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LETTER TO THE EDITOR

Labeling native bacterial RNA in live cells

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Dear Editor,

Labeling RNA molecules in their physiological environment is still a technological challenge that should be overcome to study kinetics, localization and protein interactions of these ubiquitous cellular regulators. The only non-invasive method applicable for detecting unmodified RNAs in live cells described so far used two RNA-binding pumilio proteins directly interacting with RNA that triggered complementation of the split fluorescent protein (FP) fused to pumilio proteins [1, 2]. To use this as a universal method, the pumilio proteins should be subjected to mutagenesis to bind each new target RNA. Protein mutagenesis is a time-consuming process and it limits application of this approach. Another method that used RNA-templated protein complementation has been developed but used for *in vitro* RNA detection only [3]. Therefore, detection of unmodified RNAs *in vivo* remains a daunting problem.

Here, we describe a universal method for labeling unmodified RNAs in live cells based on combination of the split aptamer approach and protein complementation. The principle of this method consists of sequence-specific binding of two RNA probes complementary to two adjacent sites on an unmodified RNA target. Each RNA probe is made of two modules connected by a flexible linker: one module is a sequence complementary to the target; the second is a fragment of the split aptamer. When target RNA is present in the cell, the head-to-tail binding of the two RNA probes to the target brings the two fragments of the split aptamer into close proximity, triggering its reassembly. The reassembled aptamer then initiates the association of two split fusion proteins, each containing a fragment of a split FP and a fragment of an RNA-binding protein. As a result, the two non-fluorescent fragments of FP reassociate and become fluorescent (Figure 1A). In all experiments, we used the split RNA-binding protein eIF4A fused with split EGFP and the eIF4A-specific aptamer, the same system we used in our previous RNA-labeling methods [4-6]. We tested two designs for RNA probes: in one case, the probes were expressed as two separate transcripts, in another they were expressed within one long transcript with an unrelated intervening sequence. We found that the second design significantly reduced the fluorescent background. This effect has been studied in detail (see Supplementary information, Data S1) and explained by the interactions of the split fusion proteins with the RNA probes containing split aptamer sequence (Supplementary information, Figure S1). Apparently, the sequestration of split fusion proteins keeps them apart and decreases their spurious association (Supplementary information, Figure S2). Modeling experiments supported the suggested mechanism (Supplementary information, Figure S3).

To test the feasibility of this approach, we targeted a 22 nt-long accessible site in rabbit β-globin mRNA [7] expressed from a plasmid (Supplementary information, Figure S4). The *E. coli* BL21(DE3) cells were transformed with three compatible plasmids expressing mRNA, RNA probes and fusion proteins. All components of the complex were expressed from the T7 promoters upon simultaneous induction with IPTG (for the details see Supplementary information, Data S1). The results showed that β-globin mRNA or its fragment was detected sequence-specifically in 20% of cells (Supplementary information, Figure S4D). It should be noted that the concentration of this plasmid-expressed transcript was ~75 molecules per cell (Supplementary information, Figure S5), which is at least one order of magnitude higher than the average concentrations of endogenous bacterial mRNAs. Therefore, the endogenous *PstC* mRNA expressed from its natural chromosomal site was chosen as the next target. PstC protein is an integral transmembrane transporter. It is a part of the *pst* operon consisting of 5 genes that mediate translocation of inorganic phosphorus, P_i, through the inner membrane [8, 9]. At normal P_i concentrations, genes from the *pst* operon are expressed at a low level from the internal promoters, but under phosphate shortage transcription is induced and is initiated at a promoter located upstream of the first gene in the operon, *PstS* [8]. Based on Mfold analysis of the secondary structure of *PstC* mRNA, the 5′ end has been chosen as a most accessible binding site (Figure 1B). The probe containing corresponding antisense sequences was transformed into *E. coli* cells together with the plasmid pMB53 expressing fusion proteins, A-F1 and B-F2. The

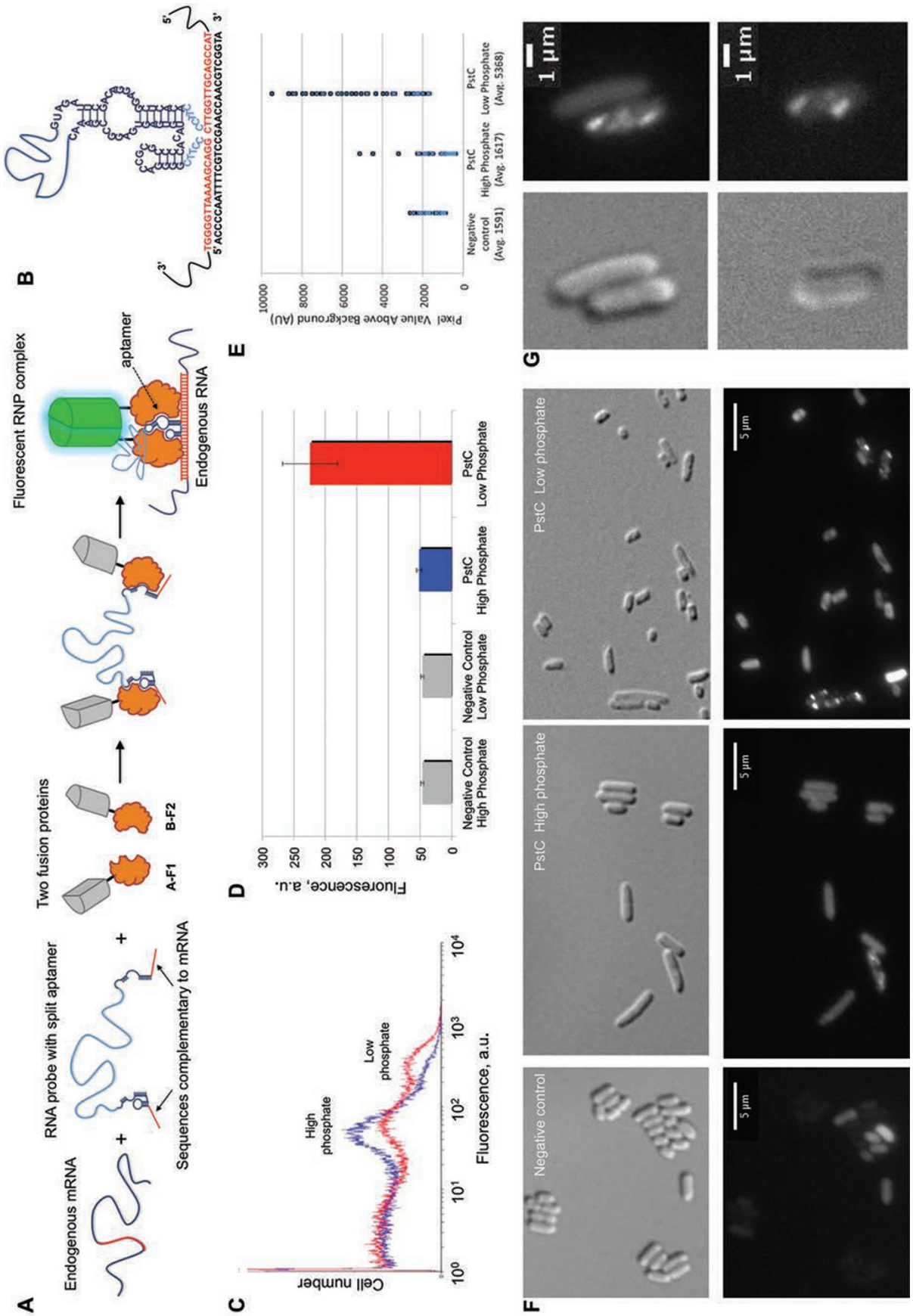
A
Endogenous mRNA
RNA probe with split aptamer
Sequences complementary to mRNA
Two fusion proteins
A-F1
B-F2
Fluorescent RNP complex
aptamer
Endogenous RNA
B
5'
3'
TGGGGTTAAAAGCAGG CTTGGTTGCAGCCAT
5' ACCCCAATTTTCGTCCGAACCAACGTCGGTA 3'
C
Cell number
High phosphate
Low phosphate
Fluorescence, a.u.
D
Fluorescence, a.u.
Negative Control High Phosphate
Negative Control Low Phosphate
PstC High Phosphate
PstC Low Phosphate
E
Pixel Value Above Background (AU)
Negative control (Avg. 1591)
PstC High Phosphate (Avg. 1617)
PstC Low Phosphate (Avg. 5368)
F
Negative control
PstC High phosphate
PstC Low phosphate
5 µm
G
1 µm

Figure 1 Detection and localization of bacterial *PstC* mRNA in live *E. coli* cells. **(A)** Schematics of native RNA recognition by the target-specific RNA probes followed by protein complementation; target RNA hybridizes with RNA probes that have two antisense sequences fused to split aptamer, and this triggers the reassembly of the eIF4A-specific aptamer; aptamer reassembly is followed by reassembly of the eIF4A protein and EGFP; A and B are the fragments of EGFP, F1 and F2 are fragments of the eIF4A protein. **(B)** Structure of the *PstC* mRNA target site and of the eIF4A-specific aptamer split at the central loop; antisense sequences are shown in red, linker sequences are shown in blue, the split site in the aptamer is marked by the curled line. **(C)** FACS analyses of *E. coli* cells with labeled *PstC* mRNA grown in high (blue) and low (red) phosphate media. **(D)** Histograms obtained from the FACS data. Average of 3 independent experiments ± SD is shown. **(E)** Total cell fluorescence obtained from the fluorescent images of single cells using fluorescent microscopy. **(F)** Fluorescent patterns of labeled *PtsC* mRNA in cells in high and low phosphate media. The left panels show cells expressing fusion proteins and the probes antisense to β-globin mRNA (negative control). Scale bar, 5 μm. **(G)** Comparison of *PtsC* mRNA patterns in live (top) and fixed cells (FISH results, bottom). Scale bar, 1 μm.

cells were grown in media with normal concentration of P_i (2.2 mM) or in media with limited phosphate (0.2 mM). Under normal phosphate conditions, cell fluorescence was only marginally higher than the negative controls (cells expressing fusion proteins and the probes to the β-globin mRNA), while under limited phosphate the average fluorescence increased by about 5-fold as compared to cells grown under normal phosphate concentrations (Figure 1D). This increase in fluorescence correlated with the increase in *PstC* mRNA concentration as determined by RT-PCR (Supplementary information, Figure S5B). At the same time, fluorescence of the negative control cells expressing probes against β-globin mRNA was the same in both high and low phosphate media. Quantitative analyses of single cell fluorescence correlated with FACS results (Figure 1D and 1E).

Microscope images revealed single or several punctate fluorescent signals in about 8% of the cells grown in normal phosphate that were not seen in the control cells (Figure 1F). When the cells were grown in low phosphate media, about 15% of cells revealed signal with a population of cells with oversaturated levels of fluorescence. Inspection of these cells with lower exposure time confirmed similar localization of RNA in small focal points (Supplementary information, Figure S6).

To verify *PstC* mRNA localization by an alternative method, we performed fluorescent *in situ* hybridization (FISH) using 38 TAMRA-labeled probes (Stellaris) specific for this mRNA (Figure 1G and Supplementary information, Table S7, Figure S7). FISH images of the cells grown in normal P_i conditions did not show any signal, while FISH images of the cells grown in phosphate-depleted media revealed characteristic signals in about 25% of the cells very similar to the signals obtained in live cells (Figure 1G).

Thus, our results demonstrate that in live cells we detected signal from *PstC* mRNA with the average RNA concentration of ≤ 1 molecule per cell. The fluorescence quenching results show abrupt bleaching of the signals (Supplementary information, Figure S8) supporting our hypothesis on single-molecule sensitivity. At the same time, FISH with Stellaris probes was unable to reveal the signal at low *PstC* mRNA concentration. These results underscore the limitations of hybridization methods that use probes with constitutive fluorescent signal, which require washing steps to reveal the specific signal. These limitations of the pre-labeled probes have motivated the efforts to develop molecular sensors that display signal in the presence of the analyte only [3].

We explain the high sensitivity of the new method by the combination of three factors. First, protein complementation reduces fluorescent background by 10-100-fold as compared to the full-size FPs [4, 10]. Second, the mechanism of split protein sequestration by the RNA probes prevents split protein reassociation in the absence of the target RNA, leading to additional 4-5-fold background reduction. Third, *PstC* mRNA is localized, therefore its fluorescence signal is not spread to the entire cell by diffusion during the image acquisition time and is not overwhelmed by cellular autofluorescence. The fact that *PstC* mRNA localization patterns revealed by FISH and our new method look similar suggests that the labeling complex does not interfere with the normal RNA localization. DNA labeling with Hoechst dye showed that *PstC* mRNA does not co-localize with the bulk DNA in both live and fixed cells (Supplementary information, Figure S9). That provides additional assurance that the live cell imaging reflects proper RNA localization. We should emphasize that our method unlike other approaches does not require any RNA modification and it targets short (25-30 nt) RNA sequences. This makes this method applicable not only for mRNA detection but also for other RNA species including short ncRNAs.

Our experiments revealed distinct localization patterns of the *PstC* mRNA in *E. coli* cells, adding more evidence to the growing data on spatial localization of several bacterial mRNAs [11-13]. Further experiments are in progress to address the mechanism of *PstC* mRNA localization.

To the best of our knowledge, this work is the first example of RNA-templated reassembly of split aptamers *in vivo*, which increases the set of tools for the regulated

manipulation of a signal depending on the presence of a user-defined RNA target.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)