Published in final edited form as:

Curr Opin Chem Biol. 2013 August ; 17(4): 651–655. doi:10.1016/j.cbpa.2013.05.014.

New approaches for sensing metabolites and proteins in live cells using RNA

Rita L. Strack^a and Samie R. Jaffrey^{a,*}

^aDepartment of Pharmacology, Weill Medical College, Cornell University. 1300 York Ave. LC 523. New York, NY. 10065, USA

Abstract

Tools to study the abundance, distribution, and flux of intracellular molecules are crucial for understanding cellular signaling and physiology. Although powerful, the current FRET-based technology for imaging cellular metabolites is not easily generalizable. Thus, new platforms for generating genetically encoded sensors are needed. We recently developed a new class of biosensors based on Spinach, an RNA mimic of GFP. In this case, RNA aptamers against a target ligand are modularly fused to Spinach that substantially induce Spinach fluorescence in the presence of ligand. We have used this approach to detect metabolites and proteins both *in vitro* and in living bacteria, thus providing an alternative to FRET-based sensors and a generalizable approach for generating fluorescent sensors to any ligand of interest.

Introduction

Methods to sense the abundance and distribution of molecules are crucial for studying cellular signaling pathways. Fluorescence-based sensing of biologically relevant small molecules and proteins in living cells has advanced tremendously in recent years, largely due to Förster resonance energy transfer (FRET)-based technology [1*,2]. Current FRET-based sensors are built by fusing a fluorescent protein (FP) donor and acceptor pair to either side of a protein recognition element that binds a ligand of interest [1]. For these sensors, ligand binding results in a change in distance and/or orientation between the FPs and in turn a change in FRET [3]. FRET-based sensors are advantageous because they are genetically encoded and can be used in living cells in real time. However, these types of sensors are not easily generalizable as each requires a unique protein that binds the target molecule and undergoes a conformational change that is sufficient to cause a change in FRET signal.

Recently, a novel class of sensors has been described based on Spinach, an RNA mimic of the green fluorescent protein (GFP). Spinach is a 98-nt RNA aptamer that binds and switches on fluorescence of a dye resembling the chromophore of GFP (Figure 1A) [4**,5]. The dye, 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), was synthesized alongside several related dyes and chosen based on photophysical properties [4]. Spinach was discovered using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technique [6*,7] against DFHBI immobilized on agarose resin [4]. Importantly,

© 2013 Elsevier Ltd. All rights reserved.

*Corresponding author: srj2003@med.cornell.edu, Address: 1300 York Ave, Box 70, New York, NY. 10065, Office phone: 212-746-6243.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Spinach RNA and DFHBI are essentially nonfluorescent when unbound. Thus, background fluorescence is low except where Spinach-DFHBI is localized. Spinach is brightly fluorescent *in vivo*, and has been used to label bacteria and to tag 5S RNA in mammalian cells [4].

Spinach-based sensors are genetically encoded and are modular in design. Thus, they can be readily modified to sense diverse biological molecules [4,8**,9]. They are built by inserting an aptamer sequence specific for any target ligand within a stem-loop region of the Spinach sequence (Figure 1B). These Spinach-based sensors are unfolded and nonfluorescent in the absence of ligand. However, in the presence of ligand, the transducer stem formed by the ligand-binding aptamer folds and stabilizes Spinach, enabling dye binding and fluorescence (Figure 1C) [8].

Constructing Spinach-based sensors

We have used this approach to develop several Spinach-based sensors. To show that these sensors can be used to study metabolite levels in cells, we generated sensors against adenosine, ADP, SAM, guanine, and GTP using previously described aptamer sequences [8]. In each case, several variants in the transducer region of Spinach were tested to generate sensors with optimal ligand-induced fluorescence. For each sensor, ~20-fold increases in fluorescence were observed upon ligand binding (Figure 2A).

We also used this approach to generate sensors of proteins. In this case, published aptamer sequences against thrombin and streptavidin were fused to Spinach and shown to be specifically activated in the presence of their cognate protein ligands. As part of the same study, the MS2 recognition element for the MS2 coat protein was fused to Spinach to generate a sensor for MCP, showing that natural protein-RNA interactions can also be used to generate Spinach-based sensors [9].

Spinach-based sensors are remarkably versatile and can be developed rapidly, because they take advantage of aptamer generation by the widely utilized SELEX technique [6,7]. During SELEX, a large library of oligonucleotides (typically $> 10^{13}$ unique molecules) is screened to enrich for aptamer sequences that bind a target ligand. RNA SELEX can be performed in 3–4 weeks and has been successfully used to generate aptamers that bind to a wide range of biologically and medically relevant molecules including ions, small molecules, metabolites, and proteins [7]. Because of the ability of SELEX to generate aptamers against diverse targets, Spinach-based sensors are expected to be able to detect a similarly diverse set of molecules.

An important consideration when generating an aptamer that will be used in a Spinach-based sensor is to select an aptamer that binds its ligand with an appropriate affinity. Usually the sensor needs to be designed so that its fluorescence is tuned to the physiological concentration of the molecule of interest. For example, if the concentration of a target varies from 50–500 μM in a cell, a Spinach-based sensor with a dissociation constant (K_D) of 10 mM would never be activated. On the other hand, a sensor with a K_D of 10 nM would exhibit maximal fluorescence regardless of the target molecule concentration. Thus, an aptamer should be chosen with a K_D that would allow changes in cellular target concentration to result in linear increases in cellular fluorescence. To achieve aptamers with an appropriate K_D , SELEX protocols can be modified to enrich for aptamers with a desired binding affinity by altering binding and washing conditions [10*].

Sensors should also be highly selective for their ligands. In some cases, desired targets may vary from other cellular metabolites by as little as a phosphate or methyl group. This selectivity can be achieved using RNA, as demonstrated by the ability of SELEX to generate

highly specific aptamers. For example, aptamers have been discovered that can selectively bind ADP even in the presence of high concentrations of ATP [11]. In some cases, SELEX protocols may include wash steps with closely related compounds to remove oligonucleotides that are nonspecific [11]. Consistent with the idea that Spinach-based sensors can be highly specific is our description of sensors that can discriminate between S-adenosylhomocysteine and S-adenosylmethionine (Figure 2B), and sensors that can discriminate between ADP, ATP, or AMP [8].

The fluorescence activation kinetics of a sensor are also crucial for imaging changes in cellular metabolite concentrations on biologically relevant timescales. Ideally, a sensor will become fluorescent instantaneously following ligand binding, ensuring that the kinetics of fluorescence acquisition accurately reflects changes in ligand concentration. However, since switching from a nonfluorescent to a fluorescence form requires conformational changes, this step has a specific activation rate. Fluorescence activation varies among sensors, however, we typically observed target-dependent increases in fluorescence within ten seconds of ligand addition, with signals plateauing around 10 minutes (Figure 2C) [8,9]. These rates likely reflect a combination of the rates of target binding, sensor folding, and DFHBI binding. Reversibility, or the ability to turn off fluorescence after ligand concentration decreases, is also an important consideration for monitoring reductions in target concentrations in a cell. Importantly, all of the sensors we have tested have shown reversibility on the scale of minutes (Figure 2D) [8], suggesting that these sensors can be used for signaling pathways in which the intracellular targets display changes in cellular concentrations on this timescale.

Using Spinach-based sensors to image metabolites in living cells

Because Spinach-based sensors are genetically encoded, they can be used to image the levels of target molecules in individual living cells in real time. Our lab has demonstrated that these sensors can be used to monitor metabolite dynamics for ADP and SAM in living bacteria. In the case of SAM sensing, *E. coli* were grown in the absence of methionine, a SAM precursor, and then treated with methionine (Figure 3). Upon methionine addition, fluorescence increased approximately six-fold over background levels over three hours, which matched an independent, ensemble measurement of cellular SAM levels. Because we were able to monitor SAM synthesis in individual cells, we observed that SAM synthesis was highly variable from cell-to-cell (Figure 3B) [8]. Further examination implicated the protein SAH hydrolase in causing this variability, as selective inhibition of this enzyme reduced variability among cells [8]. These results would have been obscured by measuring SAM levels in populations of cells, and underscore the importance of monitoring the dynamics of target molecules on the single-cell level in real time.

A Spinach-based sensor of the MS2 phage coat protein (MCP) has also been used to monitor infection kinetics of *E. coli* by MS2 [9]. In these studies, cells expressing the MCP sensor were infected by MS2 and imaged over time. These studies also revealed cell-to-cell variability in infection kinetics that would be masked in population measurements of MCP synthesis after infection, highlighting the strength of these molecules as sensors *in vivo*.

Spinach-based sensors produce robust signals in bacteria. For our imaging experiments, the sensors were transcribed from a pET-based vector under control of the strong T7 promoter, making the sensor highly expressed throughout the cell. This helps lead to a readily detectable signal enhancement in the presence of the target molecule. These signal changes can be measured using standard, commercially available fluorimeters, fluorescence plate readers, and conventional widefield microscopes. In addition, the bacterial signal is bright enough to allow for use in flow cytometry.

Like Spinach, Spinach-based sensors are remarkably photostable [4,8]. This photostability is likely due to the exchange of photobleached chromophores with unbleached chromophores from solution. This makes Spinach-based sensors extremely useful for continuous and extended live-cell imaging, as signal decreases can be tied to decreases in ligand concentration rather than photobleaching of the sensor.

An additional consideration for measuring intracellular ligand concentrations is the consistency of sensor expression in different cells. Ideally, sensor expression will be homogenous between cells, indicating that differences in signal seen among cells are due solely to differences in ligand concentration. In our bacterial imaging experiments, we have shown that sensor signal is uniform among bacterial cells by comparing the uniformity of fluorescence of cells expressing Spinach alone with the sensor under identical conditions [8]. We have also coexpressed BFP from the same vector as the sensor to allow for ratiometric measurements in individual cells [9]. Thus, controls for expression can be used to ensure consistency of sensor expression in cells.

Optimization and Future Directions

There are several approaches that can be used to improve Spinach-based sensors. The first involves increasing the overall brightness of Spinach, which would be expected to increase the brightness of Spinach-based sensors. The second is to expand the palette of available RNA aptamers, particularly in the yellow to far-red region of the spectrum, which could allow for sensing of multiple molecules at the same time in a given cell, and would enable precise ratiometric measurement of sensor concentration.

Several approaches can be envisioned to increase the brightness of Spinach. We previously described the use of Spinach to tag and image the 5S RNA in mammalian cells [4]. However, in these experiments, long imaging times were required suggesting that Spinach exhibits low fluorescence in cells despite exhibiting similar molar brightness to GFP [4]. The limited brightness of Spinach in mammalian cells seems to have many causes, including poor folding, ion sensitivity, thermal instability, and rapid degradation of tagged RNAs. We are developing novel Spinach variants with enhanced folding characteristics, increased thermostability, and reduced dependence on ions such as Mg^{2+} . These mutants should not only be more generally useful as an RNA imaging tag, but also should be useful for generating brighter sensors.

In addition, we are generating RNA-fluorophore complexes that exhibit bright yellow, orange, and red fluorescence emission. For these RNAs, fluorophores can be utilized that are similar to DFHBI, but which contain chemical modifications that alter the electronic properties of the dye resulting in spectral shifts [4]. RNAs discovered using SELEX against these fluorophores that produce bright fluorescence upon binding will result in new tags that can be used in place of Spinach. Red-shifted RNA-fluorophore complexes are desirable because cellular autofluorescence decreases at longer wavelengths. Using red-shifted RNA-fluorophore complexes will also decrease the exposure of cells to more toxic, lower wavelength excitation light and enhance signal to noise in all experiments.

The successful generation of bright red-shifted vegetable tags will greatly improve *in vivo* sensing. First, a Spinach-based sensor could be coexpressed with a red fluorescent RNA tag to allow for quantitative analysis of sensor concentration in a given cell, which would bypass the need to coexpress a fluorescent protein along with a sensor, and provide a more accurate measurement of sensor concentration.

In addition, two sensors could simultaneously be used to monitor a process in live cells. For instance, if the Spinach-based ATP sensor were coexpressed with a red fluorescent ADP

sensor, the amounts of ATP and ADP could be monitored and compared at the same time for enhanced accuracy. Moreover, cascading processes could be monitored in real time by using multiple sensors at the same time.

Conclusions

Overall, Spinach-based sensors provide a generalizable platform for monitoring the cytosolic concentrations of a target of interest. By combining the power of SELEX to generate aptamers, with the conditional fluorescence of Spinach-DFHBI, a wide array of targets can be analyzed in living cells. We expect that RNA-based sensors will extend our capacity to image molecules that are inaccessible to current protein-based imaging approaches.

References

- *1. Zhang J, Campbell RE, Ting AY, Tsien RY. Creating new fluorescent probes for cell biology. *Nat Rev Mol Cell Biol.* 2002; 3:906–918. An important review summarizing the generation of the first fluorescent protein based FRET sensors. [PubMed: 12461557]
2. Frommer WB, Davidson MW, Campbell RE. Genetically encoded biosensors based on engineered fluorescent proteins. *Chem Soc Rev.* 2009; 38:2833–2841. [PubMed: 19771330]
3. Lakowicz, JR. Principles of fluorescence spectroscopy. 3. New York: Springer; 2006. p. xxvip. 954
- **4. Paige JS, Wu KY, Jaffrey SR. RNA mimics of green fluorescent protein. *Science.* 2011; 333:642–646. The original paper describing the generation of Spinach for tagging RNAs. [PubMed: 21798953]
5. Tsien RY. The green fluorescent protein. *Annu Rev Biochem.* 1998; 67:509–544. [PubMed: 9759496]
- *6. Ellington AD, Szostak JW. *In vitro* selection of RNA molecules that bind specific ligands. *Nature.* 1990; 346:818–822. This paper describes the first *in vitro* selection of RNA aptamers. [PubMed: 1697402]
7. Stoltenburg R, Reinemann C, Strehlitz B. SELEX--a (r)evolutionary method to generate high-affinity nucleic acid ligands. *Biomol Eng.* 2007; 24:381–403. [PubMed: 17627883]
- **8. Paige JS, Nguyen-Duc T, Song W, Jaffrey SR. Fluorescence imaging of cellular metabolites with RNA. *Science.* 2012; 335:1194. The original paper describing Spinach-based sensors and demonstrating their utility in monitoring metabolites. [PubMed: 22403384]
9. Song W, Strack RL, Jaffrey SR. Imaging bacterial protein expression using genetically encoded sensors composed of RNA. 2013
- *10. Klug SJ, Famulok M. All you wanted to know about SELEX. *Mol Biol Rep.* 1994; 20:97–107. A high quality article that reviews SELEX and gives valuable tips for carrying out SELEX protocols. [PubMed: 7536299]
11. Srinivasan J, Cload ST, Hamaguchi N, Kurz J, Keene S, Kurz M, Boomer RM, Blanchard J, Epstein D, Wilson C, et al. ADP-specific sensors enable universal assay of protein kinase activity. *Chem Biol.* 2004; 11:499–508. [PubMed: 15123244]

Highlights

- We developed a system for developing genetically encoded fluorescent sensors based on Spinach.
- Modular design allows for straightforward generation of sensor to almost any target using SELEX.
- We have used these sensors to monitor biological processes in real time in living bacteria.

Figure 1. Modular strategy for Spinach-based sensors.

(a) Spinach is an aptamer that binds a small molecule dye called DFHBI (green ball). Both DFHBI and Spinach are nonfluorescent until binding occurs, which turns on fluorescence of the Spinach-DFHBI complex. Stem loop 2 of Spinach was found to tolerate insertion of additional sequences, and is the region that is modified to generate sensors. (b) To generate sensors, Spinach is modified to include a transducer region (orange) and a recognition module (blue). The recognition molecule is typically an aptamer generated against a target ligand by SELEX. Transducer length and composition can be varied for optimal sensor function. (c) In the absence of DFHBI and ligand (purple hexagon), the Spinach-based sensor displays minimal fluorescence. However, upon target binding, the recognition module of Spinach folds, which induces overall folding. The Spinach-based sensor can then bind DFHBI and activate fluorescence. This general strategy can be used to generate sensors against a vast array of targets.

Figure 2. A Spinach-based sensor of SAM

(a) A SAM sensor shows minimal fluorescence in the absence of SAM, even in the presence of DFHBI. However, upon SAM addition, fluorescence is activated over 20-fold. (b) The SAM sensor is specifically activated by SAM, and not the closely related molecules S-adenyl histidine (SAH), methionine, or vehicle. (c) Fluorescence activation occurs rapidly for the SAM sensor and reaches saturation on the order of minutes. (d) Fluorescence activation is reversible, and diminishes on the order of minutes for the SAM sensor after SAM has been removed. These data are representative of the metabolite and protein sensors generated based on Spinach, and are reproduced with permission from ref. 5.

Figure 3. SAM levels can be imaged in living cells.

(a) *E. coli* expressing the SAM sensor were imaged after treatment with methionine. Time course imaging revealed distinct patterns of SAM accumulation among individual cells. Some cells exhibited slow SAM accumulation (arrowhead) while others had higher than average (arrow) increases. A double arrow highlights a cell that initially increases then decreases SAM level. Pseudocolored images show the fold increase in fluorescence at each time point relative to 0 min (0- to 11.2-fold). Scale bar, 5 μ m. **(b)** Quantification of SAM levels in 800 individual cells revealed the large variance in SAM abundance after treatment with methionine, highlighting the importance of being able to monitor processes on the single-cell level. These data are reproduced with permission from ref. 5.