A superfolding Spinach2 reveals the dynamic nature of trinucleotide repeat–containing RNA

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Imaging RNA in living cells is a challenging problem in cell biology. One strategy for genetically encoding fluorescent RNAs is to express them as fusions with Spinach, an ‘RNA mimic of GFP’. We found that Spinach was dimmer than expected when used to tag constructs in living cells owing to a combination of thermal instability and a propensity for misfolding. Using systematic mutagenesis, we generated Spinach2 that overcomes these issues and can be used to image diverse RNAs. Using Spinach2, we detailed the dynamics of the CGG trinucleotide repeat–containing ‘toxic RNA’ associated with Fragile X–associated tremor/ataxia syndrome, and show that these RNAs form nuclear foci with unexpected morphological plasticity that is regulated by the cell cycle and by small molecules. Together, these data demonstrate that Spinach2 exhibits improved versatility for fluorescently labeling RNAs in living cells.

RNA localization is dynamically regulated in cells1,2. A major goal has been to develop genetically encoded systems analogous to GFP to enable imaging of tagged RNAs in living cells (Supplementary Note). We had previously developed Spinach, a 98-nt RNA aptamer that binds 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), a small-molecule mimic of the GFP fluorophore3. Spinach and DFHBI are essentially nonfluorescent when separate but interact to form a brightly fluorescent complex. RNAs fused to Spinach can be expressed and imaged in live cells; for example, we previously labeled the 5S RNA in mammalian cells and observed changes in localization under stress conditions3.

Detection of the 5S RNA tagged with Spinach (5S-Spinach) in mammalian cells requires 1-s exposure despite its high expression level3. In contrast, imaging abundant GFP-tagged proteins typically requires exposure of 10–100 ms under the same imaging conditions. The dimness of 5S-Spinach in cells also contrasts with the high brightness of Spinach measured in vitro3.

Here we show that Spinach exhibits thermal instability and poor folding, which reduces its brightness. Moreover, Spinach fluorescence is reduced when it is fused to target RNAs. Using systematic mutagenesis guided by brightness, thermostability and a new assay to measure folding, we identified mutations that confer thermostability and substantially increase the fraction of properly folded aptamer. The resulting RNA, Spinach2, is a ‘superfolder’ variant of Spinach that exhibits reduced context dependence and is markedly brighter than Spinach in living cells. Using Spinach2, we explored the localization and dynamics of toxic CGG repeat–containing RNAs. Imaging with short exposure times revealed that these RNAs exhibit dynamic localizations that can be readily altered by cell division and small molecules. These data show that the enhanced folding and thermal stability of Spinach2 make it a versatile tool for imaging RNA in living cells.

RESULTS

Low fluorescence of Spinach-tagged RNAs

We sought to investigate ‘toxic RNA’ localization by expressing an RNA containing 60 CGG repeats (CGG)60 previously shown to form intranuclear foci resembling those in patients with Fragile X–associated tremor/ataxia syndrome (FXTAS)4 with a 3’ Spinach tag. However, expression of the (CGG)60-Spinach construct did not result in readily detectable nuclear foci in COS-7 cells in the presence of DFHBI (Fig. 1). Although fluorescence was not detectable, Spinach-tagged RNA formed nuclear foci, as measured by fluorescence in situ hybridization (FISH; Supplementary Fig. 1a).

To test whether the Spinach tag was unstable or degraded independently from the CGG repeat–containing RNA, we carried out FISH with a probe to Spinach and confirmed its presence in these foci (Supplementary Fig. 1a). Tagged and untagged versions of (CGG)60 RNA were equally stable based on quantitative reverse-transcription PCR (qRT-PCR) (Supplementary Fig. 1b). The observation that the Spinach-tagged CGG repeat–containing RNA was abundant in foci but was not fluorescent indicated that Spinach was not fluorescent in the context of the CGG repeat–containing RNA and required modifications to enhance its fluorescence in cells.

We considered several factors that could affect the brightness of Spinach in cells. These include poor ability of DFHBI to permeate into the cell, low intrinsic brightness and poor folding in cells. Poor ability of DFHBI to enter cells is unlikely because it matches that of Hoechst in mammalian cells, with maximal fluorescence achieved in ~30 min (Supplementary Fig. 2). Additionally, in vitro measurements of Spinach-DFHBI fluorescence show that its intrinsic brightness is 80% of that of GFP and 53% of that of eGFP3, which is bright enough for imaging. We therefore

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considered the possibility that Spinach misfolds in cells, which decreases the number of Spinach-tagged RNAs that can bind and activate the fluorescence of DFHBI.

We determined the melting temperature ($T_m$) of Spinach by monitoring the fluorescence of the RNA-DFHBI complex in vitro between 20 °C and 60 °C. Spinach had a $T_m$ of 34 ± 0.6 °C (t.s.e.m.) (Supplementary Table 1), indicating that a substantial fraction of Spinach molecules may be unfolded when imaging at 37 °C.

**Mutational analysis and development of Spinach2**

We identified mutations that could increase the thermostability of Spinach by correcting bulges and mismatches in the predicted structure (Fig. 1b). These results led us to generate Spinach1.1, which has perfect complementarity in stem 1, and Spinach1.2, which has perfect complementarity in both stem 1 and stem loop 3 (Online Methods and Supplementary Fig. 3). Spinach1.1 had slightly enhanced thermostability, with a $T_m$ of 35 ± 0.5 °C and was as bright as Spinach (Fig. 2a and Supplementary Table 1). Spinach1.2 was more thermostable than Spinach and Spinach1.1, with a $T_m$ of 38 ± 0.3 °C (Supplementary Table 1). However, Spinach1.2 was 16% dimmer than Spinach (Fig. 2a), indicating that mutations in stem 1 and stem loop 3 enhance thermostability but do not improve brightness.

Mutations in Spinach1.2 can affect brightness by either reducing the extinction coefficient or quantum yield of Spinach-DFHBI or by increasing the misfolded fraction of Spinach that cannot bind DFHBI. To help distinguish between these alternatives, we developed an assay to measure the fraction of Spinach that is properly folded (Online Methods).

Using this assay with buffers that mimic cytoplasmic ion concentrations, we found that 32 ± 4.2% and 13 ± 2.8% of Spinach is folded at 25 °C and 37 °C, respectively. Spinach1.2 was also largely misfolded, with 27 ± 2.1% and 16 ± 2.3% folded at 25 °C and 37 °C, respectively (Fig. 2). Spinach thus folds poorly, and the increased thermostability of Spinach1.2 does not correspond to improved folding. We next carried out systematic mutagenesis to identify thermostable Spinach mutants with improved folding (Online Methods). We identified six positions in Spinach that maintained or enhanced brightness at 25 °C and maintained Spinach1.2 thermostability; we tested mutations in these alone and in combination (Supplementary Fig. 4 and Supplementary Table 2). The best-performing variant from this screen contained all six mutations and was 1.8- and 2.8-fold brighter than Spinach in vitro at 25 °C and 37 °C, respectively, with a $T_m$ of 38 ± 0.4 °C (Fig. 3 and Supplementary Table 1). We named this mutant Spinach2 (Fig. 1b).

**Characterization of Spinach2 fluorescence**

In our folding assay, a substantially higher fraction of Spinach2 was folded compared to Spinach; 58 ± 4.8% and 37 ± 3.3% of Spinach2 was folded at 25 °C and 37 °C, respectively (Fig. 2c). To determine whether mutations in Spinach2 affect its ability to activate the fluorescence of DFHBI, we calculated the extinction coefficient and quantum yield of Spinach2. In these experiments, we used excess RNA and a limiting concentration of 0.1 μM DFHBI to compare 0.1 μM Spinach-DFHBI against 0.1 μM Spinach2-DFHBI, regardless of any difference in the percentage of each RNA that is folded. We found that Spinach and Spinach2...
had nearly identical photophysical properties (Supplementary Table 1), and that the excitation and emission spectra as well as the dissociation constant (Kd) for DFHBI binding were nearly identical (Fig. 3d,e and Supplementary Table 1). These data suggest that the enhanced brightness of Spinach2 reflects an increase in its folding efficiency.

**Spinach2 retained fluorescence in diverse contexts**

RNA folding can be affected by flanking sequences, which may interact with the RNA aptamer. To test whether sequence context affects folding, we monitored the fluorescence of Spinach and Spinach2 inserted into different RNAs. First, we found that Spinach flanked by 50 nucleotides (nt) of RNA on both the 5′ and 3′ ends was only 20% as bright as identical concentrations (0.1 μM) of Spinach alone (Fig. 2d). Flanked Spinach2 was 90% as bright as Spinach2 alone and tenfold brighter than flanked Spinach (Fig. 2d), indicating that Spinach2 is relatively insensitive to flanking sequence.

Spinach fluorescence in *vivo* is improved by inserting Spinach into the tRNA^{Lys3} sequence, which acts as a folding scaffold. In the case of Spinach, folding increased from 32 ± 4.2% to 50 ± 3.9% at 25 °C and from 13 ± 2.8% to 24 ± 2.4% at 37 °C in the presence of the tRNA (Fig. 2c). In the case of Spinach2, folding increased from 58 ± 4.8% to 80 ± 6.1% at 25 °C and from 37 ± 3.3% to 60 ± 5.4% at 37 °C. For this reason, we used tRNA^{Lys3}-Spinach and tRNA^{Lys3}-Spinach2 in all subsequent tagged constructs and imaging experiments.

We then compared the brightness of Spinach and Spinach2 in fusions with 5S, 7SK and (CGG)60 RNA. The Spinach2 fusions to these RNAs were, respectively, 3-, 6- and 20-fold brighter than the same constructs fused to Spinach (Fig. 2d). Moreover, tagged Spinach2 retained more than 70% of its fluorescence relative to Spinach2 alone in all cases (Fig. 2d).

**Spinach2 exhibited increased fluorescence in cells**

In *Escherichia coli*, Spinach2 was 1.4-fold brighter at 25 °C and 2.1-fold brighter at 37 °C than Spinach (Fig. 3b). We normalized aptamer abundance to 16S RNA and found it to be essentially identical for all samples (Fig. 3c).

We next determined whether 5S-Spinach2 is brighter in mammalian cells. HEK293T cells expressing 5S-Spinach or 5S-Spinach2 exhibited the expected diffuse nuclear and cytoplasmic distribution (Fig. 4a) and 5S-Spinach2 showed 3.2-fold higher signal than 5S-Spinach (Fig. 4b). We also compared the brightness of Spinach-7SK and Spinach2-7SK in HeLa cells. 7SK localizes to nuclear speckles. Expression of Spinach-7SK showed no detectable signal, but Spinach2-7SK labeled intranuclear foci that colocalized with mCherry-tagged SC35, a known protein component of nuclear speckles (Fig. 4c). These data demonstrate improved RNA imaging in live cells using Spinach2.
mitosis in imaging medium containing DFHBI. With the CGG-Spinach2 vector undergoing foci. (de novo) displayed fluorescence above background. 2 h later incubated with imaging medium expressing (CGG)60-Spinach2 0 h and 48 h after transiently transfected COS-7 cells. Spinach2 fluorescence was highly (G+C)-rich, it has been proposed that they form highly stable RNA localization in the nucleus. Because these RNA complexes are highly heterogeneous in appearance (Supplementary Fig. 4b). Thus (CGG)60-Spinach2 can be used to study the dynamics of toxic RNA aggregates.

Live-cell imaging of CGG repeat–containing RNA aggregates

We next monitored the formation of (CGG)60-Spinach2 foci in transiently transfected COS-7 cells. Spinach2 fluorescence was detectable as early as 3 h after transfection. (CGG)60-Spinach2 signal was initially diffusely nucleoplasmic, with formation of foci evident within 1 h after transfection (Fig. 1a and Supplementary Video 1). Number, size and brightness of foci increased over the course of the experiment. These data indicate that CGG repeat–containing RNA aggregates rapidly after expression.

To examine the stability of (CGG)60-Spinach2 RNA, we measured fluorescence after treating cells with actinomycin D, a potent transcription inhibitor. Spinach2 signal was stable and remained unchanged for up to 8 h (Fig. 5c), at which point we observed actinomycin D–mediated cytotoxicity.

To test the stability of (CGG)60-Spinach2 foci over longer periods, we controlled (CGG)60-Spinach2 transcription using the TET-off system (Online Methods). Immediately after inhibition of transcription, 94 ± 1.7% of transfected cells contained foci. These foci were long-lived: after 24 h and 48 h, 88 ± 5.6% and 82 ± 6.5% of cells retained foci, respectively (Fig. 5d,e). These results were supported by qRT-PCR results, which demonstrate that (CGG)60 and (CGG)60-Spinach2 RNA are highly stable. The stability of these RNAs is most likely due to their incorporation into nuclear foci, since (CGG)60 RNAs, which do not form foci, are markedly less stable (Supplementary Fig. 1b).

As these repeat-containing RNAs are relatively resistant to degradation and form thermodynamically stable duplexes, we tested whether they form static foci. Time-lapse imaging revealed that foci were mobile and could merge to form larger foci (Fig. 5a and Supplementary Video 1). This dynamic behavior was also apparent in dividing cells (Fig. 5b). During division, the multiple foci typically present in the cell coalesced to form a large single aggregate that subsequently extended into a long linear structure. This long aggregate was divided between daughter cells. The RNA then became diffusely nucleoplasmic before reaggregating into foci. These results suggest that CGG repeat–containing RNA aggregate into foci and disaggregate during the cell cycle.

A small molecule can disrupt RNA aggregates

We next asked whether small molecules can induce disaggregation of foci of CGG repeat–containing RNA. No molecules have been shown to disrupt existing aggregates, although two drugs prevent the formation of CGG repeat–containing RNA foci in transfected cells. These are tautomycin and 1a, a small molecule
that binds CGG repeat–containing RNA and disrupts its binding to a CGG-binding protein, DGCR8 (refs. 4,21). We confirmed that both drugs prevented formation of \((CGG)_{60}\text{-}\text{Spinach2}\) foci (Fig. 6a,b).

To determine whether 1a can disrupt existing foci, we treated COS-7 cells expressing \((CGG)_{60}\text{-}\text{Spinach2}\) with the drug. We observed no change in foci upon imaging every 5 min for 2 h (Fig. 6c). After 48 h of treatment, the number of cells with foci only changed slightly, from \(94 \pm 2.8\%\) to \(86 \pm 3.5\%\) (Supplementary Fig. 5a). Furthermore, 48 h of treatment with 1a did not induce the dissociation of Sam68 from \((CGG)_{60}\text{-}\text{Spinach2}\) foci (Supplementary Fig. 5b). These results show that 1a can prevent formation of foci, but does not readily disrupt existing foci, even after long treatments.

In contrast, tautomycin induced disassembly of foci in as little as 1 h (Fig. 6c and Supplementary Video 2). The disaggregated \((CGG)_{60}\text{-}\text{Spinach2}\) remained as diffuse nucleoplasmic staining in cells (Fig. 6c). Removal of tautomycin after 24 h of treatment was not sufficient to restore foci (Supplementary Fig. 6), suggesting that tautomycin induces cellular changes that prevent reaggregation.

To test whether the effect of tautomycin on \((CGG)_{60}\text{-}\text{Spinach2}\) foci was due to inhibition of its known targets protein phosphatase-1 (PP1) or protein phosphatase-2A (PP2A) 22, we treated cells with okadaic acid at a concentration that also inhibits \((CGG)_{60}\text{-}\text{Spinach2}\) foci. These RNAs had been thought to form stable (G+C)-rich aggregates 17,20. Our studies show that the RNA component of these foci is highly dynamic and undergoes considerable morphologic rearrangements, especially during cell division. These results suggest that CGG repeat–containing RNAs bind to preexisting nuclear structures that are normally partitioned during cell division. This idea is supported by previous studies demonstrating colocalization of CGG repeat–containing RNAs with various intranuclear markers 4.

DISCUSSION

As Spinach exhibited poor thermal stability and folding when fused to other RNAs, we developed Spinach2, which has nearly identical photophysical properties to Spinach yet displays enhanced folding both alone and in the context of flanking RNA, at 25 °C and 37 °C. The improved folding is more apparent when Spinach2 is fused to other RNAs. For example, Spinach2 retained 80% of its fluorescence when fused to the CGG repeat–containing RNA, whereas Spinach is essentially nonfluorescent in this context. However, it is possible that other flanking sequences will affect fluorescence of Spinach2, so first, fluorescence of \textit{in vitro}–transcribed Spinach2–tagged RNA should be compared with that of untagged Spinach2. If the tagged RNA lacks fluorescence \textit{in vitro}, inserting Spinach2 at other sites may restore fluorescence by providing flanking sequences that are more compatible with folding of Spinach2.

Both \((CGG)_{60}\text{-}\text{Spinach2}\) and Spinach2-7SK formed RNA–enriched foci in the cell, which makes imaging straightforward. However, imaging RNAs that are present at lower concentrations may require longer imaging times. As multimerization of fluorescent proteins has been used to enhance the imaging of low-abundance proteins 23, an analogous strategy could be adapted to label RNAs with multiple Spinach2 sequences.

We demonstrated the utility of Spinach2 in diverse imaging experiments by imaging the localization of CGG repeat–containing RNAs in living cells, to our knowledge for the first time. These RNAs had been thought to form stable (G+C)-rich aggregates 17,20. Our studies show that the RNA component of these foci is highly dynamic and undergoes considerable morphologic rearrangements, especially during cell division. These results suggest that CGG repeat–containing RNAs bind to preexisting nuclear structures that are normally partitioned during cell division. This idea is supported by previous studies demonstrating colocalization of CGG repeat–containing RNAs with various intranuclear markers 4.

By imaging \((CGG)_{60}\text{-}\text{Spinach2}\) we identified to our knowledge the first compound that can induce disassembly of toxic RNAs. Previous studies have relied on imaging foci-associated RNA-binding proteins, such as Sam68 (refs. 4,15). Direct imaging of toxic RNA provides opportunities to identify small molecules and signaling pathways that affect localization dynamics of CGG repeat–containing RNA in living cells. Assays using \((CGG)_{60}\text{-}\text{Spinach2}\) may enable the identification of additional compounds that can disrupt foci and potentially serve as therapeutics for FXTAS.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
R.L.S., M.D.D. and S.R.J. conceived and designed the experiments, M.D.D. provided compounds and assisted in experiments using 1a and CGG foci, R.L.S. performed experiments and analyzed data, and R.L.S. and S.R.J. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Cell culture conditions. Cell lines were obtained directly from the American Type Culture Collection (ATCC) for all experiments. COS-7 (ATCC-CRL-1651), HEK-293T (ATCC-CRL-11268) and Hela (ATCC-CRM-CCL-2) cells were grown according to ATCC instructions. Cells were screened for mycoplasma contamination before passage using Hoechst 33258, according to ATCC recommendations.

Spinach2 DNA sequence. 5′-GATGTA ACTGAATGAAATGGTG AAGGACGGGTCCAGTAGGTG CTCCGCCAGCTACTTGT TGAAGTAGGTGTAAGCTCGTAACGTAGTTACATC-3′.

Reagents and equipment. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich. Commercially available reagents were used without further purification. Absorbance spectra were recorded with a Thermo Scientific NanoDrop 2000 spectrophotometer with cuvette capability. Fluorescence excitation and emission spectra were measured with a PerkinElmer LS-55 fluorescence spectrometer.

Preparation and analysis of Spinach and Spinach mutants. RNAs were created by using the appropriate single-stranded DNA templates (Integrated DNA Technologies) and PCR amplification using primers that included a 5′ T7 promoter sequence to generate double-stranded DNA templates. PCR products were then purified with PCR purification columns (Qiagen) and used in vitro T7 transcription reactions (Epicentre) as described previously.3 RNA was purified using ammonium acetate in 1 °C increments from 20 °C to 60 °C, with a 5-min incubation at each temperature to allow for equilibration. Fluorescence measurements were performed using a PerkinElmer LS-55 fluorescence spectrometer using the following instrument parameters: excitation wavelength, 460 nm; emission wavelength, 501 nm; slit widths, 10 nm. Signal from the first condition (limiting RNA) was divided by the signal from the second condition (limiting dye) to determine the fraction folded.

Thermostability measurements. Spinach or Spinach2 (1 μM) was incubated in 20 mM Hepes pH 7.4, 100 mM KCl, 1 mM MgCl2 and 10 μM DFHBI. Fluorescence values were recorded in 1 °C increments from 20 °C to 60 °C, with a 5-min incubation at each temperature to allow for equilibration. Fluorescence measurements were performed using a PerkinElmer LS-55 fluorescence spectrometer using the following instrument parameters: excitation wavelength, 460 nm; emission wavelength, 501 nm; slit widths, 10 nm. Curves were fitted using the Boltzmann sigmoidal equation in GraphPad Prism 5 software. Values presented are mean and s.e.m. from three independent measurements.

Folding assay. Our folding assay involves measurement of fluorescence under two conditions, one in which RNA is in excess relative to DFHBI, and one in which the DFHBI is in excess relative to RNA. As Spinach and DFHBI form a 1:1 stoichiometric complex, the maximum amount of complex that can be formed is determined by the limiting component. In the first condition, the fluorescence was determined by incubation of 0.1 μM DFHBI and 100-fold excess (10 μM) Spinach. This value was used to define the fluorescence of 0.1 μM Spinach-DFHBI complex. We assumed that even if nearly all Spinach is misfolded or unfolded, there will be enough properly folded Spinach to stoichiometrically bind 0.1 μM DFHBI. We confirmed this by measuring fluorescence after doubling the RNA to 20 μM, which caused no increase in fluorescence (data not shown). In the second condition, we measured the fluorescence obtained using 10 μM DFHBI and 0.1 μM Spinach. In theory, up to 0.1 μM Spinach-DFHBI can form if all the Spinach is folded. However, if a portion of Spinach is unfolded, the fluorescence will be proportionately less than the fluorescence of 0.1 μM Spinach-DFHBI. Thus, this approach can reveal the fraction of Spinach that is folded under diverse conditions.

Fluorescence was measured for each RNA under the following conditions: (i) 0.1 μM RNA and 10 μM DFHBI, and (ii) 0.1 μM DFHBI and 10 μM RNA. For each condition, the signal from DFHBI without RNA was subtracted from each signal. Fluorescence was measured in 20 mM Hepes pH 7.4, 100 mM KCl and 1 mM MgCl2 at the designated temperature. Fluorescence measurements were performed using a PerkinElmer LS-55 fluorescence spectrometer using the following instrument parameters: excitation wavelength, 460 nm; emission wavelength, 501 nm; slit widths, 10 nm. Signal from the first condition (limiting RNA) was divided by the signal from the second condition (limiting dye) to determine the fraction folded.

Generation of Spinach1.1 and Spinach1.2. Our previous work mutating Spinach and designing Spinach-based sensors demonstrated that stem 1 and stem loop 3 can tolerate various mutations and insertions.2-5. To test the possibility that mismatches in stem 1 adversely affect Spinach thermal stability, we generated a mutant of Spinach with perfect complementarity in stem 1. This mutant, called Spinach1.1, was also mutated to convert the last base pair in stem 1 from U-A to C-G, in an attempt to stabilize stem 1 (Supplementary Fig. 4). Spinach1.1 showed slightly enhanced thermostability, with a Tm of 35 ± 0.5 °C and was as bright as Spinach (Fig. 3a and Supplementary Table 1).

Next we asked whether stem loop 3 could be altered to increase Spinach thermostability. We previously found that alterations in stem loop 3 do not substantially reduce Spinach fluorescence.5 In Spinach, stem loop 3 contains three mismatches and an internal bulge. We generated Spinach1.2 by retaining the mutations in Spinach1.1 and mutating stem loop 3 to eliminate this bulge and introduce perfect complementarity (Supplementary Fig. 3).

Systematic mutagenesis of Spinach1.2. Because elevated G+C content can lead to stable misfolded structures,17,20, we reasoned that decreasing the overall G+C content could promote proper folding. We carried out scanning mutagenesis, mutating every guanidine and cytosine to adenosine or uracil, respectively. In regions where G and C residues were predicted to form a base pair, we mutated both residues to A and U, to maintain the complementarity. Each of these 35 mutants was synthesized in vitro, and the fraction folded was measured at 25 °C and 42 °C. Fluorescence signals that were equal to or greater than Spinach at 25 °C indicated equal or greater percent folded. A higher percentage signal at 42 °C indicated improved thermostability relative to Spinach.

Preparation and in vitro analysis of flanked Spinach and Spinach2 constructs. Spinach and Spinach2 constructs flanked on either side by 50 base pairs were generated by PCR. For Spinach, the forward primer was 5′-TAATACGACTCACTATAGGGCTTGAGCATTTAGTGTTACACCTTTTCTGACAAAACCTAA ACCAGCCTGGTACAGTCTGGGAAGACGGGTCCAGTAGGTGCTCCGCCAGCTACTTGT TGAAGTAGGTGTAAGCTCGTAACGTAGTTACATC-3′ and reverse primer was 5′-AAAACAAAAAACAATAAAGCCATGCGCTTTACATCTATGCTTTTCTGCGGCGCAGCAGAGATACCTGAGCTGAAATGAAATGGTG-3′.

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For Spinach2, the forward primer was 5′-TAATACGACTCACTATAGGGTCTACGGCA TACCACCTG-3′ and reverse primer was 5′-GGGATCCACGCGAGAAGATGTATAGTGAGGGAGGAC-3′. The 50-base-pair sequences were taken from the human β-actin 3′ untranslated region. Both forward primers encode the T7 RNA polymerase promoter for \textit{in vitro} transcription.

5S-Spinach and 5S-Spinach2 were amplified by PCR from pAV-5S-Spinach and pAV-5S-Spinach2, respectively, with forward primer 5′-TAATACGACTCACTATAGGGTCTACGGCA TACCACCTG-3′ and reverse primer 5′-GGGATCCACGCGAGAAGATGTATAGTGAGGGAGGAC-3′. PCR products were used as templates for \textit{in vitro} transcription by Ampliscribe T7 RNA polymerase as previously described. Fluorescence measurements were recorded for 0.1 µM RNA in the presence of 10 µM DFHBI in buffer composed of 20 mM HEPES pH 7.4, 100 mM KCl and 100 µM MgCl₂ as described above.

**Cloning Spinach2 for expression in \textit{E. coli}.** Spinach and Spinach2 were PCR-amplified with primers containing the EagI restriction sites on both the 5′- and 3′-ends of the Spinach sequence. They were then cloned into a pET28c-based plasmid containing a chimeric human tRNA Scaffold, which we previously used for Spinach and Spinach-based metabolite sensors and which has previously been shown to stabilize heterologous expression of RNA aptamers in \textit{E. coli}.

**Whole-cell fluorescence measurements of \textit{E. coli}.** BL21 cells were transformed to harbor either pET28c-tRNA-Spinach or pET28c-tRNA-Spinach2, and grown in Luria broth with 100 µg/ml kanamycin to OD₅₀₀ 0.4 at room temperature. The cells were then induced with addition of 1 mM IPTG for 2 h at room temperature. After induction, cells were normalized for cell density and split into two aliquots. One aliquot per sample was incubated at room temperature, and the other was incubated for 20 min at 37 °C. Cells were then measured for total fluorescence using a Tecan SafireII plate reader with 460 ± 10 nm excitation and emission was recorded at 510 ± 10 nm. Data shown represent mean and s.e.m. values for three independent experiments.

**Quantitative reverse transcription–PCR analysis of Spinach and Spinach2 concentration in \textit{E. coli}.** Total RNA samples were collected from \textit{E. coli} at both 25 °C and 37 °C using the RNaseasy Protect Bacteria Mini Kit (Qiagen). Reverse transcription was carried out on all samples using a reverse primer that bound in the tRNA portion of the tRNA-Spinach transcripts (5′-TGGGCGCCGACAACGGGAC-3′) and a reverse primer against 16S RNA (5′-GTATTACCGCGGCTGCTG-3′) according to the SuperscriptIII reverse transcription kit protocol. qRT-PCR was carried out according to the iQ SYBR Green Supermix (Bio-Rad) protocol with forward primer 5′-GCCGCCGATA GCTCATGCGTAG-3′ and reverse primer 5′-TGCCGCGCG AACAGGGAC-3′ to the tRNA portion of each transcript as well as forward primer 5′-CTCCTACGGGAGGCAGCAG-3′ and reverse primer 5′-GTATTACCGCGGCTGCTG-3′ to 16S RNA. In all cases, Spinach transcript levels were normalized to 16S RNA levels. Data represent mean and s.e.m. values for three independent experiments.

**Cloning of Spinach2 and Spinach2.** pAV-5S-Spinach was generated as previously described. This construct contained Spinach in the context of the tRNA₃₅ scaffold. Sequence encoding tRNA₃₅-Spinach was removed from pAV-5S by restriction digest with SalI and XbaI. Sequence encoding tRNA₃₅-Spinach2 was amplified from pET28c-tRNA-Spinach2 by PCR using forward primer 5′-TAGGCGTCGACGCCGAGGATACTCGTGTA GAGCAG-3′ and reverse primer 5′-ATATATCTAGTGCGG CCGAACAGGGACTTGACACCC-3′, and digesting the resulting PCR products with XbaI and SalI to clone into pAV-5S.

**Imaging 5S-Spinach and 5S-Spinach2.** Imaging of 5S-Spinach and 5S-Spinach2 was carried out as previously described for Spinach3. Cells were imaged for either 100 ms or 1 s. Background signals from cells expressing pAV-5S incubated with DFHBI were also taken at 100 ms and 1 s and subtracted from the corresponding images using NIS-Elements software.

For brightness quantification, fluorescence signal was measured for 20 background-subtracted cells per sample and normalized for total area using NIS-Elements AR 3.2 (Nikon). 5S-Spinach2 signal was normalized to 1.0.

**Cloning of Spinach-7SK and Spinach2-7SK.** Spinach or Spinach2 in the context of the tRNA₃₅ scaffold was amplified by PCR using forward primer 5′-ATATATGATCTCGTGCCGCCGGACGCAGC-3′ and reverse primer 5′-ATATATGATCTCGTGCCGCCGGACGCAGC-3′. The resulting PCR product was digested using BamHI and BglII. This digested PCR product was then ligated into a vector of pLPCXU6PT7SK (Addgene plasmid 27549) that was modified as follows. pLPCXU6PT7SK was used as a template along with forward primer 5′-ATATATGATCTCGTGCCGCCGGACGCAGC-3′ and reverse primer 5′-ATATATGATCTCGTGCCGCCGGACGCAGC-3′. This construct contained Spinach in the context of the tRNA₃₅ scaffold. Sequence encoding tRNA₃₅-Spinach was amplified from pET28c-tRNA-Spinach2 by PCR using forward primer 5′-TAGGCGTCGACGCCGAGGATACTCGTGTA GAGCAG-3′ and reverse primer 5′-ATATATCTAGTGCGG CCGAACAGGGACTTGACACCC-3′, and digesting the resulting PCR products with XbaI and SalI to clone into pAV-5S.

**Imaging Spinach-7SK and Spinach2-7SK.** Imaging of Spinach-7SK and Spinach2-7SK was carried out as previously described for Spinach3. Cells were imaged for either 100 ms or 1 s. Background signals from cells expressing pAV-5S incubated with DFHBI were also taken at 100 ms and 1 s and subtracted from the corresponding images using NIS-Elements software.

For brightness quantification, fluorescence signal was measured for 20 background-subtracted cells per sample and normalized for total area using NIS-Elements AR 3.2 (Nikon). 5S-Spinach2 signal was normalized to 1.0.

**Cloning of Spinach-7SK and Spinach2-7SK.** Spinach or Spinach2 in the context of the tRNA₃₅ scaffold was amplified by PCR using forward primer 5′-ATATATGATCTCGTGCCGCCGGACGCAGC-3′ and reverse primer 5′-ATATATGATCTCGTGCCGCCGGACGCAGC-3′. This construct contained Spinach in the context of the tRNA₃₅ scaffold. Sequence encoding tRNA₃₅-Spinach was amplified from pET28c-tRNA-Spinach2 by PCR using forward primer 5′-TAGGCGTCGACGCCGAGGATACTCGTGTA GAGCAG-3′ and reverse primer 5′-ATATATCTAGTGCGG CCGAACAGGGACTTGACACCC-3′, and digesting the resulting PCR products with XbaI and SalI to clone into pAV-5S.

**Imaging Spinach-7SK and Spinach2-7SK.** Imaging of Spinach-7SK and Spinach2-7SK was carried out as previously described for Spinach3. Cells were imaged for either 100 ms or 1 s. Background signals from cells expressing pAV-5S incubated with DFHBI were also taken at 100 ms and 1 s and subtracted from the corresponding images using NIS-Elements software.

For brightness quantification, fluorescence signal was measured for 20 background-subtracted cells per sample and normalized for total area using NIS-Elements AR 3.2 (Nikon). 5S-Spinach2 signal was normalized to 1.0.
with 0.3 µg of pLPC-Spinach-7SK or pLPC-Spinach2-7SK and 0.3 µg of pCDNA3.1-SC35-mCherry using FuGeneHD (Roche) per the manufacturer’s instructions in DMEM lacking penicillin and streptomycin. Cells were imaged 24 h after transfection. At 30 min before imaging, medium was supplemented with 25 mM HEPES, 5 mM MgSO4, and 20 µM DFHBI. Cells were imaged as described below using FITC and Texas Red filter sets.

Cloning of CGG60-Spinach and Spinach2. Spinach or Spinach2 in the context of the tRNA13p scaffold was amplified by PCR using forward primer 5’-ATATATATCGGCCGATAGCTGAGT CGGTTAGACCG-3’ and reverse primer 5’-ATATATGCGGCC TGGCCCGCCGACAGGGACTTGAACCC-3’ and digesting the resulting PCR products with XbaI and Apal to clone downstream of pCDNA-60CGG-Spinach or pCDNA-60CGG-Spinach2 using 1 µl of pCDNA-60CGG-Spinach or pCDNA-60CGG-Spinach2. For TET-off experiments, the entire transcript from pCDNA-60CGG-Spinach2 (CGG60-Spinach2-BGH-polyadenylation signal) was excised using NheI and EcoRV and subcloned into pTRE2-Hyg (Clontech) that was cut with Nhel and EcoRV.

Transfection of COS-7 cells and live cell imaging. COS-7 cells (ATCC-CRL-1651) were cultured and passaged in DMEM supplemented with 50 units of penicillin and 50 µg of streptomycin per milliliter. For imaging experiments, cells were grown on cells cultured on 24-well glass-bottom dishes and transfected with 0.6 µg of pCDNA-60CGG-Spinach or pCDNA-60CGG-Spinach2 using FuGeneHD (Roche) per the manufacturer’s instructions in DMEM medium lacking penicillin and streptomycin. Cells were imaged in CO2-independent medium (Invitrogen) supplemented DMEM medium lacking penicillin and streptomycin. Cells were transfected to express either pTet-off alone or pTet-off with either pTRE2-Hyg-(CGG)60, pTRE2-Hyg-(CGG)60-Spinach, pTRE2-Hyg-(CGG)60-Spinach2, or pTRE2-Hyg-(CGG)30 or pTRE2-Hyg-(CGG)30-Spinach2 in 12 wells each in 24-well plates. At 24 h after transfection, the transfection medium was replaced, and doxycycline was added to 1 µg/ml. At 0 h, 6 h, 12 h and 24 h after transfection, total RNA from three wells per sample was extracted using TRIzol (Invitrogen) according to manufacturer’s protocol.

Reverse transcription was carried out on all samples using a reverse primer that bound downstream of the CGG repeats in all constructs (5’-CTAGAGATATCGGTGATACACG-3’) and a reverse primer against GAPDH mRNA (5’-TCACCAACCCCG TGTTGCTGTA-3’) according to the SuperscriptIII reverse transcription kit protocol. qRT-PCR was carried out according to the iQ SYBR Green Supermix (Bio-Rad) protocol with forward (5’-GTCAGCTGACGCGTGCTAGC-3’) and reverse (5’-CTAGAGATATCGGTGATACACG-3’) primers against all CGG transcripts as well as forward (5’-ACCCACGTGCTG CATCAC-3’) and reverse (5’-TCCACCACTCGTTGCTG TA-3’) primers against GAPDH mRNA. In all cases, CGG transcript levels were normalized to GAPDH mRNA levels. Data represent mean and s.e.m. values for three independent experiments. We also carried out qRT-PCR of sample RNA compared to in vitro–transcribed control RNA to determine the approximate number of CGG repeat–containing RNA in a cell. We obtained roughly 0.2 ng of (CGG)60-Spinach2 RNA from 0.2 × 10⁶ transfected cells. We estimated the molecular weight of polyadenylated (CGG)60-Spinach2 to be roughly 280 kDa. Using these values, we calculated that each transfected cell contained roughly 2,000 copies of (CGG)60-Spinach2. On average, each cell contained 10–15 foci, indicating that each aggregate contains roughly 150–200 RNA molecules. It should be noted that foci vary in size in different cells, and foci that are much smaller than the ‘average’ size are readily detectable in cells. Moreover, we observed some Spinach2 signal in the nucleoplasm that is not in foci. So 150–200 RNA molecules is unlikely to be the limit of detection at 50 ms; however, the precise limit will require more precise quantification methods of these foci that are closer to the limits of detection. Because foci were typically imaged at 50 ms, it is likely that smaller numbers of RNAs would be detectable at longer imaging times such as 500 ms or 1 s.