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# Imaging single mRNAs to study dynamics of mRNA export in the yeast *Saccharomyces cerevisiae*

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ABSTRACT

Regulation of mRNA and protein expression occurs at many levels, initiated at transcription and followed by mRNA processing, export, localization, translation and mRNA degradation. The ability to study mRNAs in living cells has become a critical tool to study and analyze how the various steps of the gene expression pathway are carried out. Here we describe a detailed protocol for real time fluorescent RNA imaging using the PP7 bacteriophage coat protein, which allows mRNA detection with high spatial and temporal resolution in the yeast *Saccharomyces cerevisiae*, and can be applied to study various stages of mRNA metabolism. We describe the different parameters required for quantitative single molecule imaging in yeast, including strategies for genomic integration, expression of a PP7 coat protein GFP fusion protein, microscope setup and analysis strategies. We illustrate the method's use by analyzing the behavior of nuclear mRNA in yeast and the role of the nuclear basket in mRNA export.



## 1. Introduction

Translating the genetic information from DNA to proteins requires the synthesis of a messenger RNA molecule, the mRNA. Eukaryotic cells have separated the site of protein production and transcription by storing DNA in a separate cellular organelle, the nucleus, requiring mRNAs to transit to the cytoplasm to meet with ribosomes for translation. Nucleocytoplasmic exchange is mediated by nuclear pore complex (NPC), a large multiprotein complex embedded in the nuclear membrane [1]. To pass through nuclear pores, mRNA interacts with specific transport receptors through association with adaptor proteins that mediate the interaction and translocation through the NPC [2].

After transcription by RNA polymerase II and assembly into RNA–protein complexes (mRNPs), mRNPs are released from the DNA template into the nucleoplasm. Various studies have shown that mRNAs show diffusional behavior within the nucleoplasm and no directed transport towards the periphery has been documented [3–6]. mRNP movement is, however, influenced by the chromatin environment and restricted movement of mRNAs in chromatin dense regions have been observed in higher eukaryotes [3,6]. Thus, the time required for mRNAs to reach the nuclear periphery is influenced by the size of the nucleus, chromatin environment as well as by the position of the gene it is transcribed from. In yeast, a number of genes are transcribed at the nuclear periphery, however, whether gene targeting to the nuclear periphery stimulates mRNA export, serves other regulatory processes such as transcription regulation, or both, is not yet fully understood (reviewed in [7,8]).

To ensure rapid and efficient protein expression, it seems reasonable for cells to optimize the kinetics of the different steps leading the nucleocytoplasmic export. This includes facilitating mRNA association with NPCs upon reaching the periphery, and ensuring that such association leads to translocation to the cytoplasm. In higher eukaryotes, the release of mRNPs from the nuclear periphery back into the nucleoplasm is likely to significantly prolong the time it takes for an mRNA to reach the cytoplasm, as the diffusive behavior of mRNAs in the nucleoplasm will result in a delay for the mRNA to re-associate with the periphery and thus the potential for subsequent export. Stabilizing interactions with the periphery therefore facilitates export and studies in yeast as well as in higher eukaryotes have shown that mRNPs frequently show a scanning behavior a the nuclear periphery prior to export [4,9]. In budding yeast, components of the NPC and factors associated with mRNAs are required for this process [9]. In particular, the myosin like protein Mlp1, a structural component of the nuclear

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basket that extents from the central scaffold of the NPC into the nucleoplasm, is required for perinuclear mRNA scanning in budding yeast. In this book chapter, we describe the experimental setup and image analysis methods used to show that the nuclear basket is implicated in maintaining mRNPs at the periphery by providing an interaction platform for mRNPs at the NPC, possibly allowing mRNP arrangement required for export to occur before mRNA enter the NPC for translocation. These examples illustrate how single molecule resolution imaging using the PP7 system in combination with sub diffraction resolution particle tracking is used to study mRNA export in budding yeast.

Budding yeast has been used extensively to study mRNA export. Genetic, biochemical and microscopy based approaches have identified many factors involved in the different steps of the mRNA export pathway, including components of the NPC, RNA binding proteins and the export receptor Mex67 [1]. With most players identified, a next step towards a better mechanistic understanding of mRNA export is to study how the different factors affect specific steps, such as the docking of mRNPs to the NPC or the translocation process. These processes reflect dynamic interactions and are difficult to study using biochemical and genetic approaches. Studying such dynamic processes therefore requires the ability to visualize individual mRNA in high spatial and temporal resolution in living cells. Such approaches can further be combined with yeast genetic approaches, such as the use of a large number of mutant yeast strains affecting mRNA export, making yeast an powerful system to study this complex process.

Different methods have been developed allowing mRNA detection in cells, including *fluorescent in situ hybridization* (*FISH*), molecular beacons, labeling of RNA binding proteins and various aptamer based techniques (reviewed in [10]). A subset of these techniques have the sensitivity to detect single RNA molecules, and only a few allow single mRNA detection in real time in living cells. Some of these approaches require the injection of labeled proteins into cells or use plasmid based expression systems to introduce reporter constructs for single molecules studies. Quantitative single molecule studies, however, are ideally performed studying mRNA and proteins expressed at endogenous levels, reducing the risk of altered mRNA behavior due to overexpression or altered expression patterns caused by exogenous expression.

The yeast *Saccharomyces cerevisiae* provides a powerful experimental system to study gene expression from a single molecule perspective. Targeted genomic integration using homologous recombination allows expression of tagged versions of endogenous RNAs and proteins in their proper genomic context [11,12]. Furthermore, many mRNAs in yeast are expressed at levels of only a few copies per cell, facilitating the study of individual molecules [13]. However, from a microscopy perspective, yeast does also have disadvantages. The presence of a thick cell wall in yeast introduces significant light scattering affecting signal-to-noise ratio. Single molecule resolution imaging in yeast therefore requires microscope setups optimized for visualizing low intensity mRNA signals.

Aptamer-based mRNA labeling, where RNA-stem loop sequences recognized by fluorescent protein fused RNA binding proteins are introduced into an mRNA of interest, is one of the most frequently used techniques for *in vivo* RNA detection [14]. In this chapter, we describe the use of an RNA labeling strategy that uses the addition of binding sites for the bacteriophage PP7 coat protein to a selected RNA in order to visualize single mRNA molecules in high spatial and temporal resolution in yeast. We will illustrate the use of the approach by studying the behavior of nuclear mRNAs prior to their export to the cytoplasm.

## 2. Overview of the method

The use of bacteriophage coat proteins to label mRNAs was initially developed in the Singer laboratory showing that insertion of binding sites for the MS2 coat-protein in the 3′ untranslated region (UTR) of the *ASH1* mRNA allowed visualization of the targeting of the *ASH1* mRNA to the bud tip in dividing yeast cells [15]. Different RNA binding proteins have since been used to label mRNAs, including lambda N, U1A, PP7 coat protein and others, which enabled the study of RNA dynamics in different organisms [16–20]. The PP7 coat protein (PCP) has recently been introduced as an RNA imaging tool to study the dynamics and complexity of different molecular mechanism of mRNA metabolism. Becoming established as a widely used tool to study RNA dynamics, it is often used in combination with the MS2 system, allowing for multiplex mRNA visualization in livings cells, as demonstrated in budding yeast, Drosophila and mammalian cells [9,12,21–26].

The PCP, like the MS2 coat protein (MCP), is derived from a single-stranded (ss) RNA bacteriophage. Although sharing only 15% sequence identity with MCP, PCP has a very similar structure than MCP, and both proteins bind to an RNA stem-loop as a homodimer, thus doubling the number of labels that are added per binding site introduced to an RNA of interest [27]. Comparing MCP and PCP ability to bind to RNAs in mammalian cells *in vivo* showed that MCP forms a weaker dimer than PCP. Furthermore, these *in vivo* studies showed that not all MS2 stem-loop get bound by MCP–GFP in cells, however, all binding sites for PP7–GFP were occupied on a PP7 stem-loop containing RNA, concluding that the PP7 system performs better for RNA labeling than the MS2 system [28]. Comparing the stability of 24 MS2 and PP7 stem-loops integrated into the yeast genome showed that PP7 stem-loops are less frequently lost through recombination than MS2 stem-loops, further suggesting that the PP7 system is easier to use than the MS2 system [12]. However, it should be noted that loss of stem-loops through recombination can be minimized by modifying the nucleotide sequences to make the stem-loops nonrepetitive [29]. Wu and co-workers further showed that dimer formation and consequently signal-to-noise ratio can be increased by expression of MCP or PCP as single chain tandem dimers [28]. Furthermore, an almost background free variation of the MS2 and PP7 system has been described where a chimeric RNA containing alternating MCP and PCP binding sites and split versions of GFPs fused to MCP and PCP were constructed [30]. However, complement fluorescent protein formation is slow and it needs to be seen if this system is useful in yeast where most RNAs have short half-lives. Furthermore, all direct comparisons of the MS2 and PP7 RNA labeling system that tested the ability to detect RNA have been performed in mammalian cells. Therefore, it has yet to be shown whether there is a significant difference between MS2 and PP7 for their ability to detect RNAs in yeast.

To visualize RNA *in vivo*, insertion of a single binding site for PCP does not allow detection of individual RNAs, and binding sites for PCP have to be multimerized to enable visualization. Early studies have used 24 stem-loops to allow single molecule detection, however, more recent studies showed that fewer copies are sufficient [9,22,26]. The number of stem loops will determine the fluorescent intensity of RNA signals, and insertion of fewer loops decreases the signal-to-noise ratio. This requires either more sensitive detection tools or longer exposure times during image acquisition, which in turn can limit the ability to detect fast diffusing RNA molecules. Furthermore, adding multiple RNA stem-loops, each bound to a dimer of PCP–GFP, adds significant size and mass to an mRNP, but it has not yet been systematically determined whether increasing the number of stem-loops affects RNA