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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

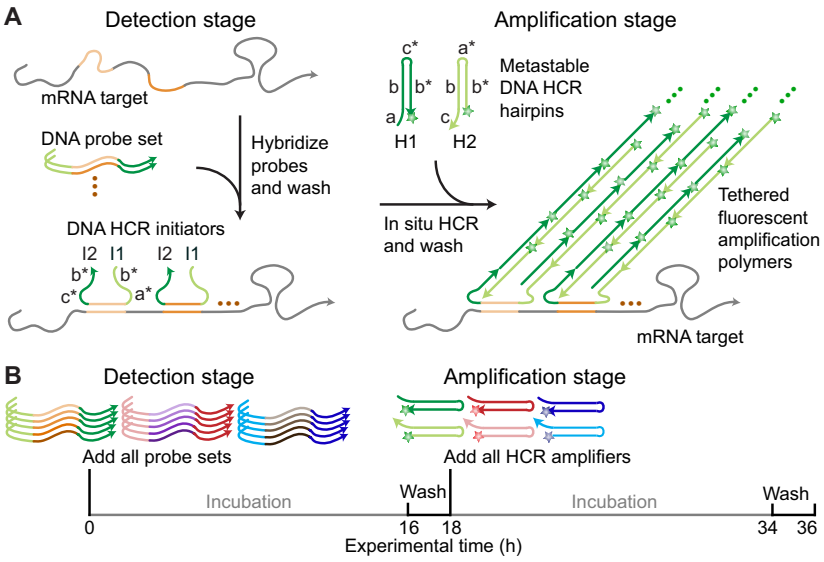

Fig. 1. Multiplexed *in situ* hybridization chain reaction (HCR). (A) Two-stage *in situ* HCR protocol (Choi et al., 2014). Detection stage: DNA probes carrying DNA HCR initiators (I1 and I2) hybridize to mRNA targets and unused probes are washed from the sample. Amplification stage: metastable DNA HCR 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.

Development (2016) 143, 3632-3637 doi:10.1242/dev.140137

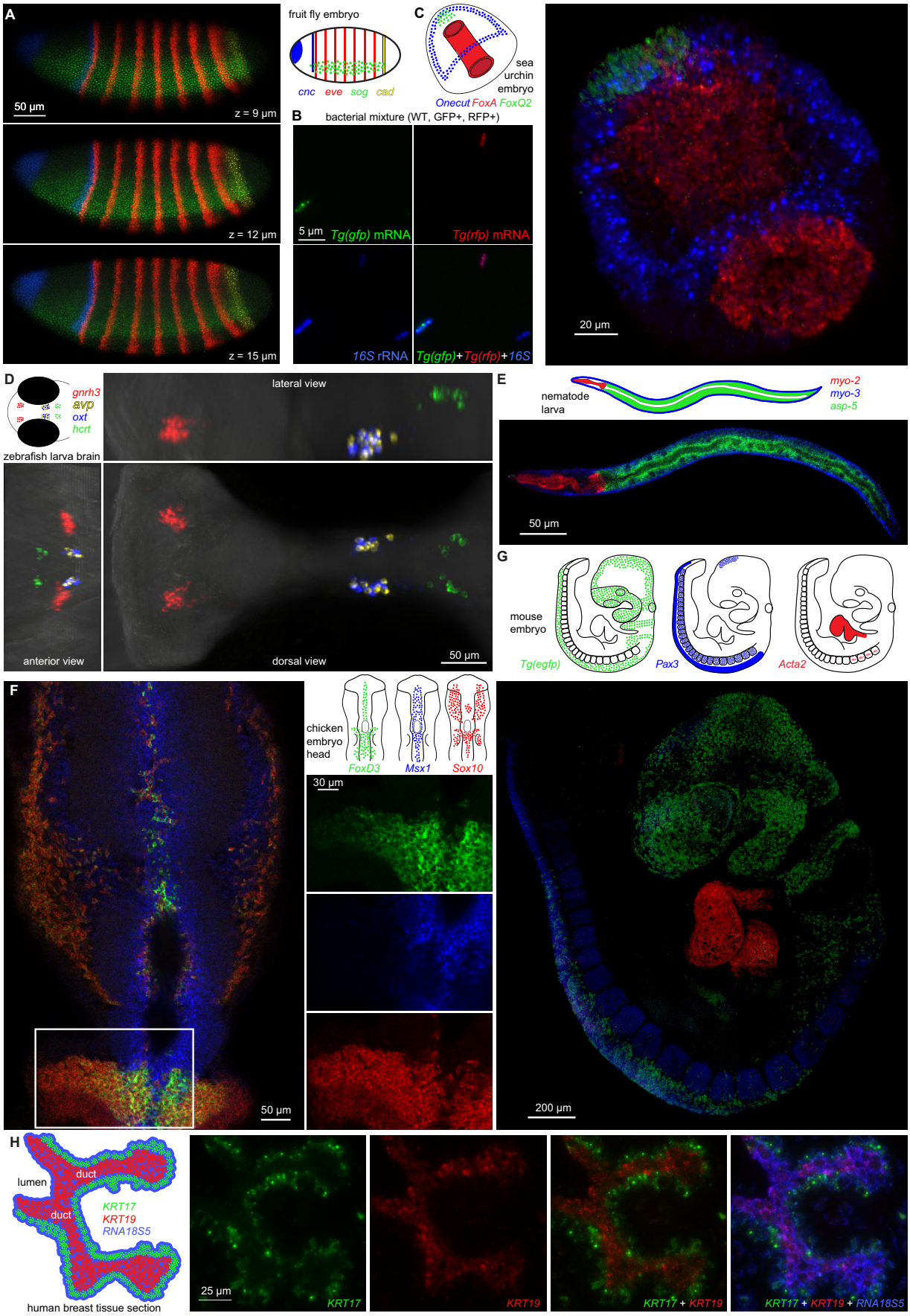

Fig. 2. See next page for legend.

Development (2016) 143, 3632-3637 doi:10.1242/dev.140137

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.

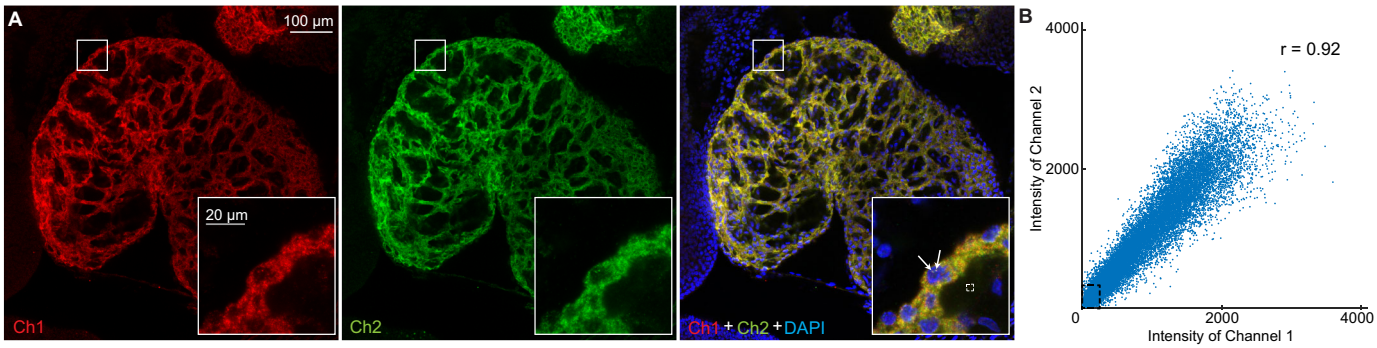

Fig. 3. Subcellular resolution using *in situ* HCR. (A) Redundant two-channel mapping of target mRNA *Acta2* in the heart of a whole-mount mouse embryo. Arrows denote putative sites of active transcription. Probe sets: two probes per channel. Pixel size: 69×69 nm. Embryo fixed: E9.5. (B) Highly correlated 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.

Development (2016) 143, 3632-3637 doi:10.1242/dev.140137

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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