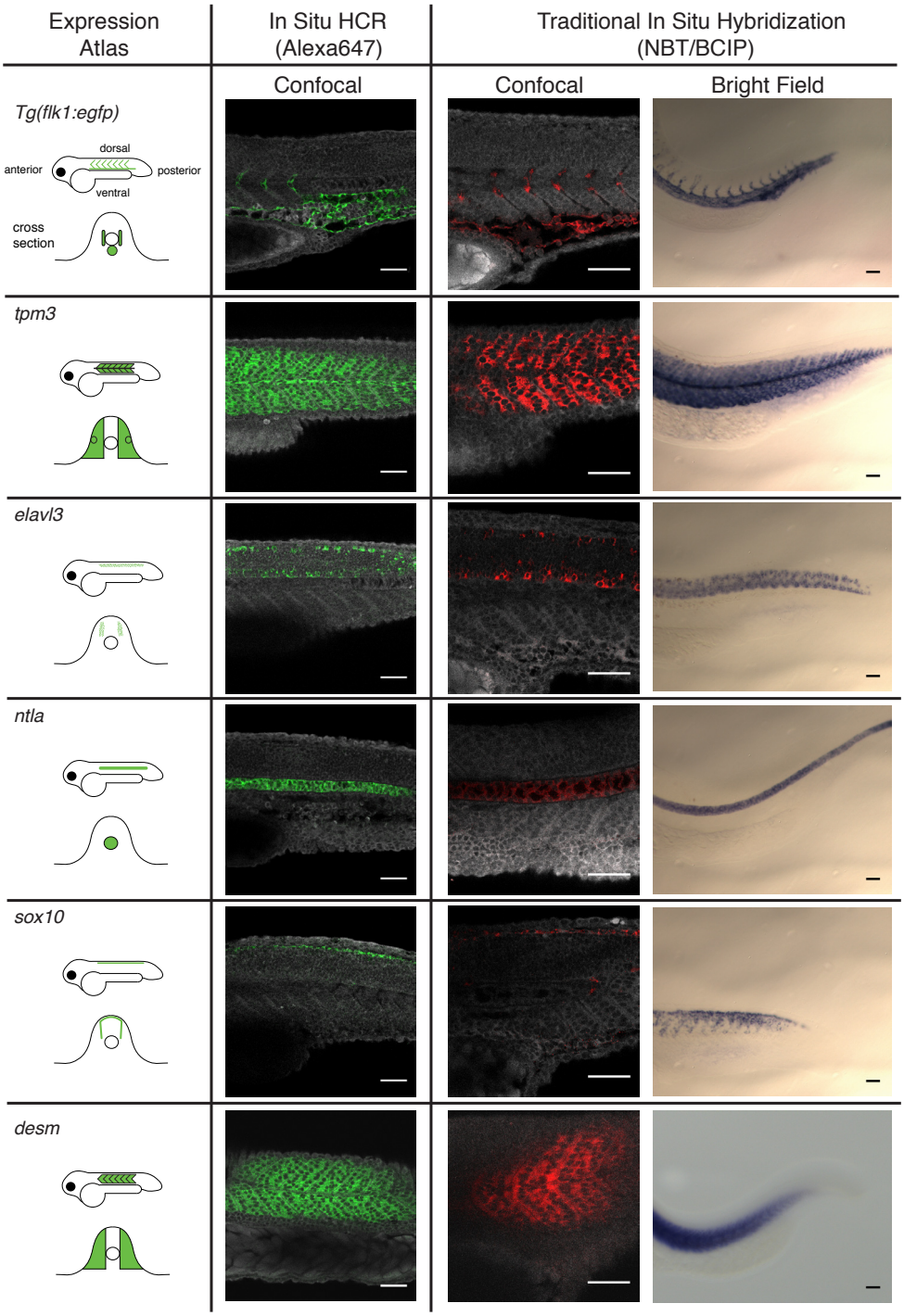
S8 Expression patterns for target mRNAs



Supplementary Figure 8. Comparison of mRNA expression patterns observed using fluorescent in situ HCR and traditional in situ hybridization for the six targets used in Figures 2-4. Traditional in situ hybridization experiments were performed as described previously.[2] Embryo fixed at 26 hpf. Scale bars: 50 μm.

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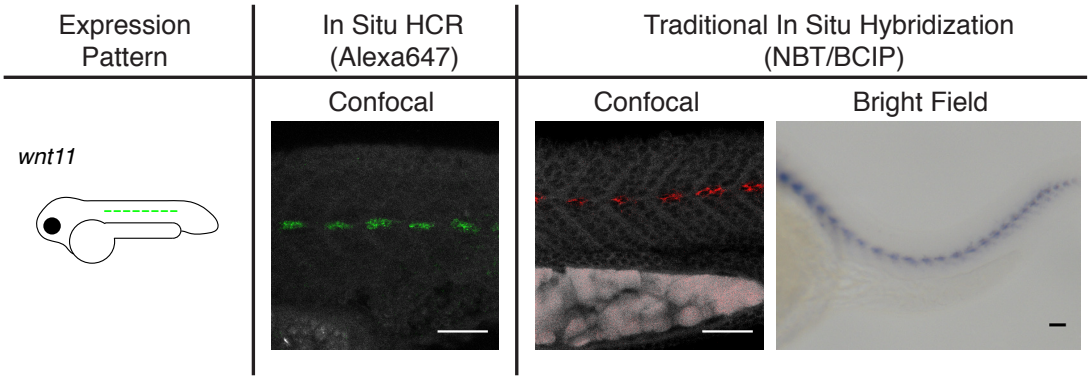
S9 Expression pattern for a less abundant target mRNA

Using a traditional in situ hybridization protocol based on catalytic deposition of reporter molecules, less abundant target mRNAs must be developed for a longer period of time to increase the signal in the vicinity of the probe. For example, using a traditional in situ protocol,[2] we required 6 hr of development for *wnt11*, compared to 1hr for *sox10*, 1.5 hr for *desm*, and 2 hr for *ntla*. Here, we tested the performance of fluorescent in situ HCR on *wnt11* using a probe set with 14 probes. High signal-to-background was observed using our standard in situ protocol (Section S1.2) without modification.



Supplementary Figure 9. Comparison of *wnt11* expression using fluorescent in situ HCR and traditional in situ hybridization. Embryos fixed at 26 hpf. Scale bars: 50 μm.

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S10 Probe sequences

Sequences for the seven target mRNAs used in this paper were obtained from the Zebrafish Information Network (ZFIN).[3]

S10.1 SP6 transcription construct

To enable in vitro transcription, a 19-nt SP6 promoter sequence was placed in front of the initiator sequence of the probe. Three additional random nucleotides were added before the promoter to increase the yield for these short probe syntheses. Depending on the initiator sequence, the transcribed probes vary in length from 81-83 nt based on the properties of SP6 (Epicentre Biotechnologies). The construct is:

5′-Three Random Nucleotides - SP6 Promoter - HCR Initiator - Spacer - Probe Sequence-3′

Three Random Nucleotides: `CAg`
SP6 Promoter: `ATTTAggTgACACTATAgA`

S10.2 Probes for Figures 2a-i and Supplementary Figure 2

A single probe was used to detect the *egfp* target mRNA and trigger polymerization of HCR1 (Figs 2a-g). Figures 2h,i employ a probe with a modified initiator (Probe′). Figures 2g-i employ amplification hairpins with modified stem sequences (HCR1′).

Target mRNA: **enhanced green fluorescent protein (*egfp*)**
Amplifier: **HCR1**
Fluorophore: **Alexa Fluor 647**

Probe #	Initiator	Spacer	Probe Sequence
1	gACCCUAAgCAUACAUCgUCCUUCAU	UUUUU	gUUCUUCUgCUUgUCggCCAUgAUAUAgACgUUgUggCUgUUgUAgUUgU

Target mRNA: **enhanced green fluorescent protein (*egfp*)**
Amplifier: **HCR1′**
Fluorophore: **Alexa Fluor 647**

Probe #	Initiator	Spacer	Probe Sequence
1′	CCAgUUAUCAgUAgUCCgUCCUUCAU	UUUUU	gUUCUUCUgCUUgUCggCCAUgAUAUAgACgUUgUggCUgUUgUAgUUgU

S10.3 Probes for Figures 2j-l and Supplementary Figure 3

Three adjacent *desm* probes, three adjacent *egfp* probes, and amplifier HCR3 were used for the penetration study. All probes have identical initiator and spacer sequences.

Target mRNA: **desmin (*desm*)**
Amplifier: **HCR3**
Fluorophore: **Alexa Fluor 647**

Probe #	Initiator	Spacer	Probe Sequence
1	UACGCCCUAAGAAUCCGAACCCUAUG	AAAUA	CUCACUCAUUUgCCUCCUCAgAgACUCAUUggUgCCCUUgAgAgAgUCAA
2			gCAgCAUCgACAUCAgCUCUgAAAgCAgAAAggUUgUUUUCAgCUUCCUC
3			CUUCgUgAAgACCCUCgAUACgUCUUUCCAggUCCAgCCUggCCAgAgUg

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Target mRNA: **enhanced green fluorescent protein (*egfp*)**
Amplifier: **HCR3**
Fluorophore: **Alexa Fluor 647**

Probe #	Initiator	Spacer	Probe Sequence
1	UACGCCCUAAGAAUCCGAACCCUAUG	AAAUA	gUUCUUCUgCUUgUCggCCAUgAUAUAgACgUUgUggCUgUUgUAgUUgU
2			ACUCCAgCUUgUgCCCCAggAUgUUgCCgUCCUCCUUgAAgUCgAUgCCC
3			UUCAgCUCgAUgCggUUCACCAgggUgUCgCCCUCgAACUUCACCUCggC

S10.4 Probes for Figure 2m and Supplementary Figures 4-6

Probe sets with 1, 3, or 9 adjacent probes were used to address each mRNA target. HCR3 was used for all probe sets. Probe set 1: probe # 1. Probe set 3: probes # 1-3. Probe set 9: probes # 1-9.

Target mRNA: **desmin (*desm*)**
Amplifier: **HCR3**
Fluorophore: **Alexa Fluor 647**

Probe #	Initiator	Spacer	Probe Sequence
1	UACGCCCUAAGAAUCCGAACCCUAUG	AAAUA	CUCACUCAUUUgCCUCCUCAgAgACUCAUUggUgCCCUUgAgAgAgUCAA
2			gCAgCAUCgACAUCAgCUCUgAAAgCAgAAAggUUgUUUUCAgCUUCCUC
3			CUUCgUgAAgACCCUCgAUACgUCUUUCCAggUCCAgCCUggCCAgAgUg
4			CUgCAgCUCACggAUCUCCUCCUCAUgAAUCUUCCUgAggAAUgCAAUCU
5			ggUUUggACAUgUCCAUUUggAUCUgCACCUgACUCUCCUgCAUCUggUU
6			CgAUAgCCUCgUACUgCAggCgAAUgUCUCUgAgggCCgCAgUCAggUCU
7			UgAAACCUUAgACUUAUACCAgUCCUCggCCUCgCUgAUAUUCUUggCAg
8			UCUCgCAggUgUAggACUggAgCUggUgACggAACUgCAUggUCUCCUgC
9			UUggCUUCUCUgAgAgCCUCgUUAUUCUUgUUCACUgCCUggUUCAAAUC

S10.5 Probes for Figures 3b,d and Supplementary Figure 8

The probe sets for each target mRNA contain different numbers of probes as described below. All probes in a given probe set contain the same initiator and are amplified using the same HCR hairpins.

Target mRNA: **enhanced green fluorescent protein (*egfp*)**
Amplifier: **HCR3**
Fluorophore: **Alexa Fluor 488**

Probe #	Initiator	Spacer	Probe Sequence
1	UACgCCCUAAgAAUCCgAACCCUAUg	AAAUA	gUUCUUCUgCUUgUCggCCAUgAUAUAgACgUUgUggCUgUUgUAgUUgU
2			ACUCCAgCUUgUgCCCCAggAUgUUgCCgUCCUCCUUgAAgUCgAUgCCC
3			UUCAgCUCgAUgCggUUCACCAgggUgUCgCCCUCgAACUUCACCUCggC
4			ACgCUgCCgUCCUCgAUgUUgUggCggAUCUUgAAgUUCACCUUgAUgCC
5			CggggCCgUCgCCgAUgggggUgUUCUgCUggUAgUggUCggCgAgCUgC
6			UUUgCUCAgggCggACUgggUgCUCAggUAgUggUUgUCgggCAgCAgCA
7			gCgggUCUUgUAgUUgCCgUCgUCCUUgAAgAAgAUggUgCgCUCCUggA
8			CgUAgCCUUCgggCAUggCggACUUgAAgAAgUCgUgCUgCUUCAUgUgg
9			UCggggUAgCggCUgAAgCACUgCACgCCgUAggUCAgggUggUCACgAg
10			ggUgggCCAgggCACgggCAgCUUgCCggUggUgCAgAUgAACUUCAggg

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Target mRNA: **tropomyosin 3 (*tpm3*)**
Amplifier: **HCR2**
Fluorophore: **Alexa Fluor 514**

Probe #	Initiator	Spacer	Probe Sequence
1	CCgAAUACAAAgCAUCAACgACUAgA	AAAAA	UCCUCAACCAgCUggAUACgCCUgUUCAgAgAAgCCACCUCUgCCUCAgC
2			CCAgCUUUUgCAgggCUgUggCCAgUCUCUCCUgAgCACgAUCCAACUCC
3			AAUCACCUUCAUCCCUCUCUCgCUCUCAUCUgCggCCUUCUCggCUUCCU
4			UggAUCUCCUgCAgCUCCAUCUUCUCCUCAUCCUUCAgAgCCCUgUUCUC
5			CUUCAUAUUUgCggUCAgCCUCCUCAgCAAUgUgCUUggCCUCCUUAAgC
6			CUCUgUACgCUCCAACUCUCCCCUCAACgAUCACCAgCUUACgAgCCACCU
7			UggUUUUCUCCAgUUUggCCACAgACCUCUCAgCAAACUCUgCACgggUC

Target mRNA: **ELAV (Embryonic Lethal, Abnormal Vision, Drosophila)-like 3 (Hu antigen C) (*elavl3*)**
Amplifier: **HCR4**
Fluorophore: **Alexa Fluor 546**

Probe #	Initiator	Spacer	Probe Sequence
1	gACUACUgAUAACUggAUUgCCUUAg	AAUUU	CCUUgUCggCgUCgUUgggAUCCACAUAgUUUACAAAgCCAUAUCCCAAg
2			CACCUUgAUUgUUUUggUCUgCAgUUUgAgACCgUUgAgCgUgUUgAUAg
3			ACAUACAggUUggCAUCgCggAUggAAgCUgAgCUgggCCUggCgUAAgA
4			AAAACAACUgCUCCAUgUCUUUCUgACUCAUggUUUUgggCAggCCgCUC
5			UgUgACCUggUUUACCAggAUgCgUgAggUgAUgAUCCUUCCAUACUggg
6			gCUUCgUUCCgUUUgUCgAACCgAAUgAAACCUACCCCgCgCgAUAUACC
7			CAgCUgCUCCUAgUggCUUCUgACCgUUCAggCCCUUgAUggCCUCCUCU
8			CUgUCCUgUCUUCUgACUggggUUgUUggCgAACUUUACggUgAUgggCU
9			gggCCAgUgUAgCggCgAgCggCUgUCUggUAgAgCUgggUCAgCAgAgC
10			UgUCAAUggUUAUgggggAgAAUCUgAAgCgCUgggUCUggUggUgCAgA
11			gCCggCUCCAgUgggCCCggUCAggUUgACCCCggCAAgACUAgUCAUgC
12			AggACACUUUCgUCAgCUUCCggggACAggUUgUAgACgAAgAUgCACCA
13			ggAUgACCUUgACgUUUgUgACggCgCCAAAAggCCCgAAgAgCUgCCAC
14			ggUCAUggUgACgAAgCCAAAgCCCUUACAUUUgUUggUggUgAAgUCAC
15			AggCggUAgCCAUUCAgACUggCgAUAgCCAUggCUgCCUCgUCgUAgUU
16			CUCUggCCUgUgAUCUUgUCUCUgACCAAUUUgCAggACUCgAUUUCCCC
17			gAUgCUgCCAAAgAggCUCUUgAACUCUUCCUgggUCAUgUUCUgAggCA
18			ggUAgUUgACgAUCAggUUAgUUUUgCUgUCAUCUgUggCgCCgUUAgUg

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Target mRNA: **no tail a (*ntla*)**
Amplifier: **HCR1**
Fluorophore: **Alexa Fluor 594**

Probe #	Initiator	Spacer	Probe Sequence
1	gACCCUAAgCAUACAUCgUCCUUCAU	UUUUU	gCAgCUCUgUggUUCCUCAAgCUggAgUAUCUCUCACAgUACgAACCCgA
2			UgUAgUUAUUggUggUAgUgCUgCggUgggAgUAAUggCUgggAUAUggA
3			CAgggCUgACCAgCUgUCAUgAgACgCAAgACUUCCggAAgAgUUgUCCA
4			gUgUUUgUggUgUgggCCAgggUUCCCAUCCCgCUggAgUUggggAUCUg
5			UCgUCCCUgCAACUgACCACAgACUUgggUACUgACUggUgUUggAggUA
6			UgUCAggCCACCUgUAAUggAgCCCgAUgCUgAgCCUgAUggggUgAgAg
7			gAggAggUCAgACCCgAgUAggACAUCgAAgAACCgCgUAggAACUgAgA
8			CCUCgCUUAggCCUggAUCgUACAUUgAggAgggAgAggACACAggCAgC
9			UgCUgUgAgCCgggCgAUggAgCUCUCgAACUgggCAUCUCCAACgCCAA
10			UCCUUAAAUgUgAAgCgAUCUCAgUAgCUCUgAgCCACAggCgCCCAUgA
11			UUCUAgAUUUCCUCCUgAAgCCAAgAUCAAgUCCAUAACUgCAgCAUCAg
12			gACUUUUAUAgUAAAUCAACCCgUUUUCUgAUUgUCAAAUCAAgAAgCUC
13			ggAgUgAACAggggCCCCAUUgAACUgAggAgggCUgCUgCUggggCCCA
14			UggggCCgUUACUgggCAggAACCAgCCACCgAgUUgUgAAUAUCCAgAU
15			UgCUggUUgUCAgUgCUgUggUCUgggACUUCCUUgUggUCACUUCUCUC
16			UUUggCAUCgAggAAAgCUUUggCAAAAggAUUgUgUUUgAUUUUCAgAg
17			CggUAAUCUCUUCAUUCUgAUAUgCUgUgACUgCAAUAAACUgUgUCUCA
18			ggAAAAgACUgACUgCUgAUCAUUUUCUgAAUCCCACCgACUUUCACgAU
19			gUgUAUCCUgggUUCgUAUUUgUgCAAUgAgUUUAACAUAAUCUgUCCUC
20			CUCCgUUgAgUUUAUUggAgAgUUUgACUUUgCUgAAAgAUACgggUgCU
21			UUCAUCCAgUgCgCgCCgAAgUUgggUgAgUCCgggUggAUgUAgACgCA
22			gCUCgggCUUUggggUUCgggUUUCCCACCgggCACCCAUUCACCgUUCA
23			CgUAUUUCCACCgAUUAUUAUCggCCgCCACAAAAUCCAgCAggACCgAg
24			UACAUUgCAUUAgggUCgAgACCggUgACACUggCUCUgAgCACgggAAA
25			CAUUCgUCUCCCAgUCUUggUgACAAUCAUUUCAUUggUgAgCUCUUUAA
26			AUUUggUCCACAACUCCgCgUCUUCAAgCgAAAgUUUAAUAUCCCgCUCg
27			gACgCgUCCCCUUUCUCgCUgCCCUUCUgAAAUUCgCUCUCCACggCgCU
28			AAggAgAUgAUCCAggCgCUggUCgggACUUgAggCAgACAUAUUUCCgA
29			UCAAAUAAAgCUUgAgAUAAgUCCgACgAUCCUACUAAAUCCCgUUggAU
30			UAAAUgAUgUCAAAAUUUUCUUUUUUgCAAgAACUAACCCUUUAAUUgAU

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Target mRNA: **SRY-box containing gene 10 (*sox10*)**
Amplifier: **HCR5**
Fluorophore: **Alexa Fluor 647**

Probe #	Initiator	Spacer	Probe Sequence
1	gCAUUACAgUCCUCAUAAgUAUCUCg	UUUUU	AUAAACggCCgCUUAUCCgUCUCgUUCAgCAgUCUCCACAgCUUCCCCAg
2			ACUCgggAUAAUCUUUCUUAUgCUgCUUCCUCAAgCgCUCggCCUCCUCg
3			UCUgAgCUggAACCCggUUUgCCgUUCUUgCgUCgACgUggCUggUACUU
4			gUgCgCCACCUCCAggUgCAggCUCUUgUAAUgCgAUUggCUgUggCUgA
5			UgUgACUCUgACCUgUAgCgUgAgggUggUgUCCAUCACCCAAUggUgAC
6			CCAgUCCACUCCgAgAggCUCCgCCCUCACgCUUgCCCUCgCCUgAUUUU
7			UCCACgUUACCgAAgUCgAUgUgCggUUUCCCgCUggCAgACgAUgAggC
8			CgUCgAACggCUCCAUgUUggCCAUCACgUCAUggCUgAUUUCgCCAAUg
9			ggACgCCUgCgggUggCCAUUgggUgggAgAUACUggUCgAACUCgUUCA
10			AgUggCCACUAgCggCCgCUAgCgCgCUggAgAUgCCgUAUgUAUACgAU
11			UCUgCgUUUUCCCgCCAUCUgCgCCCAAAUgCUgCUgggACggCAgUUgC
12			gUgUgAACCgCUCgCCgCUgUAUCCCCAgggAAgUgUgUUUCACUCUUUA
13			gAggggAAggCggAgCUgUAgUgCggCAgUgUUAgCggCgUgUAUgUgAC
14			UAgUAggAUCCCgAggCCUggUgCUCggCgUAUUCggCgAAUUgUgCgCg
15			AgUgUggUgUAUACgggCUgCUCCCAAUgCgUAgggCUgUgUgACUgCgg
16			UUggACCUUUAgUgACUggUCAUCUUggUAgAgUgUgUCACggUCgAgAC
17			UgCAggCgAgUgUUUCgAUgAUUUUUAgCACACACACACACACCUUACgg
18			ACACACACACACACACUCgUUUCUCAgAUCUCAgUUUgUgUCgAUUgUgg
19			UCUggACggUggUCgUCUgAggCACgUgAgAAUAUUUCCCUgCAgAUCUC
20			CgUCUUUUUCgAAAAUACUACUggUgUCAAAUUggCgUUgAgggAgCAgg

S10.6 Probes for Figure 4 and Supplementary Figure 7

Two probe sets target *desm*. Two probe sets target *Tg(flk1:egfp)*. Each probe set initiates a different HCR amplifier. The four HCR amplifiers are labelled with spectrally distinct fluorophores.

Target mRNA: **enhanced green fluorescent protein (*egfp*)**
Amplifier: **HCR1**
Fluorophore: **Alexa Fluor 488**

Probe #	Initiator	Spacer	Probe Sequence
1	gACCCUAAgCAUACAUCgUCCUUCAU	UUUUU	UUgUggCCgUUUACgUCgCCgUCCAgCUCgACCAggAUgggCACCACCCC
2			UCAgCUUgCCgUAggUggCAUCgCCCUCgCCCUCgCCggACACgCUgAAC
3			ggUgggCCAgggCACgggCAgCUUgCCggUggUgCAgAUgAACUUCAggg
4			UCggggUAgCggCUgAAgCACUgCACgCCgUAggUCAgggUggUCACgAg
5			CgUAgCCUUCgggCAUggCggACUUgAAgAAgUCgUgCUgCUUCAUgUgg
6			gCgggUCUUgUAgUUgCCgUCgUCCUUgAAgAAgAUggUgCgCUCCUggA
7			UUCAgCUCgAUgCggUUCACCAgggUgUCgCCCUCgAACUUCACCUCggC
8			ACUCCAgCUUgUgCCCCAggAUgUUgCCgUCCUCCUUgAAgUCgAUgCCC

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Target mRNA: **enhanced green fluorescent protein (*egfp*)**
Amplifier: **HCR2**
Fluorophore: **Alexa Fluor 514**

Probe #	Initiator	Spacer	Probe Sequence
1	CCgAAUACAAAgCAUCAACgACUAgA	AAAAA	gUUCUUCUgCUUgUCggCCAUgAUAUAgACgUUgUggCUgUUgUAgUUgU
2			ACgCUgCCgUCCUCgAUgUUgUggCggAUCUUgAAgUUCACCUUgAUgCC
3			CggggCCgUCgCCgAUgggggUgUUCUgCUggUAgUggUCggCgAgCUgC
4			UUUgCUCAgggCggACUgggUgCUCAggUAgUggUUgUCgggCAgCAgCA
5			gCggUCACgAACUCCAgCAggACCAUgUgAUCgCgCUUCUCgUUggggUC
6			CgCggCCgCUUUACUUgUACAgCUCgUCCAUgCCgAgAgUgAUCCCggCg

Target mRNA: **desmin (*desm*)**
Amplifier: **HCR4**
Fluorophore: **Alexa Fluor 594**

Probe #	Initiator	Spacer	Probe Sequence
1	gACUACUgAUAACUggAUUgCCUUAg	AAUUU	gCUgAAUAUUUCgUgCUCAUgACUgggCCUgUUgggUUUgUACgCUgUgU
2			AAAAUAgAggAgCCCAAACCUgAgCCAAAggUgCggCggUAAgAggACgC
3			UAAACUCUggAggUCAgUCUUgAggAgCCAgAggAACCUgAggAACCgUg
4			AAAgAgCCggACgCACggUggCUggAAAAAUggggAgAAgCggAgCUCUU
5			AUCAgCCAgAUUgAAAUCCAgCUUCUCACCAAggCCAgCgUAggAACggA
6			UggAgCUCggCCUUCUCAUUAgUACgCgUgUUgAggAAgUCCUggUUUAU

Target mRNA: **desmin (*desm*)**
Amplifier: **HCR3**
Fluorophore: **Alexa Fluor 647**

Probe #	Initiator	Spacer	Probe Sequence
1	UACgCCCUAAgAAUCCgAACCCUAUg	AAAUA	UAggUUgUCCCUCUCgAUCUCCACACgggAUCUCUgAUUggUCAgUgCCU
2			UggUggAUCUCCUCUUgAAgUCUgAgCUUUAgUUUCUgUAggUCAUCgAC
3			gCAgCAUCgACAUCAgCUCUgAAAgCAgAAAggUUgUUUUCAgCUUCCUC
4			CUUCgUgAAgACCCUCgAUACgUCUUUCCAggUCCAgCCUggCCAgAgUg
5			CUgCAgCUCACggAUCUCCUCCUCAUgAAUCUUCCUgAggAAUgCAAUCU
6			CgAUAgCCUCgUACUgCAggCgAAUgUCUCUgAgggCCgCAgUCAggUCU
7			UgAAACCUUAgACUUAUACCAgUCCUCggCCUCgCUgAUAUUCUUggCAg
8			UUggCUUCUCUgAgAgCCUCgUUAUUCUUgUUCACUgCCUggUUCAAAUC
9			UCUCgCAggUgUAggACUggAgCUggUgACggAACUgCAUggUCUCCUgC
10			CUCACUCAUUUgCCUCCUCAgAgACUCAUUggUgCCCUUgAgAgAgUCAA

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S10.7 Probes for Supplementary Figure 9

All probes in the probe set contain the same initiator and are amplified using the same HCR hairpins.

Target mRNA: **wingless-type MMTV integration site family, member 11 (*wnt11*)**
Amplifier: **HCR3**
Fluorophore: **Alexa Fluor 647**

Probe #	Initiator	Spacer	Probe Sequence
1	UACgCCCUAAgAAUCCgAACCCUAUg	AAAUA	CUCCAGUGAAGUUUUUCCACAACGGUCAAGAUACACCACGGACUGUCUGA
2			CCGAGCUCCCGUUUAUCGUCAAACCAAGCCAUGAUAUUCCUGUGCAUGGA
3			CGCGCGUACAAUGCUGUGCAUGAGCUCGAGGUUGCGCUUGCAGAGCUGCU
4			UUCCAGCGCAUAUCACUGAAGGAGCUCGUGCACGCGCUCUUGGUGAGUCU
5			GUAGCGAAGGUUAUCUCCACAUCCACCCCAGCGGAAAUCAGGAGCCGCCU
6			UCUGCCAACAGCAUUGUUGUGAAGCUGCAUGAGUCUAAAGGCCUGUGGGC
7			CGGCGGAGAUGGUGCUGAUGUCUUGAAGACCCUUCCAACAGGUCUUUACA
8			CCAAUCUGACGCGGAAUCACCUUGGUGGCCGACAGGUAUUUAGACUUGAG
9			AAAGGGUCACACAAGGUCUAUUGGUGAAGACGGUCUGUCACUUGCAGACG
10			CUUUCUCCACUUUCCUGCCUGGGAAAGGAUUAUCCACUCACUUAUGGCUC
11			ACAGCCAUUCAUUAACUCUUUCACCAUCCCAUAUUCCUCAACUCCGGCUC
12			CUCUGGUUUUCGGUGGGGAGCCUGAAGGGUCUCACUGCAGGGUUCUUUGU
13			GAGUGCAGAGAACAUCUGACGUCAACCGAACUAAUCUGAUGUCCAUCACG
14			CACAACUAGAAGUUGUUAAUUUCCAAGCAGUCUCGUUUGUCCUAUUGCUG

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S10.8 Traditional probes for Supplementary Figures 8 and 9

The following RNA probes were used to perform the traditional in situs of Supplementary Figures 8 and 9.

Target mRNA: ***egfp***
Probe Sequence: gACgUAAACggCCACAAgUUCAgCgUgUCCggCgAgggCgAgggCgAUgCCACCUACggCAAgCUgACCCUgAAgUUCAUCUg
CACCACCggCAAgCUgCCCgUgCCCUggCCCACCCUCgUgACCACCUUCggCUACggCCUgAUgUgCUUCgCCCgCUACCCCgACCACAUgAAgCAgC
ACgACUUCUUCAAgUCCgCCAUgCCCgAAggCUACgUCCAggAgCgCACCAUCUUCUUCAAggACgACggCAACUACAAgACCCgCgCCgAggUgAAg
UUCgAgggCgACACCCUggUgAACCgCAUCgAgCUgAAgggCAUCgACUUCAAggAggACggCAACAUCCUggggCACAAgCUggAgUACAACUACAA
CAgCCACAACgUCUAUAUCAUggCCgACAAgCAgAAgAACggCAUCAAggUgAACUUCAAgAUCCgCCACAACAUCgAggACggCAgCgUgCAgCUCg
CCgACCACUACCAgCAgAACACCCCCAUCggCgACggCCCCgUgCUgCUgCCCgACAACCACUACCUgAgCUACCAgUCCgCCCUgAgCAAAgACCCC
AACgAgAAgCgCgAUCACAUggUCCUgCUggAgUUC

Target mRNA: ***tpm3***
Probe sequence: UgUACAAgACCggUCCUUCAAACAUUgggCUACAgUAUUCCCAgAAggAggACAAgUAUgAggAAgAAAUCAAgAUCCUCACU
gAUAAgCUgAAggAggCUgAgACCCgUgCAgAgUUUgCUgAgAggUCUgUggCCAAACUggAgAAAACCAUUgAUgAUUUggAAgAUgAgCUUUAUgC
UCAgAAACUCAAgUAUAAggCCAUUAgUgAggAgUUggAUCAUgCUCUCAACgACAUgACCUCUAUAUAAAgAgUUUCUggACUgUUCUgUggCUgAC
UgUgACUUCAAgAAAUgCUUCCUCgUCUUCUCUgACUgUCCAUAUUUgUUgCUUUUUUUCUUCUUUgUACACUUCCUgUUUUgUgUgUUUUUCCgUgU
ACUCAUgUCUgUAgUgCCAgUUUCUUUAUUCUgUUUCUgCUUCUgUUUUUUAgAUAUUCAUUAUCUgCCCCAACAUCUUCCUCUUAUCAggAUCUgUU
gUUCUUAUgUCCCUgCUCUUgCUCUUCUgUgACCUUUUgCUgUAUUUUUCAUgCCUUgCgUCCAUgUUUAUUgAAgggAggAgAAAAAACggCUCUgC
UCUCUUUUgAAUgUCUgCUUgUCUCUCUUUAUUgCAAUggACUggUgUUgggCAACCAAgCAUUUACCCAUCUUCAAUUgCACAUgUAUUAUAUCUCA
UggUUgAAAgAUAAAAggCUUgAUUAAAUUCUCCgUCACUAAUUgUgAUUAAAAUCgAAUUCCCgCggCCgCCAUggCggCCggAg

Target mRNA: ***elavl3***
Probe sequence: ggAUAUggCUUUgUAAACUAUgUggAUCCCAACgACgCCgACAAggCUAUCAACACgCUCAACggUCUCAAACUgCAgACCAA
AACAAUCAAggUgUCUUACgCCAggCCCAgCUCAgCUUCCAUCCgCgAUgCCAACCUgUAUgUgAgCggCCUgCCCAAAACCAUgAgUCAgAAAgACA
UggAgCAgUUgUUUUCCCAgUAUggAAggAUCAUCACCUCACgCAUCCUggUAgACCAggUCACAgCAggUAUAUCgCgCggggUAggUUUCAUUCgg
UUCgACAAACggAACgAAgCAgAggAggCCAUCAAgggCCUgAACggUCAgAAgCCACUAggAgCAgCUgAgCCCAUCACCgUAAAgUUCgCCAACAA
CCCCAgUCAgAAgACAggACAggCUCUgCUgACCCAgCUCUACCAgACAgCCgCUCgCCgCUACACUggCCCUCUgCACCACCAgACCCAgCgCUUCA
gACUCgACAAUUUACUAAACgCCAgCUACggAgUCAAgAgAUUCUCCCCCAUAACCAUUgACAgCAUgACUAgUCUUgCCggggUCAACCUgACCggg
CCCACUggAgCCggCUggUgCAUCUUCgUCUACAACCUgUCCCCggAAgCUgACgAAAgUgUCCUgUggCAgCUCUUCgggCCUUUUggCgCCgUCAC
AAACgUCAAggUCAUCCgUgACUUCACCACCAACAAAUgUAAgggCUUUggCUUCgUCACCAUgACCAACUACgACgAggCAgCCAUggCUAUCgCCA
gUCUgAAUggCUACCgCCUgggCgACCgCgUgCUgCAggUCUCgUUCAAgACCAgCAAgCAgCACAAggCUUgAAggAAggCCUAgUCACUAUUgCUC
UUUAACAUgCAgggggAgCUACUgAgCUCCCUgUACAUUCACUCUACAUgggCCUggACUgAgUCUCUCUCUAACAUACAUUCgACACACACACA

Target mRNA: ***ntla***
Probe sequence: gAAUUCCCgCUgUCAAAgCAACAgUAUCCAACgggAUUUAgUAggAUCgUCggACUUAUCUCAAgCUUUAUUUgAUCggAAAU
AUgUCUgCCUCAAgUCCCgACCAgCgCCUggAUCAUCUCCUUAgCgCCgUggAgAgCgAAUUUCAgAAgggCAgCgAgAAAggggACgCgUCCgAgCg
ggAUAUUAAACUUUCgCUUgAAgACgCggAgUUgUggACCAAAUUUAAAgAgCUCACCAAUgAAAUgAUUgUCACCAAgACUgggAgACgAAUgUUUC
CCgUgCUCAgAgCCAgUgUCACCggUCUCgACCCUAAUgCAAUgUACUCggUCCUgCUggAUUUUgUggCggCCgAUAAUAAUCggUggAAAUACgUg
AACggUgAAUgggUgCCCggUgggAAACCCgAACCCCAAAgCCCgAgCUgCgUCUACAUCCACCCggACUCACCCAACUUCggCgCgCACUggAUgAA
AgCACCCgUAUCUUUCAgCAAAgUCAAACUCUCCAAUAAACUCAACggAggAggACAgAUUAUgUUAAACUCAUUgCACAAAUACgAACCCAggAUAC
ACAUCgUgAAAgUCggUgggAUUCAgAAAAUgAUCAgCAgUCAgUCUUUUCCUgAgACACAgUUUAUUgCAgUCACAgCAUAUCAgAAUgAAgAgAUU
ACCgCUCUgAAAAUCAAACACAAUCCUUUUgCCAAAgCUUUCCUCgAUgCCAAAgAgAgAAgUgACCACAAggAAgUCCCAgACCACAgCACUgACAA
CCAgCAAUCUggAUAUUCACAACUCggUggCUggUUCCUgCCCAgUAACggCCCCAUgggCCCCAgCAgCAgCCCUCCUCAgUUCAAUggggCCCCUg
UUCACUCCUCgggUUCgUACUgUgAgAgAUACUCCAgCUUgAggAACCACAgAgCUgCUCCAUAUCCCAgCCAUUACUCCCACCgCAgCACUACCACC
AAUAACUACAUggACAACUCUUCCggAAgUCUUgCgUCUCAUgACAgCUggUCAgCCCUgCAgAUCCCCAACUCCAgCgggAUgggAACCCUggCCCA
CACCACAAACACUACCUCCAACACCAgUCAgUACCCAAgUCUgUggUCAgUUgCAgggACgACUCUCACCCCAUCAggCUCAgCAUCgggCUCCAUUA
CAggUggCCUgACAUCUCAgUUCCUACgCggUUCUUCgAUgUCCUACUCgggUCUgACCUCCUCgCUgCCUgUgUCCUCUCCCUCCUCAAUgUACgAU
CCAggCCUAAgCgAggUUggCgUUggAgAUgCCCAgUUCgAgAgCUCCAUCgCCCggCUCACAgCAUCAUgggCgCCUgUggCUCAgAgCUACUgAgA
UCgCUUCACAUUUAAggACUgAUgCUgCAgUUAUggACUUgAUCUUggCUUCAggAggAAAUCUAgAAgAgCUUCUUgAUUUgACAAUCAgAAAACgg

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gUUgAUUUACUAUAAAAgUCACAUCUgUAUCAUACCgAggCAUACgUAUUUACAAUCAAgAUgAgAgACAAUCAAUUAAAgggUUAgUUCUUgCAAAA
AAgAAAAUUUUgACAUCAUUUACUCACCUUUgUUUUAAACAUUgUUAAgUUUUUAUUCUgUUAAACACAAAAgAAgAUAUUUUgAAgAAUgUUCAAAA
CUggUAACCAUUgCAUAgAAgCUgUUUUACUUAUggAAgUAAAUggUUACAggUUAUCAgCAUUUUUUUAAAUAUAUUUUUUAgUUCAACAgAAgAAA
gAAACUCUUUAAAgUUUggAACAACUUgAgggUgAgUAAAUUgAgUAAAAgUACgUUUUUgggUUAACUAUCCCUUUAACUAUCAgAUUUUAgCCAUA
CAUUUUggggCAAUUAUAgUgUUUAUUCUUgAUAAUAUUAUCUAAAAgAUUAAUAAAAUCAAAAUUgUgCUgUUgACUCACUAAAAgUgUAUAUgUgU
gUAAAUAAAUAgAAAUUAACgUCCggUUUCAUUgUAUCACAgAAgAAUgUAACAgUCUUACAUgUgCUUUCUgUAgAACgAgAgAAAgACAgACUUUg
CUgUUUCgUUUgAgAAAgUgAAUACgCUUUgAAAAgUgACCgUAUAgUUUUgUCUgCUAUUCgUCCUAUAgAgAAACCAUUUgUACAUAUCUAUCUAU
UUgUAUUUgUUgggCUCUUUgAgUUUUAUUUAUgUCAUUUUAAUAAUAAAUUAAAUUUCUUUUUUUUUUCUgUCAAAAAAAAggAgUUCCggAAUUC

Target mRNA: ***sox10***

Probe sequence: gUCgACgCAAgAACggCAAACCgggUUCCAgCUCAgAggCCgACgCCCACUCUgAgggCgAggUCAgCCACAgCCAAUCgCAU
UACAAgAgCCUgCACCUggAggUggCgCACggCggggCUgCAgggUCACCAUUgggUgAUggACACCACCCUCACgCUACAggUCAgAgUCACAgCCC
UCCAACgCCCCCUACCACCCCCAAgACggAACUgCAgggAggAAAAUCAggCgAgggCAAgCgUgAgggCggAgCCUCUCggAgUggACUgggggUgg
gAgCAgAUggAAgCUCCgCCUCAUCgUCUgCCAgCgggAAACCgCACAUCgACUUCggUAACgUggACAUUggCgAAAUCAgCCAUgACgUgAUggCC
AACAUggAgCCgUUCgACgUgAACgAgUUCgACCAgUAUCUCCCACCCAAUggCCACCCgCAggCgUCCgCCACUgCCAgCgCAggAUCUgCAgCgCC
AUCgUAUACAUACggCAUCUCCAgCgCgCUAgCggCCgCUAgUggCCACUCCACCgCAUggCUgUCCAAgCAgCAACUgCCgUCCCAgCAgCAUUUgg
gCgCAgAUggCggAAAAACgCAgAUAAAgAgUgAAACACACUUCCCCggggAUACAgCggCgAgCggUUCACACgUCACAUACACgCCgCUAACACUg
CCgCACUACAgCUCCgCCUUCCCCUCgCUggCgUCCCgCgCACAgUUCgCCgAAUACgCCgAgCACCAggCCUCgggAUCCUACUACgCCCACUCCAg
CCAgACCUCAggCCUCUACUCCgCCUUCUCCUACAUgggCCCCUCACAgCggCCCCUgUACACCgCCAUUCCggAUCCgggAUCCgUgCCgCAgUCgC
ACAgCCCCACgCACUgggAgCAgCCCgUAUACACCACACUgUCUCgACCgUgACACACUCUACCAAgAUgACCAgUCACggAAggUCCAACCgUAAAg
UgUgUgUgUgUgUgUgUgCUAAAAAUCAUCgAAACACUCgCCUgCACCACAAUCgA

Target mRNA: ***desm***

Probe sequence: AggAgAAAAUACAgUgAAUUUUAUUgUUUUUgUUgCUgUggAACAgUgUCUCAgAUAUgACCCAACCACACACACACgCACAC
AUACAUUCAUUAAAUCAgAAACUCUgAgCUCAUCUggCCACUUUCUgAACgCUCAgCAUCACACCAUUCCACUUUUCCUCCCCUCUggUUUUUCAgCUC
ACUUUCCUUUUgUCUCCAUgCgUCAUCCACACAUgCggUAAUgggUCACggCAAUACUgggUgUgUgCUUAAAAAAgCCUgAggCCAUCAgUgAAAAU
gCAAggCAUUCUgUAAACUCgAUCUgUUUCUCAAgCUUUACAUgACgUCCUgCUggUgCUgUgUggACUCgCUgACgAC

Target mRNA: ***wnt11***

Probe sequence: UAUUUUUGCAUUAAAUAUUUUAAUAAAAUAAAUACAAAUUUUCAUUUUUUACAGACAAUGUACAAGUUUUUUUUCAGGCUGAA
AUGCAUGGCUUUCAUUUUUCUUUUUAUGAAUGCAAUGAAAAUAUUAAUAGACAUCACAAAAAUAGCUAUGGAAAAAUGUUUUUCAAACAGUUUUUUUU
UCUAUUUAUGUGCUUGAGUAAAAUGUUAAUAUCAAAAUUAUUCUGUAAUUUGAGAAAUUAAAUAUGGUCAUUUACAGUGUUUUUUUCUCUCUCUAAAU
GACAACUGGUCAGAAUAACAAAGUGUCUACAAAUCCAGGGUAUUCCACUGUAAUGACUUAUCUGAAAUUAAAAUAUUGGAAAAAUUAACAUACACAUG
GCAUUUUUUUUUGUUAUUUAAAACAAUUUAAAAUAUCAUAAUCAUUUUCUGCUCUGAAUGACUACAAAAUCAUCAGUAUUUUGCAAAACAAAACUAUU
UUUUACUCAAACACACAACUAGAAGUUGUUAAUUUCCAAGCAGUCUCGUUUGUCCUAUUGCUGUAAGUUACUUUACUAUAAAAAUAGUGUCGUCAACC
UUUCAUAAAUCAAUACUGAGUGCAGAGAACAUCUGACGUCAACCGAACUAAUCUGAUGUCCAUCACGUCCAGCUCGUGAAAUUCAUCUGACCAAAACA
AAACCGAUCCGGGCAGGCCAUCAACGCGUGAAGUUUUACCACAGUAAAGUAAGUGUCUUCCCAGCACAGUCCCUUCCCCCAGUCUCUUCCCCUCAGUG
CCGGAGUUGAUGAGUUUUGCCUCUACUUAUCUCUGGUUUUCGGUGGGGAGCCUGAAGGGUCUCACUGCAGGGUUCUUUGUCUACAGCCAUUCAUUAAC
UCUUUCACCAUCCCAUAUUCCUCAACUCCGGCUCUUCUUUCUCCACUUUCCUGCCUGGGAAAGGAUUAUCCACUCACUUAUGGCUCUAAAAAAAAAGG
GUCACACAAGGUCUAUUGGUGAAGACGGUCUGUCACUUGCAGACGUAUCUCUCGACGGUGCGCUUGCAGGUUUUGCAGGACACGUAACAGCACCAGUG
GUAUUUACACUGGCAGCGCUCCACCAGCACCUCUGUGUAGGCAUUAUAACCCCGUCCACAACACAUCAGCCCACAGCUCUCACUGCCGCUCGCCGUCU
UGUUACACUGCCUGUCUGUGGUCCCCAGUGACCCCUGUUUGGCGUUCUGUGUGCAGUAAUCCGGUGAGCUGACCAGGUAGACUAGUUCAUUCUCUCCA
ACCGGCCUCACCUCCAUCUCUCGGGGCACAAGCUGCCGGCGUGUGCCAAUCUGACGCGGAAUCACCUUGGUGGCCGACAGGUAUUUAGACUUGAGGUC
GGCGGAGAUGGUGCUGAUGUCUUGAAGACCCUUCCAACAGGUCUUUACAGAGCAUGAGCCAGAAACGCCAUGACAUUUGCACUUCAUCUCUAGAGAGU
CCAUAAGCACCUGUCUGCCAACAGCAUUGUUGUGAAGCUGCAUGAGUCUAAAGGCCUGUGGGCCCGAGCGCCGGUUCCUUAUUGGUGCAUCUGAGAAA
GCGGAGCCCAUCUGUAGGCCGUAGCGAAGGUUAUCUCCACAUCCACCCCAGCGGAAAUCAGGAGCCGCCUGCUCUGACGGCAUUGCAGCACAGGAACA
GCUGGGCAGGUCUCCAGAUGCACAGGCACGAGCUAUGGCAUGACUGACCACCGCAGCAGCCAGAGAAAACACAAACGCUGCCUCACGGGUCCCUUUGG
CCAGGUCAGGGGUGAAGUGUGGCGCGCUCUCGAUGGACGAGCAGUUCCAGCGCAUAUCACUGAAGGAGCUCGUGCACGCGCUCUUGGUGAGUCUGGCC
GCGCGUACAAUGCUGUGCAUGAGCUCGAGGUUGCGCUUGCAGAGCUGCUGCUGAUCGGGAACGAGCCCGUCCAGAAGUUUACAGUGGUGCGUCUGAUU
CCAGCCCACCGAGCUCCCGUUUAUCGUCAAACCAAGCCAUGAUAUUCCUGUGCAUGGAUAAAUGACGCUCAGUGACGUGAUGAAAAGCAGAAGAAAGU
UCCUGUAUUCUGUCAUGUCGCUCUUUUGCUGUUUAGAAACUCCAGUGAAGUUUUUCCACAACGGUCAAGAUACACCACGGACUGUCUGA

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S11 HCR amplifier sequences

RNA initiator and hairpin sequences for the six HCR amplifiers used in this paper. Each amplifier has an initiator (I) and two hairpins (H1 and H2).

– :	Hairpin ligation site
/5′-dye-C12/:	5′ Alexa Fluor modification with a C12 spacer
/C9-dye-3′/:	3′ Alexa Fluor modification with a C9 spacer

HCR1

I	gACCCUAAgCAUACAUCgUCCUUCAU
H1	AUgAAggACgAUgUAUgCUUAgggUCgACUUCCAUAgACCCU–AAgCAUACAU /C9-dye-3′/
H2	/5′-dye-C12/ gACCCUAAgC–AUACAUCgUCCUUCAUAUgUAUgCUUAgggUCUAUggAAgUC

HCR1′

I′	CCAgUUAUCAgUAgUCCgUCCUUCAU
H1′	AUgAAggACggACUACUgAUAACUgggACUUCCAUACCAgU–UAUCAgUAgUC /C9-dye-3′/
H2′	/5′-dye-C12/ CCAgUUAUCAgUAgUCCgUCCUUCAUgACUAC–UgAUAACUggUAUggAAgUC

HCR2

I	CCgAAUACAAAgCAUCAACgACUAgA
H1	UCUAgUCgUUgAUgCUUUgU–AUUCggCgACAgAUAACCgAAUACAAAgCAUC /C9-dye-3′/
H2	/5′-dye-C12/ CCgAAUACAAAg–CAUCAACgACUAgAgAUgCUUUgUAUUCggUUAUCUgUCg

HCR3

I	UACgCCCUAAgAAUCCgAACCCUAUg
H1	CAUAgggUUCggAUUCUUAgggCgUAgCAgCAUCAAUACgC–CCUAAgAAUCC /C9-dye-3′/
H2	/5′-dye-C12/ UACgCCCUAAgAAUCCgAACCCUAUgggAUUC–UUAgggCgUAUUgAUgCUgC

HCR4

I	gACUACUgAUAACUggAUUgCCUUAg
H1	CUAAggCAAUCCAgUUAUCAgUAgUCUgACACgACUgACUAC–UgAUAACUgg /C9-dye-3′/
H2	/5′-dye-C12/ gACUACUgAUA–ACUggAUUgCCUUAgCCAgUUAUCAgUAgUCAgUCgUgUCA

HCR5

I	gCAUUACAgUCCUCAUAAgUAUCUCg
H1	CgAgAUACUUAUgAggACUgUAAUgCAAgUCgUUCAgCAUU–ACAgUCCUCAU /C9-dye-3′/
H2	/5′-dye-C12/ gCAUUACAgUC–CUCAUAAgUAUCUCgAUgAggACUgUAAUgCUgAACgACUU

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References

1. Adler, J. & Parmryd, I. Quantifying colocalization by correlation: The Pearson correlation coefficient is superior to the Mander's overlap coefficient. *Cytometry Part A* **77A**, 733–742 (2010).
2. Trinh, L. A. *et al.* Fluorescent in situ hybridization employing the conventional NBT/BCIP chromogenic stain. *BioTechniques* **42**, 756–759 (2007).
3. Sprague, J. *et al.* The Zebrafish Information Network: the zebrafish model organism database. *Nucleic Acids Res* **34**, D581–D585 (2006).

Nature Biotechnology: doi:10.1038/nbt.1692