SUPPORTING INFORMATION

Spectral tuning by a single nucleotide controls the fluorescence properties of a fluorogenic aptamer

Grigory S. Filonov,[†‡] Wenjiao Song,[†] Samie R. Jaffrey[†*]

[†]Department of Pharmacology, Weill Medical College, Cornell University, New York, NY 10065, United Sates.

[‡]Present address: Essen BioScience, part of Sartorius Group, Ann Arbor, Michigan, 48108, United States

*Correspondence should be addressed to S.R.J. (srj2003@med.cornell.edu).

SUPPLEMENTARY FIGURES

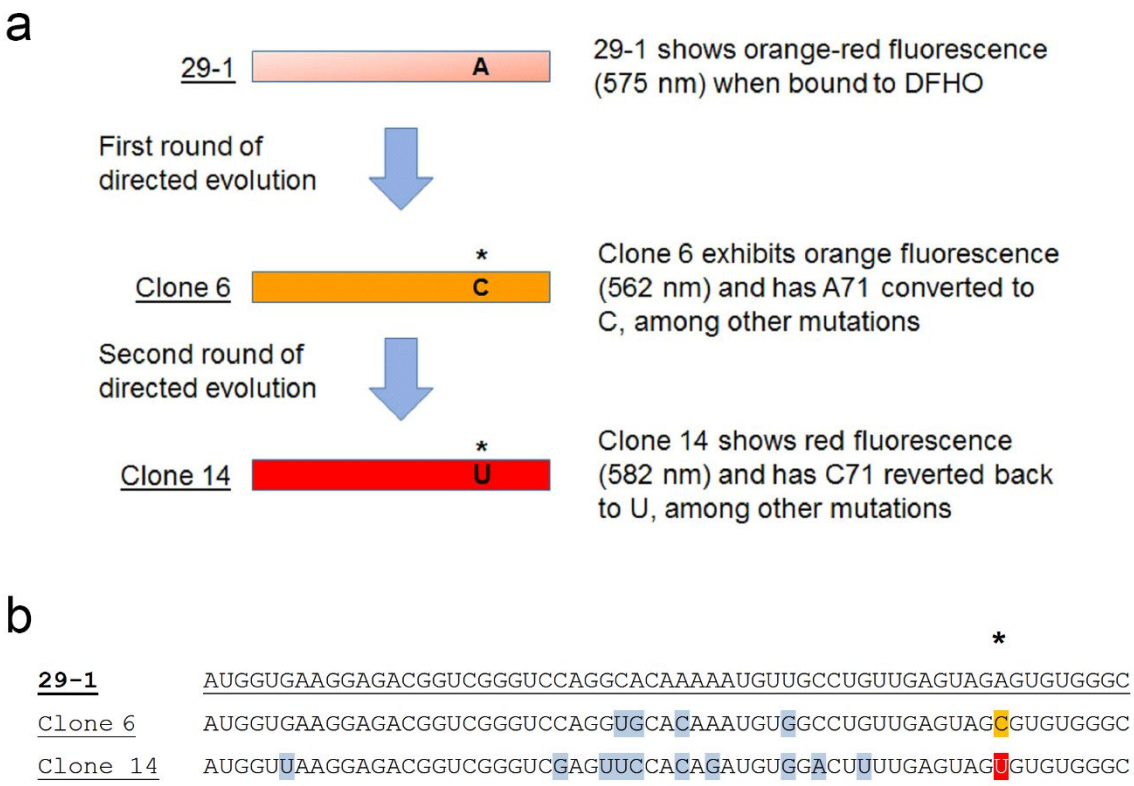

Figure S1. Revertant phenotype additionally supports the importance of the nucleotide at position 71.

(a) More evidence about the importance of residue 71 came from directed evolution of an orange-fluorescent aptamer which resulted in a revertant with red fluorescence. Shown is a schematic of how a red fluorescent mutant was generated from the directed evolution of an orange aptamer, which helped us to pinpoint residue 71 as critical for spectra tuning.

Clone 6, which had the brightest orange fluorescence signal in bacteria, was subjected to another round of directed evolution. While no brighter orange aptamer was identified, one clone reverted back to red fluorescence (emission maximum 582 nm). In agreement with our hypothesis, this aptamer (clone 14) has C71 mutated back to U, among other mutations. This further supported the importance of position 71.

(b) Sequence of the revertant. Shown is the sequence of 29-1, as well as the orange clone 6. Also shown is clone 14 which came from the directed evolution of clone 6, but which showed red

fluorescence. Mutations at position 71, which were hypothesized to be the key determinants of the fluorescence emission, are highlighted with orange and red. Grey indicates other mutations in the sequence compared to 29-1. These other mutations may influence aptamer folding, quantum yield, or extinction coefficient, but do not appear to substantially tune the fluorescence emission wavelength. All sequences are presented for the mutagenized part of the aptamers only. The full sequences include constant domains, which were used for library member amplification. These constant domains were described before[1] and are shown for Orange Broccoli and Red Broccoli in Figures 2 and 4a.

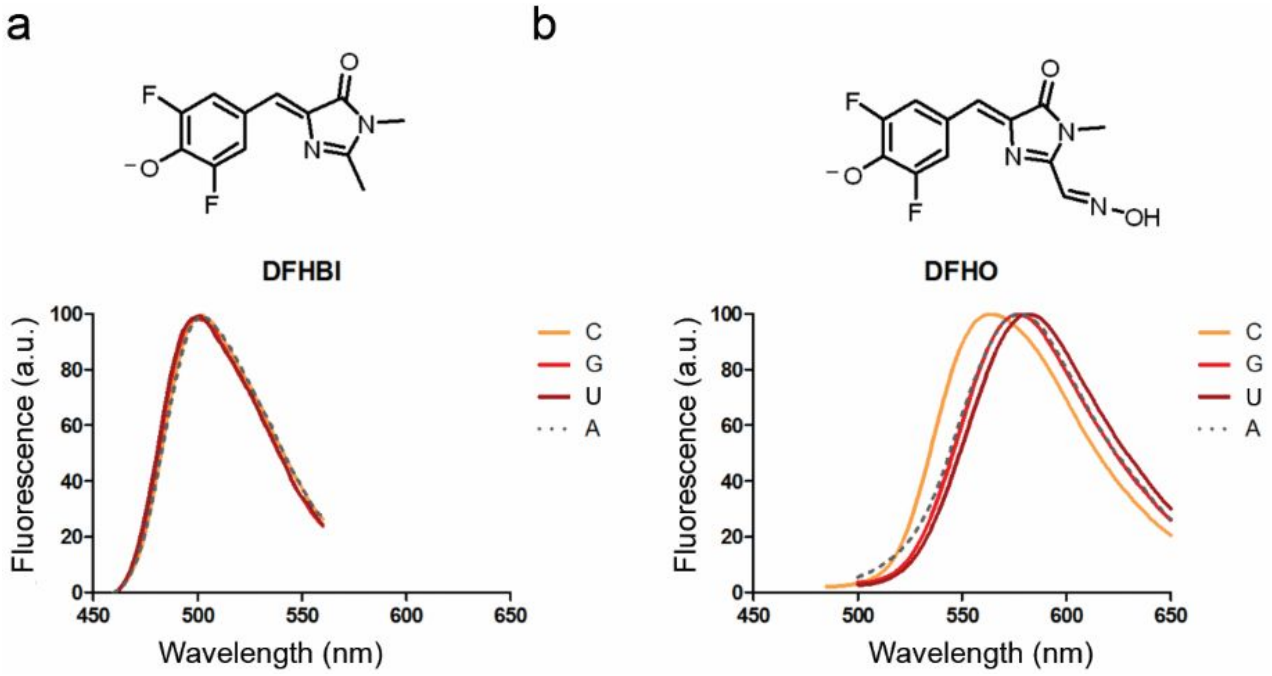

Figure S2. The oxime substituent in DFHO is the critical determinant of DFHO ability to be spectrally tuned.

The purpose of this experiment was to determine if the oxime substituent confers DFHO's ability to be spectrally tuned. To test this, we compared the tuning of DFHBI and DFHO, which only differ by the presence of the oxime in DFHO. We tested 29-1 aptamers which differ by the nucleotide identity at position 71. 29-1 normally contains an A residue at position 71. Although the 29-1 mutants weakly activated DFHBI fluorescence, there was no spectral tuning by the different mutants (left). However, these mutants spectrally tuned the fluorescence emission of DFHO (right). Thus, the oxime moiety is required for the spectral tuning of these mutants.

(a) Fluorescence emission spectra of A71 mutants of 29-1 bound to DFHBI. No mutation-dependent spectral tuning is observed.

(b) Fluorescence emission spectra of A71 mutants of 29-1 bound to DFHO. A single nucleotide mutation spectrally tunes the fluorescence emission maxima of DFHO-binding aptamer. This panel is the same as Figure 3.

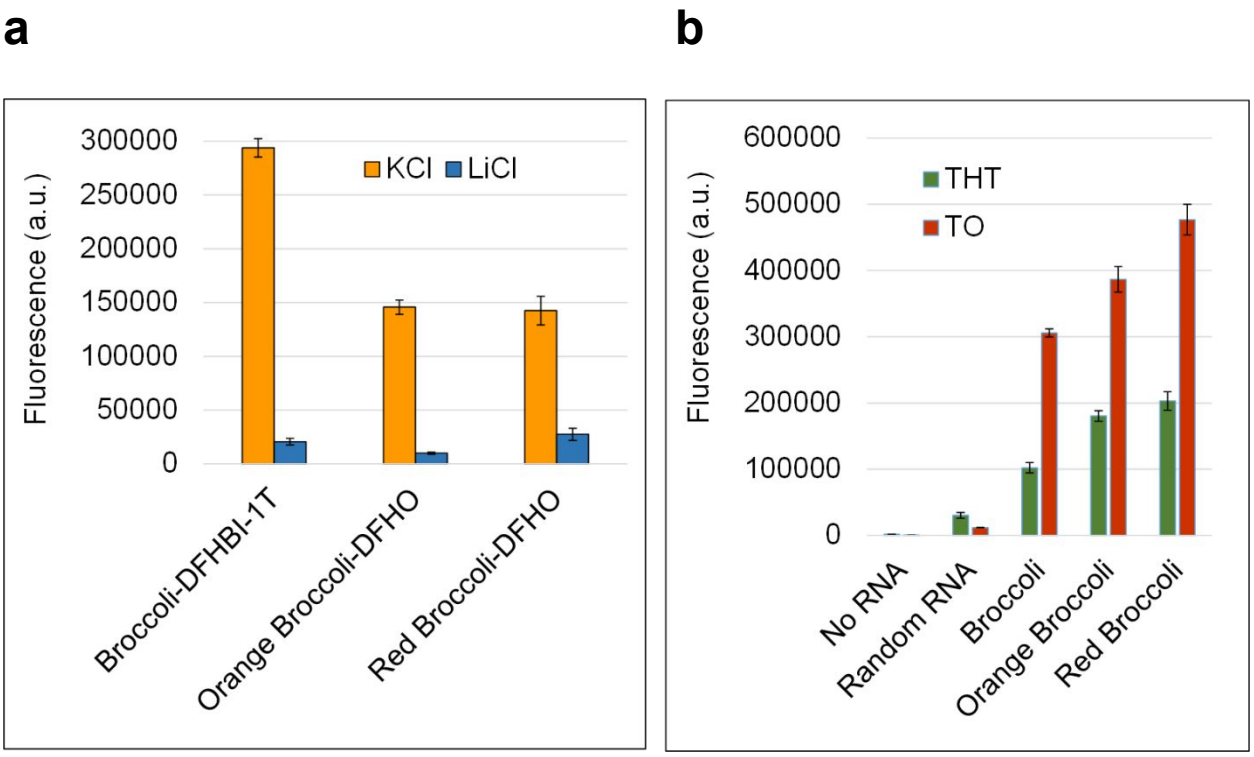

Figure S3. Broccoli, Orange Broccoli and Red Broccoli likely have G-quadruplex structures.

(a) Test for potassium dependence for aptamer-induced fluorescence. In these experiments, we took advantage of the fact that G-quadruplexes typically highly dependent on potassium to form their structure. Other monovalent ions, in particular the commonly used control cation lithium, fail to promote G-quadruplex folding. Thus, we tested the potassium dependence on RNA aptamer-induced fluorescence. We prepared the indicated RNA-fluorophore mixtures (1 μM of RNA and 10 μM fluorophore) in a buffer containing 40 mM HEPES (pH 7.4), 5 mM $MgCl_2$ and 100 mM KCl or 100mM LiCl. The Broccoli aptamer, which has essentially the same core structure, as Spinach and thus likely contains the same G-quadruplex, was used as a positive control. Broccoli showed the expected dependence on potassium for its fluorescence. Notably, each of the DFHO-binding aptamers also showed strong dependence on potassium, suggesting that they each contain a G-quadruplex structure. Fluorescence was measured using a FluoroMax-4 spectrofluorometer (Horiba Scientific).

(**b**) Test for G-quadruplexes using G-quadruplex-specific fluorescent dyes. Another way to assess the presence of G-quadruplex is to use dyes that fluoresce only when bound to this structure. We used two well-characterized and structurally distinct G-quadruplex-binding dyes:

thioflavin T and thiazole orange[2,3]. Again, Broccoli was used as a positive control while a pool of random RNA from the first round of SELEX was used an unstructured RNA control. RNAs were compared at identical concentrations. Thioflavin T (ThT) (0.05 μM) or thiazole orange (TO) (0.5 μM) were mixed with 1 μM (3 μg) of random RNA or with 1 μM of aptamer RNA in a buffer containing 40 mM HEPES pH 7.4, 100 mM KCl, 5 mM $MgCl_2$. Fluorescence was measured using a FluoroMax-4 spectrofluorometer (Horiba Scientific) using the excitation and emission wavelengths commonly used for these dyes (thioflavin T: excitation 430nm, emission 485nm; thiazole orange: excitation 490nm, emission 530nm). All aptamers, but not random RNA, showed a significant enhancement in fluorescence upon incubation with the G-quadruplex-binding dyes, further suggesting that these aptamers contain a G-quadruplex in their structure.

Error bars represent 95% confidence interval (n=3 technical replicates).

METHODS

Reagents and equipment

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich. DFHBI fluorophores were obtained from Lucerna Technologies (New York, NY) or were synthesized as described previously[1,4,5]. Fluorescence was measured on FluoroMax-4 spectrofluorometer (Horiba Scientific). Fluorescence was also measured on Safire II or Genios Pro plate readers (Tecan)

DFHO was synthesized as described elsewhere.[6] Briefly, DFHO (3,5-difluoro-4-hydroxybenzylidene-imidazolinone-2-carbaldehyde oxime) was synthesized using DFHBI (3,5-difluoro-4-hydroxybenzylidene-imidazolinone) as a precursor. A suspension of DFHBI (1.01 g, 4 mmol) was prepared as described previously[4], and selenium dioxide (0.45 g, 4 mmol, 1.0equiv) in anhydrous dioxane (25 mL) was stirred at reflux for 1 hour and carefully decanted from the red solid while hot. The solution was concentrated in *vacuo*, the crude product was dissolved in ethanol (80 mL), treated sequentially with hydroxylamine hydrochloride (0.56 g, 8 mmol, 2.0equiv) and sodium acetate (0.66 g, 8 mmol, 2equiv). After stirring for overnight at room temperature, the volatiles were removed in *vacuo* and the residue was purified by column chromatography (silica, 3:2 hexane:ethylacetate) to afford the DFHO as an red solid (0.67 g, 60%).

In vitro RNA characterization

RNA was synthesized off double stranded DNA templates utilizing the AmpliScribe T7-Flash Transcription Kit (Epicentre Biotechnologies). After treatment with DNase (Epicentre Biotechnologies) for 1 h, RNAs were purified using Bio-Spin columns (Bio-Rad), and quantified using both absorbance values at NanoDrop 2000 and the Quant-iT RiboGreen RNA Assay Kit (Life Technologies). All RNAs used in the paper were transcribed within the tRNA scaffold to improve folding.[7]

All *in vitro* RNA properties were measured in 40 mM HEPES pH 7.4, 100 mM KCl, 5 mM $MgCl_2$ buffer unless specified.

Absorption, excitation and emission spectra were measured for solutions using “excess RNA” conditions and limiting amount of fluorophore to ensure that no free fluorophore contributes to the absorbance or fluorescence signal[8]. This approach also allows us to have a fixed concentration

of RNA-fluorophore complex which is equal to the concentration of the fluorophore that was initially added. The RNA concentration was 20 μM (for the fluorescence measurements) and 50 μM (for the absorbance measurements) while the DFHO concentration was 2 μM and 5 μM respectively.

For the monovalent cation (potassium vs. lithium) dependence assay, the indicated RNA was heated to 95°C for 2 min in 200 mM HEPES, pH 7.5, 25 mM $MgCl_2$, 500mM KCl or 500mM LiCl and cooled on ice for 2 min. DEPC-treated water was added to adjust the final concentrations of RNA to 1 μM, HEPES to 40 mM and KCl or LiCl to 100 mM. DFHBI-1T or DFHO was added to a final concentration of 10 μM from a 400 μM stock in DMSO. To refold the RNA, the solution was heated to 65°C for 5 min and cooled to 25°C for over 15 min. The final concentrations were 40 mM HEPES, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 μM RNA and 10 μM DFHBI-1T or DFHO.

To observe G-quadruplex-specific fluorogen activation, thioflavin T (0.05 μM) or thiazole orange (0.5 μM) were mixed with 1 μM (3 μg) of random RNA or with 1 μM of aptamer RNA in a buffer containing 40 mM HEPES pH 7.4, 100 mM KCl, 5 mM $MgCl_2$. Fluorescence was measured using a FluoroMax-4 spectrofluorometer (Horiba Scientific) using the excitation and emission wavelengths commonly used for these dyes (thioflavin T: excitation 430nm, emission 485nm; thiazole orange: excitation 490nm, emission 530nm).

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