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# Methods for studying RNA localization in bacteria

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ABSTRACT

The subcellular localization of RNA transcripts provides important insights into biological processes. Hence, understanding the mechanisms underlying RNA targeting is a high priority aim of modern cell biology. The advancements in imaging techniques, such as *in situ* hybridization and live-cell imaging, coupled with the evolution in optical microscopy led to the discovery that bacterial RNAs, despite the lack of nucleus, are specifically localized. Here we describe the methods used to study RNA localization in bacteria and their applications and discuss their advantages and limitations.



**Contents**



## 1. Introduction

RNA is one of the most versatile molecules in nature: it serves as the genetic material of retroviruses, it transfers information from DNA to proteins in most living organisms known today, it can act as a regulator of gene expression, it may execute enzymatic functions once attributed only to proteins, etc. Hence, studying the spatial and temporal regulation of RNA is obviously essential for understanding basic cellular processes. However, owing to the short-half life and the unstable nature of RNA molecules, it took longer time to develop tools and methods for monitoring RNA localization than for protein localization. Needless to say that the extremely small size of the bacterial cell made this challenge even trickier in prokaryotes. Nevertheless, there are currently a handful of methods that can effectively track RNA in bacterial cells.

Based on the requirements of the study, the methods for observing RNA within the cell can be divided into two main categories – fixed cell imaging and live cell microscopy. The first method is primarily *in situ* hybridization and it requires that the

*Abbreviations:* RNA, ribonucleic acid; sRNA, small RNA; FISH, fluorescence *in situ* hybridization; DEPC, diethylpyrocarbonate; GFP, green fluorescent protein.

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cells are fixed, which implies that they are dead, but the RNA can be monitored at its native state. On the other hand, in live cell imaging, the cells are not fixed, which allows for monitoring real-time dynamics of RNA, but in most cases additional sequences are added to the RNA. One of the best examples for the latter strategy is aptamer-fluorescent protein approaches. The two categories are described and discussed below.

## 2. *In situ* hybridization

*In situ* hybridization was one of the first approaches adapted for studying RNA localization in bacteria. The cells are fixed and the probes against the RNA target are delivered into the fixed cell. The probes may be of two types: probes directly labeled with fluorophores, in which case the technique is called fluorescence *in situ* hybridization (FISH), and high affinity molecule-labeled probes, which are detected by a secondary antibody attached to a dye, for example digoxigenin-labeled probes. The second type of probes is seldom used these days and the fluorescent ones are the probes of choice, as their use reduces the complexity associated with additional steps required to detect the non-fluorescent probes. There are many standardized protocols available for performing FISH [1].

There are several disadvantages associated with the probes used for *in situ* hybridization. Their delivery into the cell and their nuclease-sensitive nature are major concerns in terms of the overall efficiency; also, once delivered they give non-specific background signal.

To circumvent the problem of the background signal, Sando and Kool [2] took advantage of the phenomenon of quenching. Quenchers are molecules that suppress the fluorescent signal by binding to a fluorescent molecule in a process called quenching. The authors used quenched probes called QUAL to visualize ribosomal RNAs (rRNA) in *Escherichia coli*. To ensure complete quenching, the fluorescein-labeled QUAL probes have dabsyl quencher at their 5′-end, few base pairs from the fluorescein-labeled nucleotides. Another probe, which contains thioate is used to revert the quenching effect. Both probes share complementarity with adjacent sequences in rRNAs and do not give any signal when administered singly. However as a pair, the thioate probe, which hybridized adjacent to the QUAL probe, unquenches the fluorophore thus giving a strong fluorescence. Using this combination of probes, the authors could distinguish between rRNAs from closely related bacteria [2].

Singer and colleagues developed another variant of FISH probes for a technique called single molecule FISH (smFISH). They designed five fluorophore-labeled probes, each about 50 bases long, which hybridize to the same mRNA target, and each fluorophore gives a bright spot [3]. The advantage of this method is that the light emitted by each probe is calibrated, so one can get quantitative molecular information on a single cell by using FISH. The disadvantage of this method is that when one or two fluorophores falls off the RNA target, the target signal and the unbound single fluorophore's fluorescence are undistinguishable.

Because the signals obtained when using a small number of heavily labeled probes are more prone to variability, Tyagi and co-workers modified smFISH by using a large number of short probes, each about 20 bases long, which are complementary to adjacent sequences on the target mRNA. This greatly improved the fluorescent signal coming from the target mRNA, making it highly quantitative [4]. To test their approach, they added 32 tandem repeats, each 80-nucleotide-long, to the 3′ untranslated region (UTR) of the *gfp* gene and expressed it in human cells. They then targeted the coding region with 48 short probes labeled with Alexa 594 (red signal) and each repeat in the 3′ UTR with 4 probes labeled with tetramethyl rhodamine (green signal). They could count the exact number of transcripts and the red and green signals showed higher than 80% colocalization. This encouraged the authors to test the possibility of simultaneously detecting three different mRNAs using probes coupled to spectrally distinct fluorophores, Cy5, Alexa 594 and TMR. Individual spots, which corresponded to the different mRNAs, were visible in the three different fluorescence channels, without any overlap. The efficiency and the sensitivity of this method was tested by Raj et al. and proven successful also in yeast, *Caenorhabditis elegans*, fruit fly and mammalian neurons [4].

The probe design rules set by Raj et al. [4] were followed by several other groups. The first application of this method in bacteria was described by Werner and co-workers. They used this technology to detect small RNA in *Yersinia pestis* [5]. However, the short length of sRNAs decreased the efficiency of their detection compared to mRNAs, since only a limited number of fluorescent probes can hybridize simultaneously to a single sRNA target, whose length can vary between tens to hundreds of nucleotides. This could be improved by increasing the concentration of delivered probes, but this also increased the background signal. To solve the high background problem, the authors used complementary quencher probes that nullified the signal from unbound and non-specifically bound probes, but not from the strongly hybridized, specifically bound probes.

Golding and co-workers used smFISH to measure the transcript copy number in *E. coli*. They developed a protocol that uses the fluorescence intensity as a measure to calculate the copy number of a target mRNA in a cell population, instead of counting the bright spots of mRNA as done in most other protocols which neglects the fluorescence signal yielded by overlapping or closely spaced mRNAs. Another modification introduced by these authors is that the entire procedure, from cell fixation to hybridization, is performed in a test tube to maintain uniform conditions and to avoid the cell-to-cell heterogeneity created on microscopic slides [6].

Several studies employed the various FISH methods described above to study RNA localization in bacteria. Russell and Keiler observed the localization pattern of the tmRNA *ssrA*, which paralleled with the helix-like localization of the tmRNA-binding protein SmpB [7], and Jacobs-Wagner and co-workers used FISH to look at several mRNAs in *Caulobacter crescentus* and one in *E. coli*, all of which were found in the vicinity of their transcription site [8]. Using FISH, Kuhlman and Cox observed that the distribution of both the *lacI* mRNA and LacI protein depends on the position of the encoding gene on the chromosome and correlates with the spatial distributions of the source genes [9].

## 3. Live cell imaging

The numerous variations of the FISH methodology, although powerful, are inadequate for studying real time dynamics of RNAs, since they do not have a temporal resolution component. Live cell imaging techniques not only provide a platform to study the kinetics associated with the spatial distribution of RNA, but they also avoid most of the drawbacks associated with the use of oligonucleotide probes in hybridization techniques. Also, most live imaging techniques do not involve the addition of any exogenous reagents to the cells and, thus, the fluorescent proteins, the probes, and the RNAs are expressed under native physiological conditions. Hence, live cell imaging can enable researchers to follow each and every stage in the life cycle of RNA molecules, from their synthesis en route their degradation, in real time. Below and in Fig. 1 we describe the methods used to observe RNA in live bacterial cells.

### 3.1. The MS2–GFP system

The coat protein of bacteriophage MS2 tightly binds to an MS2-encoded RNA to form the viral capsid structure. Singer and co-