Mapping a multiplexed zoo of mRNA expression

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TECHNIQUES AND RESOURCES

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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 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., 1996; 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; Thissee 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; Thissee and Thissee, 2008; Weizsammann 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; Thissee et al., 2004; Denkers et al., 2004; Clay and Ramakrishnan, 2005; Barroso-Chinea et al., 2007; Acloque et al., 2008; Piette et al., 2008; Thissee and Thissee, 2008; Weizsammann 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; Thissee 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
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 resolved 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.
Fig. 2. See next page for legend.
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)]. 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).

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.

TECHNIQUES AND RESOURCES

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.

Materials and methods

Probes, 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.
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. (mice), J.L.A., and S.E.R. (human); the Gordon and Betty Moore Foundation [GBMF2809]; the Beckman Institute for Advanced Science and Technology; the National Science Foundation Molecular Programming Project [NSF-CCF-0727366]; and the National Institutes of Health (NIH) [5R01EB006192]; the NSF Science and Technology Center for Engineered Biological Systems [NSF-ECC-0501096]; and the California Institute of Technology [0727366]. The authors declare competing financial interests in the form of patents and pending patent applications.

Supplementary information
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