jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

TITLE: Determination of *in vitro* and cellular turn-on kinetics for fluorogenic RNA aptamers

AUTHORS AND AFFILIATIONS:
Madeline M. Mumbleau*, Madeline R. Meyer*, and Ming C. Hammond[¥]
Affiliations: Department of Chemistry and Henry Eyring Center for Cell & Genome Science, University of Utah
Email: m.mumbleau@utah.edu, madeline.meyer@utah.edu, mingch@chem.utah.edu
* Equal contributions
[¥] Corresponding author

KEYWORDS: fluorescence, flow cytometry, platereader, Spinach2 aptamer, Broccoli aptamer, *E. coli*

SUMMARY: Two methods are presented to determine the kinetics of the fluorogenic RNA aptamers Spinach2 and Broccoli. The first method describes how to measure fluorogenic aptamer kinetics in vitro with a plate reader, while the second method details the measurement of fluorogenic aptamer kinetics in cells by flow cytometry.

ABSTRACT: Fluorogenic RNA aptamers have been applied in live cells to tag and visualize RNAs, to report on gene expression, and to activate as part of fluorescent biosensors that detect levels of metabolites and signaling molecules. In order to study dynamic changes in each of these systems, it is desirable to obtain real-time measurements, but the accuracy of the measurements depends on the kinetics of the fluorogenic reaction being faster than the sampling frequency. Here we describe methods to determine the *in vitro* and cellular turn-on kinetics for fluorogenic RNA aptamers using a platereader equipped with a sample injector and a flow cytometer, respectively. We show that the *in vitro* kinetics for fluorescence activation of the Spinach2 and Broccoli aptamers can be modeled as two-phase association reactions and have differing fast phase rate constants of 0.56 and 0.35 s^{-1}, respectively. In addition, we show that the cellular kinetics for fluorescence activation of Spinach2 in *E. coli*, which is further limited by dye diffusion into the Gram negative bacteria, is still sufficiently rapid to enable accurate sampling frequency on the minute timescale. These methods to analyze fluorescence activation kinetics are applicable to other fluorogenic RNA aptamers that have been developed.

INTRODUCTION:

Fluorogenic reactions are chemical reactions that generate a fluorescence signal. Fluorogenic RNA aptamers typically perform this function by binding a small molecule dye to enhance its fluorescence quantum yield (Fig. 1A)[1]. Different fluorogenic RNA aptamer systems have been developed and consist of specific RNA aptamer sequences and the corresponding dye ligands[1]. Fluorogenic RNA aptamers have been appended to RNA transcripts as fluorescent tags that enable live cell imaging of mRNAs and non-coding RNAs[2–4]. They also have been placed after promoter sequences as fluorescent reporters of gene expression, similar to the use of green fluorescent protein (GFP) as a reporter except the reporting function is at the RNA level[5, 6]. Finally, fluorogenic RNA aptamers have been incorporated into RNA-based fluorescent biosensors, which

are designed to trigger the fluorogenic reaction in response to a specific small molecule. RNA-based fluorescent biosensors have been developed for live cell imaging of various non-fluorescent metabolites and signaling molecules[7–11].

There is growing interest in the development of fluorogenic RNA aptamers to visualize dynamic changes in RNA localization, gene expression, and small molecule signals. For each of these applications, it is desirable to obtain real-time measurements, but the accuracy of the measurements depends on the kinetics of the fluorogenic reaction being faster than the sampling frequency. Here we describe methods to determine the *in vitro* kinetics for fluorogenic RNA aptamers Spinach2[12]and Broccoli[13] using a platereader equipped with a sample injector, and to determine the cellular turn-on kinetics for Spinach2 expressed in *E. coli* using a flow cytometer. These two RNA aptamers were chosen because they have been applied to study RNA localization[2–4] and have been used in reporters[5, 6] and biosensors[7–11], and the corresponding dye ligands (DFHBI or DFHBI-1T) are commercially available. A summary of their *in vitro* properties determined in the literature is given in Table 1[4, 13, 14], which informed the protocol development, e.g. wavelengths and dye concentrations used. These results demonstrate that the fluorogenic reactions effected by RNA aptamers are rapid and should not impede accurate measurements for the desired cell biological applications.

PROTOCOL:

1. *IN VITRO* KINETICS EXPERIMENT

1.1. Preparation of DNA templates by PCR

1.1.1. Set up PCR reaction(s)

To prepare PCR reactions, combine the following reagents in a thin-walled PCR tube:

- 33 µL double-distilled water (ddH_2O)
- 10 µL 5X buffer for high-fidelity DNA polymerase
- 5 µL of 2 mM each deoxyribonucleoside triphosphate (dNTP)
- 0.5 µL of 40 µM forward primer
- 0.5 µL of 40 µM reverse primer
- 0.5 µL (10-100 ng) DNA template (for Spinach2 PCR only; Broccoli primers overlap)
- 0.5 µL high-fidelity DNA polymerase (add last)

NOTE: Synthetic oligonucleotides are often shipped dry. To prepare stock solutions, add a known volume (100 µL recommended) of ddH_2O, and measure the A_{260} of that solution to determine the concentration by Beer's law, where the extinction coefficient can be calculated by nearest neighbor rules online. This stock solution can then be used to make dilutions appropriate for use in PCR.

1.1.2. Run thermocycler protocol

Use the following thermocycler protocol to amplify full-length Spinach2 and Broccoli DNA templates:

Initial denaturation: 98 °C for 2 min

35 cycles:
Denaturation: 98 °C denaturation for 15 s
Annealing: 72 °C for 30 s
Extension: 72 °C for 30 s
Final extension: 72 °C for 5 min.

After the reaction, analyze a small aliquot of the product by 2% agarose gel along with a low molecular weight ladder (size range: 25-766 nt) to confirm the presence of the desired DNA product. Purify the product by commercially available gel extraction or PCR clean-up kits and elute with ddH_2O or buffer provided by the manufacturer.

NOTE: Be sure when choosing a PCR clean-up kit that the column molecular weight cutoff is low enough to retain T7-Broccoli (81 nt), or else the PCR product will be lost.

OPTIONAL PAUSE POINT: DNA can be stored overnight before purification or after purification for as long as needed at -20 °C.

1.2. Preparation of Spinach2 and Broccoli RNA by IVT

1.2.1. Set up transcription reaction(s)

To prepare a 100-µL transcription reaction, combine the following reagents in a 1.5 mL microcentrifuge tube:

- 10 µL 10x transcription buffer
- 20 µL of 10 mM ribonucleoside triphosphates (rNTPs)
- 1-64 µL DNA template (total of 1 µg)
- 2 µL inorganic pyrophosphatase
- ddH_2O to 98 µL
- 2 µL T7 RNA polymerase (add last)

Incubate this reaction for 4 h at 37 °C. Quench the reaction by adding 100 µL of 2 X Urea gel loading buffer (2 X ULB), comprised of 20% sucrose, 0.1% sodium dodecyl sulfate (SDS), 1 X Tris-Borate-EDTA (1 X TBE) buffer, and ~18 M urea.

OPTIONAL PAUSE POINT: The quenched reaction can be stored overnight at -20 °C.

1.3. Polyacrylamide gel electrophoresis (PAGE) purification of RNA

1.3.1 PAGE purification of Spinach2 and Broccoli RNA

CAUTION: Non-polymerized (liquid or powdered) acrylamide is extremely toxic. If weighing out powdered acrylamide, do so in a fume hood. Always wear proper protective equipment and immediately remove gloves contaminated with acrylamide powder or solution, washing hands thoroughly. If acrylamide is in direct contact with skin, wash the exposed area for at least 15 minutes with soap and water. If acrylamide is in direct contact with eyes, flush them with water for 15 minutes.

1.3.1.1. Prepare PAGE gel

To remove unwanted abortive transcripts and unreacted rNTPs from full-length product, prepare a 6% urea-polyacrylamide gel. In general, 28 cm long by 16.5 cm wide and 1.5 mm thick gels can be used with an 8-well comb. Set up the gel and electrophoresis equipment, using 1 X TBE buffer to fill reservoirs.

1.3.1.2. Load RNA sample(s) in PAGE gel

Load the gel with one quenched 200 µL reaction per lane. In a separate lane, load 2 X ULB with tracker dyes xylene cyanol and bromophenol blue, which migrate in the gel at 106 and 26 nucleotides, respectively[15]. Leave an empty lane between each sample to avoid potential contamination in next steps.

1.3.1.3. Run the PAGE gel

To separate 95-nt Spinach2 and 49-nt Broccoli from their respective truncated products, run the gel for 1.5-2 hours at 25 W, at which point the bromophenol blue dye will have migrated ~5/6 of the gel length.

1.3.1.4. Visualize RNA sample(s) in PAGE gel

Disassemble the glass plates around the gel and cover the gel in plastic wrap on both sides, labeling the lanes on the wrap. Visualize RNA bands in a dark room by UV shadowing by placing the wrapped gel on a fluorescent TLC plate under UV light. Quickly outline the edge of the RNA bands corresponding to product with a marker and switch off the UV lamp to minimize damage from UV exposure.

1.3.1.5. Excise and extract RNA sample(s) from PAGE gel

With a fresh razor blade for each sample, excise the desired product bands, dice into ~1 mm cubes, and add the gel pieces to a 2 mL microcentrifuge tube with 500 µL crush soak buffer to extract RNA on a rotator for either 2 h at room temperature or overnight at 4 °C.

OPTIONAL PAUSE POINT: After the above procedure, the sample may be stored at -20 °C for a few months.

1.3.1.6. Precipitation of RNA

To separate gel pieces from the extracted RNA in buffer, centrifuge the sample at 13,000 x g for 20 min at 4 °C, then use a narrow-tip pipette to extract the supernatant and load into a new 2 mL microcentrifuge tube. To precipitate RNA, add 1.5 mL ice cold ethanol and 1 µL of 20 mg/mL glycogen, vortex, and store for at least 1 h at -20 or -80 °C.

OPTIONAL PAUSE POINT: The sample may be stored in this way for a few months.

1.3.1.7. Collection of RNA precipitate

Pellet the precipitated RNA by centrifugation 13,000 x g for 20 min at 4 °C. Remove the

supernatant and allow the remaining ethanol to evaporate under open air (~1 h) before resuspending the pellet in 30 µL ddH_2O or 1 X TE buffer.

NOTE: This process typically results in a final RNA concentration of ~10 µM.

OPTIONAL PAUSE POINT: The sample may be stored at -20 °C for a few months.

1.3.2. Determine RNA stock concentrations

1.3.2.1. Prepare RNA aliquot for hydrolysis reaction

To perform this assay, first use a UV/Vis nanospectrophotometer to determine the A_{260} of the stock RNA sample and make a diluted aliquot of the sample to ~10 absorbance units (AU) in ddH_2O. Prepare the following reaction in a 0.5 mL PCR tube:

16 µL ddH_2O
2 µL 10 X Na_2CO_3 buffer
2 µL RNA aliquot diluted to ~10 AU

Incubate the reaction for 90 min at 95 °C, then allow to cool to room temperature.

1.3.2.2. Determine RNA concentration using nucleotide absorbances

Measure the A_{260} of this sample with a UV/Vis nanospectrophotometer and calculate the RNA concentration using the following formula:

$$c_{RNA} = \frac{A_{260,hydrolyzed}}{b \sum_i^{A,C,G,U} n_i \varepsilon_{260,i}}$$

where *c* is the concentration of RNA, *b* is the path length, *i* is the specific nucleotide (A, C, G, or U), n_i is the frequency of nucleotide *i* in the RNA sequence, and ε_i is the molar extinction coefficient of nucleotide *i*. To determine the original stock RNA concentration, multiply *c* by the dilution factor.

OPTIONAL PAUSE POINT: Stock RNA can be stored at -20 °C for a few months.

1.4. Perform *in vitro* plate reader kinetics assay

1.4.1. Set up RNA renaturation program

Create the following thermocycler protocol by selecting “create new program,” “add new phase,” then “add new step” multiple times to add each of the following steps before pressing “save”:

70 °C for 3 min
65 °C for 45 s
60 °C for 45 s
55 °C for 45 s

50 °C for 45 s
45 °C for 45 s
40 °C for 45 s
35 °C for 45 s
30 °C for 45 s

1.4.2. Renature RNA

To renature Spinach2 and Broccoli RNAs, prepare 2 µM stocks of each RNA in ddH_2O in a 0.5-mL thin-walled PCR tube, then add an equal volume of 2X renaturation buffer (80 mM HEPES, pH 7.5 (KOH), 250 mM KCl, 6 mM $MgCl_2$) to make 1 µM RNA solutions. Add the tubes to the thermocycler, open the saved renaturation program, and press "run."

NOTE: If a thermocycler is unavailable, the RNAs may instead by incubated on a 70 °C heat block for 3 min and then allowed to cool slowly to room temperature on the bench.

1.4.3. Prepare binding reaction buffer

To prepare buffer for one aptamer-dye binding reaction, make a master mix containing buffer components with the following reagents:

69.5 µL ddH_2O
4 µL 1 M HEPES, pH 7.5 (KOH)
6.2 µL 2 M KCl
0.3 µL 1 M $MgCl_2$

Depending on how many samples and replicates are needed, multiply these values by the number of samples plus one. Generally, 3 replicates per RNA sample is satisfactory.

1.4.4. Prepare the plate reader

1.4.4.1. Set up plate reader injector program

On the fluorescence platereader, select "temp" and set the desired temperature to 37 °C, making sure the temperature has equilibrated to this value well before starting kinetics experiments. Open the platereader software, select "settings," then "acquisition view" and input the following program for kinetics measurements:

1. **Loop:** For each well
2. **Baseline setting:** 60 baseline reads
3. **SmartInject settings:** 10 µL injection (which will happen after the baseline reads)
4. **Fluorescence (or FL) reads:**
    Excitation: 448 nm (bandwidth: 9 nm)
    Emission: 506 nm (bandwidth: 15 nm)

Cartridge: MONO (s/n 3297)
Timing:
    Total read time: 10 min
    Read interval: 0.5 s
    PMT and Optics: 6 flashes per read

5. **Loop:** next well

1.4.4.2. Prepare the injector

1.4.4.2.1. Wash and aspirate the injector

To prepare the plate reader injector, first select "inject" on the platereader, supply a waste collection plate to the platereader when directed, then select "wash," and clean the injection tube following instructions on the platereader with 1 mL volumes of ddH_2O, 75% ethanol in ddH_2O, then ddH_2O. Next, select "aspirate," which will allow the injector to eject excess liquid.

1.4.4.2.2. Prime the injector

After exiting to the previous screen, select "prime" to prime the injector with two 260 µL volumes of ligand to be injected to ensure pure, concentrated ligand will be added to samples during experiments- in this case, prime with 100 µM DFHBI in ddH_2O.

1.4.5. Perform *in vitro* kinetics experiments

To perform one kinetics experiment, first add 80 µL of previously prepared binding buffer master mix to one well of a 96-well clear-bottom plate, followed by 10 µL of renatured RNA. Allow both this plate and DFHBI solution in the injector to equilibrate to 37 °C for 15 min in the plate reader. In the platereader software "settings" under "read area," select the well to be analyzed, then under the "home" tab select "run" to execute the kinetics program described previously. Repeat this process until all experiments are complete.

NOTE: Critically, kinetics experiments should be performed one well at a time to ensure RNA kinetics are measured under identical conditions between replicates and samples.

1.4.6. Wash the injector

To remove remaining DFHBI solution from the injection tube, wash the injection tube as described in step 1.4.3.2 with 1 mL volumes of ddH_2O, 75% ethanol in ddH_2O, then ddH_2O.

OPTIONAL PAUSE POINT

1.5. Analyze *in vitro* kinetics of fluorogenic RNA aptamers

1.5.1. Input data into analysis software

Export experimental data as a spreadsheet to easily copy over data for processing. In the analysis software, create a new data table in XY format. In the X column, enter each reading timepoint, with t = 0 being the time of DFHBI injection. In the Y column, enter the average fluorescence values between replicates at that respective timepoint starting at t = 0.

1.5.2. Normalize and graph the data

To normalize fluorescence values, click “Analyze” tab > “Data Processing” > Normalize, then click “OK.” Choose to use the smallest and largest values of the dataset for normalization, to present results as fractions and to “Graph the results,” then click “OK.” To create a graph of the normalized averaged fluorescence over time, click the “Normalize of [Dataset Name]” icon and select Graph Family: XY with “Points Only.”

NOTE: Error bars can be useful to see in cases where a small number of timepoints are graphed. If these are desired, when choosing an XY format data table, select the option under “Y” to enter replicate values in side-by-side columns. Graphing the resulting table with default options selected will produce a graph with error bars.

1.5.3. Perform curve fitting to obtain kinetic parameters

To fit a curve to the kinetics data, click “Analyze” > “Analyze Data” and select “Nonlinear regression (curve fit)” under the “XY analyses” tab. Under the “Model” tab, click “Exponential” > “Two phase association.” This will fit the kinetics data with the following two-phase association equation:

$$Y = Y_0 + SpanFast\left(1 - e^{(-K_{Fast}*X)}\right) + SpanSlow(1 - e^{(-K_{Slow}*X)})$$

where Y is the fluorescence at time X, Y_0 is the fluorescence at t = 0, K_{Fast} and K_{Slow} are fast and slow rate constants, respectively, and SpanFast and SpanSlow are the ranges of fluorescence turn-on accounted for by the fast and slow rates, respectively (see representative results Figure 1). Click the “Nonlin Fit” tab to view rate constants, $t_{1/2}$ values, and PercentFast values.

NOTE: To obtain a standard deviation for all these values, individual plate reader experiment replicates can be processed in the same way as described above.

2. CELLULAR KINETICS EXPERIMENT

2.1. Preparation of *E. coli* strains

2.1.1. Transform BL21 Star (DE3) *E. coli* cells with ~100 ng of pET31b tRNA-Spinach2 construct following the manufacturer’s protocol.

NOTE: Plasmid construct is commercially available (Plasmid #79783).

2.1.2. Plate the cells on LB agar containing carbenicillin (Carb: 50 mg/ mL) plates and incubate at 37 °C for 12-16 h. Colonies should contain plasmid and plates can be stored at 4 °C for a week.

OPTIONAL PAUSE POINT: Transformed BL21 Star plates can be stored at 4 °C wrapped in parafilm for a week.

2.2: Grow cells and induce expression of fluorogenic RNA aptamer

2.2.1. Inoculate a 2 mL culture of noninducing media (NI) containing carbenicillin (Carb: 50 mg/ mL) with a single colony of the transformed BL21 Star cells. Repeat this for at least three biological replicates. Incubate the cultures at 37 °C in an incubator /shaker at 250 rpm for 22-24 h.

OPTIONAL PAUSE POINT: Cells grown in NI media retain the plasmid and can be stored at 4 °C for a week.

2.2.2. After growth in NI media, dilute the culture 100x into fresh 3 mL ZYP-5052 autoinduction media (AI) containing carbenicillin (Carb: 50 mg/ mL). Grow the cells at 37 °C in an incubator/ shaker at 250 rpm for 16-18 h to induce expression. The typical OD_{600} for cultures will range from 2.0-3.3 after 18 h of growth. The optimum cell density range is between 2.5-3.0.

2.3. Perform cellular kinetics experiment

2.3.1. Set up Flow Cytometer

2.3.1.1. Turn on flow cytometer and computer connected to instrument. Once logged into the software for the flow cytometer, under the "Instrument" tab, click on the "Startup" icon. Follow the steps indicated on the software screen to ensure proper instrumentation initialization.

NOTE: Some flow cytometers call the instrument startup sequence as "Priming". Make sure to follow the manufacturer's protocol for the flow cytometer that will be in use for the experiment.

2.3.1.2. Run performance test (if applicable). Under the "Main Menu" tab, click on "Performance Test". In a culture tube, add 3 drops of the manufacturers' Performance Tracking Beads into 3 mL of Focusing Fluid. Place culture tube into sample tube lifter and raise the lifter. Prior to clicking "Run Performance Test", make sure the Lot # of the tube of the Tracking Beads is the same as what is indicated on the Performance Test Setup screen. Click run "Performance Test".

2.3.1.3. Set up the flow cytometer software for this experiment with the following acquisition parameters for single-cell fluorescence:

Excitation Laser: 488 nm
Emission Channel: GFP (also called FITC)
Acquisition Volume: 40 µL (with a total draw volume of 90 µL)
Flow Rate: 200 µL/ min
Cell Counts for each measurement: 30,000

Instrument Settings:
Voltage

FSC: 480
SSC: 400
BL1: 540
BL2: 392
BL3: 422

2.3.2. Set up Experimental Files

2.3.2.1. Create a new experiment file within the "Experiment Explorer" tab by right clicking flow cytometer username. Select "New Experiment" in the dropdown window. When a new window on the computer screen pops up, select "OK".

2.3.2.2. In the new experiment file, right click the "Group" folder and select "Add New Sample Tube". Label sample tubes for each specific time point and replicate by right clicking "Sample" and select "Rename" in the drop-down menu. Repeat this step for the total number of replicates and time points for the intended time course study.

2.3.3. Prepare dilute cell solution

In a new culture tube, add 1.5 mL of 1X PBS solution. Next, add 3 µL of induced cells in AI media into the 1X PBS solution to make a dilute cell solution. Repeat this step for each biological replicate in different culture tubes.

2.3.4. Measure background fluorescence of cells

Before adding dye, take readings of each biological replicate culture tube containing cells in 1X PBS solution. This is so that the fluorescent background of the cells is measured to observe the fold turn on over time once the dye is added.

2.3.4.1. To take a reading, place the culture tube into the sample tube lifter, raise the lifter by hand to the sample injection needle. Select the proper sample file and within the "Collection Panel" tab click "Record".

2.3.4.2 When the run is complete, lower sample tube lifter with culture tube by hand. This will initiate a "Rinse" step that will flush the fluidic system and minimize carryover between each biological replicate sample. The data will automatically be saved to the computer after the run is completed.

2.3.4.3. Repeat the steps within **2.3.4.** to measure the cellular fluorescent background for at least three biological replicates. To move to the next sample file, select the next sample file by clicking the right arrow icon near the sample tube name, which is underneath the "Record" icon.

2.3.5. Measure fluorescence at time points for cells with dye

2.3.5.1. Add 1.4 µL of a concentrated dye stock (50 mM DFHBI-1T in DMSO) into

1X PBS solution with cells to give a final of concentration of 50 µM DFBHI-1T. Next, secure the culture tube lid, then invert culture tubes 3 to 5 times to mix the solution evenly before taking the first time point reading.

NOTE: Total percentage of DMSO within culture tubes for *E. coli* should not exceed 10 % as this can affect cell viability[16].

2.3.5.2 Remove lid and place culture tube into sample tube lifter, raise the holder by hand to the sample injection needle and under the proper sample file click the "Record" icon. Additionally, begin a timer by pressing "Start" for the experiment.

2.3.5.3. Lower the tube lifter by hand after the run is completed, and repeat steps **2.3.5.1** through **2.3.5.2.** (with the timer running) by adding 1.4 µL of concentrated DFHBI-1T, inverting culture tubes, and taking readings for all remaining biological replicates. These first recordings will be the readings obtained at "0 minutes" for all biological replicates. Do this one at a time for each biological replicate.

NOTE: Make note of the time when the record flow cytometry acquisition is pressed. Adhere to this time staggering for time points over the course of the experiment.

2.3.5.4. Continue to take readings by raising the culture tubes in the sample tube lifter to the sample injection needle, selecting the proper sample file, clicking "Record" and lowering the lifter by hand after each run is complete. Do this for all additional time points and biological replicates being tested. Repeat steps until experiment is completed.

NOTE: Keep samples out of light to avoid the photobleaching of DFHBI-1T in solution by covering samples with aluminum foil.

2.3.6. Measure fluorescence at time points for cells without dye (Control)

2.3.6.1. Run the appropriate cleaning protocols for flow cytometer before repeating experiment again with negative controls following the manufacturer's protocol. This is done to minimize any carryover from the previous experiment into the control time point analysis experiment. Below are the steps followed for the flow cytometer between experiments:

2.3.6.1.1. Place an empty culture tube in the tube lifter by hand, raise the tube holder and click on the "Unclog" icon in the "Instrument" tab. This will run a backflush the fluidics system to clean up any sticky samples. Lower the tuber holder by hand and remove the tube once the "Unclog" sequence is done.

2.3.6.1.2. With a new culture tube, add 3 mL of a 10 % Bleach solution and place culture tube into tube holder and raise the holder by hand to the sample injection needle. Additionally, place a clean 96 well plate into Autosampler if applicable to flow cytometer.

2.3.6.1.3. Click on "Sanitize SIP/ Sanitize Autosampler SIP" icon to run a cleaning sequence with 10 % Bleach throughout the fluidics system. Lower the tube holder to complete cleaning sequence.

2.3.6.2. Set sample files for the control time point analysis run following the directions within **2.3.3.**

2.3.6.3. In a new culture tube, prepare a dilute cell solution in 1.5 mL of 1X PBS solution. Add 3 µL of induced cells in AI media into the 1X PBS solution to make a dilute cell solution. Repeat this step for each biological replicate.

2.3.6.4. Add 1.4 µL of DMSO into culture tube one at a time 1X PBS solution with cells and testing the same time points. Secure the culture tube lid, then invert culture tubes 3 to 5 times to mix the solution evenly before taking the first time point reading. Do this one at a time for each biological replicate.

2.3.6.5. Follow the same protocol for analysis of control cells as for cells with dye, using steps **2.3.5.2** to **2.3.5.4.**

2.3.7. Shutdown the flow cytometer

Follow the manufacturer's protocol for proper shutdown of instrumentation. For the flow cytometer, the instrument is prepared for shutdown in the following manner:

2.3.7.1. Place an empty culture tube in the tube lifter, raise the tube holder and click on the "Unclog" icon in the "Instrument" tab. This will run a backflush the fluidics system to clean up any sticky samples. Lower the tuber holder and remove the tube once the "Unclog" sequence is done.

2.3.7.2. With a new culture tube, add 3 mL of a 10 % Bleach solution and place culture tube into tube holder and raise the holder by hand to the sample injection needle. Additionally, place a clean 96 well plate into Autosampler if applicable to flow cytometer. Click on "Sanitize SIP/ Sanitize Autosampler SIP" icon to run a cleaning sequence with 10 % Bleach throughout the fluidics system. Lower the tube holder to complete cleaning sequence.

2.3.7.3. Replace the culture tube with 10 % Bleach solution with a culture tube with 3 mL Focusing Fluid. Raise the tube holder by hand and under the "Shutdown" icon click the on the drop-down menu and select "Thorough".

OPTIONAL PAUSE POINT

2.4. Analysis of flow cytometry data

2.4.1. Export all FCS files for analysis. Open the FCS files with a flow cytometry analysis software.

2.4.2. Using one of the Cell only FCS files, generate a gate from the forward scatter (FSC) and side scatter (SSC) dot plot (FSC-Area/SSC-Area) using both log axes to exclude any signals from debris. To create this gate, click on the "AutoGate" icon on the flow cytometry analysis software and name it "Gate 1". Apply this same gate to all samples tested under the "All Samples" tab in the data processing workspace. This will result in "Gate 1" underneath all FCS files being processed.

2.4.3. Create a new subset file with the Cell only FCS file used in step **2.4.2.** by double clicking it. Change the axis settings to FSC-Area/ FSC-Height with both using log axes. Click on the "AutoGate" icon on the flow cytometry analysis software to generate an oval gate naming it "Gate 2". Apply this gate as a subset underneath the gate set in step **2.4.2.** to all samples tested. This will result in all samples having "Gate 1" > "Gate 2" associated with each FCS file.

2.4.4. Create another subset file with both gates set in steps **2.4.2.** and **2.4.3.** applied by double clicking "Gate 2". Change the axis settings to FSC-Area/ Histogram. Apply this histogram gate as a subset to all samples tested resulting all samples having "Gate 1" > "Gate 2" > "Gate 2" associated with each FCS file.

NOTE: The Histograms can be renamed from "Gate 2" to "Histogram" to assist with moving the histograms into the layout window as well as create more organization with data processing.

2.4.5. To analyze the mean fluorescence intensity (MFI) values, open the layout window. Click and drag histogram gates for each time point into the layout window.

2.4.6. Perform statistical analysis for "∑ Mean: BL1-A" (GFP) for each sample tested to display the MFI results onto the layout window.

2.4.7. Calculate the standard deviation for the MFI values per time point analysis for at least three independent biological replicates.

2.4.8. Save Histograms and MFI values for each time point by exporting layout window as a PDF file.

OPTIONAL PAUSE POINT

2.5 Graph flow cytometry data

2.5.1. Open PDF file containing the histograms and MFI values for each time point. The MFI

values will be copied over into a data analysis software. In data graphing software., create a new data table in the XY format.

2.5.1.1. Select to create an XY Table with the following selected:

Data Table: Enter or import data into a new table

Options: X: Elapsed Times
Y: Enter (3-4) replicate values in side-by-side subcolumns

2.5.1.2. Label on the X axis all the time points for the experiment and control runs.

2.5.1.3. In Group A, input MFI values for all biological replicates into each time point for fluorescence analysis of cells with dye added.

2.5.1.4. In Group B, input MFI values for all biological replicates into each time point for analysis of cells with no dye (DMSO) added.

2.5.1.5. To observe results, click on "[Insert Data Set Name]" under the "Graphs" tab. This will display data points as a mean with error bars that represent the standard deviation (s.d.) at each time point. The X axis represents the elapsed time and the Y axis is the MFI values.

REPRESENTATIVE RESULTS:

***In vitro* kinetics.** The sequences of the DNA templates and primers, which are purchased as synthetic oligonucleotides, are shown in Table 2 and reagent recipes are shown in Supplementary Information. PCR amplification is used to scale-up the amount of DNA template with the T7 promoter, which is required for the subsequent *in vitro* transcription (IVT) reaction. In addition, PCR amplification can used for two purposes in the same reaction, to generate the full-length Broccoli DNA template by primer extension as well as scale-up the DNA template.

After the IVT reaction to synthesize RNA, PAGE purification will remove any unwanted truncated transcripts, degraded products, and unreacted rNTPs from the full-length RNA product. This type of purification is preferred because truncated or degraded RNAs will cause inaccurate determination of RNA concentrations. Since nucleotide bases absorb UV light, RNA bands and rNTPs on the gel can be visualized under UV as shadows against a fluorescent TLC plate. Thus, bands corresponding to the correct product size can be selectively extracted.

The nearest-neighbor method overestimates extinction coefficients and thus concentrations of structured RNAs since it does not account for hypochromicity due to base pairing[17]. Therefore, to determine accurate RNA concentrations, neutral pH thermal hydrolysis assays were performed to hydrolyze RNA to individual NMPs[18]. The accurate extinction coefficient was calculated as a sum of the extinction coefficients of NMPs in the RNA sequence.

The kinetics of DFHBI binding to Spinach2 and Broccoli was determined using a plate reader assay. RNA was first renatured to ensure it will be in the correct conformation for dye binding. Reaction conditions for the plate reader kinetics assay consist of 40 mM HEPES, pH 7.5 (KOH), 125 mM KCl, 3 mM $MgCl_2$, 100 nM renatured RNA, and 10 µM DFHBI, and the reaction was measured at 37 °C. This temperature and concentration of $MgCl_2$ was chosen to mimic physiological conditions[19] though conditions of 28 °C and 10 mM $MgCl_2$ may also be used for improved aptamer folding.

Both fluorogenic aptamers Spinach2 and Broccoli display two-phase association kinetics for binding to the DFHBI dye (Fig. 2). The kinetics data was better fit by two-phase association than one-phase association for both aptamers (Fig. S1). Rate constants and $t_{1/2}$ values for the fast and slow associations was determined by the best fit curve (Table 3). PercentFast, which describes what percent of the fluorescence turn-on magnitude is accounted for by the faster DFHBI-binding RNA population, also was determined.

Spinach2 in the binding-competent state shows faster turn-on than Broccoli ($t_{1/2}$ = 1.2 s vs 2.0 s). The second phase kinetics for both aptamers are similar ($t_{1/2}$ = 180 s), and likely correspond to a common rate-limiting step for a sub-population of the sample (PercentFast = 68% and 60% for Spinach2 and Broccoli, respectively). Overall, these results show that well-folded Spinach2 and Broccoli aptamers exhibit very fast turn-on kinetics, with initial half-maximal turn-on within 1-2 sec of dye addition.

Cellular kinetics. The sequences of the DNA constructs, which are cloned into the pET31b plasmid, are shown in Table 2 and reagent recipes are shown in Supplementary Information. The DNA constructs of fluorogenic RNA aptamers are typically contained within a tRNA scaffold for cellular experiments. The BL21 Star (DE3) *E. coli* strain is an expression strain with a mutation in RNase E that increases RNA stability.

Fluorescence time point measurements were recorded every 5 minutes for the first 45 minutes followed by readings at 1 h, 1.5 h and 2 h, giving a total of 12 time points plus the cell only reading. Having the shortest time interval being 5 minutes permitted multiple biological replicates to be measured at each time point, with regular spacing between replicate measurements of 30 seconds to 1 minute. The total volume of cells diluted in 1x PBS solution used for the time course experiment was 1.5 mL.

The cells were gated prior to determining the mean fluorescence intensity (MFI) of the population of single cells. Gating selects an area on the scatter plot to determine the cell population that will be analyzed. This process gets rid of any debris or multiplet readings from being included in the analysis. For the flow cytometry analysis shown, 30,000 events were recorded that resulted in 10,000-20,000 events analyzed after gating.

The cellular fluorescence kinetics is a function of both dye diffusion into *E. coli* and dye binding kinetics to the RNA aptamer within the cellular environment (Fig. 1B). For cells expressing Spinach2-tRNA, it is observed that the mean fluorescence intensity (MFI) increases immediately

at the '0' timepoint, due to the short time lag (in seconds) between dye addition and sample analysis (Fig. 3). Furthermore, cellular fluorescence has already reached its maximal equilibrated MFI value (40,441 ± 990) at the first timepoint of 5 min. In contrast, control cells show low background fluorescence (416) and no change in MFI value with DMSO addition. Comparison between cells with dye and cells with no dye reveals that fluorescence activation is 98-fold $\pm$ 2 in cells. Overall, these results show that Spinach2-tRNA expressed in *E. coli* cells exhibit fast turn-on kinetics, with maximal turn-on within less than 5 min of dye addition.

FIGURE AND TABLE LEGENDS:

Figure 1. Fluorescence activation occurs upon RNA aptamers binding to dye molecules (A) *in vitro* and (B) in cells.

Figure 2. Representative in vitro kinetics of the fluorogenic aptamers Spinach2 (A and B) or Broccoli (C and D) modeled by two-phase association, with t = 0 s being the timepoint of DFHBI addition (final DFHBI concentration: 10 µM). Experiments were performed in triplicate. All error bars represent standard deviations from the mean. From the fit, $t_{1/2}$ values were obtained for both the fast and slow association reaction components.

Figure 3. Representative cellular kinetics of Spinach2 in an tRNA scaffold. (A) Time point analysis of tRNA-Spinach2 dye uptake over the course of 2 hours. A cell only baseline was taken prior to adding in DFHIB-1T or DMSO. Time points were taken every 5 minutes for the first 45 minutes, followed by a time point reading at 1 h, 1.5 h, and 2 h. Arrow represents when the addition of DFHBI-1T or DMSO was added into 1 X PBS solution with BL21 Star cells. Final concentration of DFHBI-1T for analysis is at 50 µM. For the DMSO control, the addition of DMSO was at equal volume (1.4 µL) used for DFHBI-1T dye addition. (B) A close-up of the DMSO control time point analysis with BL21 Star *E. coli* cells. Mean Fluorescence Intensity (MFI) indicates the overall fluorescent readout of BL21 Star cells with dye or DMSO. Data represents the mean $\pm$ standard deviation of three biological replicates.

Table 1. Previously published photophysical and biochemical properties of Spinach2-DFHBI-1T[4, 13, 14] and Broccoli-DFHBI-1T[13].

Table 2. DNA Sequence Table contains DNA sequences and primers used for in vitro and cellular kinetics studies. **Bold**= T7 promoter; <u>UNDERLINED</u>= tRNA scaffold; CAPS= Spinach2; lowercase= Broccoli; ***BOLD ITALICS***= T7 terminator.

Table 3. In vitro kinetics values of the Spinach2 and Broccoli aptamers derived from fitted data, reported as the mean ± standard deviation of three replicates.

DISCUSSION:

Modifications and troubleshooting. For the *in vitro* kinetics experiment, the same general protocol can be modified to measure the *in vitro* kinetics of an RNA-based fluorescent biosensor

containing both a ligand-binding and fluorophore-binding domain[8]. In this case, the RNA should be incubated with the fluorophore prior to measurements upon injecting the ligand in order to obtain ligand response kinetics. If high variability is observed between replicates, one can troubleshoot by checking that each sample is allowed to equilibrate for the same amount of time in the 96-well plate before measurement. Each sample or replicate should be individually prepared in a well and measured right after the 15-minute equilibration step, rather than preparing all samples at once.

For the cellular kinetics experiment, the protocol can be modified for shorter or longer time courses, but it is critical to plan out the number of biological replicates and adjust the needed cell solution volume. It is recommended to space out each biological replicate reading between 30 seconds to 1 minute to have adequate time to perform the steps carefully. Another modification is to test a different fluorogenic dye that does not bind the RNA aptamer as an alternative negative control, which should not show fluorescence activation over background. If inconsistent results are observed, one can troubleshoot by checking that the flow cytometer is properly cleaned following the manufacturer's protocol between different experimental runs to prevent any bleed over of cells or dyes from the previous run to the next.

Limitations. While the *in vitro* method presented is useful for comparing kinetics between fluorogenic aptamers or RNA-based fluorogenic biosensors, the kinetic values obtained may change depending on the temperature, magnesium concentration, or other buffer components used. Also, while this method provides well-defined conditions that have been used previously to characterize different fluorogenic RNA systems, the intracellular environment cannot be perfectly represented due the presence of other biological macromolecules.

Whereas the fluorescence platereader equipped with a programmable injector has no dead time for data acquisition, the flow cytometer instrument has limited temporal accuracy due to observable dead time. There is a ~5 second lag between when the "Record" button is clicked and when data acquisition starts. An additional ~5 second lag occurs for the instrument to measure 30,000 events; this sample acquisition time will vary slightly depending on how dilute the cells are in 1X PBS.

Another potential limitation to cellular experiments is cell viability in 1X PBS. For extended time point analysis, cell viability can be checked using propidium iodide to stain dead cells[20]. Dye aggregation also can limit the accuracy of fluorescent measurements made by the flow cytometer. Dyes with very limited solubility in aqueous solutions can aggregate and appear as particles large enough to be counted as cells on the flow cytometer. Thus, it is important to run dye-only experimental controls to check for aggregates in the gated region.

Significance. Previously it was shown that Broccoli has comparable brightness to Spinach2 at 1 mM Mg^{2+} *in vitro*, but Broccoli-tRNA exhibits ~2-fold greater fluorescence intensity in live *E. coli* compared to Spinach2-tRNA[13]. To our knowledge, the dye-binding kinetics for Spinach2 and Broccoli fluorogenic aptamers have not been compared before and modeled by two-phase association. The initial fast rate constants for both RNA aptamers support that the dye binding

pocket is pre-folded and no structural changes are needed for the dye to bind, which is consistent with X-ray crystallography and UV-melting experiments[21, 22]. The second phase with a slower rate constant has not been previously reported because other experiments such as stopped-flow and fluorescence lifetime measurements analyzed the Spinach aptamer for shorter duration (20 and 300 seconds)[23,24]. The much slower second phase results in an observed biexponential increase in fluorescence when data are analyzed for 600 seconds. This slow step can be attributed to either a rate-limiting refolding step from a binding-incompetent to binding-competent RNA state or a rate-limiting photoconversion step from *trans* to *cis* forms of the bound dye. The latter mechanism was previously modeled to give a biexponential fluorescence profile[24] and is supported by recent analysis of the difference between the absorption and excitation spectra on the related aptamer, Baby Spinach[25].

The overall significance of the *in vitro* findings is that they show that dye association to the fluorogenic RNA aptamer does not limit real-time RNA localization and gene expression studies. For RNA-based fluorescent biosensors that employ Spinach2, the measured turn-on kinetics are similar to the second phase kinetics measured here[10], because the biosensors require a refolding step, so should be sufficiently rapid to enable near-real-time signaling studies.

It was expected that the cellular kinetics will be different from the observed *in vitro* kinetics for Spinach2. One key difference is that there is an additional step of dye diffusion into the *E. coli* cells, which involves crossing the outer and inner membranes. In addition, the cellular environment poses different conditions for dye-aptamer association, in terms of molecular crowding, ion composition and concentrations, as well as RNA and dye concentrations.

The overall significance of the results is that cellular fluorescence reaches maximum signal in less than 5 min and remains stable for at least 2 h, which enables real-time RNA localization and gene expression studies in this time range. For an RNA-based biosensor that employs Spinach2, we previously showed that significant fluorescence response could be observed within 4-5 min of ligand addition, but reaching maximal signal takes longer (15 to 30 min)[8]. Taken together, these findings indicate that dye diffusion into cells is not the practical rate-limiting step for *in vivo* experiments with RNA-based biosensors. Finally, this experimental protocol can be applied to analyze other fluorogenic RNA systems in cells.

Application to other fluorogenic aptamer systems. The experimental protocols presented here can be applied to analyze other fluorogenic RNA systems. Beyond the two aptamers analyzed in this study, Spinach2 and Broccoli, other fluorogenic RNA systems have been developed that provide different emission profiles, improved photostability, tighter binding affinities, and the ability to change fluorophores (recently reviewed[1]). In addition to their fluorescence properties, benchmarking the turn-on kinetics for these systems *in vitro* and in cells is important to assess suitability for different cell biological applications and may support structural pre-folding or rearrangement of the aptamer. As discussed, with some modifications, these protocols also have been applied to analyze RNA-based biosensors[8].

ACKNOWLEDGMENTS:

Funding sources: NIH R01 GM124589 and T32 GM122740, NSF-BSF 1815508

DISCLOSURES:

No conflicts of interest.

REFERENCES:

1. Su, Y., Hammond, M.C. RNA-based fluorescent biosensors for live cell imaging of small molecules and RNAs. *Current Opinion in Biotechnology*. **63**, 157–166, doi: https://doi.org/10.1016/j.copbio.2020.01.001 (2020).
2. Zhang, J., Fei, J., Leslie, B.J., Han, K.Y., Kuhlman, T.E., Ha, T. Tandem Spinach Array for mRNA Imaging in Living Bacterial Cells. *Scientific Reports*. **5** (1), 17295, doi: 10.1038/srep17295 (2015).
3. Wang, Z. *et al.* In Situ Spatial Complementation of Aptamer-Mediated Recognition Enables Live-Cell Imaging of Native RNA Transcripts in Real Time. *Angewandte Chemie (International ed. in English)*. **57** (4), 972–976, doi: 10.1002/anie.201707795 (2018).
4. Strack, R.L., Disney, M.D., Jaffrey, S.R. A superfolding Spinach2 reveals the dynamic nature of trinucleotide repeat–containing RNA. *Nature Methods*. **10** (12), 1219–1224, doi: 10.1038/nmeth.2701 (2013).
5. Thavarajah, W., Silverman, A.D., Verosloff, M.S., Kelley-Loughnane, N., Jewett, M.C., Lucks, J.B. Point-of-Use Detection of Environmental Fluoride via a Cell-Free Riboswitch-Based Biosensor. *ACS Synthetic Biology*. **9** (1), 10–18, doi: 10.1021/acssynbio.9b00347 (2020).
6. You, M., Litke, J.L., Jaffrey, S.R. Imaging metabolite dynamics in living cells using a Spinach-based riboswitch. *Proceedings of the National Academy of Sciences of the United States of America*. **112** (21), E2756–E2765, doi: 10.1073/pnas.1504354112 (2015).
7. Kellenberger, C.A., Wilson, S.C., Sales-Lee, J., Hammond, M.C. RNA-Based Fluorescent Biosensors for Live Cell Imaging of Second Messengers Cyclic di-GMP and Cyclic AMP-GMP. *Journal of the American Chemical Society*. **135** (13), 4906–4909, doi: 10.1021/ja311960g (2013).
8. Manna, S., Truong, J., Hammond, M.C. Guanidine Biosensors Enable Comparison of Cellular Turn-on Kinetics of Riboswitch-Based Biosensor and Reporter. *ACS Synthetic Biology*. **10** (3), 566–578, doi: 10.1021/acssynbio.0c00583 (2021).
9. Bose, D., Su, Y., Marcus, A., Raulet, D.H., Hammond, M.C. An RNA-Based Fluorescent Biosensor for High-Throughput Analysis of the cGAS-cGAMP-STING Pathway. *Cell Chemical Biology*. **23** (12), 1539–1549, doi: https://doi.org/10.1016/j.chembiol.2016.10.014 (2016).
10. Wang, X.C., Wilson, S.C., Hammond, M.C. Next-generation RNA-based fluorescent biosensors enable anaerobic detection of cyclic di-GMP. *Nucleic acids research*. **44** (17), e139, doi: 10.1093/nar/gkw580 (2016).
11. S., P.J., Thinh, N.-D., Wenjiao, S., R., J.S. Fluorescence Imaging of Cellular Metabolites with RNA. *Science*. **335** (6073), 1194, doi: 10.1126/science.1218298 (2012).
12. Paige, J.S., Wu, K.Y., Jaffrey, S.R. RNA mimics of green fluorescent protein. *Science (New York, N.Y.)*. **333** (6042), 642–646, doi: 10.1126/science.1207339 (2011).
13. Filonov, G.S., Moon, J.D., Svensen, N., Jaffrey, S.R. Broccoli: rapid selection of an RNA mimic of green fluorescent protein by fluorescence-based selection and directed

evolution. *Journal of the American Chemical Society*. **136** (46), 16299–16308, doi: 10.1021/ja508478x (2014).
14. Song, W., Strack, R.L., Svensen, N., Jaffrey, S.R. Plug-and-Play Fluorophores Extend the Spectral Properties of Spinach. *Journal of the American Chemical Society*. **136** (4), 1198–1201, doi: 10.1021/ja410819x (2014).
15. Sambrook, J., Fritsch, E., Maniatis, T. *Molecular Cloning: A Laboratory Manual*. N.Y.: Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY. (1989).
16. Basch, H., Gadebusch, H.H. In vitro antimicrobial activity of dimethylsulfoxide. *Applied microbiology*. **16** (12), 1953–1954, doi: 10.1128/am.16.12.1953-1954.1968 (1968).
17. Kallansrud, G., Ward, B. A comparison of measured and calculated single- and double-stranded oligodeoxynucleotide extinction coefficients. *Analytical biochemistry*. **236** (1), 134–138, doi: 10.1006/abio.1996.0141 (1996).
18. Wilson, S.C., Cohen, D.T., Wang, X.C., Hammond, M.C. A neutral pH thermal hydrolysis method for quantification of structured RNAs. *RNA* . **20** (7), 1153–1160, at <http://rnajournal.cshlp.org/content/20/7/1153.abstract> (2014).
19. Szatmári, D. *et al.* Intracellular ion concentrations and cation-dependent remodelling of bacterial MreB assemblies. *Scientific Reports*. **10** (1), 12002, doi: 10.1038/s41598-020-68960-w (2020).
20. Boulos, L., Prévost, M., Barbeau, B., Coallier, J., Desjardins, R. LIVE/DEAD® BacLight™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *Journal of Microbiological Methods*. **37** (1), 77–86, doi: https://doi.org/10.1016/S0167-7012(99)00048-2 (1999).
21. Huang, H. *et al.* A G-quadruplex-containing RNA activates fluorescence in a GFP-like fluorophore. *Nature chemical biology*. **10** (8), 686–691, doi: 10.1038/nchembio.1561 (2014).
22. Jeng, S.C.Y., Chan, H.H.Y., Booy, E.P., McKenna, S.A., Unrau, P.J. Fluorophore ligand binding and complex stabilization of the RNA Mango and RNA Spinach aptamers. *RNA* . **22** (12), 1884–1892, doi: 10.1261/rna.056226.116 (2016).
23. Han, K.Y., Leslie, B.J., Fei, J., Zhang, J., Ha, T. Understanding the Photophysics of the Spinach–DFHBI RNA Aptamer–Fluorogen Complex To Improve Live-Cell RNA Imaging. *Journal of the American Chemical Society*. **135** (50), 19033–19038, doi: 10.1021/ja411060p (2013).
24. Wang, P. *et al.* Photochemical properties of Spinach and its use in selective imaging. *Chemical Science*. **4** (7), 2865–2873, doi: 10.1039/C3SC50729G (2013).
25. Dao, N.T., Haselsberger, R., Khuc, M.T., Phan, A.T., Voityuk, A.A., Michel-Beyerle, M.-E. Photophysics of DFHBI bound to RNA aptamer Baby Spinach. *Scientific Reports*. **11** (1), 7356, doi: 10.1038/s41598-021-85091-y (2021).