Supporting Materials and Methods

FCS Volume of Detection Estimation. To determine the FCS probed volume in our experimental setup, we calibrated the autocorrelation functions with fluorescent spherical polystyrene beads with a diameter of 44 nm (G40, Duke Scientific, Palo Alto, CA) (Fig. 4). We fit the autocorrelation functions with $G(t) = 1/\{N[(1 + (4Dt/\omega^2)]\}$, which describes a two-dimension translational diffusion of fluorescent molecules (1, 2). N is the number of fluorescent beads in the detection volume, D is the two-dimensional diffusion constant of the fluorescent beads, and t is the time variable. Using the Stokes-Einstein relationship $D = k_B T/6\Pi\eta R$, where k_B is the Boltzman constant, T is temperature in Kelvin (300K), η is the viscosity of water (0.01 cm^2/s), and R is the radius of the beads (0.22 nm), we determined that the diffusion constant (D) of the beads is 3.6 $\mu m^2/s$. We inferred ω (0.19 μm) using the equation $D = \omega^2/(4\zeta)$, where ω is the radius of the detection volume and ζ is the diffusion time of the polystyrene beads (0.9 ms; Fig. 4).

The FCS volume of detection is calculated with the equation $V_{probe} = \pi\omega^2 h$, where h is the height of this volume. Under our conditions and for *in vitro* FCS measurements, h was estimated to be 8.8 μm (1). The probe volume *in vitro* was therefore found to be ~1 fl. For FCS measurements in a living *E. coli* cell, the thickness of an *E. coli* cell was estimated to be 0.4 μm. The probe volume in a living cell was calculated to be ~0.045 fl. One fluorescent molecule in the probe volume for *in vitro* measurements represents a concentration of 7 nM. One MS2-GFP molecule in the detection volume within a living bacterium represents a concentration of 37 nM. Finally, one ms2-RNA molecule, which binds to two MS2-GFP molecules, represents a concentration of 18 nM.

ms2-RNA and MS2-GFP Protein Purification. The ms2-RNA-binding sites from plasmid pZE31ms2 were amplified by PCR with primers carrying a T7 promoter and purified on 1.2% agarose gel (3). ms2-RNA was transcribed by using the AmpliScribe T7 transcription kit from Epicentre (Madison, WI). The concentration of ms2-RNA was determined with absorbance at 260 nm.

DNA sequence coding for MS2-GFP mutant was cloned into pRSETA vector (Invitrogen) and transformed into BL21 (DE3PlysS)-competent cells. Cells were grown to 0.5 OD and induced with 1 mM IPTG overnight at 30°C. The N terminus 6x histidine-tagged MS2-GFP was purified on a nickel column (Qiagen, Valencia, CA) and dialyzed in 1 mM PBS buffer overnight. The 6x histidine tag was removed with enterokinase at 0.25 mg/ml (Roche) for 4 h at 30°C. The flow-through fraction of MS2GFP was collected from the nickel column, dialyzed in 1 mM PBS buffer overnight, and stored at 4°C. The concentration of MS2-GFP was determined by absorbance at 280 nm.

ms2-RNA Ribosome Association Assay. Cells carrying both reporter and expression plasmids were streaked on agar plates overnight. A single colony was picked and grown in 200 ml of M9 medium. At A_{600} of 0.2, anhydrotetracycline was added to a final concentration of 400 ng/ml, and the cells were incubated for 20 min. Cells were collected by centrifugation, lysed with a French press at 16,000 psi, and treated with RQ1 DNase. Cell lysate was spun at 30,000 × *g* for 15 min at 4°C. The supernatant containing ribosome was collected as fraction 1 (S30 fraction). S30 fraction was further spun at 100,000 × *g* for 4 h at 4°C. The supernatant containing no ribosome was designated fraction 2, and the pellet containing ribosome was designated fraction 3 (S100 fraction). Fraction 3 was run on a sucrose gradient to separate the ribosomal subunits 30S (fraction 4), 50S (fraction 5), and 70S (fraction 6). RNA from these six fractions was extracted with RNA extraction buffer (0.5% SDS/12 mM EDTA/0.3M NaOAc, pH5/heparin 50 units/ml) and with phenol and chloroform. One microgram of RNA from each fraction was used in ms2-RNA primer extension assay (details below) to probe for the presence of ms2-RNA (Fig. 5*A*).

Theoretical Analysis of ms2-RNA Association with Ribosome. To estimate the fraction of ms2-RNA transcripts (*R*) not bound to ribosomes (*B*), we solve the following simple kinetic equilibrium system:

$$BR = K_A\ B\ R$$
$$B_T = B + BR + BO$$
$$R_T = R + BR$$

Here, *B* is the concentration of free ribosome and *R* is the concentration of free ms2-RNA transcripts. *BR* is the concentration of ribosome-ms2-RNA complexes and *BO* is the concentration of ribosomes being used elsewhere in the cell. B_T and R_T stand for the total concentrations of ribosome and ms2-RNA in the cell. K_A is the ribosome-ms2-RNA association constant. We calculate the fraction $y = R / R_T$ of ms2-RNA transcripts not bound to ribosome as a function of the fraction $x = 1 - BO / B_T$ of ribosomes available to bind ms2-RNA:

$$y = -\frac{1}{2}\left(\frac{B_T}{R_T}x + \frac{1}{R_T K_A} - 1\right) + \frac{1}{2}\sqrt{\left(\frac{B_T}{R_T}x + \frac{1}{R_T K_A} - 1\right)^2 + \frac{4}{R_T K_A}}$$

We plotted *y* as function of *x* in Fig. 6. We assume $R_T = 50$ ms2-RNA molecules and $B_T = 10{,}000$ ribosomes. We use $V = 1.41 \times 10^{-15}$ liter for the cell volume. The two curves correspond to $K_A = 20\,\mu\text{M}^{-1}$ and 2 μM^{-1}.

Fig. 6 shows that >90% of ms2-RNA is bound to ribosome when <20% of total cellular ribosome is available. This finding concurs with our experimental results where we show that nearly all the ms2-RNA transcripts are bound to ribosome (Fig 5*A*, lane 2).

Determination of RNA Concentration in Living Cells. The fraction of free and bound MS2-GFP within a living cell was determined by fitting the autocorrelation functions with $G(t) = \frac{1}{N}\frac{1}{[(1-y)+2y]^2}\left(\frac{1-y}{(1+t/\zeta_{free})} + \frac{4y}{(1+t/\zeta_{bound})}\right)$

(2, 4). To derive RNA concentration, we multiply the fraction of bound MS2-GFP at any given time with the total number of MS2-GFP molecules (N_0) measured at the initial time point (0 s). One RNA transcript in this detection volume represents 18 nM. We assume that the MS2-GFP concentration within a cell remains constant during FCS

measurements. After one division, we reduced the initial number of MS2-GFP molecules N_0 by the ratio of body lengths of daughter to mother cells. The errors in estimating RNA concentration are calculated by using the equation $(\delta y/y + \delta N_0/N_0)$[RNA], where δy and δN_0 are the fitting errors of the fraction bound and of the initial MS2-GFP concentration inside the detection volume, respectively.

Fitting of the FCS Autocorrelation Functions with Two Components. There are several fluorescent species that we have to consider: (*i*) free monomeric MS2-GFP protein; (*ii*) free homodimeric MS2-GFP protein, which is the ms2-RNA-binding unit; (*iii*) ms2-RNA transcripts bound to one MS2-GFP homodimer; (*iv*) ms2-RNA transcripts bound to two MS2-GFP homodimers; (*v*) the ms2-RNA–ribosome complex bound to one MS2-GFP homodimer; (*vi*) ms2-RNA–ribosome complex bound to two MS2-GFP homodimers. To simplify data fitting, we made the following assumptions:

- Peabody and colleagues (5) found that the mutant MS2 protein used in our experiment (dlFG) existed predominantly in homodimeric form. In addition, the diffusion times of free GFP and MS2-GFP molecules (either monomeric or homodimeric) are indistinguishable in living cells as measured with FCS, ~1 ms (data not shown). Therefore, we can safely treat free MS2-GFP protein, both monomeric and dimeric, as unique free component.

- *In vitro* experiments demonstrated (Fig. 5*A*) that all ms2-RNA transcripts were bound to ribosomes (lane 2, Fig. 5*A*). Given that ribosomes exist in large quantity in a living cell (~10 μM) and that the binding affinity of a ribosome to a typical mRNA is 0.05-0.5 μM, it is reasonable to assume that all ms2-RNA transcripts are associated with ribosomes. In addition, we performed a simulation to calculate the concentration of ribosome needed to bind to all ms2-RNA transcripts produced and found that only a small fraction of total ribosome concentration in a living cell is sufficient (Fig. 6). Therefore, we can safely assume that all ms2-RNA transcripts are bound to ribosome.

• One ms2-RNA–ribosome complex bound to one MS2-GFP homodimer has a total molecular weight of ~2.8 MDa. One ms2-RNA–ribosome complex bound to two MS2-GFP homodimers has a total molecular weight of ~2.9 MDa. A 3% change in molecular weight translates into a negligible change in diffusion time, thus indistinguishable by FCS measurement. Therefore, we can safely treat the ms2-RNA–ribosome bound to one or two MS2-GFP homodimers as one component. (MS2-GFP homodimer, ~88 kDa; ms2-RNA, ~124 kDa; ribosome, ~2,600 kDa) (6, 7).

For the former reasons, we can simplify the analysis of our FCS data with a two-component fitting. One component is the free MS2-GFP homodimers and the other is the ms2-RNA–ribosome-bound MS2-GFP homodimers.

Alternative Fitting of FCS Data. If one does not take into account the difference in brightness, one can use a simpler formula that gives very similar (Fig. 7) results. One possibility is to fit the FCS autocorrelation functions, with the following two-component equation (fit 1):

$$G(t) = \frac{1}{N} \bullet \left\{ \frac{(1-y)}{(1+\frac{t}{\tau_{free}})} + \frac{y}{(1+\frac{t}{\tau_{bound}})} \right\},$$

where y, the only fit parameter, is the fraction of MS2-GFP molecules bound to ms2-RNA–ribosome complex. To calculate the number of MS2-GFP molecules bound, we multiply y by N, the number of fluorescent molecules in the detection volume. The concentration of ms2-RNA transcript is one-half of the products of y and N as two MS2-GFP molecules bind to one mRNA (see above).

Alternatively, we can fit our data with the following two-component fit that takes into account the difference in brightness of free homodimers and two homodimers bound to the RNA recognition sequence (fit 2):

$$G(t) = \frac{1}{N}\frac{1}{[1+y]^2}\left(\frac{1-y}{(1+t/\zeta_{free})} + \frac{4y}{(1+t/\zeta_{bound})}\right)$$

where *y*, the only fit parameter, is the fraction of MS2-GFP molecules bound to ms2-RNA–ribosome complex. In this fit, however, the concentration of ms2-RNA is simply the product of *y* and *N*.

The ms2-RNA concentration obtained from fit 1, in general, is slightly higher than that obtained with fit 2 (Fig. 7). The actual ms2-RNA concentration in a living cell might be somewhere in between.

Effect of Photobleaching on RNA Profiling. We assumed that the fractions of bound (*y*) and free (1 – *y*) MS2-GFP molecules are not affected by the bleaching of MS2-GFP (see below), which can reach 70% at the end of repeated measurements within one cell. Because MS2-GFP molecules bound to RNA diffuse slower, they are exposed longer to laser excitation light and could bleach more than the free MS2-GFP molecules diffusing faster. However, we collected data at 10-min intervals, and k_{off} of MS2 protein from ms2-RNA is 1 min^{-1} (7). Bleached molecules have been fully recycled before the next measurement. Consequently, we hypothesize that the fraction of bound and free MS2-GFP is independent of the photobleaching of MS2-GFP. To experimentally support this hypothesis, we compared the mean concentration of RNA transcripts obtained from 10 continuous RNA profiles (where bleaching is important; Fig. 8*A*) with the mean RNA concentration obtained from 62 cells that were exposed to the laser only once [where bleaching is very low, <5% (Fig. 8*B*)]. Fig. 8*C* demonstrates that RNA profiling is insensitive to bleaching because the two approaches produced the same result.

In vitro Population Profiling of ms2-RNA Expression

Experimental Conditions. Overnight cultures of *E. coli* cells carrying the dual plasmid system were diluted 20-fold in fresh M9 media and grown for 2 h (~0.18 OD).

Anhydrotetracycline (aTc) (400 ng/ml final concentration) was added (at time t = 0 min) to induce ms2-RNA expression. At 10-min intervals after induction, total RNA was extracted in triplicate from 5-ml cells each using RNAwiz (Ambion, Austin, TX). Cell density was maintained constant at 0.18 OD by continuous addition of fresh M9 medium premixed with 400 ng/ml aTc. One microgram of total RNA from each collection was used for ms2-RNA primer extension (details below) to determine the relative concentration of ms2-RNA in a population after induction.

Detection and Quantitation of ms2-RNA by Primer Extension. The amount of ms2-RNA in total RNA mixture or the ribosomal fractions was measured by primer extension using the primer: 5'-TGA GGA TCA CCC ATG TCT GCA, complementary to a 3' portion of the MS2-binding site. It was designed such that reverse transcription reaction with dCTP, dTTP, dGTP, and ddATP generated a terminated 30-mer extension product, allowing better quantification.

To allow hybridization, 0.4 pmol 5' ^{32}P-labeled primer in 1.4 μl was mixed with 2 μl of total *E. coli* RNA (1 μg) and heated at 93°C for 1 min, followed by incubation on ice for 4 min. The hybridization buffer contained 20 mM Tris•HCl, pH 7.5/1.2 mM $MgCl_2$/1.2 mM 2-mercaptoethanol. To this mixture, 1.6 μl of reverse transcriptase (RT) reaction concentrate was added, and the final reaction mixture contained 50 mM Tris•HCl, pH 8.3/8 mM $MgCl_2$/50 mM NaCl/1 mM DTT/0.2 mM each dCTP, dTTP, and dGTP/0.1 mM ddATP/0.1 units/μl AMV-RT (USB). The RT mixture was incubated for 15 min at 42°C. The reaction was stopped upon the addition of an equal volume of 9 M urea/100 mM EDTA. The mixture was boiled for 2 min before loading on 15% denaturing polyacrylamide gels containing 7 M urea. The amount of reverse transcription product and the primer remaining was quantified by using a Fuji phosphorimager.

To convert the amount of RT product from the population measurements into the same unit as the RNA concentration determined by FCS, two additional parameters are needed, one experimental and one empirical. The experimental factor (*Ex*) calibrates the amount of RT product to a concentration of nM/μg total RNA. In this case, reverse transcription

was carried out under the same conditions as described above including 0.5 μg of total *E. coli* RNA with varying, known amounts of a purified RNA standard containing the same two MS2-sites. Upon plotting the amount of RT product versus the known concentration of the RNA standard, a reaction efficiency of 0.56% RT product/nM RNA transcript was obtained (data not shown). The amount of RNA transcript in any total of RNA samples then corresponds to

Ex = %RT product per μg total RNA/0.56 (unit: nM/μg total RNA). [**1**]

The empirical factor (*Em*) converts the number of MS2-site-containing RNA (~400 nt) per cell into the same concentration unit using a reasonable assumption that ~80% of the total RNA are ribosomal RNAs and there are ~10,000 ribosomal RNAs per cell (8). The length of the three *E. coli* rRNA is 1,542, 2,904, and 120 nt (a total of ~4,570 nt), corresponding to 4.57×10^7 nucleotides of rRNA per cell. The calculation below is for a single ms2-RNA per cell:

Em = [μg MS2-RNA/μg total RNA]/(molecular weight of MS2-RNA)/(reaction volume) = $[(400/4.57 \times 10^7)/0.8]/(400 \times 310)/5 = 1.76 \times 10^{-2}$ nM/μg total RNA [**2**]

Because one ms2-RNA molecule represents a concentration of 18 nM in our FCS measurement, the RNA concentration from the primer extension assay is converted as below:

[RNA] = (*Ex*/*Em*)*0.018 μM [**3**]

***In Vivo* Transcription Assay in Polylysine Immobilized Cells.** Cells were immobilized on a polylysine-coated coverslip in a reaction chamber. The reaction chamber was made of an aluminum slide with a hollow ring at the center. A polylysine-coated glass coverslip was sealed on one side of the ring with beeswax to create a chamber that can hold up to 300 μl of sample. A moveable uncoated coverslip was placed on top of the chamber to prevent evaporation of the sample during measurements.

The chamber was filled with 200 μl of M9 media and placed on a thermo-controlled microscope stage set at 30°C. In our experiments, MS2-GFP was preexpressed from an inducible promoter controlled by LacI. We used FCS to select for cells that preexpressed MS2-GFP protein at the level of ~9 μM. We replaced M9 media in the chamber with 200 μl of fresh M9 media premixed with inducer (400 ng/ml aTc). FCS data were collected from cells at 5-min intervals for the first 25 min and 10-min intervals afterward (Fig. 8*A*).

***In Vivo* Transcription Assay of Single Cells in a Population.** In population measurements, FCS data were collected from 62 single cells immobilized with polylysine on the glass surface over time after induction with 400 ng/ml aTc (Fig. 8*B*). The microscope stage was moved in one direction such that each cell was exposed once to the laser beam for 2 s. Photobleaching was <5% in each cell during this exposure. When the cells were immobilized with polylysine, we noticed that they responded synchronously to transcriptional induction by anhydrotetracycline (Fig. 8*A*). RNA profiles measured from cells to cells indiscriminately (Fig. 8*B*) were comparable to those measured within individual cells (Fig. 8*C*). This observation validated our assumption on the effect of photobleaching in RNA profiling.

1. Eigen, M. & Rigler, R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5740-5747.
2. Rauer, B., Neumann, E., Widengren, J. & Rigler, R. (1996) *Biophys. Chem.* **58,** 3-12.
3. Lutz, R. & Bujard, H. (1997) *Nucleic Acids Res.* **25,** 1203-1210.
4. Cluzel, P., Surette, M. & Leibler, S. (2000) *Science* **287,** 1652-1655.
5. Peabody, D. S. & Ely, K. R. (1992) *Nucleic Acids Res.* **20,** 1649-1655.
6. Moore, P. B. (1998) *Annu. Rev. Biophys. Biomol. Struct.* **27,** 35-58.
7. Johansson, H. E., Dertinger, D., LeCuyer, K. A., Behlen, L. S., Greef, C. H. & Uhlenbeck, O. C. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 9244-9249.
8. Dong, H., Nilsson, L. & Kurland, C. G. (1996) *J. Mol. Biol.* **260,** 649-663.