Supporting Information

A fluorescence *in situ* hybridization method to quantify mRNA translation by visualizing ribosome-mRNA interactions in single cells.

Kelly S. Burke, Katie A. Antilla, and David A. Tirrell*

Division of Chemistry and Chemical Engineering, California Institute of Technology, 1200 East California Boulevard, Pasadena, California 91125, United States

*Email: tirrell@caltech.edu

Table of Contents

Materials and Methods

Dulbecco's Modified Eagle Medium (DMEM) (#12491015), Fetal Bovine Serum (FBS) (#10438026), Penicillin-Streptomycin (5,000 U/mL) (#15070063), Trypsin-EDTA (0.05%) (#25300054), RIPA buffer (#89900), and Human Plasma Fibronectin (#33016015) were purchased from ThermoFisher (Tustin, CA). SecureSeal hybridization chambers (8 well, 7mm x 7mm x 0.8mm, SKU 621503) were purchased from Grace BioLabs (Bend, OR), and 22mm x 50mm No. 1 glass coverslips were purchased from VWR (Brisbane, CA). All DNA oligonucleotide probes were designed using Stellaris Probe Designer version 4.2 (LGC Biosearch Technologies) and purchased from Integrated DNA Technologies (San Diego, CA). HCR hairpins were purchased pre-coupled to fluorophores from Molecular Instruments (Pasadena, CA). Formamide (SKU F9037), dextran sulfate (SKU D8906), puromycin (SKU P8833), hemin (SKU 51280), Benzonase Nuclease (SKU E1014) and cOmplete Protease Inhibitor Cocktail (SKU 4693116001) were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies for FTH1 (ab183781) and ACTB (ab8227) were purchased from Abcam (Burlingame, CA). A goat anti-rabbit IgG secondary antibody coupled to Alexa 488 (A-11034) was purchased from Life Technologies (Carlsbad, CA).

Images were acquired on a Zeiss LSM 800 laser scanning confocal microscope operated by the Biological Imaging Facility of the Beckman Institute at Caltech. Imaging data were analyzed with the FISH-quant program written by Florian Mueller, the Cell Profiler program developed by the Broad Institute Imaging Platform, and the XPIWIT software tool developed by Johannes Stegmaier and the Center for Advanced Methods in Biological Image Analysis at the Beckman Institute (CAMBIA).

Cell Culture. NIH 3T3 fibroblasts were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, and 50 μg/mL streptomycin at 37˚C in 5% CO_2. Cells were passaged 2-3 times per week. In preparation for each imaging experiment, cells were trypsinized and transferred to glass coverslips pre-coated with 10 μg/mL human plasma fibronectin in 1X PBS, then grown on the coverslips overnight.

For puromycin and hemin treatments, cells were grown on coverslips overnight and then the appropriate reagent was added directly to the cell culture medium. Cells were incubated with the reagents at 37˚C and 5% CO_2. Puromycin was added at a final concentration of 200 μg/mL (from a stock 50-mg/mL solution in water) and left for 1 h. Hemin was added at a final concentration of 25 μM (from a 50-mM stock solution in DMSO) and left for 4 h, 12 h, or 24 h. A vehicle control sample was incubated with 0.2% DMSO for 24 h. Hemin stocks were prepared fresh before each experiment.

***In Situ* Hybridization and Hybridization Chain Reaction (HCR)**. Cells were fixed with 4% formaldehyde in 1X PBS for 30 min at room temperature (25°C). The reaction was quenched with 0.1 M glycine in 1X PBS for 5 min and then cells were washed once with 1X PBS. Fixed cells were permeabilized with 0.1% SDS in 1X PBS for 10 min with gentle rocking. Cells were washed once with 0.1% Triton in 1X PBS, then twice with 1X PBS. Cells were stored in 70% ethanol at 4˚C for at least 2 h and up to 3 days before hybridization.

For hybridization, coverslips were removed from ethanol and air dried. Secure-Seal hybridization chambers were attached to each coverslip. Cells were incubated overnight at 37˚C in a humid chamber with 1-2 nM/oligo of probes for ribosomes and mRNA in a hybridization buffer of 10% formamide and 10% dextran sulfate in 2X SSC.

The next day, the probe solutions were removed, and cells were washed three times with a solution of 35% formamide and 0.1% Triton in 2X SSC, then three times with 2X SSC, to remove excess probes not bound to RNA. Cells were then incubated for 30 min at 40˚C with a pre-heated solution of 10 nM linker probe in a hybridization buffer consisting of 35% formamide and 10% dextran sulfate in 2X SSC ("linker hybridization buffer"). After incubation, cells were washed three times with 0.1% Triton in 2X SSC and twice with 2X SSC, then were left to cool for 15-20 min at room temperature. Cells were finally washed three times with 35% formamide and 0.1% Triton in 2X SSC and three times with 2X SSC to remove any unbound or partially-hybridized linker probes.

For HCR, the basic protocol of Choi and coworkers was followed with modifications[1]. Fluorescently labeled HCR hairpins were first snap-cooled (heated to 95˚C for 90 sec, then allowed to cool at room temperature for 30 min). The cells were then incubated for 1 h at room temperature (25˚C) with 85 nM hairpins in a hybridization buffer of 10% dextran sulfate in 2X SSC. Following HCR, the cells were washed twice with 0.1% Triton in 2X SSC, incubated with 1 μg/mL DAPI for 1 min, washed twice with 2X SSC, and then kept in 2X SSC for imaging.

Western Blot. Cells were left untreated, incubated with 50-μM hemin (from a 50-mM stock solution in DMSO) for 4 h, 12h, or 24h, or incubated with 0.2% DMSO for 24 h as a vehicle control. Cells were then washed with cold PBS and lysed in RIPA buffer supplemented with one cOmplete protease inhibitor tablet per 10 mL of buffer for 30 min on ice with gentle rocking. Lysates were collected using a cell scraper and were treated with 1 μL of Benzonase Nuclease per 500 μL of lysate for 10 min at 37˚C. Lysates were heated in a loading buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 5% BME at 95˚C for 10 min and immediately loaded onto a pre-cast SDS PAGE gel. Each lane was loaded with

15 μg of protein. After electrophoretic separation, proteins were transferred to a nitrocellulose membrane, which was blocked with 5% nonfat dry milk (NFDM) in PBS and 0.1% Tween-20 (PBST) for 1 h. The membrane was then incubated with a 1/1000 dilution each of the FTH1 antibody and ACTB loading control in 5% NFDM in PBST overnight at 4˚C with gentle rocking. The next day, the membrane was washed three times with PBST for 10 min each with gentle rocking. The membrane was then incubated with an Alexa Fluor 488-conjugated secondary antibody at 1/10,000 dilution in 5% NFDM in PBST at room temperature for 1 h. The membrane was washed three times with PBST for 5 min each and imaged with a Typhoon Trio Imager.

Fluorescence Imaging. All images were collected with a Zeiss LSM 800 laser scanning confocal microscope, using a 63X, NA 1.4 Plan-Apochromat objective. Resolution was set to 1024 x 1024 with a digital zoom of 0.7X and line averaging of 2. Excitation laser sources and emission ranges were 405 nm/400-510 nm (DAPI, shown as blue), 488 nm/510-560 nm (Alexa 488, shown as red), and 561 nm/560-700 nm (Alexa 546, shown as green). Each image was collected as a 3D stack of 15-30 images with a spacing of 0.4 μm in the z-direction between slices.

Image Analysis. Images were analyzed using the MATLAB-based program FISH-quant[2]. Outlines of cells and nuclei were drawn manually in FISH-quant or automatically using Cell Profiler[3]. A 3D dual-Gaussian filter was used in FISH-quant for background subtraction. Spots corresponding to ribosome-mRNA interactions or mRNA transcripts were identified by fitting with a 3D Gaussian function. The intensity and the width of the 3D Gaussian were thresholded to exclude autofluorescence and non-specific signals. Ribosome-mRNA interaction spots and mRNA transcript spots were identified independently and then analyzed for colocalization. The distance threshold for colocalization was set at 420 nm, which is equal to 3 pixels in all of our

images. Raw intensity values were used for measurements of fluorescence intensity. For measuring colocalization of nuclear transcripts, a custom processing pipeline was implemented in the XPIWIT software tool to create a 3D mask based on DAPI fluorescence for each image[4]. The pipeline consisted of a median filter for noise reduction, a binarization of the image using Otsu's method and a morphological closing to remove holes in the mask. The final 3D mask was multiplied with the raw images and thus, only the spots within the 3D mask were kept for analysis. Any remaining spots on the edges of the nuclear mask were removed manually in FISH-quant before colocalization analysis.

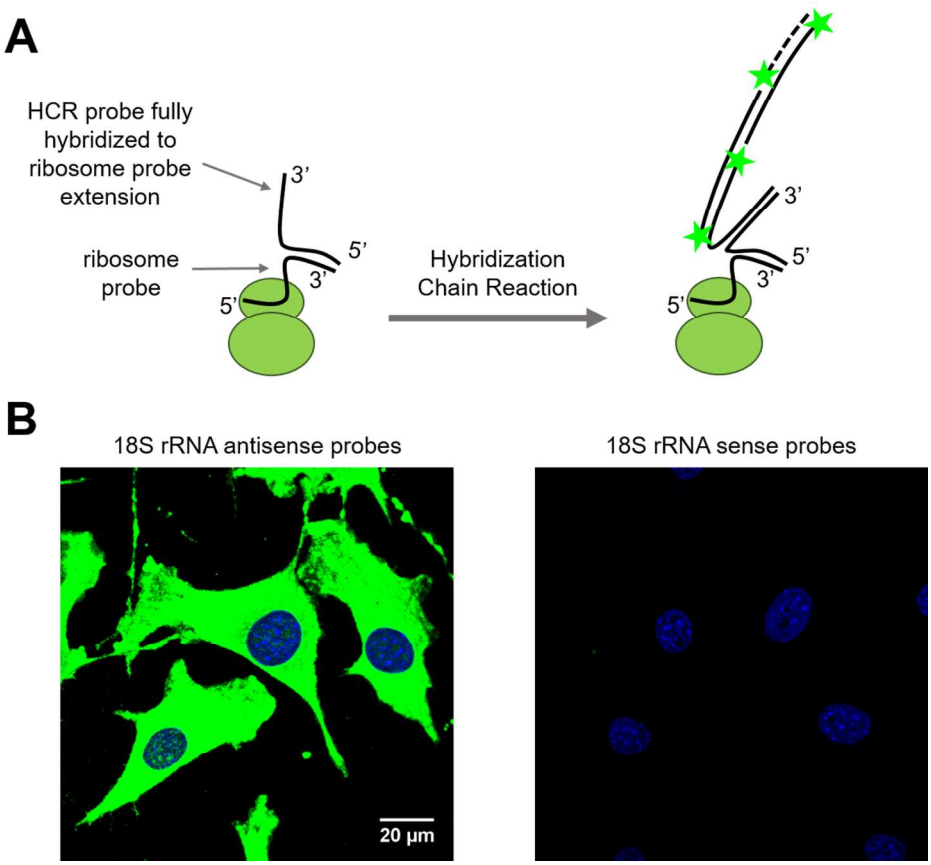

Figure S1. (A) Schematic of experimental design to measure fluorescence from ribosome probes. Multiple ribosomes probes are hybridized to the 18S rRNA, but for illustration purposes, only a single probe is shown. (B) Fluorescence from antisense or sense probes for 18S rRNA. Green = ribosome signal, Blue = DAPI. Bright fluorescence signal is detected throughout cells with antisense probes. No signal is detected with sense probes.

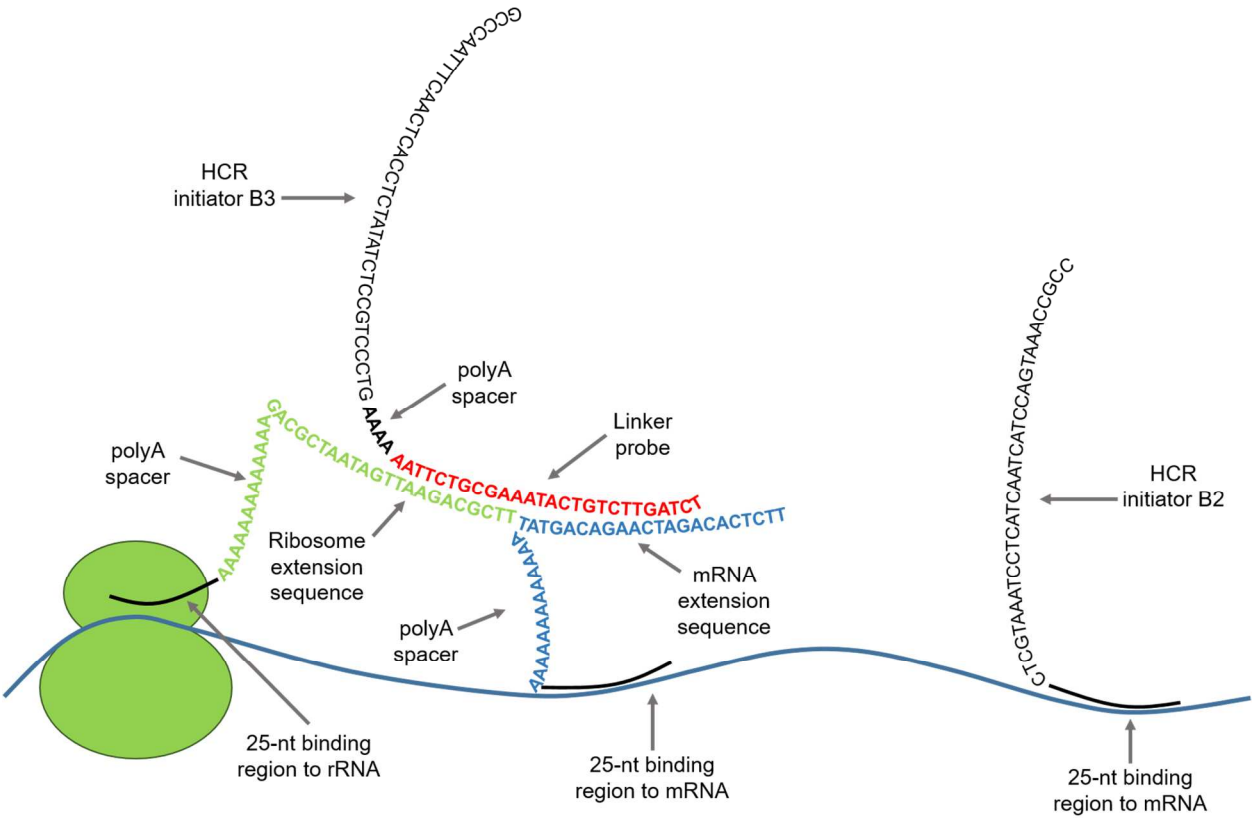

Figure S2. Schematic showing shared nucleotide sequences of ribosome probes and mRNA probes, as well as the sequence and binding location of the linker probe.

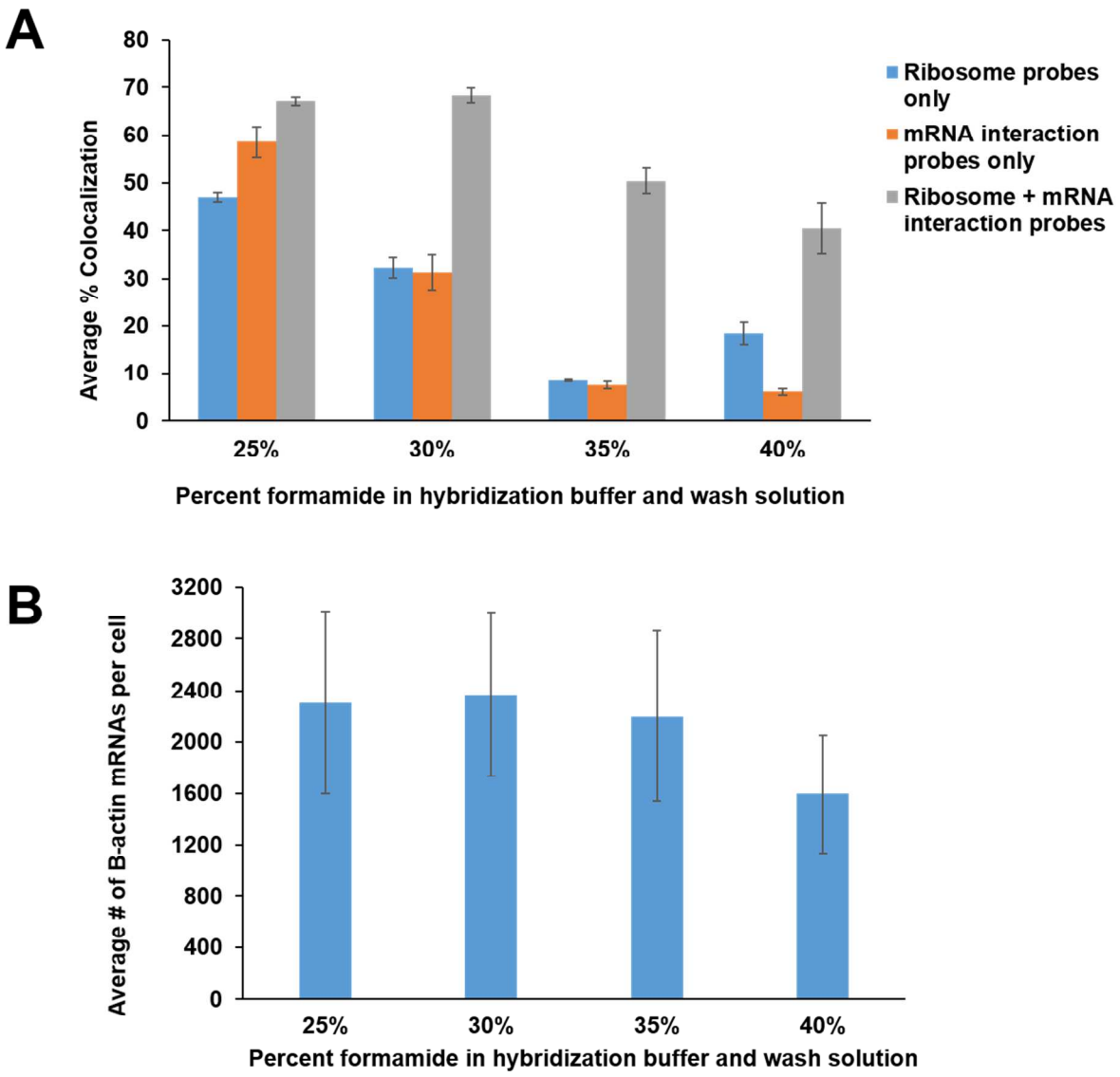

Figure S3. Effect of formamide concentration in the linker hybridization buffer and wash solution on (A) colocalization of β-actin mRNA transcript signals to background signal relative to ribosome-mRNA interaction signal and (B) hybridization of the β-actin mRNA transcript probes. In solutions with 25% and 30% formamide, the colocalization of β-actin transcript signals to ribosome or mRNA interaction probes alone may contribute significantly to the colocalization measured with both probes added. The buffer and wash solution with 40% formamide compromises hybridization of mRNA transcript probes, resulting in a significantly lower measure of mRNA transcripts per cell. Hence, we chose 35% formamide for all experiments in this study. Error bars, standard deviation. For (A) and (B), n = 3-9 cells or 20-27 cells per measurement, respectively.

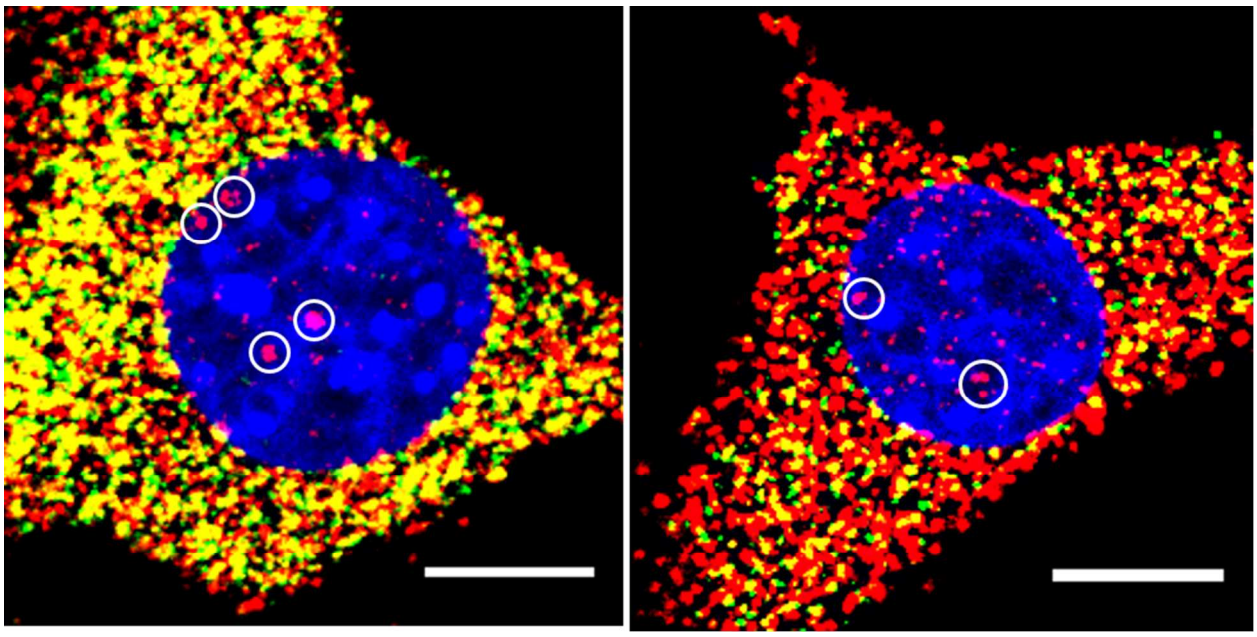

Figure S4. Interaction of β-actin and FTH1 with ribosomes in cell nuclei. Confocal images of ribosome-mRNA interactions for β-actin (left) and FTH1 (right). Images show merged signals from Alexa 488 and Alexa 546 channels. Red = Alexa 488 fluorescence from mRNA transcript probes. Green = Alexa 546 fluorescence from mRNA interaction probes. Yellow = colocalization of red and green indicating an mRNA with bound ribosomes. Circled red spots in the nucleus illustrate lack of ribosome interaction with these transcripts. Scale bar = 10 μm. The fraction of β-actin transcript spots colocalized with ribosomes in the nucleus is 0.12 ± 0.12 (n = 10 cells). The fraction of FTH1 transcript probes colocalized with ribosomes in the nucleus is 0.12 ± 0.08 (n = 15 cells).

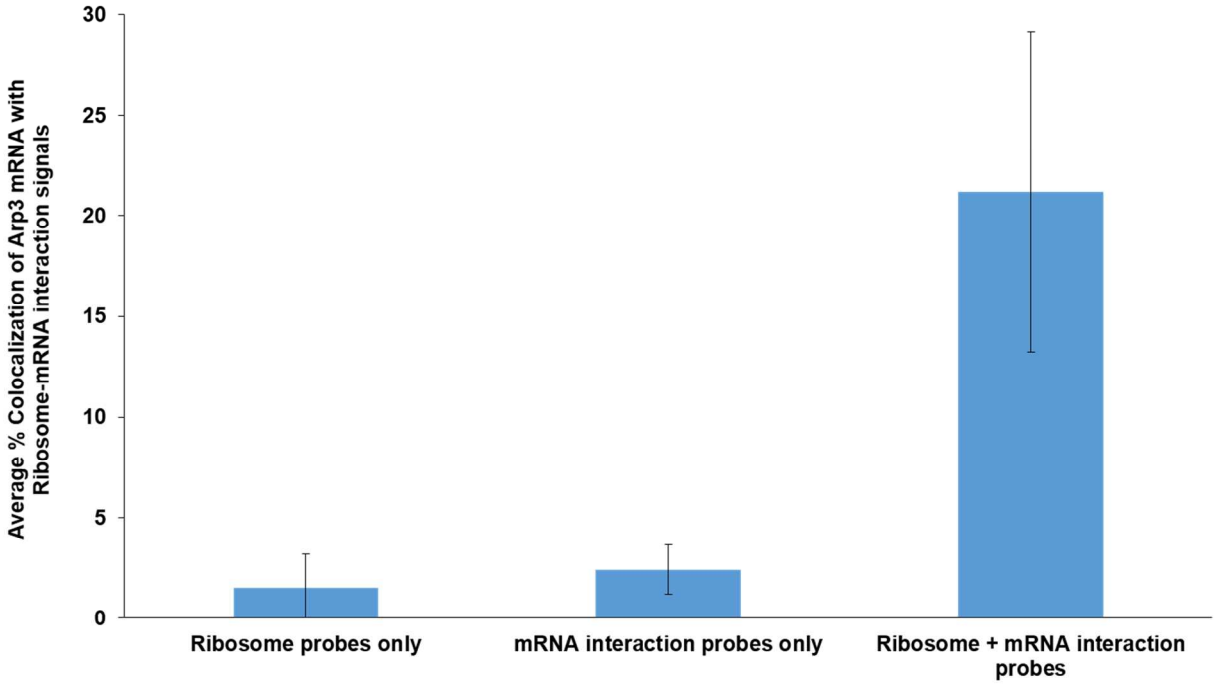

Figure S5. Fraction of Arp3 transcript spots colocalizing with ribosome-mRNA interaction spots, when different probe sets are added: ribosome probes only, Arp3 mRNA interaction probes only, or the combination of ribosome and Arp3 mRNA interaction probes. Error bars, standard deviation. Data represents three independent experiments, n = 20 – 31 cells per measurement.

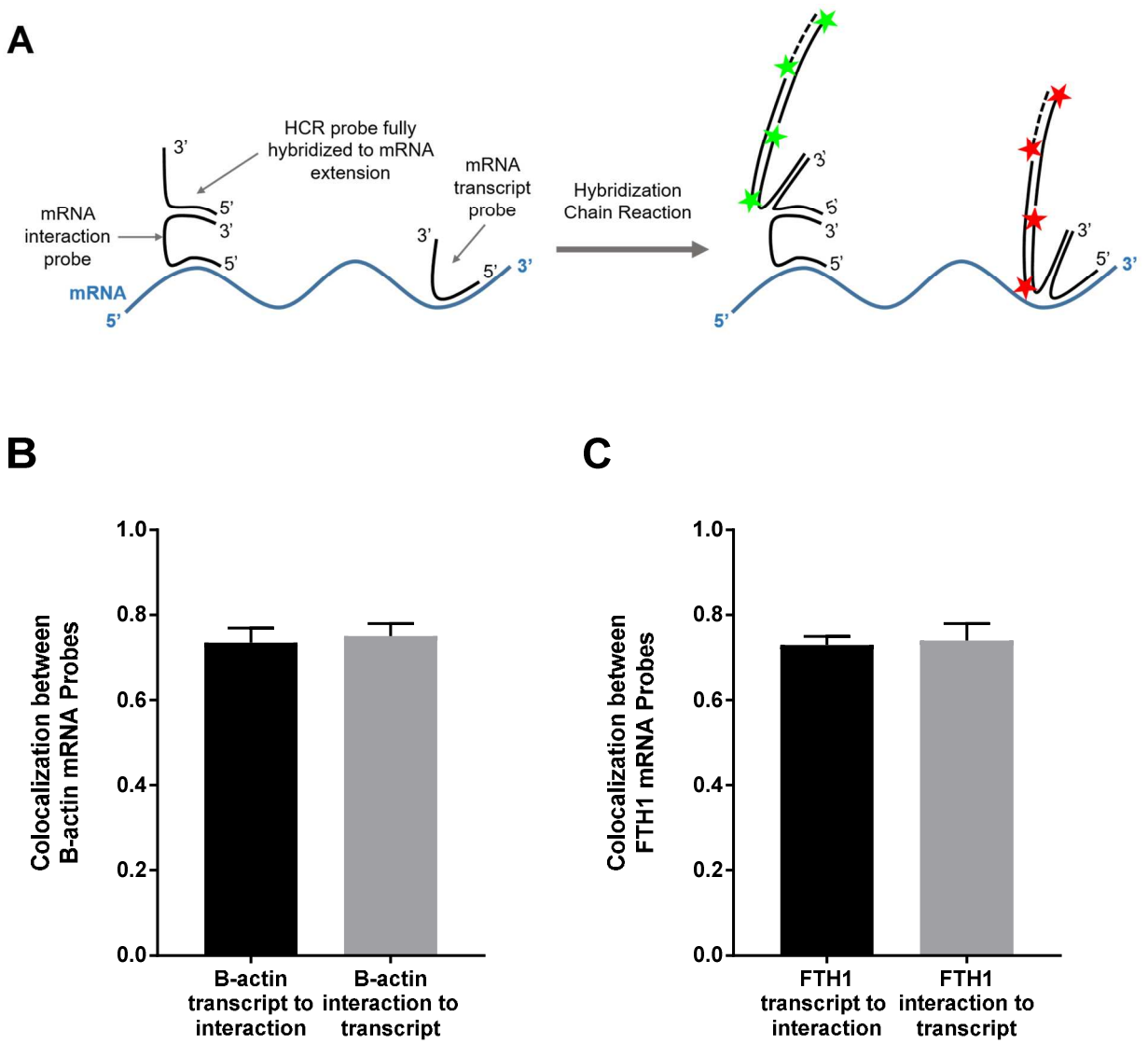

Figure S6. Colocalization between transcript probes and interaction probes for β-actin and FTH1. (A) Schematic of experimental design to test extent of colocalization between mRNA interaction and transcript probes. Multiple interaction and transcript probes are hybridized to a single mRNA, but for illustration purposes, only a single probe per type is shown. (B) Average colocalization between β-actin mRNA probes in the cytoplasm. The fraction of β-actin transcript probes colocalized with interaction probes is 0.74 ± 0.03. The fraction of interaction probes colocalized with transcript probes is 0.75 ± 0.03. Data represent two independent experiments, n = 8 cells. (C) Average colocalization between FTH1 mRNA probes in the cytoplasm. The fraction of FTH1 transcript probes colocalized with interaction probes is 0.73 ± 0.02. The fraction of interaction probes colocalized with transcript probes is 0.74 ± 0.04. Data represent three independent experiments, n = 20 cells.

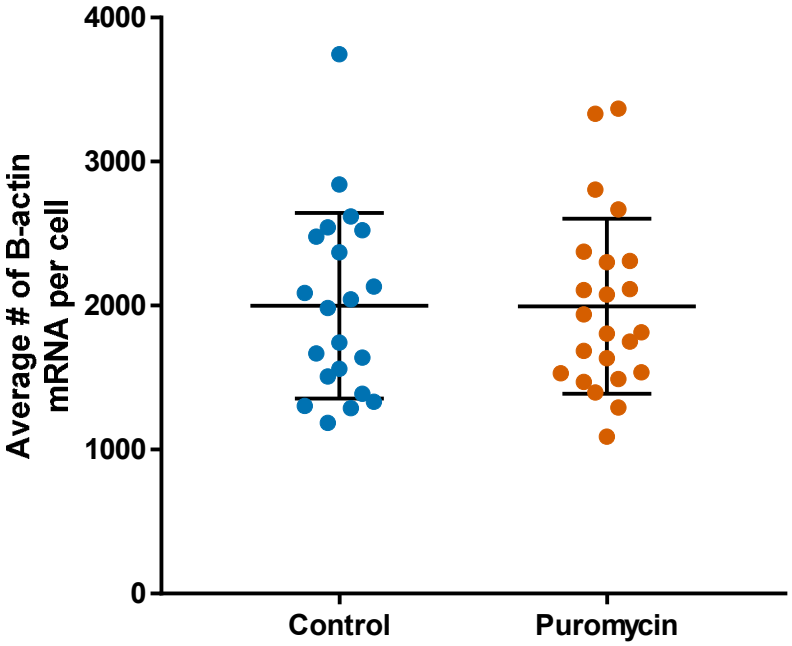

Figure S7. Levels of β-actin mRNA in control and puromycin-treated NIH 3T3 fibroblasts. The average numbers (± standard deviation) of β-actin mRNAs per cell are 1999 ± 645 (n = 21 cells) and 1995 ± 608 (n = 23 cells) for control and puromycin-treated cells, respectively. Differences in the values are not statistically significant, P = 0.9843.

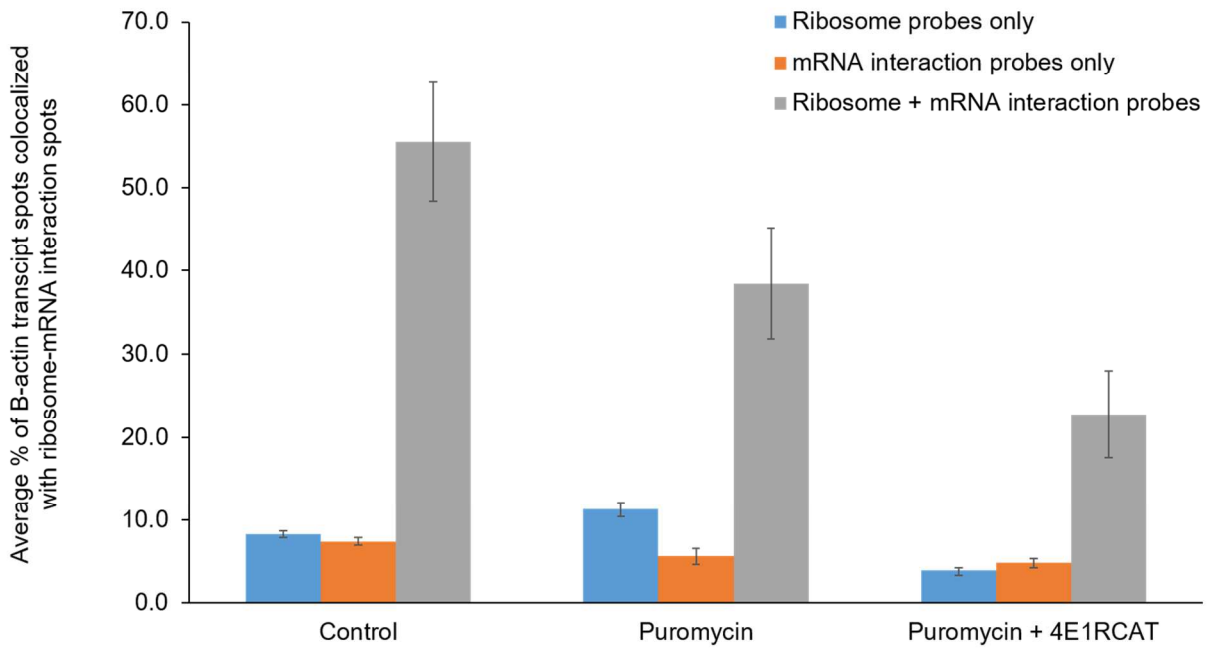

Figure S8. Fraction of β-actin mRNA transcript spots per cell colocalized with a ribosome-mRNA interaction spot after no treatment (Control), treatment with 200 ug/mL of puromycin for 1 h, or treatment 200 μg/mL of puromycin and 5 μM 4E1RCat for 1 h. n = 3-14 cells per measurement. Error bars, standard deviation. We thank a reviewer for suggesting this experiment.

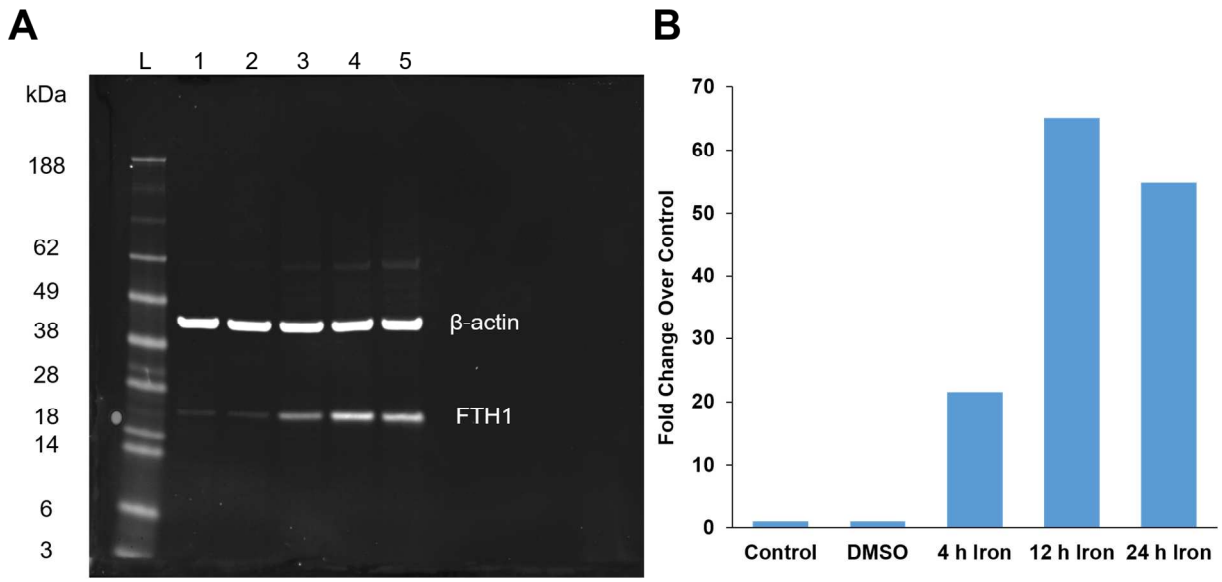

Figure S9. (A) Western blot and (B) quantification of increased FTH1 protein levels in NIH 3T3 cells treated with iron in the form of hemin at 50 μM for 4, 12, and 24 h. Lysates were first blotted against primary antibodies for FTH1 and β-actin and then blotted against a goat anti-rabbit IgG secondary antibody coupled to Alexa 488. β-actin was used as a loading control. L = ladder, 1 = No treatment, 2 = 0.2% DMSO for 24 h (vehicle), 3 = 4 h hemin, 4 = 12 h hemin, 5 = 24 h hemin. We used ImageQuant TL software to quantify the fold change in FTH1 protein level per treatment condition compared to the control. We first performed a background subtraction of the Western blot with the rolling ball method to remove the baseline intensity. We then measured the integrated intensity of each band and determined the ratio of the FTH1 band intensity to the β-actin band intensity per lane. The fold change for each treatment condition was calculated by dividing its band intensity ratio for by the ratio of the control lane.

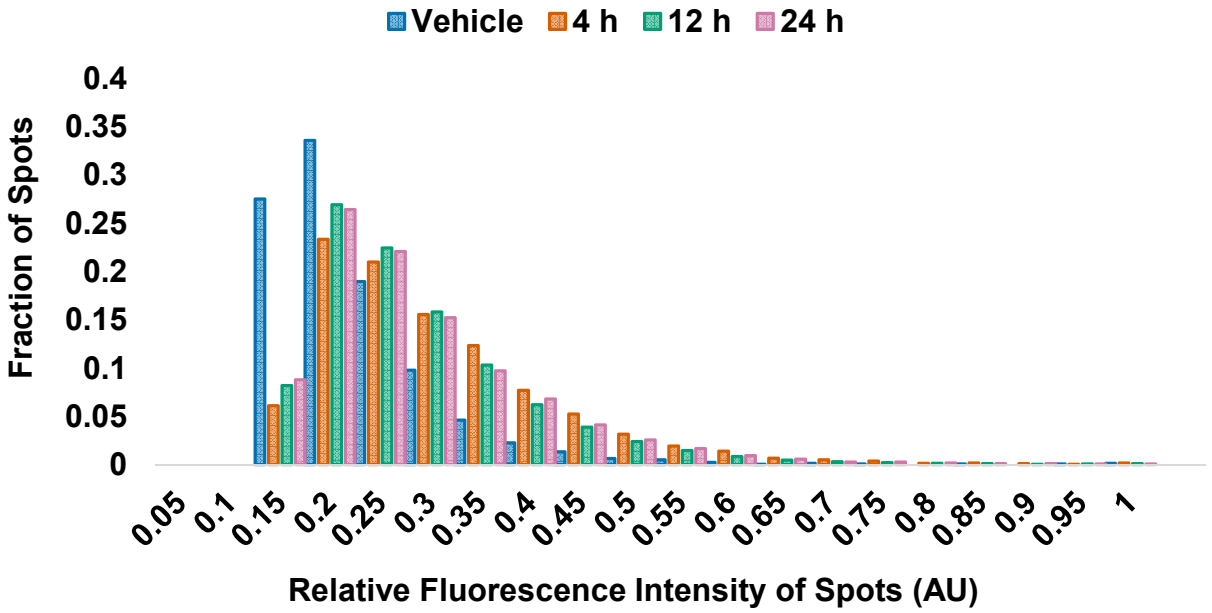

Figure S10. Distribution of fluorescence intensities of ribosome-mRNA interaction spots for FTH1 in cells treated with iron for 4 h, 12 h, or 24 h compared to a vehicle control. Representative results from one experiment. Vehicle, n = 10 cells and 2770 spots; 4 h, n = 16 cells and 13360 spots; 12 h, n = 16 cells and 11392 spots; 24 h, n = 15 cells and 11890 spots.

Table S1. Sequences of all oligonucleotide probes used in this study. See attached spreadsheet.

Table S2. Probe sequences for human 18S rRNA. See attached spreadsheet.

Table S3. Fraction (average ± standard deviation) of putative β-actin transcripts (Alexa 488 spots) in the cytoplasm colocalized with Alexa 546 spots, which may correspond to linker probes or dye-labeled HCR hairpins, when only β-actin interaction probes, only ribosomes probes, or neither are added. Data represent two independent experiments. n = 4-6 cells per measurement per experiment.

	Ribosome probes only	mRNA interaction probes only	Neither ribosome nor mRNA interaction probes (Linker + HCR background)
Control	0.08 ± 0.01	0.07 ± 0.02	0.007 ± 0.003
Puromycin	0.09 ± 0.03	0.09 ± 0.03	0.004 ± 0.002

Table S4. Fraction (average ± standard deviation) of putative FTH1 transcripts (Alexa 488 spots) in the cytoplasm colocalized with Alexa 546 spots, which may correspond to linker probes or dye-labeled HCR hairpins, when FTH1 interaction probes, ribosomes probes, or both are omitted. Data represent three independent experiments. n = 5-9 cells per measurement per experiment.

	Ribosome probes only	mRNA interaction probes only	Neither ribosome nor mRNA interaction probes (Linker + HCR background)
Vehicle	0.06 ± 0.02	0.02 ± 0.02	0.006 ± 0.004
4h	0.06 ± 0.02	0.04 ± 0.01	0.005 ± 0.003
12h	0.05 ± 0.02	0.04 ± 0.02	0.005 ± 0.003
24h	0.07 ± 0.01	0.04 ± 0.01	0.005 ± 0.002

Table S5. Fraction of ribosome-mRNA interaction spots colocalizing with β-actin transcript spots with or without puromycin treatment. Data represent two independent experiments. n = 4-6 cells per measurement per experiment.

	Fraction of spots
Control	0.68 ± 0.04
Puromycin	0.68 ± 0.03

Table S6. Fraction of ribosome-mRNA interaction spots colocalizing with FTH1 transcript spots after different treatments with iron. Data represent three independent experiments. n = 5-9 cells per measurement per experiment.

	Fraction of spots
Vehicle	0.54 ± 0.05
4h	0.57 ± 0.04
12h	0.55 ± 0.05
24h	0.58 ± 0.03

References

(1) Choi, H. M. T.; Beck, V. A.; Pierce, N. A. Next-Generation in Situ Hybridization Chain Reaction : Higher Gain , Lower Cost , Greater Durability. *ACS Nano* **2014**, *8*, 4284–4294 DOI: 10.1021/nn405717p C2014.

(2) Mueller, F.; Senecal, A.; Tantale, K.; Marie-nelly, H.; Ly, N.; Collin, O.; Basyuk, E.; Bertrand, E.; Darzacq, X.; Zimmer, C. FISH-Quant : Automatic Counting of Transcripts in 3D FISH Images. *Nat. Methods* **2013**, *10*, 277–278 DOI: 10.1038/nmeth.2406.

(3) Carpenter, A. E.; Jones, T. R.; Lamprecht, M. R.; Clarke, C.; Kang, I. H.; Friman, O.; Guertin, D. A.; Chang, J. H.; Lindquist, R. A.; Moffat, J.; Golland, P.; Sabatini, D. M. CellProfiler: Image Analysis Software for Identifying and Quantifying Cell Phenotypes. *Genome Biol.* **2006**, *7*, R100 DOI: 10.1186/gb-2006-7-10-r100.

(4) Bartschat, A.; Hubner, E.; Reischl, M.; Mikut, R.; Stegmaier, J. XPIWIT - An XML Pipeline Wrapper for the Insight Toolkit. *Bioinformatics* **2016**, *32*, 315–317 DOI: 10.1093/bioinformatics/btv559.