Dynamic visualization of transcription and RNA subcellular localization in zebrafish

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ABSTRACT
Live imaging of transcription and RNA dynamics has been successful in cultured cells and tissues of vertebrates but is challenging to accomplish in vivo. The zebrafish offers important advantages to study these processes – optical transparency during embryogenesis, genetic tractability and rapid development. Therefore, to study transcription and RNA dynamics in an intact vertebrate organism, we have adapted the MS2 RNA-labeling system to zebrafish. By using this binary system to coexpress a fluorescent MS2 bacteriophage coat protein (MCP) and an RNA of interest tagged with multiple copies of the RNA hairpin MS2-binding site (MBS), live-cell imaging of RNA dynamics at single RNA molecule resolution has been achieved in other organisms. Here, using a Gateway-compatible MS2 labeling system, we generated stable transgenic zebrafish lines expressing MCP, validated the MBS-MCP interaction and applied the system to investigate zygotic genome activation (ZGA) and RNA localization in primordial germ cells (PGCs) in zebrafish. Although cleavage stage cells are initially transcriptionally silent, we detect transcription of MS2-tagged transcripts driven by the βactin promoter at ∼3.5 h post-fertilization, consistent with the previously reported ZGA. Furthermore, we show that MS2-tagged nanos3 3′UTR transcripts localize to PGCs, where they are diffusely cytoplasmic and within larger cytoplasmic accumulations reminiscent of those displayed by endogenous nanos3. These tools provide a new avenue for live-cell imaging of RNA molecules in an intact vertebrate. Together with new techniques for targeted genome editing, this system will be a valuable tool to tag and study the dynamics of endogenous RNAs during zebrafish developmental processes.

KEY WORDS: In vivo RNA labeling, Transcription, MS2, Transgenic zebrafish

INTRODUCTION
Given the vital roles coding and noncoding RNAs play in cell biology, it is not surprising that regulatory mechanisms coordinate every step of RNA metabolism, from transcription to translational capacity and, ultimately, degradation (Garneau et al., 2007; Holt and Bullock, 2009). To fully understand RNA regulation as it occurs in vivo requires detection and visualization of RNA in the living organism. Many tools have been used to study RNA dynamics in cell culture (Armitage, 2011; Santangelo et al., 2012); however, they have yet to be widely extended to studies in living vertebrates. One tool, the MS2 RNA-labeling system, based on the high-affinity binding of the bacteriophage MS2 coat protein (MCP) to its RNA hairpin binding site (MBS), has been used in model organisms (Bertrand et al., 1998; Belaya and St Johnston, 2011; Lionnet et al., 2011). By labeling a gene of interest with multiple copies of MBS and coexpressing a fluorescent MCP, live-cell imaging of RNA dynamics with single RNA molecule resolution is possible (Hocine et al., 2013). Tagging the fluorescent MCP with a nuclear localization signal (NLS) primes the MCP for interactions with nascent RNAs in the nucleus and reduces cytoplasmic background fluorescence, allowing enhanced visualization of cytoplasmic transscripts. This method has been used to study Drosophila oogenesis (Belaya and St Johnston, 2011) and embryogenesis (van Gemert et al., 2009), trafficking in Xenopus oocytes (Gagnon et al., 2013) and recently in brain slices of mice (Park et al., 2014) but has not been applied to vertebrate development. We have developed and applied a Gateway-based MS2-MCP system (Hartley et al., 2000; Walhout et al., 2000; Kwan et al., 2007; Villefranc et al., 2007) for the easy generation of expression vectors and stable transgenic zebrafish lines expressing fluorescent MCPs (FP-MCP). We have validated its use in zebrafish and have used it to study the onset of zygotic βactin transcription and nanos3 localization in PGCs. Together with established methods of transgenesis and mutagenesis, these tools should facilitate future studies of RNA regulation in living vertebrates.

RESULTS AND DISCUSSION
Transgenic NLS-4dMCP-GFP lines
We developed a set of Gateway-compatible plasmids to facilitate generation of MCP expression vectors (Fig. 1A). Using these plasmids and Tol2-mediated transgenesis (Kawakami et al., 1998, 2004; Kawakami, 2007), we generated stable transgenic zebrafish lines expressing MCP as a tandem dimer (tdMCP) (Wu et al., 2012) fused to a NLS and eGFP under the control of the ubiquitous βactin (Higashijima et al., 1997) and inducible hsp70 (Halloran et al., 2000) promoters (supplementary material Fig. S1). In all lines, the labeled cells displayed the expected nuclear fluorescence with minimal cytoplasmic background (supplementary material Fig. S1).

Validation of MCP-MBS interaction in zebrafish
To validate and test the feasibility of this system to visualize RNA molecules in zebrafish, we transiently and mosaically expressed cherry RNA tagged with MS2 hairpins using the βactin promoter (βactin:cherry-24xMBS) by injecting DNA into Tg(βactin:NLS-tdMCP-GFP) lines (Fig. 1B). Live imaging of zebrafish embryos revealed tdMCP-GFP cytoplasmic puncta in cells expressing the Cherry protein (Fig. 1C-E), likely representing cherry-24xMBS RNA species, as previously reported in other systems (Bertrand et al., 1998; van Gemert et al., 2009; Lionnet et al., 2011;
Schonberger et al., 2012; Gagnon et al., 2013; Park et al., 2014). Furthermore, time-lapse analysis of these cells revealed highly dynamic cytoplasmic puncta (supplementary material Movies 1 and 2) that were not detected in neighboring cells lacking the Cherry reporter (n=43 cells; 6 embryos). These experiments suggested that MS2-RNA labeling is feasible in zebrafish.

**Visualization of zygotic genome activation**

An advantage of MS2 labeling is the ability to track transcripts throughout their lifetime, because nuclear puncta appear soon after transcriptional activation (Larson et al., 2011; Lionnet et al., 2011; Park et al., 2014). We utilized this property to investigate the time course of zygotic genome activation (ZGA) in zebrafish. The zebrafish genome is quiescent until ∼3 h post-fertilization (hpf) (Giraldez et al., 2006; Schier, 2007; Dalle Nogare et al., 2009; Tadros and Lipshitz, 2009). In addition, time-lapse analysis revealed that these transcriptional events are dynamic, because both appearing and disappearing puncta were detected in uninjected embryos at any time point assayed (supplementary material Fig. S2; n=9 embryos each at 3, 3.5, 4 and 4.5 hpf) nor in embryos injected with the β-actin:cherry-24xMBS control plasmid DNA lacking MBS (Fig. 2C,D; n=13, 13, 11 and 14 at 3, 3.5, 4 and 4.5 hpf, respectively). By contrast, at 3.5 hpf and stages thereafter nuclear puncta were present in subsets of cells in most embryos injected with β-actin:cherry-24xMBS (embryos with nuclear puncta at 3.5 hpf, n=11/11; at 4 hpf, n=9/10; at 4.5 hpf, n=16/17) but not at earlier stages (Fig. 2E,F; 3 hpf embryos with nuclear puncta n=1/17). Furthermore, cherry-24xMBS expressed from a promoter element that is not activated at ZGA yielded no nuclear puncta at 4.5 hpf (supplementary material Fig. S3), further suggesting that the puncta represent transcriptional events. Where nuclear puncta were detected, the number ranged from one to more than ten per nucleus. Because DNA was injected, the number of puncta could reflect the plasmid copy number and the transcriptional activity of the cell. Consistent with this notion, injection of 100 pg of β-actin:cherry-24xMBS DNA yielded more nuclear puncta at 4.5 hpf (supplementary material key Fig. S3), further suggesting that the puncta represent transcriptional events. Where nuclear puncta were detected, the number ranged from one to more than ten per nucleus. Because DNA was injected, the number of puncta could reflect the plasmid copy number and the transcriptional activity of the cell. Consistent with this notion, injection of 100 pg of β-actin:cherry-24xMBS DNA yielded more nuclear puncta at 4.5 hpf (n=36 nuclei; 7 embryos; 9.28 puncta per nucleus) than injection of 25 pg (n=58 nuclei; 8 embryos; 3.12 puncta per nucleus; P=8.14×10^−7). Our results are consistent with previous work suggesting that ZGA occurs at ∼3 hpf (Giraldez et al., 2006; Schier, 2007; Dalle Nogare et al., 2009; Tadros and Lipshitz, 2009). In addition, time-lapse analysis revealed that these transcriptional events are dynamic, because both appearing and disappearing puncta were detected (supplementary material Movie 3). We also captured fluorescence signals exiting the nucleus in cells with nuclear puncta, suggesting that the MBS-MCP complex can be exported from the nucleus (supplementary material Movie 4).

Conventional in vivo promoter reporters generally use a fluorescent protein to readout transcriptional activity. Using the
structures were qualitatively different and easily distinguishable. Genomic translocations that became prominent, predominantly in skin cells (supplementary material Fig. S1). Of note, in uninjected (C-F) Animal pole view of fixed embryos showing that β-actin:cherry-injected control embryos at (C) 3 hpf and (D) 3.5 hpf have no nuclear puncta. Animal pole views of fixed embryos injected with β-actin:cherry-24xMBS at (E) 3 hpf and (F) 3.5 hpf showing that nuclear puncta (arrows) are not detected at 3 hpf but are apparent at 3.5 hpf and beyond. Both injected (C-F) and uninjected (supplementary material Fig. S2) embryos display accumulations of MCP-GFP at cell membranes (arrowheads). Scale bars: 25 μm.

Fig. 2. MS2-labeling reveals the onset of transcription in zebrafish embryos. (A) Immediately after fertilization, the RNA present is exclusively comprised of maternal products. After ZGA, zygotic transcripts begin to accumulate and replace maternal transcripts. (B) Embryos were injected with DNA encoding MS2-tagged-cherry RNA expressed from the β-actin promoter and assayed for nuclear puncta around the time of ZGA (3-4.5 hpf). (C-F) Animal pole view of fixed embryos showing that β-actin:cherry-injected control embryos at (C) 3 hpf and (D) 3.5 hpf have no nuclear puncta. Animal pole views of fixed embryos injected with β-actin:cherry-24xMBS at (E) 3 hpf and (F) 3.5 hpf showing that nuclear puncta (arrows) are not detected at 3 hpf but are apparent at 3.5 hpf and beyond. Both injected (C-F) and uninjected (supplementary material Fig. S2) embryos display accumulations of MCP-GFP at cell membranes (arrowheads). Scale bars: 25 μm.

From the transcriptional puncta observed in cells expressing MBS-tagged RNAs (supplementary material Fig. S3A,B). Combined with advances in targeted mutagenesis and targeted insertions into the zebrafish genome (Chang et al., 2013; Hruscha et al., 2013; Hwang et al., 2013a,b; Auer et al., 2014; Shin et al., 2014), it should be possible to insert MBS tags into endogenous loci to probe endogenous transcription in the future.

Visualization of germ granule-like nanos3 RNA accumulations

To determine whether the MS2 system could recapitulate the localization of an endogenous zebrafish RNA, we fused MS2 tags to the nanos3 3′ UTR (Koprunner et al., 2001). nanos3 is required to maintain PGCs and localizes to them beginning after ZGA (Koprunner et al., 2001; Draper et al., 2007). This localization is achieved through 3′ UTR-mediated stabilization of nanos3 transcripts in PGCs and microRNA (miRNA)-mediated degradation in somatic cells (Koprunner et al., 2001; Giraldez et al., 2006; Mishima et al., 2006). We injected cherry-24xMBS-nanos3 3′ UTR RNA (hereafter called cherryMBSnos3′utr) into Tg(β-actin:NLS-tdMCP-GFP) embryos and analyzed fixed embryos at time points before and after clearance of nanos3 RNA from somatic cells (Fig. 3A). Before complete clearance of the RNA, MCP-GFP was cytoplasmic in cells expressing the Cherry label (Fig. 3D,G), although no cytoplasmic signals were detected in uninjected embryos (Fig. 3B,E). Of note, MCP-GFP foci were also detected at the membrane between adjacent cells (Fig. 3D,G). Similar accumulations were detected in embryos injected with cherry-24xMBS RNA lacking a UTR (Fig. 3C,F), and in uninjected Tg(β-actin:NLS-tdMCP-GFP) embryos, although they were less prominent (supplementary material Fig. S2). Thus, these accumulations represent a background artifact of expressing the NLS-tdMCP-GFP that becomes more prominent upon MBS-tagged RNA expression.

Similar to other fluorescent proteins tagged with the nanos3 3′ UTR, cherryMBSnos3′utr, as indicated by Cherry expression by PGCs alone, was later specifically stabilized in the PGCs (Fig. 3I), reminiscent of endogenous nanos3, indicating that the MBS hairpins did not disrupt 3′ UTR-mediated clearance and stabilization of the RNA (Mishima et al., 2006). In Cherry-positive PGCs, diffuse punctate cytoplasmic MCP-GFP revealed persisting RNA (n=99/122 Cherry-positive PGCs, 21 embryos), which was not visible in somatic cells (Fig. 3H-K). Diffuse cytoplasmic MCP-GFP was never observed in PGCs of uninjected embryos (n=0/101 PGCs, 15 embryos), although one or two small MCP-GFP accumulations were infrequently visible in perinuclear regions (n=19/101 PGCs, 15 embryos), which probably represent nuclear breakdown associated with mitosis, based on nuclear morphology. Similar punctate signals have been observed for nuclear membrane proteins in dividing PGCs in other studies (Strasser et al., 2008).

To test whether cherryMBSnos3′utr localization revealed by the cytoplasmic MCP-GFP resembled that of endogenous nanos3, we used RNAscope technology (Gross-Thebing et al., 2014) to probe for nanos3 RNA. Similar to MS2-tagged RNA, nanos3 was diffusely cytoplasmic in PGCs but not in somatic cells (Fig. 4A,B). We also occasionally observed larger perinuclear accumulations of nanos3 (Fig. 4B). Closer examination of Cherry-positive PGCs from cherryMBSnos3′utr-injected embryos revealed that, in addition to diffuse cytoplasmic MCP-GFP, a fraction of Cherry-positive PGCs displayed perinuclear accumulations of MCP-GFP (Fig. 4C-F). To test whether these accumulations colocalized with endogenous nanos3, we again utilized RNAscope technology.
Gross-Thebing et al., 2014) to examine nanos3 RNA. Importantly, to avoid detecting the injected cherryMBSnos3′utr, this probe specifically recognizes the open-reading frame of nanos3 and not the 3′UTR (Gross-Thebing et al., 2014). In injected embryos, endogenous nanos3 was again cytoplasmic in PGCs with occasional perinuclear accumulations (Fig. 4I). Based on MCP-GFP colocalization with endogenous nanos3 (Fig. 4G-J), we conclude that cherryMBSnos3′utr recapitulates endogenous nanos3 localization in PGCs and that the MCP-GFP fluorescence signal can serve to read out this RNA.

The nanos3 accumulations resembled the localization patterns of other germ cell markers that promote germ cell survival or differentiation and localize to perinuclear germ granules, such as Vasa (Knaut et al., 2000; Hartung et al., 2014) and Dead end (Weidinger et al., 2003; Slanchev et al., 2009). To test whether MCP-GFP colocalized with germ granules, cherryMBSnos3′utr-injected transgenic animals were immunostained for Vasa protein. In Cherry-positive PGCs some cytoplasmic accumulations of MCP-GFP coincided with Vasa-granules and others did not (Fig. 4K-N), which might have functional significance given the role of nanos3 in PGC maintenance (Draper et al., 2007). Moreover, this result suggests that distinct populations of germ granules exist and is consistent with previous findings that germ plasm RNAs differentially accumulate during blastula cleavage stages (Theusch et al., 2006).

Our studies illustrate the feasibility of using MS2 RNA labeling to study transcription and RNA localization in vivo in zebrafish. Given that a growing number of biologically important processes, including the regulation of developmental transitions such as ZGA and MBT, involve regulation by non-coding RNA species such as miRNAs and long non-coding RNAs (lncRNAs) (Giraldez et al., 2006; Pauli et al., 2011, 2012), this system provides the potential to detect such molecules in vivo. Furthermore, with a growing number of targeted genetic manipulation techniques and an increasing wealth of transgenic disease models, these tools should permit future studies of RNA dynamics in development and disease.

MATERIALS AND METHODS

Animals
AB strain wild-type zebrafish embryos were obtained from natural matings and reared according to standard procedures (Westerfield, 2000). Embryos and larvae were raised in 1× Embryo Medium at 28.5°C and were staged...
as described previously (Kimmel et al., 1995). All procedures and experimental protocols were in accordance with NIH guidelines and approved by the Einstein Institutional Animal Care and Use Committee (protocol number 20140502).

Plasmids
All primers are listed in supplementary material Table S1.

MCP expression plasmids
NLS-HA-tdMCP-GFP and NLS-HA-tdMCP-tagRFP were amplified by PCR from phage-UBC-NLS-HA-tdMCP-GFP (Addgene, 40649) (Wu et al., 2012) and phage-UBC-NLS-HA-tdMCP-tagRFP (Wu et al., 2012) using NLS-HA-tdMCP-FP-F+R primers. NLS-tdMCP was amplified by PCR from phage-UBC-NLS-HA-tdMCP-GFP using NLS-tdMCP-F+R primers. To add flanking attL1 and attL2 sites (Hartley et al., 2000; Walhout et al., 2000), PCR fragments were TOPO cloned into pCR8/GW/TOPO (K250020, Invitrogen) to make pME-NLS-tdMCP-eGFP. PCR fragments were TOPO cloned into pCR8/GW/TOPO. To add flanking attL1 and attL2 sites (Hartley et al., 2000; Walhout et al., 2000), PCR fragments were TOPO cloned into pCR8/GW/TOPO (K250020, Invitrogen) to make pME-NLS-tdMCP-eGFP. PCR fragments were TOPO cloned into pCR8/GW/TOPO.

MBS-RNA expression plasmids
pME-24xMBS was created from pCR4-24xMS2-SL (Addgene, 31865), which was digested with EcoRI, to release 24xMBS, which was then ligated into pCR8/GW/TOPO. pME-mCherry-24xMBS was created by amplifying mCherry with flanking BamHI primers. NLS-tdMCP was amplified by PCR from NLS-HA-tdMCP-GFP and NLS-HA-tdMCP-tagRFP were amplified by PCR from MCP expression plasmids. All primers are listed in supplementary material Table S1. Plasmids (protocol number 20140502). experimental protocols were in accordance with NIH guidelines and as described previously (Kimmel et al., 1995). All procedures and experimental protocols were in accordance with NIH guidelines and approved by the Einstein Institutional Animal Care and Use Committee (protocol number 20140502).

MCP stable transgenic lines
To2 Transposase RNA, transcribed from pCS2FA-transposase (Kwan et al., 2007), and pTol-Promoter-NLS-tdMCP-eGFP circular DNA were combined (25 ng/μl each). Embryos were injected with 1 nl of this solution at the one-cell stage. GFP-expressing embryos were selected at 5 dpf and raised to generate founders (supplementary material Methods).

Transient assays
25 ng/μl each of circular pTol-factin-mCherry-24xMBS DNA and To2 Transposase RNA were combined and injected as described above. For live imaging, embryos were scored for mCherry expression and imaged. For the time-course analysis, embryos were fixed in 4% paraformaldehyde every half hour from 3 hpf to 4.5 hpf.

mCherry-24xMBS and mCherry-24xMBS-nanos3 3’ UTR RNAs were transcribed from pCS-mCherry-24xMBS and pCS-mCherry-24xMBS-nanos3 3’ UTR, respectively. Plasmids were linearized with NoI and transcribed with the mMESSAGE mMACHINE T7 transcription kit.
RNAscope in situ hybridization

Zebrafish whole-mount staining using the RNAscope Multiplex Fluorescent Reagent Kit (ACD Bio) was performed as described previously (Gross-Thebing et al., 2014). Briefly, 30 hpf embryos were fixed for 30 min in 4% paraformaldehyde, washed with PBS with 0.1% Tween, and dehydrated in MeOH at −20°C overnight. Embryos were dried for 30 min at room temperature before pretreatment 3. RNAscope Blank Probe C1 and Dr-nanos3-CDs Probe C3 (ACD Bio 431191-C3) were hybridized overnight at 40°C at a 50:1 ratio. Embryos were postfixed with 4% paraformaldehyde and washed with 0.2× SSC before the amplification steps (Ampl1-4). Amp4-Alb was used for the final reaction. Nuclei were stained with DAPI.

Immunostaining

For whole-mount immunofluorescence, embryos were fixed in 4% paraformaldehyde overnight at 4°C and permeabilized in acetone for 12 min. Anti-Vasa antibody (Knaut et al., 2000) was diluted at 1:5000. Alexa Fluor 633-conjugated (Molecular Probes) secondary antibody was used for the Western blot.

Competing interests

The authors declare no competing or financial interests.

Author contributions

P.D.C. performed experiments and analysis, which were conceived and designed by P.D.C. and F.L.M. and manuscript. P.D.C. performed experiments and analysis, which were conceived and designed by P.D.C. and F.L.M. wrote the manuscript.

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Supplementary material

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