
CHAPTER 12

Using Fluorescent Proteins to Study mRNA Trafficking in Living Cells

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Abstract

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Abstract

This chapter presents the MS2-GFP system, a method to study the trafficking of RNA molecules in living cells. This system is based on two components: a fusion of the MS2 coat protein to a fluorescent protein and a reporter mRNA containing multimers of the RNA stem-loop recognized by the MS2 coat protein. The MS2-GFP protein bound to the RNA stem-loops acts as a beacon that allows the detection of this mRNA within a cell by epifluorescence or confocal microscopy. This chapter focuses on the use of this system in mammalian fibroblast cells and in

yeast cells, and discusses several technical considerations of the MS2-GFP system. Detailed protocols for validating the MS2-GFP signal in fixed cells by fluorescent *in situ* hybridization of the target RNA using fluorophore-labeled oligonucleotide probes are also provided.

I. Introduction

The “RNA World” theory of evolution posits that life originated from RNA, a molecule that can be both a gene and an enzyme. The past few years have certainly seen tremendous excitement in the RNA world of research, with the discovery of a new class of small noncoding RNAs that play a regulatory role in mRNA translation and transcription. The study of RNA trafficking, the movement and intracellular localization of RNAs, has revealed another level of regulation of gene expression. Splicing and mRNA nuclear export are tightly coupled and regulated processes (reviewed in [Cullen, 2003](#)). Once in the cytoplasm, mRNA transport and local translation have now been documented in vertebrates, invertebrates, and unicellular organisms (reviewed in [Kindler *et al.*, 2005](#)). Polarized cells in particular localize RNAs to locally translate specific proteins, which restricts their distribution to a particular subregion of the cytoplasm. This mechanism of protein sorting is involved in major biological processes such as asymmetric cell division, oogenesis, cellular motility, and synapse formation. Complex machineries are also involved in the transport of noncoding RNAs, like snRNAs, snoRNAs, and rRNAs (reviewed in [Cullen, 2003](#)). To properly study RNA trafficking, new methods have been developed to visualize RNA in living cells, and this chapter presents one such method, the MS2-GFP system. The first part of this chapter introduces general considerations and the use of this system in mammalian cells, while the second part focuses on its utilization in the budding yeast.

II. The MS2-GFP System

The coat protein from the MS2 bacteriophage binds with high specificity to an RNA stem-loop structure of 19 nucleotides containing the initiation codon of the phage replicase gene ([Bernardi and Spahr, 1972](#)). By binding to a unique site in the RNA genome of the phage, the coat protein represses translation of the RNA replicase gene and also guides packaging into phage particles. Over the years, the MS2 coat protein has been engineered so that it can be fused to any protein and tethered to any RNA containing the MS2 stem-loop motif. This approach is notably at the core of the yeast three-hybrid system, which has been developed to study RNA–protein interactions ([Jaeger *et al.*, 2003](#)).

The MS2-GFP system involves the expression of two constructs: a fusion of the MS2 coat protein to the green fluorescent protein (GFP) or any fluorescent

protein, and a multimer of the MS2 stem-loop sequence in the mRNA to be tracked. When the MS2-GFP protein binds to the RNA stem-loops, it acts as a beacon that allows the detection of this mRNA within a cell by microscopy. The MS2-GFP protein is highly specific for RNAs containing MS2 stem-loops in bacteria, plant, and higher eukaryote cells (Bertrand *et al.*, 1998; Fusco *et al.*, 2003; Golding *et al.*, 2005; Rook *et al.*, 2000; Zhang and Simon, 2003). The advantage of using this system over the expression of a GFP-tagged endogenous RNA-binding protein is that MS2-GFP is specific to the RNA containing MS2 stem-loops, while the endogenous RNA-binding protein may bind several mRNAs and reflect the behavior of all of them. Therefore, this system offers two benefits: the specific detection of MS2-tagged mRNAs by standard epifluorescence microscopy and the study of RNA dynamics in living cells.

A. Designing the MS2 Fluorescent Protein Fusion

The MS2 phage coat protein has been studied extensively over the past 30 years and its structure, binding specificity, and affinity are well known. Once bound to the phage RNA, the native MS2 coat protein multimerizes to form the phage capsid. Several mutations to the wild-type MS2 protein sequence have been created in order to decrease multimer formation and enhance specificity and avidity of binding to the MS2 RNA stem-loop. Mutant versions that inhibit multimer formation should preferentially be used in order to avoid the aggregation of the MS2-GFP proteins in the cell. Among these mutations, the dIFG mutation, described in Fig. 1, is a deletion in the 15-amino acid FG loop that prevents the MS2 coat protein dimers from assembling into a phage capsid (Peabody and Ely, 1992). The FG loop is involved in the interdimer interactions that permit capsid assembly. Another mutation in the FG loop, W82R, can be used, which also inhibits capsid assembly (Peabody and Ely, 1992; Rook *et al.*, 2000). The replacement of valine at position 29 with isoleucine (V29I) results in tighter binding of the MS2 dimer to the RNA stem-loop (Peabody and Ely, 1992). The V29I-dIFG double mutant illustrated in Fig. 1 decreases the K_d of the RNA-protein interaction to 40 nM *in vitro* (Lim and Peabody, 1994). The formation of an MS2-MS2 protein dimer is essential for binding to the RNA stem-loop (Valegard *et al.*, 1994), and mutations that disrupt dimer formation should be avoided.

Expression of very high levels of MS2-GFP can cause many problems when using this system, by making it nearly impossible to discriminate between signals coming from the unbound versus the RNA-bound MS2-GFP protein. To eliminate this problem, a nuclear localization signal (NLS) can be added to the fusion protein which will result in the nuclear accumulation of the MS2-GFP protein. To accumulate in the cytoplasm, the MS2-GFP protein must be exported from the nucleus via its interaction with an mRNA containing MS2 stem-loops (Fusco *et al.*, 2003). In the absence of a target transcript, the MS2-GFP protein stays in the nucleus. This approach has been very useful to visualize single RNA molecules in the cytoplasm, as demonstrated in the study by Fusco *et al.* (2003).

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ATG GCT TCT AAC TTT ACT CAG TTC GTT CTC GTC GAC AAT
M  A  S  N  F  T  Q  F  V  L  V  D  N

GGC GGA ACT GGC GAC GTG ACT GTC GCC CCA AGC AAC TTC
G  G  T  G  D  V  T  V  A  P  S  N  F

GCT AAC GGG ATC GCT GAA TGG ATC AGC TCT AAC TCG CGT
A  N  G  I  A  E  W  I  S  S  N  S  R

TCA CAG GCT TAC AAA GTA ACC TGT AGC GTT CGT CAG AGC
S  Q  A  Y  K  V  T  C  S  V  R  Q  S

TCT GCG CAG AAT CGC AAA TAC ACC ATC AAA GTC GAG GTG
S  A  Q  N  R  K  Y  T  I  K  V  E  V

CCT AAA|GGC GCC TGG CGT TCG TAC TTA AAT ATG GAA CTA
P  K  |G  A  W  R  S  Y  L  N  M  E  L

ACC ATT CCA ATT TTC GCC ACG AAT TCC GAC TGC GAG CTT
T  I  P  I  F  A  T  N  S  D  C  E  L

ATT GTT AAG GCA ATG CAA GGT CTC CTA AAA GAT GGA AAC
I  V  K  A  M  Q  G  L  L  K  D  G  N

CCG ATT CCC TCA GCA ATC GCA GCA AAC TCC GGC ATC TAC
P  I  P  S  A  I  A  A  N  S  G  I  Y

GCG GAT CC
A  D

```

Fig. 1 Sequence of the V29I dIFG mutant MS2 protein used for this study. The V29I mutated amino acid is highlighted in bold, and the site of the FG loop deletion is indicated by a vertical line. A linker sequence added at the C-terminus of the protein that includes a BamHI site is shown in gray.

For the visualization of transcripts in the nucleus, it is of course preferable to not include an NLS ([Shav-Tal et al., 2004](#)). The MS2-GFP protein will be distributed throughout the cell without accumulating in any compartment (see [Fig. 3B](#)). Different approaches can be used to carefully time and dose the expression of MS2-GFP so that the nuclear MS2-tagged mRNA can be visualized successfully, and some of these approaches are described in [Section III.A](#) on mammalian expression systems.

An ingenious new method to eliminate background from the unbound MS2-GFP fusion protein was recently described in the laboratory of Chris Brown, who used split Venus yellow fluorescent protein (YFP) fusions ([Rackham and Brown, 2004](#)). In their system, each half of the split Venus fluorescent protein was fused to a different RNA-binding protein (MS2 and either IMP1 or FMRP), and

coexpressed with a reporter mRNA containing an MS2 stem-loop and the IMP1- or FMRP-binding motif in proximity. The recruitment of both MS2-Venus and IMP1- or FMRP-Venus proteins on the reporter mRNA brings the two portions of the split Venus in proximity and result in a functional fluorescent protein. This system has a very low background of fluorescence in the absence of the reporter mRNA and is very specific since it requires the simultaneous binding of both fusion proteins in order to produce fluorescence.

B. Designing the Reporter mRNA

The MS2 RNA hairpin is composed of 19 nucleotides; a seven-base-paired stem containing a bulged adenine and a four-nucleotide loop. When the uracil at position -5 of the loop is replaced by a cytosine (illustrated in bold in Fig. 2), it results in a 15-fold increase in binding affinity of the MS2 coat protein (Talbot *et al.*, 1990; Valegard *et al.*, 1994). This C-variant mutant was thus used for the MS2-GFP system. Figure 2 shows the sequence of two MS2 stem-loops separated by a linker containing common restriction enzyme sites designed to express a convenient MS2 stem-loop tandem array (from the plasmid pSL-MS2-6; Bertrand *et al.*, 1998). Multimerization of the tandem MS2 stem-loops can be achieved using the compatible BamHI/BglII sites.

The location of the MS2 stem-loop repeats in the RNA must be considered. If the stem-loop cassette is placed in the 5'untranslated region (UTR) of the transcript, it gives the advantage that the MS2-GFP protein may locate the site of transcription of the reporter transcript on the genomic DNA, as shown in Fig. 4. However, the possibility of interference with translation needs to be considered if the stable MS2 stem-loop repeats are placed in the 5'UTR or coding sequence of the message. If the MS2 RNA repeats are placed in the 3'UTR of the transcript, as is commonly done, the GFP signal will correlate with the mature free mRNA.

The need to place multiple stem-loops in the transcript may limit which kind of RNA one can study with the MS2-GFP system. Visualization of snoRNAs with this system was recently achieved in yeast (Verheggen *et al.*, 2001), and this may

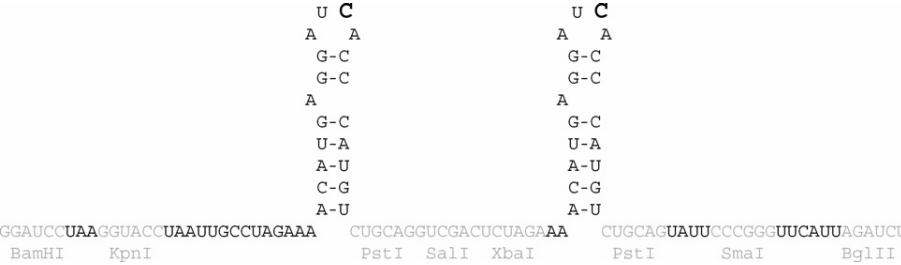


Fig. 2 Sequence of the C-variant MS2 stem-loop tandem array used for this study. The mutated cytidine residue is shown in bold.

represent the smallest sized RNA to be detectable. Cassettes of either 1 (Zhang and Simon, 2003), 2 (Verheggen *et al.*, 2001), 6 (Bertrand *et al.*, 1998), 8 (Rook *et al.*, 2000), 12 (Fusco *et al.*, 2003), 24 (Janicki *et al.*, 2004), or 96 (Golding *et al.*, 2005) MS2 stem-loops have been used successfully in the MS2-GFP system, depending on the organism and the level of expression of the RNA to be visualized. In general, increasing the number of binding sites in the RNA augments signal strength due to a greater number of fluorescent domains bound per RNA molecule. However, one needs 24 MS2 repeats in order to detect single RNA molecules (Fusco *et al.*, 2003). An obvious drawback of the MS2-GFP system is that the need to put stem-loop repeats in the transcript means one cannot study an endogenous mRNA. On a technical note, the MS2 stem-loops are repetitive sequences in a plasmid and care must be taken to avoid deletions and recombination during propagation in bacteria. We have used the HB101 strain grown at 30 °C to successfully amplify plasmids with a low frequency of recombination events.

III. RNA Trafficking in Fibroblasts

A. Mammalian Expression Systems

The protocols discussed in this section can be applied to many cell types, but they have mainly been optimized for common tissue culture cells, hence referred to as fibroblasts for brevity. To use the MS2-GFP system in fibroblast cells, one of the main concerns is the level of expression of the MS2-GFP protein one will obtain. Transient transfection will result in high levels of expression from plasmids that are not integrated into the cell genome. Adapting the promoter and the timing of expression is thus a crucial optimization step. One approach is to use a promoter with a lower level of expression for the MS2-GFP protein and a high expressing promoter for the mRNA construct. One can then do an optimization step by cotransfecting both vectors, and can select vector amount ratios that will result in the desired expression level. Rackham and Brown (2004) transiently transfected COS-7 cells with four times the amount of RNA reporter compared to the MS2 fluorescent protein expression plasmids. Fusco *et al.* (2003) also cotransfected all the necessary expression plasmids into COS-7 cells for their experiments.

When experimental goals include the detection of nuclear RNA, a low level of expression of MS2 fluorescent protein such as that seen in Fig. 3B and C is even more critical. Janicki *et al.* (2004) electroporated an MS2-YFP expressing plasmid into U2OS cells and were able to detect appropriate low levels of fluorescent proteins 2.5 h later. Additionally, for nuclear RNA tracking, the stable integration of the MS2-tagged RNA expression construct into chromosomal DNA is advisable (Janicki *et al.*, 2004; Shav-Tal *et al.*, 2004). Retroviral vectors offer a quick and convenient method for stable integration into mammalian cells that can be maintained with selectable markers.

The experiment presented here was designed to visualize the nuclear accumulation of mRNA containing the mutant 3'UTR of dystrophin myotonia protein

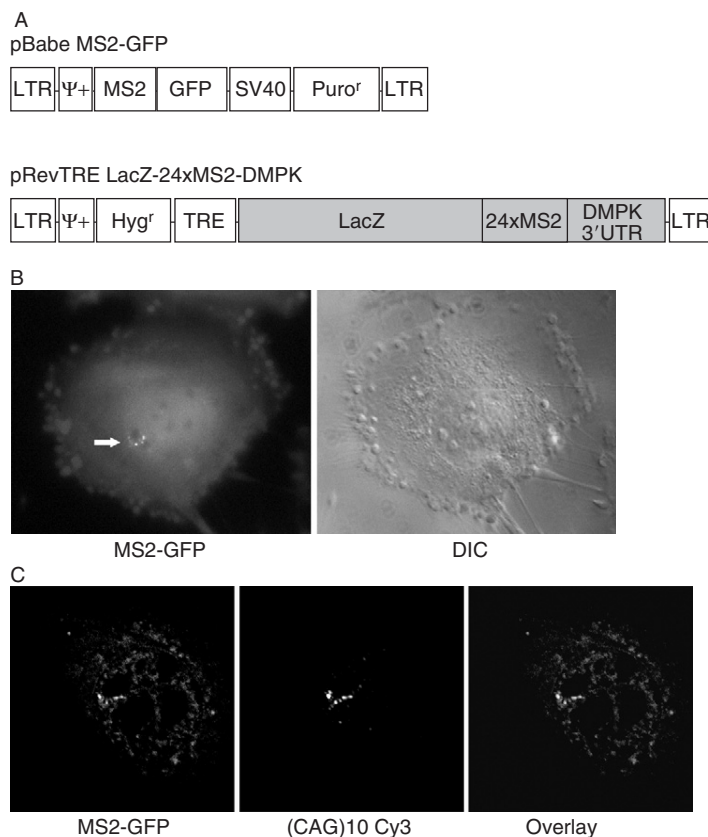


Fig. 3 Application of the MS2-GFP system in myoblast cells to study myotonic dystrophy. (A) Schematic representation of the two retroviral vectors used. (B) GFP-tagged LacZ-MS2-DMPK mRNA foci in living cells. Arrow points to nuclear foci. (C) Colocalization of the MS2-GFP signal with FISH on the LacZ-MS2-DMPK mRNA (Cy3 CAG probe).

kinase (DMPK) with expanded CUG repeats as a model system to study type 1 myotonic dystrophy. While the DMPK 3'UTR normally contains 5–37 CUG repeats, patients with an expanded repeat allele may express several thousand CUG repeats (reviewed in Machuca-Tzili *et al.*, 2005). In Fig. 3A, a schematic representation of the two retroviral vectors used for this experiment is shown. C2C12 myoblast clones stably expressing the desired level of LacZ-24× MS2-DMPK 3'UTR mRNA were derived by hygromycin selection. In the pRevTRE retroviral vector (BD Biosciences Clontech, Palo Alto, CA), the MS2-tagged mRNA is expressed from the strongly inducible tetracycline-responsive element promoter. The MS2-GFP cDNA was cloned into pBabe puro, with the low expressing long terminal repeat promoter. Myoblasts were infected only once with diluted retroviral supernatant, to promote single integration events. The cells

were visualized as soon as 24 h postinfection with pBabe MS2-GFP retrovirus, in a Focht Live-Cell Chamber System 2 (FCS2) (Bioptechs, Inc., Butler, PA). A single time-point image is shown in Fig. 3B, both in the differential interference contrast and in the GFP channel, showing a cluster of nuclear RNA foci that were clearly visible in the focal plane shown.

B. Fluorescent *In Situ* Hybridization on Fibroblasts

Because there is the possibility of unbound MS2-GFP protein being visualized in this system, it is important to confirm that the GFP signal one is tracking actually colocalizes with the MS2-tagged reporter mRNA. To do this, one should perform fluorescent *in situ* hybridization (FISH) as part of the experimental setup. In Fig. 3C, C2C12 myoblasts expressing the mRNA reporter with expanded CUG repeats, as in Fig. 3B, were fixed, and FISH was performed with a Cy3-labeled DNA probe consisting of 10 CAG repeats. This allowed us to verify the colocalization of the MS2-GFP foci that we observed with CUG repeat nuclear mRNA.

The following protocol is modified from Chartrand *et al.* (2000) and allows the simultaneous detection of a specific mRNA and the GFP signal. Paraformaldehyde fixation does not affect the fluorescence intensity or the distribution of the MS2-GFP fusion protein. When designing FISH probes to detect a nonrepetitive sequence in an mRNA, one may need to combine four to seven probes specific for different regions of the mRNA, in order to achieve sufficient fluorescence intensity. Probes consist of oligonucleotides of 50 nucleotides in length containing four to five amino-modified C6 thymidine residues. Labeling the probes with fluorophores, like Cy3, occurs on the amino-modified C6-dT residues. To image LacZ transcripts (as shown in Fig. 4C), we use a mix of five oligonucleotides spread across the coding sequence. The sequences of the LacZ probes can be found in the study by Long *et al.* (1995). For the detection of the transcript containing MS2 stem-loops by FISH, a probe that targets the MS2 stem-loops present in the reporter mRNA can be used (Fusco *et al.*, 2003). It has the advantage that it can be used to detect any MS2-tagged RNA.

MS2 probe: 5' AT*GTCGACCTGCAGACAT*GGGTGATCCTCAT*GTTT TCTAGGCAATT*A 3'

1. Labeling Amino-Modified Oligonucleotides Probes with Cy3

Important: All solutions for fixation and *in situ* hybridization should be diethyl pyrocarbonate (DEPC) treated or prepared with DEPC-treated distilled water, and gloves should be worn at all times to avoid RNase contamination.

1. Dissolve 10 µg of oligonucleotide in 35 µl of freshly made sodium carbonate buffer pH 8.8.
2. Dissolve the contents of one foil pack of Cy3 fluorophore (Amersham Biosciences, United Kingdom) in 30 µl of ddH₂O. See package insert for additional details (kit no. PA23001). Ambient light in the room should be dimmed during manipulation of Cy3 dye to minimize bleaching.

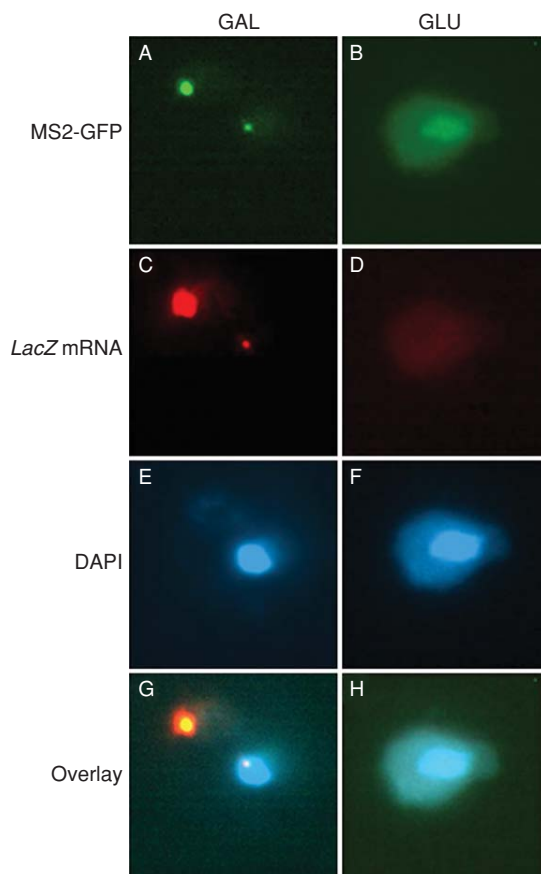


Fig. 4 Visualization of a specific mRNA in the yeast nucleus using the MS2-GFP system. Yeast cells expressing an MS2-GFP protein without nuclear localization signal (NLS) were transformed with a plasmid containing the *LacZ* gene with 24 copies of the MS2 RNA motif in its 5'UTR. The gene also contained an *ASH1* bud-localization zipcode. GAL: galactose induction of the single *LacZ-MS2* gene (panels A, C, E, and G). GLU: glucose repression of the *LacZ-MS2* gene (panels B, D, F, and H). MS2-GFP: Signal from the MS2-GFP protein bound to the *LacZ-MS2* mRNA (panel A) or free (panel B). *LacZ* mRNA: FISH signal from *LacZ*-specific probes. DAPI: Chromosomal DNA marker. Overlay: Overlap between MS2-GFP, *LacZ* probes, and DAPI signals. These images are of formaldehyde-fixed yeast cells.

3. Add 15 μ l of monoreactive dye to the oligo (we use one foil pack to label two probes). Incubate for 24–36 h in the dark at room temperature, mixing vigorously at regular intervals.
4. Remove unincorporated dye by passing the dye/oligo on a commercial (RNase free) Sephadex G-25 or G-50 column.

5. Determine the labeling efficiency (dye/oligo ratio):
 - a. Dilute the labeled oligonucleotide and measure OD₂₆₀ and OD₅₅₂.
 - b. Calculate the total molar extinction coefficient (MEC) of the oligonucleotide. The individual base values are adenosine 15,400 Mol⁻¹ cm⁻¹, cytidine 7400 Mol⁻¹ cm⁻¹, guanine 11,500 Mol⁻¹ cm⁻¹, and uracil/thymidine 8700 Mol⁻¹ cm⁻¹; or one can consider that the average MEC is 10,000 Mol⁻¹ cm⁻¹ per base for DNA in aqueous solution. The MEC of the Cy3 dye at 552 nm is 150,000 Mol⁻¹ cm⁻¹.
 - c. Use the following equations to measure labeling efficiency:

$$[\text{Cy3 dye}] = \frac{A_{552}}{150,000}$$

$$[\text{Oligo}] = \frac{(A_{260} - 0.08A_{552})}{\text{oligo MEC}}$$

$$\text{Dye/oligo ratio} = \frac{[\text{Cy3 dye}]}{[\text{Oligo}]}$$

While 100% labeling efficiency is ideal, we have obtained good results with calculated labeling efficiencies of around 70% of amino-reactive residues per probe.

Sodium carbonate buffer

106 mg Na₂CO₃

84 mg NaHCO₃

Up to 10 ml ddH₂O

Adjust pH to 8.8 with HCl

2. Gelatin-Coated Coverslips for FISH

1. Set up a beaker with 0.1 N HCl on a hot plate in a fume hood. Boil 22 × 22 mm glass coverslips for 30 min then let cool at room temperature.
2. Rinse with ddH₂O 10 times.
3. Prepare 0.5% gelatin in ddH₂O in a large beaker. The volume of liquid must not be higher than halfway up the beaker.
4. Autoclave the coverslips in the beaker, then store at 4 °C.

3. FISH Protocol to Colocalize RNA with GFP Signal

a. Fixation

1. Plate fibroblasts on 22×22 mm glass coverslips coated with gelatin.
2. When the cells are ready, remove the growth media and replace with 4% formaldehyde in $1 \times$ PBS. Allow fixation to proceed for 15–30 min at room temperature.
3. After fixation, wash the coverslips twice with $1 \times$ PBS.
4. Dehydrate in 70% EtOH DEPC at 4°C for more than 2 h.
5. Coverslips stored in 70% EtOH should be protected from light and can remain usable for FISH for several weeks.

Notes: The quality of the formaldehyde is crucial for the preservation and detection of small details by FISH. We always use electron microscopy grade ultrapure, single-usage sealed ampoules of formaldehyde from Electron Microscope Sciences (Fort Washington, PA). A concentration of 10% acetic acid in the fixation buffer may be optimal for some FISH experiments; however, acetic acid is omitted here because it negatively affects GFP fluorescence. Additional details of the FISH procedure can be found in the study of [Chartrand *et al.* \(2000\)](#).

b. Hybridization

1. Prepare FISH probes by diluting them to $1 \text{ ng}/\mu\text{l}$ in ddH_2O . If a combination of probes is used, prepare a mix so that each individual probe has a final concentration of $1 \text{ ng}/\mu\text{l}$. Ambient light in the room should be dimmed during the FISH procedure to minimize bleaching of the Cy3 probes.
2. In a tube, combine $10 \mu\text{l}$ of probe mix and $20 \mu\text{g}$ of tRNA.
3. Lyophilize completely on medium heat. Cover from light in Speed-Vac and at all times.
4. Add $10 \mu\text{l}$ of $2 \times$ solution A and denature for 3 min at 95°C .
5. Let the tube cool down to room temperature and add $10 \mu\text{l}$ of $2 \times$ solution B. Mix carefully, the solution will be very viscous and bubbles should be avoided.

Notes: The final concentration indicated here for the probe solution and the washes is 40% formamide. This is a good starting point, but concentrations ranging from 25% to 50% formamide may need to be tested.

$2 \times$ Solution A

$4 \times$ SSC

80% Formamide

$2 \times$ Solution B

20% Dextran sulphate (weigh powder directly in 50-ml tube, add ddH₂O DEPC gradually, dissolve by agitation at 37 °C, and adjust final volume then keep at 4 °C)

0.4% BSA (commercial 2% BSA solution certified RNase free)

4 mM Vanadyl ribonucleotide complex

6. Rehydrate coverslips in 2× SSC 40% formamide for 5 min at room temperature.
7. Incubate coverslips with 20 µl of probe overnight at 37 °C.

Notes: We use Parafilm[®] to create an RNase-free-humidified chamber that will protect the probe from drying out in these conditions. Cover a 16 × 20 cm glass plate with a layer of Parafilm by placing its paper side up and rubbing firmly to make it adhere to the glass. Ultrafine tip tweezers should be used to manipulate the coverslips. Flame tweezer tips prior to RNase-free use. Place a 20 µl drop of probe mix on the Parafilm and lay the coverslip on the drop, making sure no bubbles are trapped. The surface of the coverslip containing the cells should face the drop. Cut another length of Parafilm, remove paper, and place the protected side down onto the coverslips. Do not move the coverslips after they have been laid down. Seal all four sides of the chamber by rubbing firmly with a blunt object. Wrap the glass plate in aluminum foil and place in a 37 °C incubator.

8. Wash coverslips twice in 8 ml of 2× SSC 40% formamide for 30 min at 37 °C.

Notes: To remove the inverted coverslips from the Parafilm without damaging the cells, first pinch and raise up the Parafilm near the coverslip to break the seal, then carefully lift the coverslip and place in a Coplin jar containing 8 ml of preheated wash solution. Wrap the Coplin jar in foil during washes.

9. Wash once with 1× PBS DEPC [containing 4',6-diamidino-2-phenylindole (DAPI) if desired] at room temperature.
10. Mount with fresh antifade mounting media and visualize as soon as possible. Fluorescence microscopy followed by deconvolution is the preferred method to visualize both Cy3 and GFP/YFP signal. Some FISH Cy3 signal can be intense enough to be successfully imaged by laser confocal microscopy, but this is often not the case.

C. Visualization of RNA Movement in Living Cells

The experimental details described here are for the FCS2 closed perfusion system (Bioptechs, Inc.), but may apply to the use of other systems as well. A closed chamber system enables the acquisition of data from cells in optimal viability conditions, and the perfusion of fresh media will help minimize photo-damage. For short-term observation of living cells, a closed perfusion chamber may not be needed. One can simply invert a 22 × 22 mm coverslip onto a slide and

image the cells immediately. Phenol red-free media or PBS can be used as the imaging media. However, to film the movement of mRNP particles, a more elaborate setup is required. Movement of mRNPs can be temperature dependent (Shav-Tal *et al.*, 2004) and fibroblast cells should be in optimal conditions for accurate experiments.

1. Plate Cells on FCS2 Coverslips

1. Place 40-mm-round Biopetechs coverslips in 60 mm dishes for cell culture. For typical fibroblasts no special coating of the glass is needed.
2. Cell placement can be restricted to the center of the coverslip by using glass culture cylinders (Biopetechs, PA). Before adding media, position one or two cylinders on the coverslip.
3. Dilute fibroblasts to the desired concentration and drop them into the culture cylinders, then apply media to the rest of the 60 mm dish. This will restrict cell growth to only one side of the coverslip, and allow a proper seal between the edges of the coverslip and the chamber gaskets. A different population of cells can also be plated in each of two cylinders on the same coverslip.
4. Allow at least 12 h for the fibroblasts to grow and settle on the glass.

2. Prepare Equipment in the Microscope Room

1. Turn on the 37 °C warmer oven in the microscope room so that it has time to reach the set temperature (Boekel Desk Top Warmer, Biopetechs, PA). This small no-humidity oven can be used to keep perfusion media, assembled chambers, objectives, extra cells, etc. in proximity to a microscope.
2. Calibrate the chamber system controller and turn it off—this controller must be off when the assembled chamber is connected to it.
3. Mount the objective heater and turn it on so that the temperature stabilizes at 37 °C.

3. Prepare Perfusion Media

1. Prepare phenol red-free media by adding tissue culture grade 1 M HEPES to a final concentration of 25 mM and preheating the media to 37 °C. Alternatively, the pH of the media can be regulated with a CO₂-bubbling apparatus.
2. To avoid contamination and evaporation, place the perfusion media in a small Erlenmeyer capped with a two-hole rubber stopper.
3. Thread 1/16 in. ID Tygon tubing through one hole so that the intake is kept permanently submerged in media. The second hole acts as a vent.

4. Place the perfusion media container in the Boekel warmer keeping the door slightly open for the tubing.

4. Set up the Perfusion System

1. Install the single-channel tube in the microperfusion pump (Instech peristaltic pump, Biopetechs, PA).
2. Position the pump at the same height and close to the microscope stage.
3. Connect tubing from the perfusion media to the pump intake port.
4. Attach tubing to the outflow port of the pump. Keep all tubing lengths as short as possible.
5. Calibrate the pump to a low-flow rate, such as 2 ml/h. Fill up all the inflow lines with perfusion media to remove air bubbles from the tubing.

5. Assemble the FCS2 Chamber

1. Remove the glass culture cylinder(s) and rinse the coverslip three times in phenol red-free media.
2. Mount the coverslip into the chamber according to manufacturer's instructions.
3. Immediately attach inflow and outflow tubing and start infusing the chamber with fresh media. Check the assembly for leaks at this time.
4. Securely mount the chamber onto the stage adapter.
5. Plug the chamber into the FCS2 controller and turn it on.
6. Tape the inflow and outflow tubing to the stage to avoid displacement when moving in *xy*-direction. The outflow tubing should extend only as far as necessary to reach the edge of the microscope stage.
7. Tape a cotton strip to the end of the outflow tube to allow the exiting media to reach a collection container by smoothly flowing through the hydrophilic material. This should prevent the formation of drops that create a vacuum that could cause flexing of the coverslip. This is especially important for prolonged imaging sessions where maintaining precise focus over time is critical.
8. The chamber must not be left unattended when the microperfusion pump is running. For obvious reasons, extreme care must be taken to avoid leaks.

6. Observe Living Cells

1. Focus on the cells using brightfield illumination.
2. Switch to the appropriate filter set and find a cell with the desired characteristics, taking care not to bleach the cell during focusing.

3. Determine the optimal duration and frequency of data acquisition for time-lapse imaging by trial and error. [Shav-Tal et al. \(2005\)](#) have recently published an outline of procedures to track and quantify single mRNP particles with the MS2-GFP system.

IV. Following RNA Trafficking in Living Yeasts

The MS2-GFP system to observe mRNA trafficking was first developed in yeast cells ([Bertrand et al., 1998](#); [Chartrand et al., 1999](#)). Since then, several studies have used this system to follow the transport and/or localization of specific transcripts in living yeast cells. Other systems to track mRNA trafficking in living yeasts have been developed subsequently, using GFP fusion with either the U1A RNA-binding protein ([Brodsky and Silver, 2000](#); [Takizawa and Vale, 2000](#)) or the MS2 coat protein ([Beach et al., 1999](#)), but they all are based on the same principle.

A. Expression in the Budding Yeast

1. The MS2-GFP Expression Vectors

For the expression of the MS2-GFP protein in yeast, the original expression vector (pG14-MS2-GFP) developed by the Singer laboratory was based on the pG14 vector (2 μ , *LEU2*) containing the MS2-GFP fusion under the control of the *GPD1* promoter ([Bertrand et al., 1998](#)). An SV40 NLS and an HA tag were inserted upstream of the MS2-GFP open reading frame (ORF). This plasmid allows constitutive expression of a nuclear-localized MS2-GFP protein. Only the MS2 fluorescent protein tethered to an mRNA containing MS2 stem-loops can be exported out of the nucleus and accumulate in the cytoplasm. New versions of this expression plasmid are now available, with lower expression levels (a centromere, *LEU2* plasmid YCP111-MS2-GFP) or without NLS(YCP111-MS2-GFP- Δ NLS) for detection of transcripts in the nucleus (see [Fig. 4](#)). Another version with the RedStar fluorescent protein has been recently published ([Schmid et al., 2006](#)).

2. Tagging an mRNA with MS2 Stem-Loops

For the expression of the mRNA with MS2 stem-loops, two approaches can be used. In both cases, expression of the reporter transcript with an inducible promoter, like the galactose-inducible *GAL1* promoter, is strongly suggested. The use of an inducible promoter makes it possible to follow the trafficking of the reporter mRNA immediately after induction rather than at steady state. One approach is to use a fusion reporter mRNA containing a heterologous ORF with MS2 stem-loops in its 3' UTR. For instance, the vector expressing a *lacZ*-6 $\times$ MS2 reporter (YEP195-*lacZ*-6 $\times$ MS2) can be used to study the role of specific RNA zipcodes or localization elements involved in RNA trafficking. Besides the *lacZ* ORF and

six MS2 stem-loops, it contains an *ADH2* 3' UTR with a multiple cloning site for insertion of heterologous DNA. The other approach is to directly insert the MS2 stem-loops in the 3'UTR of the ORF in the mRNA of interest. In this case, one can use the six MS2 stem-loops from the pSL-MS2–6 plasmid, which contain several restriction sites for subcloning. The MS2 stem-loops can also be inserted in the 5' UTR of the gene of interest, as is shown in Fig. 4. The *LacZ* gene was expressed with 24 copies of the MS2 stem-loop in its 5'UTR as well as the *ASH1* bud-localization zipcode in its 3'UTR (Chartrand *et al.*, 1999). The site of transcription in the nucleus is visible in Fig. 4 A, as well as the mature MS2-tagged mRNA localized to the bud tip.

3. Induction and Visualization in Yeast

Yeast cells are transformed with the plasmids encoding the MS2-GFP fusion protein and the mRNA with MS2 stem-loops using standard yeast transformation procedures (Schiestl and Gietz, 1989). Yeasts are grown overnight in culture tubes at 30 °C in 5 ml of the appropriate selection media with either 2% raffinose/0.02% glucose or 2% lactic acid/3% glycerol/0.05% glucose. These carbon sources neither repress nor activate the *GALI* promoter, so the reporter mRNA is not transcribed. The following day, the cultures are diluted to $OD_{600} = 0.1$ in the same medium but without glucose (glucose, even in small amount, represses the *GALI* promoter) and the yeasts are grown to mid-log phase to an OD_{600} between 0.2 and 0.4. Galactose is added to a final concentration of 3% to activate the transcription of the MS2-tagged RNA (as seen in Fig. 4). The yeast cells can then be directly put under the microscope (for kinetic analysis) or maintained in culture for the desired period before imaging.

An important aspect of the growth medium is the yeast autofluorescence. Several yeast strains are *ade2⁻* and a red pigment (an oxidized metabolite of phosphoribosylaminoimidazole) accumulates in the vacuole. This pigment is fluorescent in the green emission channel and may interfere with the GFP signal. Yeast cells grown in yeast peptone dextrose medium, which is poor in adenine, show more autofluorescence. Use selection medium with extra adenine (~20 µg/ml) to avoid this problem. Also, use yeast cultures at OD_{600} below 0.5, since yeasts grown to high concentration (~1 OD_{600}) show more autofluorescence.

For acquisition of videos or time-lapse images with the microscope, a 10 µl sample of the yeast culture can be spotted on a microscope slide, covered with a coverslip (do not seal with nail polish) and put directly under the microscope. However, the yeasts float in the medium and they can rapidly move out of the focal plane, making acquisition a difficult task. To acquire images from the same yeast cell over a long period, it is better to immobilize the yeasts on a solid medium. To do so, prepare a 2% agarose solution containing the appropriate yeast selection medium with 3% galactose. Deposit 200 µl of the melted agarose solution on a microscope slide and drop a second slide over the agarose solution in order to

create a sandwich of two slides separated by the thin agarose layer. Once solidified, the sandwich can be open, leaving a slide coated with a thin layer of agarose gel. On this layer of gel, the sample of yeast culture can be spotted and covered with a coverslip (do not seal). The yeasts will be immobilized on the agarose gel, making it easier to perform time-lapse imaging. Moreover, the gel is transparent enough not to interfere with image acquisition. Finally, the presence of selection medium in the agarose gel maintains the yeasts actively dividing over several hours.

B. *In Situ* Hybridization on Yeast Cells

Since the MS2-GFP system allows the visualization of the MS2-tagged RNA, this method provides an indirect tracking of the GFP-bound transcripts. To confirm that the GFP signal is associated with the MS2-tagged mRNA, a FISH can be performed to detect the transcript. In [Fig. 4C and D](#), the cells were hybridized with a Cy3-labeled mix of five probes directed against LacZ ([Long et al., 1995](#)). In [Fig. 4G](#), the overlay shows that the MS2-GFP and the Cy3 signal colocalize in the bud and at the mRNA transcription site in the nucleus.

The following protocol is modified from [Chartrand et al. \(2000\)](#) and allows the simultaneous detection of a specific mRNA and the GFP signal. Paraformaldehyde fixation has no effect on the fluorescence intensity of the MS2-GFP fusion protein or on its distribution. The probes used are the same as described in [Section III.B](#).

Important: All solutions for fixation, spheroplasting, and *in situ* hybridization should be DEPC-treated or prepared with DEPC-treated distilled water. Individuals must also wear gloves to avoid RNase contamination.

1. Preparation of the Coverslips

To maintain the yeast cells at the surface of the coverslip during the hybridization and the washing steps, the coverslips must be coated with poly-L-lysine.

1. Boil type 1 coverslips (22 × 22 mm) in 250 ml of 0.1 N HCl for 30 min. Let the coverslips cool down at room temperature.
2. Rinse 10 times with distilled water in a beaker.
3. Autoclave in distilled water. Coverslips can be stored at 4 °C for several months.
4. Put one coverslip in each well of a six-well tissue culture plate and drop 200 µl of poly-L-lysine 0.01% on each coverslip.
5. Incubate 2 min at room temperature, aspirate the excess, and let dry at room temperature (about 2–3 h).
6. When dry, wash each well three times with distilled water for 10 min.
7. Rest each coverslip on the wall of the wells, the face treated with poly-L-lysine on the top, and let dry (do not let the coverslips air-dry on the bottom of the wells, they will stick to the plastic).

2. Fixation and Spheroplasting of the Yeast Cells

1. Yeasts are grown in 50 ml cultures in the appropriate media until they reach early log phase (OD_{600} between 0.2 and 0.4, about 10^8 cells).
2. Fix cells for 45 min at room temperature by directly adding to the medium 6.25 ml of 32% formaldehyde.
3. Spin down cells for 5 min at 3500 rpm at 4 °C.
4. Wash cells three times with 10 ml of ice-cold 1× buffer B (1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5) and centrifugation (5 min at 3500 rpm at 4 °C).
5. Resuspend cells (do not vortex) in 1 ml of buffer B containing 20 mM vanadyl ribonucleoside complex, 28 mM β -mercaptoethanol, 0.06 mg/ml phenylmethylsulfonyl fluoride, 5 μ g/ml of pepstatin, 5 μ g/ml of leupeptin, 5 μ g/ml of aprotinin, and 120 U/ml of RNase Inhibitor.
6. Transfer cells to a tube containing 60 μ g of dried Zymolyase 100T (Seikagaku, Japan).
7. Incubate the cells for 20 min at 30 °C.
8. Centrifuge for 4 min at 3500 rpm at 4 °C.
9. Wash with 1 ml of ice-cold buffer B and spin.
10. Resuspend spheroplasts in 750 μ l of buffer B.
11. Add 100 μ l of spheroplasts on each poly-L-lysine-coated coverslips in the six well tissue culture plates.
12. Cells are left to adhere to the coverslips by incubating for 30 min at 4 °C.
13. Wash spheroplasts carefully with 3 ml of buffer B and remove by aspiration.
14. Add 5 ml of 70% ethanol in each well. Incubate at least 15 min at -20 °C before performing the *in situ* hybridization. At this stage, the coverslips can be stored for few weeks at -20 °C.

Notes: The Zymolyase 100T should be resuspended in 1× buffer B, aliquoted at 60 μ g per tube, and lyophilized. These aliquots can be stored at -20 °C in a dessicator.

3. *In Situ* Hybridization

a. Preparation of the Probes

For each coverslip used in the hybridization, prepare one tube of probes (we suggest the use of two coverslips per experiment in order to have a duplicate if one is broken during manipulations).

1. Mix 10 μ l of a 1 ng/ μ l probes solution with 4 μ l of a 5 mg/ml solution of 1:1 sonicated salmon sperm DNA:*Escherichia coli* tRNA.
2. Lyophilize and resuspend in 12 μ l of 80% formamide, 10-mM sodium phosphate pH 7.0.
3. Heat the probe solution at 95 °C for 3 min. Keep covered at room temperature.

b. Hybridization

1. Rehydrate the cell-coated coverslips in a Coplin jar with two washes in 8 ml of $2\times$ SSC for 5 min at room temperature.
2. Incubate coverslips in 8 ml of 40% formamide, $2\times$ SSC for 5 min at room temperature.
3. Add to the probes 12 μ l of $4\times$ SSC, 20-mM vanadyl ribonucleoside complex, 4 μ g/ μ l of RNase-free BSA, 50 U of RNase inhibitor.
4. Incubate the coverslips with 24 μ l of the probe solution overnight at 37 °C in a humidified chamber, as described in [Section III.B](#).

c. Washing

1. After the incubation, remove the coverslips from the Parafilm sheet and put them back in the Coplin jar.
2. Wash the coverslips twice with 8 ml of 40% formamide, $2\times$ SSC (preheated at 37 °C) for 15 min at 37 °C.
3. Wash with 8 ml of $2\times$ SSC, 0.1% Triton X-100 for 15 min at room temperature.
4. Wash twice with 8 ml of $1\times$ SSC for 15 min at room temperature.
5. Add 8 ml of $1\times$ PBS containing 1 ng/ml of DAPI.
6. Mount coverslips on a glass slides (1-mm thick). Drop 5 μ l of mounting medium on the slide, lay down the coverslip on the drop (the surface of the coverslip containing the cells should face the drop), and remove the excess of medium with Kimwipes[®].
7. Seal the sides of the coverslips with nail polish. Image cells as soon as possible.

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