Supporting Information

Materials and Methods

FCS volume of detection estimation

To determine the FCS probed volume in our experimental set-up, we calibrated the autocorrelation functions with fluorescent spherical polystyrene beads with a diameter of 44nm (G40, Duke scientifics, Palo Alto, Calif.) (fig. S1). We fit the autocorrelation functions with $G(t) = 1/\{N[(1+ (4Dt/\omega^2)]\}$, which describes a two-dimension translational diffusion of fluorescent molecules. 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 $D = k_BT/6\Pi\eta R$, 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 (0.22nm), 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. S1).

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 8.8μm (*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 7nM. One MS2-GFP molecule in the detection volume within a living bacterium represents a concentration of 37nM. Finally, one ms2-RNA molecule, which binds to two MS2-GFP molecules, represents a concentration of 18nM.

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. ms2-RNA was

transcribed using the AmpliScribe T7 transcription kit from Epicentre (Madison, WI). The concentration of ms2-RNA was determined with absorbance at 260nm.

DNA sequence coding for MS2-GFP mutant was cloned into pRSETA vector (Invitrogen, Carlsbad, CA) and transformed into BL21 (DE3PlysS) competent cells. Cells were grown to 0.5 O.D. and induced with 1 mM IPTG overnight at 30^{o}C. The N-terminus 6X Histidine tagged MS2-GFP was purified on Nickel column (Qiagen, Valencia, CA) and dialyzed in 1mM PBS buffer overnight. The 6X Histidine tag was removed with enterokinase at 0.25 mg/ml (Roche, Palo Alto, CA) for 4 hours at 30^{o}C. The flow-through fraction of MS2GFP was collected from the Nickel column, dialyzed in 1mM PBS buffer overnight, and stored at 4^{o}C. The concentration of MS2-GFP was determined by absorbance at 280nm.

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 200ml M9 medium. At A_{600} of 0.2, anhydrotetracycline was added to a final concentration of 400ng/ml and the cells were incubated for 20 minutes. Cells were collected by centrifugation, lysed with a French press at 16,000 psi, and treated with RQ1 DNase. Cell lysate was spun at 30,000g for 15 minutes at 4^{o}C. The supernatant containing ribosome was collected as fraction 1 (S30 fraction). S30 fraction was further spun at 100,000g for 4 hours at 4^{o}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, 12mM EDTA, 0.3M NaOAc pH5, Heparin 50U/ml) and with phenol and chloroform. 1µg of RNA from each fraction was used in ms2-RNA primer extension assay (details below) to probe for the presence of ms2-RNA (fig. S2A).

Theoretical analysis of ms2-RNA association with ribosome

To determine the fraction of ms2-RNA transcripts not bound to the ribosomes, we wrote a simple kinetic model. We plotted the results of numerical simulations (fig.

S3) for two distinct ms2-RNA/ribosome binding constants. The simulations showed that more than ninety percent of ms2-RNA is bound to ribosomes when less than twenty percent of total cellular ribosome is available. This numerical result concurs with our experimental results where we show that all ms2-RNA transcripts are bound to ribosome (fig. S2A, lane 2).

The association between ribosomes (B) to ms2-RNA transcripts (R) is given by the equation

$$B + R \leftrightarrow BR$$

where $K_A = \frac{[BR]}{[B][R]}$ = 20 μM^{-1} or 2 μM^{-1} is the association constant.

We assume conservation of cellular ribosome and ms2-RNA :

$B_T = B + BR + BO$ is the total cellular ribosome, ~10,000 ribosomes per *E. coli* cell; where BO is the concentration of ribosome being used elsewhere in the cell.

And $R_T = R + BR$ is the total concentration of ms2-RNA transcripts at a given time point, ~50 ms2-RNA transcripts per *E. coli* cell.

By rearranging the parameters, we have:

$$B = B_T - BO - BR = B_T - R_T - BO + R$$

$$BR = R_T - R$$

$$K_A = \frac{[R_T - R]}{[B_T - R_T - BO + R][R]}$$

$$K_A R^2 + K_A(B_T - R_T - BO)R - R_T + R = 0$$

$$K_A R^2 + K_A(B_T - R_T - BO + \frac{1}{K_A})R - R_T = 0$$

$$R^2 + (B_T - R_T - BO + \frac{1}{K_A})R - \frac{R_T}{K_A} = 0$$

By defining $y = \frac{R}{R_T}$ as the percentage of ms2-RNA transcripts not bound to ribosome and $x = 1 - \frac{BO}{B_T}$ as the ratio of total ribosome available to bind to ms2-RNA transcripts, we derive the relationship between y and x:

$$y^2 + (\frac{B_T}{R_T} - 1 - \frac{BO}{R_T} + \frac{1}{R_T K_A})y - \frac{1}{R_T K_A} = 0$$

$$y^2 + [\frac{B_T}{R_T}(1 - \frac{BO}{B_T}) + \frac{1}{R_T K_A} - 1]y - \frac{1}{R_T K_A} = 0$$

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

The relationship between y and x plotted on fig. S3.

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

In order 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_o) measured at the initial time-point (0 seconds). 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_o by the ratio of body lengths of daughter to mother cells. The errors in estimating RNA concentration are calculated using the equation ($\delta y/y + \delta N_o/N_o$)[RNA], where δy and δN_o are the fitting errors of the fraction bound and of the initial MS2-GFP concentration inside the detection volume, respectively.

Assumptions for two components fitting of the FCS autocorrelation functions

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 homodimer, (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:

1. Peabody and colleagues 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, ~ 1ms (data not shown). Therefore, we can safely treat free MS2-GFP protein, both monomeric and dimeric, as unique free component.

2. In-vitro experiments demonstrated (fig. S2A) that all ms2-RNA transcripts were bound to ribosomes (lane 2, fig. S2A). Given that ribosomes exist in large quantity in a living cell (~10μM) and the binding affinity of 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. S3). Therefore, we can safely assume that all ms2-RNA transcripts are bound to ribosome.
3. One ms2-RNA- ribosome complex bound to one MS2-GFP homodimer has a total molecular weight of ~2.8 MD. One ms2-RNA- ribosome complex bound to two MS2-GFP homodimers has a total molecular weight of ~2.9 MD. 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 homodimer as one component. (MS2-GFP homodimer ~ 88kD; ms2-RNA ~ 124kD; ribosome ~ 2600 kD).

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 which gives very similar (Fig. S4) 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 with 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 free homodimers and two homodimers bound to the RNA recognition sequence(fit 2):

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

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. S4). The actual ms2-RNA concentration in a living cell might be somewhere in between.

Effect of photo-bleaching on RNA profiling

We assumed that the fraction 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 exposed longer to laser excitation light and could bleach more than the free MS2-GFP molecules diffusing faster. However, we collected data at ten-minute intervals, and k_{off} of MS2 protein from ms2-RNA is 1 min^{-1}(7). Bleached molecules have fully been recycled before the next measurement. Consequently, we hypothesize that the fraction of bound and free MS2-GFP is independent of the photo-

bleaching 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. S5A) with the mean RNA concentration obtained from 62 cells that were exposed to the laser only once (where bleaching is very low, less than 5% (fig. S5B)). The figure S5C 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 culture of *E. coli* cells carrying dual plasmid system were diluted 20-fold in fresh M9 media and grown for two hours (~0.18 O.D.). Anhydrotetracycline (aTc, 400ng/ml final concentration) was added (at time t=0 min) to induce ms2-RNA expression. At 10-minute intervals after induction, total RNA was extracted in triplicate from 5ml cells each using RNAwiz (Ambion Inc., TX). Cell density was maintained constant at 0.18 O.D. by continuous addition of fresh M9 medium premixed with 400ng/ml aTc. 1µg 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 the 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 pmole 5' ^{32}P-labeled primer in 1.4 µl was mixed with 2 µl of total *E. coli* RNA (1 µg), heated at 93 °C for 1 min followed by incubation on ice for 4 min. The hybridization buffer contained 20 mM trisHCl, pH 7.5, 1.2 mM $MgCl_2$ and 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,

dGTP, 0.1 mM ddATP and 0.1 U/µl AMV-RT (USB, Cleveland, OH). The RT-mixture was incubated for 15 minutes 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 minutes prior to loading on 15% denaturing polyacrylamide gels containing 7 M urea. The amount of reverse transcription product and the primer remaining was quantified 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:

$$Ex = \%\text{RT-product per µg total RNA} / 0.56 \text{ (unit: nM/µg total RNA).} \quad (1)$$

The empirical factor (*Em*) converts the number of MS2-site containing RNA (~ 400 nucleotides) 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 1542, 2904 and 120 nucleotides (a total of ~ 4,570 nucleotides), corresponding to 4.57×10^7 nucleotides of rRNA per cell. The calculation below is for a single ms2-RNA per cell:

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

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

$$[\text{RNA}] = (Ex / Em) * 0.018\ \mu M \qquad (3)$$

In vivo transcription assay in poly-lysine immobilized cells

Cells were immobilized on a poly-lysine coated cover slip in a reaction chamber. The reaction chamber was made of an aluminum slide with a hollow ring at the centre. A poly-lysine coated glass cover slip was sealed on one side of the ring with bees wax to create a chamber which can hold up to 300µl of sample. A moveable uncoated cover slip was placed on top of the chamber to prevent evaporation of the sample during measurements.

The chamber was filled with 200µl of M9 media and placed on a thermo-controlled microscope stage set at 30^{o}C. In our experiments, MS2-GFP was pre-expressed from an inducible promoter controlled by LacI. We used FCS to select for cells that were pre-expressed MS2-GFP protein at the level of ~9µM. We replaced M9 media in the chamber with 200µl of fresh M9 media premixed with inducer (400ng/ml aTc). FCS data were collected from cells at 5 minute intervals for the first 25 minutes and 10 minute intervals afterwards (fig. S5A).

In-vivo transcription assay of single cells in a population

In population measurements, FCS data were collected from sixty two single cells immobilized with poly-lysine on the glass surface over time after induction with 400ng/ml aTc (fig. S5B). The microscope stage was moved in one direction such that each cell was exposed once to the laser beam for 2 seconds. Photo-bleaching was less than 5% in each cell during this exposure. When the cells were immobilized with poly-lysine, we noticed that responded synchronously to transcriptional induction by anhydrotetracycline (fig. S5A). RNA profiles measured from cells to cells indiscriminately (fig. S5B) were comparable to those measured within individual cells (fig. S5C). This observation validated our assumption on the effect of photo-bleaching in RNA profiling.

Supporting figures

Figure S1 Diffusion of fluorescent beads in buffer solution monitored with FCS. FCS instrument calibration with 44nm green fluorescent beads (G40, Duke Scientifics Corp.) in PBS buffer (11.9 mM $KH_2PO4.H_2O$, 137 mM NaCl, 2.7 mM KCl). Autocorrelation function (black line) was obtained with a two-second acquisition. We fit this function (grey line) with $G(t) = 1/(N[1 + (4Dt/\omega^2)])$, which describes two-dimension translational diffusion.

Figure S2 Ribosome association with ms2-RNA probed with primer extension. (A) Equal amount of total RNA as measured by UV absorbance was used for all samples. (+), total *E. coli* RNA containing ms2-RNA (positive control); (-), total *E. coli* RNA lacking ms2-RNA (negative control); lane 1, ribosomal fraction (supernatant) collected at 30,000g; lane 2, no ribosome fraction (supernatant) collected at 100,000g; lane 3, ribosome fraction (pellet) collected at 100,000g; lanes 4-6, fractions from sucrose gradient; lane 4: 30S ribosome; lane 5, 50S ribosome, lane 6, 70S ribosome. The absence of ms2-RNA in lane 2 suggests that all ms2-RNA transcripts are bound to ribosome. (B) Ribosome fractions purified on the sucrose gradient. The total amount of the 70S ribosome is at least 10 times the total amount of the 30S ribosome, and the absence of polysome indicates that there is only one ribosome bound to a ms2-RNA transcript.

Figure S3 Theoretical analysis of ms2-RNA association with ribosome. Red curve, K_A= 20 μM^{-1} , blue curve K_A= 2 μM^{-1}. Simulation performed with Mathematica.

Figure S4 Alternative fittings of FCS autocorrelation functions. Blue, ms2-RNA concentration derived by fitting the autocorrelation functions with G(t) = (1/N)[(1-y)/(1+t/ ζfree) + y/(1+ t/ ζbound)]. Red, ms2-RNA concentration derived by fitting the autocorrelation functions with G(t) = (1/N)[(1-2y)/(1+t/ ζfree) + 4y/(1+ t/ ζbound)]. Error bars represent the uncertainties in the fitting parameters of the autocorrelation function. Cell division events are indicated with a dark horizontal bar. Data are the same as in Fig 2A.

Figure S5 ms2-RNA expression profiles in single cells and in a population of single cells immobilized on poly-lysine coated glass surface. ms2-RNA concentration profiles as a function of time, cells were exposed to inducer at time point zero (400ng/ml aTc). (**A**) Profiles of 10 individual cells, (**B**) population of 62 single cells. In (B) each cell was exposed only once to the laser beam for two seconds to obtain a single ms2-RNA concentration time point measurement. Photo-bleaching in each cell was less than 5%. Error bars represent uncertainties in the fitting parameters of the autocorrelation function. (**C**) Overlay of average ms2-RNA concentration profiles in ten individual cells (black) and of a population of 62 single cells (grey). ms2-RNA concentrations from a population of single cells were averaged by binning ms2-RNA concentrations from 1-5, 6-10, 11-15, 16-20, 21-25, 26-35, 36-45, 46-55, 56-65, 66-75, and 76-85 minutes to yield ms2-RNA concentrations at 5, 10, 15, 20, 25, 35, 45, 55, 65, 75, 85 minutes, respectively. Error bars represent the standard deviations from the ms2-RNA concentration distributions.

Figure S6 ms2-RNA expression profiles from four single Frag1A (Δ*acrAB*) mutant cells induced with 400ng/ml aTc. Error bars represent the uncertainties in the fitting parameters of the autocorrelation function. Cell division events are indicated with horizontal bars of corresponding colors.

References

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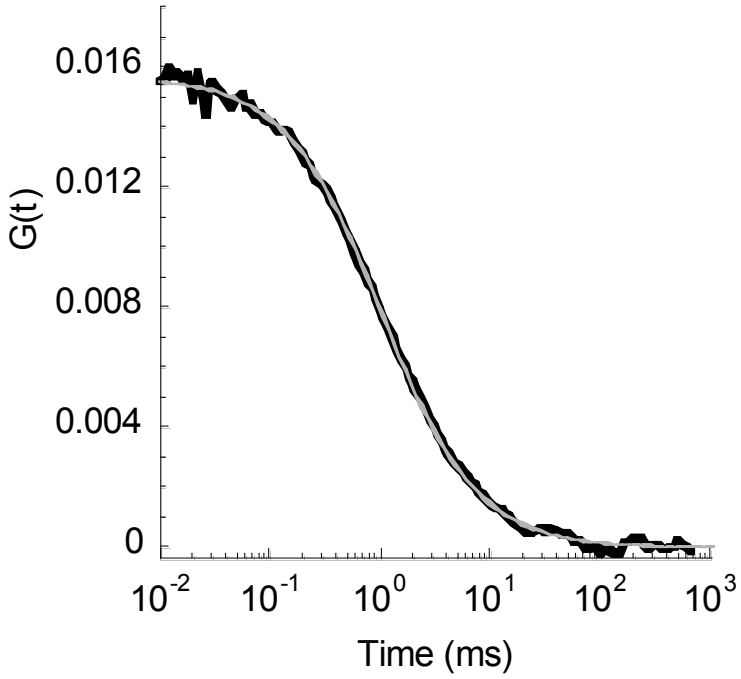
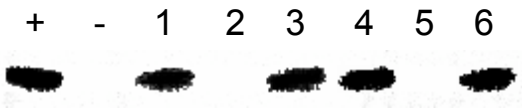


Figure S1

A



B

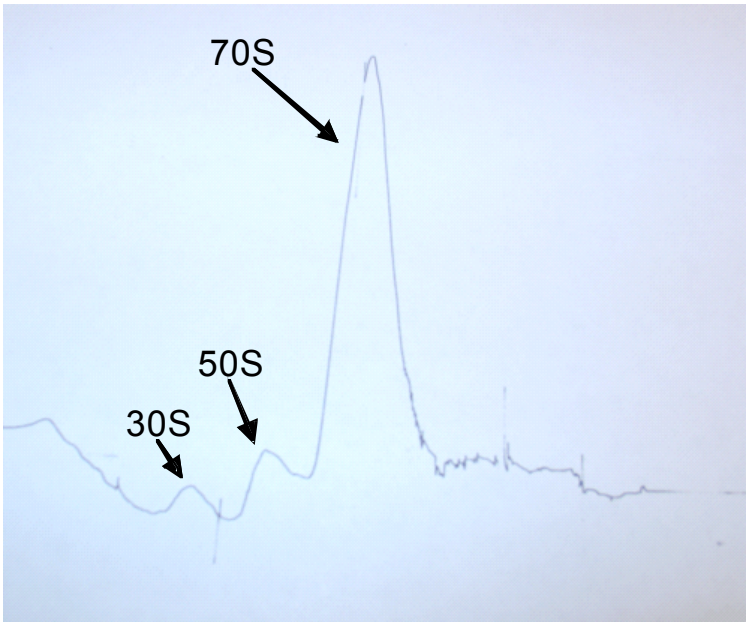


Figure S2A,B

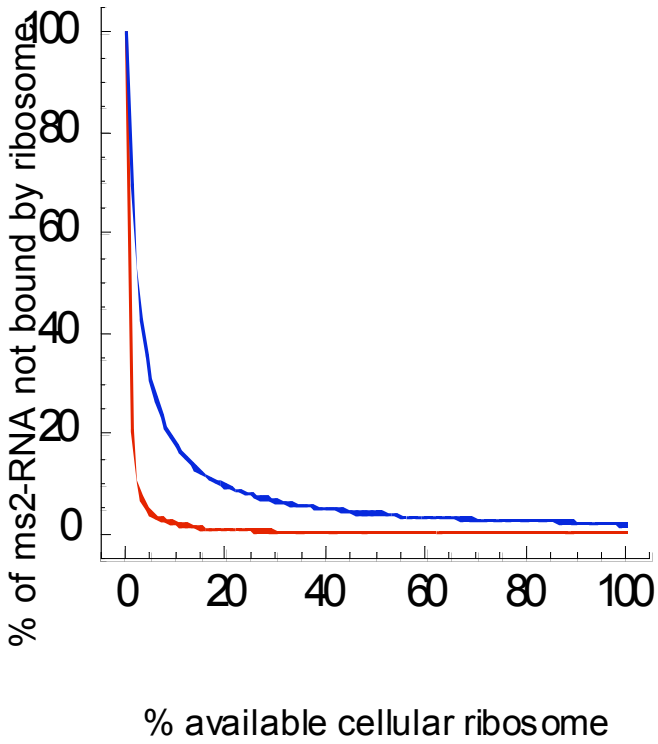

% of ms2-RNA not bound by ribosome
100
80
60
40
20
0
0
20
40
60
80
100
% available cellular ribosome

Figure S3

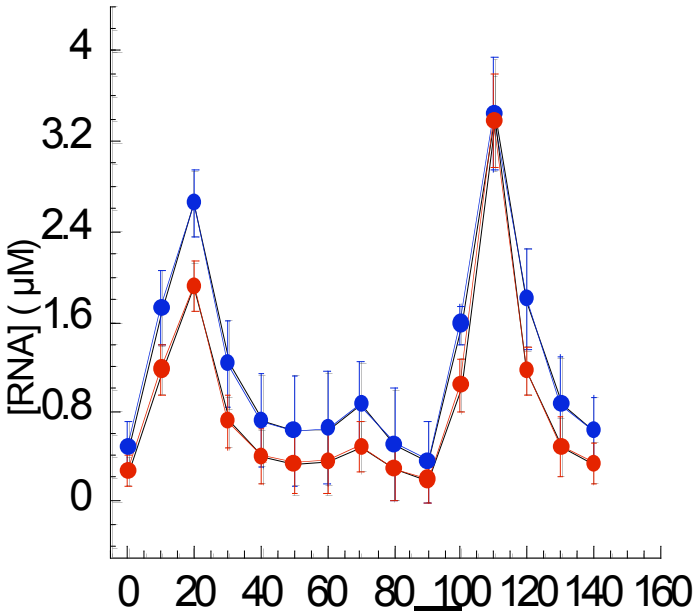

4
3.2
2.4
1.6
0.8
0
[RNA] (μM)
0
20
40
60
80
100
120
140
160
Time after induction (min)

Figure S4

A

B

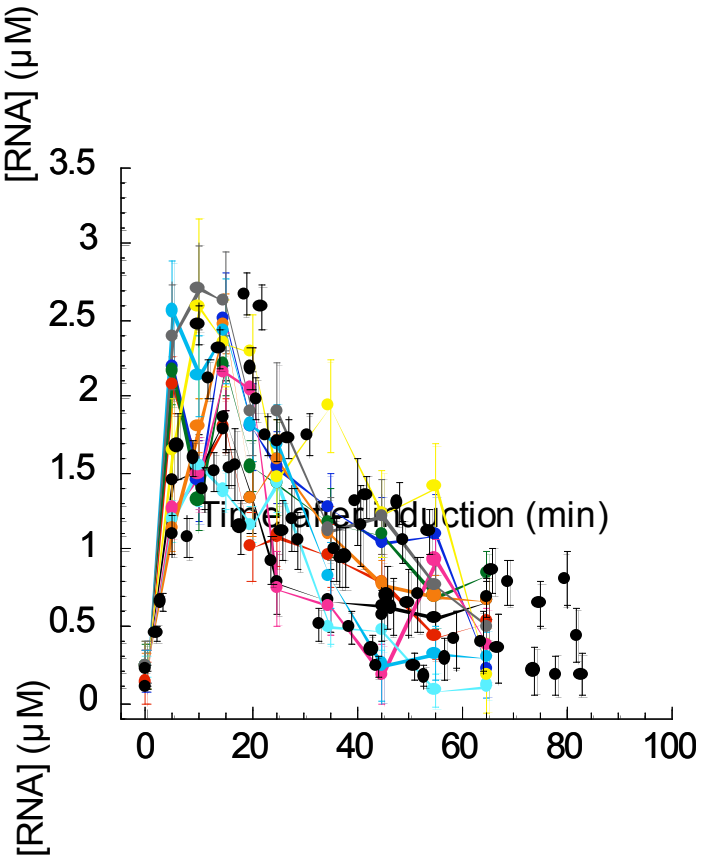


Figure S5A,B

C

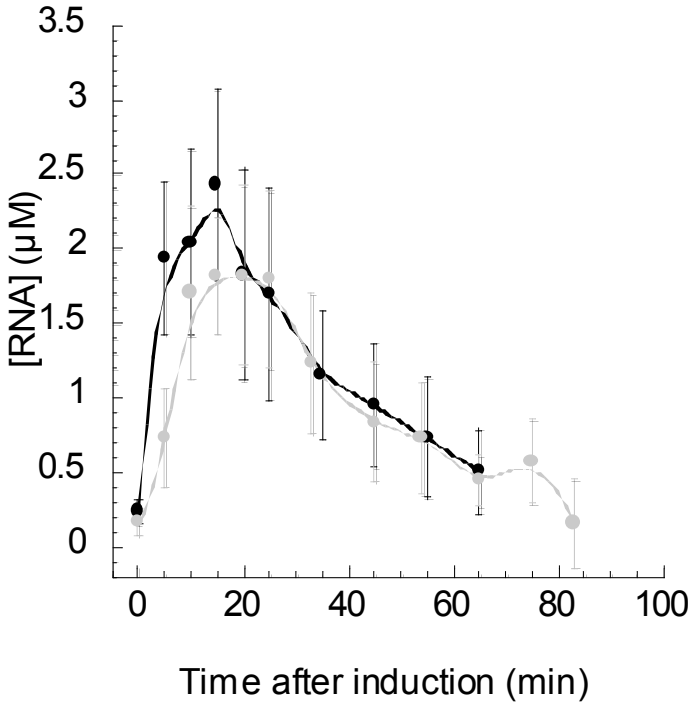

3.5
3
2.5
2
1.5
1
0.5
0
[RNA] (µM)
0
20
40
60
80
100
Time after induction (min)

Figure S5C

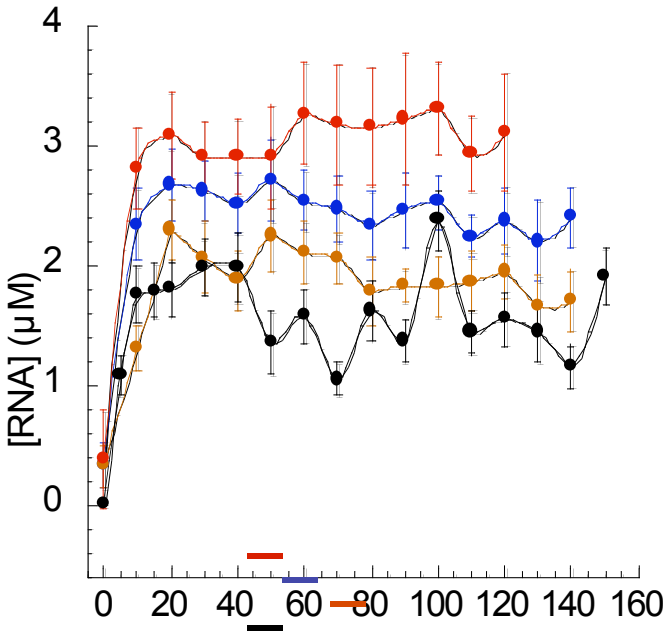

4
3
2
1
0
[RNA] (µM)
0
20
40
60
80
100
120
140
160
Time after induction (min)

Figure S6