

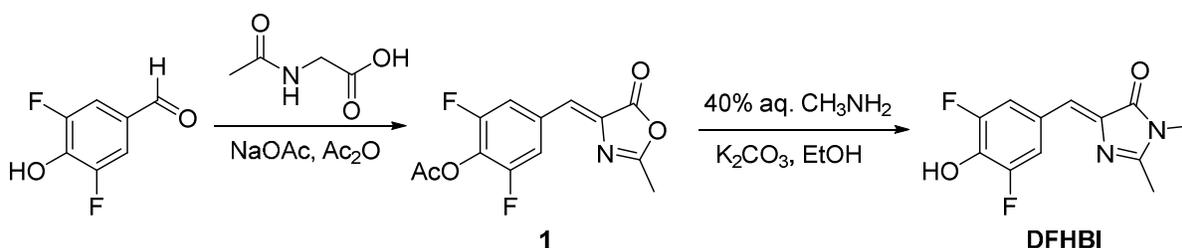
Supporting Information

Understanding the Photophysics of the Spinach-DFHBI RNA Aptamer-Fluorogen Complex to Improve Live Cell RNA Imaging

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Chemical synthesis of DFHBI

DFHBI was synthesized following the procedure of Paige and coworkers in Scheme 1.¹ All reagents were purchased from Sigma-Aldrich, except 4-hydroxy-3,5-difluorobenzaldehyde, which was procured from Oakwood Products, and all reagents were used without further purification.



Scheme S1

(Z)-2,6-difluoro-4-((2-methyl-5-oxooxazol-4(5H)-ylidene)methyl)phenyl acetate (**1**). In a 40 mL reaction vial with PTFE-faced silicone septum (ChemGlass) charged with dry Ar, *N*-Acetylglycine (316 mg, 2.7 mmol), anhydrous NaOAc (221 mg, 2.7 mmol), and 4-hydroxy-3,5-difluorobenzaldehyde (425 mg, 2.7 mmol) were suspended in acetic anhydride (2 mL). The vial was capped and stirred in an oil bath at 110 °C. After two minutes of stirring, all solids were fully dissolved, providing a yellow solution. After 2 h stirring at 110 °C, the vial was removed from heat, 10 mL EtOH was added, and the vial was recapped and stirred overnight at 4 °C to fully precipitate **1** as a yellow crystalline solid. Compound **1** was washed serially with -20 °C ethanol, 70 °C water, and hexanes over a sintered glass funnel and dried under vacuum to provide 323 mg of **1** (50%) as a yellow solid, which was used without further purification.

(Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-1,2-dimethyl-1H-imidazol-5(4H)-one (**DFHBI**). Compound **1** (158 mg, 0.56 mmol) and K₂CO₃ (95 mg, 0.7 mmol) were suspended in 4 mL EtOH at RT in a stirred 30 mL round bottom flask. Dropwise addition of 160 μL 40% aq. methylamine turned the yellow solution to orange and caused immediate dissolution of **1**. The flask was fitted with a water-cooled reflux condenser and the solution was refluxed for 2.5 h. The reaction mixture was then cooled to 4 °C, forming a dark yellow precipitate which was washed briefly with -20 °C EtOH and redissolved in EtOAc, then washed with 500 mM NaOAc, pH 3, in a separatory funnel. The organic layer was separated, dried over anhydrous Na₂SO₄ and dried under vacuum to provide 80 mg **DFHBI** as a bright yellow solid (57% yield).

which was used without further purification. HR-ESI calculated for $C_{12}H_{11}N_2OF_2$ 253.0789; found 253.0790.

Fluorescence detection by confocal microscope

We used a custom built confocal microscope to detect fluorescence of GFP and Spinach, unless specified otherwise.² A spatially filtered and collimated beam from CW diode laser (473 nm, CNI laser) was reflected by dichroic beam splitters (z473rdc, Chroma). After the beam was focused onto the sample plane by an oil immersion objective lens (NA = 1.4 HCX PL APO 100x, Leica Microsystems), the fluorescence signal was collected by the same objective, spectrally filtered (Chroma) over the wavelength range 500-550 nm, and imaged onto a multimode fiber (M31L01, Thorlabs). The fluorescence signal was detected by an avalanche photo diode (SPCM-AQR-14-FC, Perkin Elmer), and further processed by a multi-channel scaler (P7882, Fast ComTec). The illumination time and level of light intensity were controlled by acousto-optical filters (MT200-A0.5-VIS, AA Opto-Electronic) synchronized by a digital pulse generator (Model 9514+, Quantum Composers). If necessary, positioning and scanning of the sample was accomplished by a piezo-controlled stage (NanoMax, Thorlabs). The full-width-at-half-maximum (FWHM) values of the excitation foci were ≈ 200 nm. The laser intensity $I_{exc} = P/(\pi (0.5 \times FWHM)^2)$ was calculated from the measured power P .

Time-resolved fluorescence measurement

The fluorescence lifetime measurements were performed by the same microscope described above. An ultrafast laser (MaiTai HP, Spectra Physics) was used to generate supercontinuum light with the use of a photonic crystal fiber (FemtoWhite 800, NKT Photonics). The generated light was spectrally selected by a narrow excitation filter (Z473/10x, Chroma) and stretched up to ~ 10 ps by a pre-stretcher (N-SF57, CASIX) and single-mode fiber (Thorlabs). The fluorescence signal was detected by an avalanche photo diode (Micro Photon Devices) and registered by a time correlated single photon counting module (SPC630, Becker Hickl). The fluorescence lifetime was extracted by deconvolution with an instrumental response function (FWHM ~ 80 ps) measured on 80-nm gold particles (BBI Solutions).³

Supplementary Note 1: Fluorescence decay in a freely diffusing solution

In a diffusing sample in a cuvette, it is not easy to detect an initial drop in fluorescence intensity under ordinary light intensity. The reason is that the signal drop is the outcome of the balance between fluorescence decay (k_{off}) arising from irreversible photobleaching and/or reversible photoconversion, and diffusion controlled net inflow (k_{influx}) where fast recovery such as $T_1 \rightarrow S_0$ may be also involved. Here, the amount of drop is roughly proportional to $k_{off}/(k_{influx} + k_{off})$. Generally, as k_{influx} is much faster than k_{off} , very high excitation intensity is required to observe a signal drop in a freely diffusing sample. (Please notice that k_{influx} of confocal microscopy is by far faster than that of fluorometer due to smaller excitation focal volume). In the case of GFP, its time trace is almost flat even at $I_{exc} = 160$ kW/cm², a value two or three orders of magnitude higher than what is used in typical wide-field and confocal imaging (Figure 2). Of course, at very high intensity, the fluorescence signal drop can be seen (Figure S1). On the other hand, Spinach solution shows initial signal drop even at $I_{exc} = 1.6$ kW/cm² (Figure 2). This suggests that the

weaker power-dependence of the Spinach-DFHBI complex in the immobilized sample would be due to a saturation effect of the highly efficient photoconversion process at the ordinary excitation intensity.

Supplementary Note 2: Measurement of recovery rates

Recently Wang *et al.* have reported fluorescence recovery rates of Spinach based on fluorescence time profiles for a diffusing sample.⁴ With 1.8 – 20 μM of RNA aptamer and 0.1 μM of DFHBI, or when using 0.1 μM Spinach with 1 μM DFHBI, their recovery rates were universally $\sim 0.0027 \text{ s}^{-1}$ corresponding to a half-time of $\sim 260 \text{ s}$. We used an immobilized sample of Spinach to compare these values. The surface density of the immobilized RNA was between 5 to 20 molecules per an illuminated area. Assuming our focal volume is about 0.2 fL, it corresponds to an aptamer concentration of 40 – 160 nM in the focal volume. In sharp contrast with their results, as [DFHBI] increases from 1 to 20 μM in our experiment, our measured recovery rate changes from 0.08 to 0.74 s^{-1} , thus displaying a strong dependence on [DFHBI], in disagreement with their results. Specifically, at our most similar reaction conditions (1 μM DFHBI), our measured recovery rate is 30 times faster (assuming a 100 nM aptamer concentration). Additionally, compared the recovery rates at 20 μM of substrate concentrations, our measured recovery rate is 190-fold faster than Wang *et al.*'s.

If one were to conduct this kind of experiment for the diffusing sample in a cuvette or small flow chamber, the effect of diffusion must be carefully considered. (In our experiments, we used an immobilized sample to measure fluorescence recovery rates of the Spinach-DFHBI complex). Unless the entire solution volume is illuminated, (c.a. 10 – 500 μL), photoconverted molecules in an excitation focal volume will be always replaced by new ones from the surrounding solution. As a result, instead of measuring recovery rates, one would simply record the replenishment rate by diffusion, not the recovery rate by reversible photoconversion. For example, with a smaller focal volume provided by a confocal microscope, recovery time gets faster because the diffusion time (t_D) is proportional to the square of the characteristic length scale (l_D) of the focal volume, where D is a diffusion coefficient (eq. 1).

$$l_D = (6 \times D \times t_D)^{1/2} \quad (1)$$

This can explain why (i) the recovery rates of Spinach-DFHBI and that of DFHBI alone were the same and (ii) the recovery rate was independent of [RNA] in their data. These are misinterpretations originating from the use of a diffusing sample. Thus, one must either use an immobilized sample or illuminate the whole volume for recovery rate measurements. To illuminate a cross section of 1 mm \times 1 mm with 10 W/cm^2 , a 100 mW collimated laser light source would be required.

References

- (1) Paige, J. S.; Wu, K. Y.; Jaffrey, S. R. *Science* **2011**, 333, 642.
- (2) Han, K. Y.; Kim, S. K.; Eggeling, C.; Hell, S. W. *Nano Lett.* **2010**, 10, 3199.
- (3) Han, K. Y.; Wildanger, D.; Rittweger, E.; Meijer, J.; Pezzagna, S.; Hell, S. W.; Eggeling, C. *New J. Phys.* **2012**, 14.
- (4) Wang, P.; Querard, J.; Maurin, S.; Nath, S. S.; Le Saux, T.; Gautier, A.; Jullien, L. *Chem. Sci.* **2013**, 4, 2865.

Supporting Figures

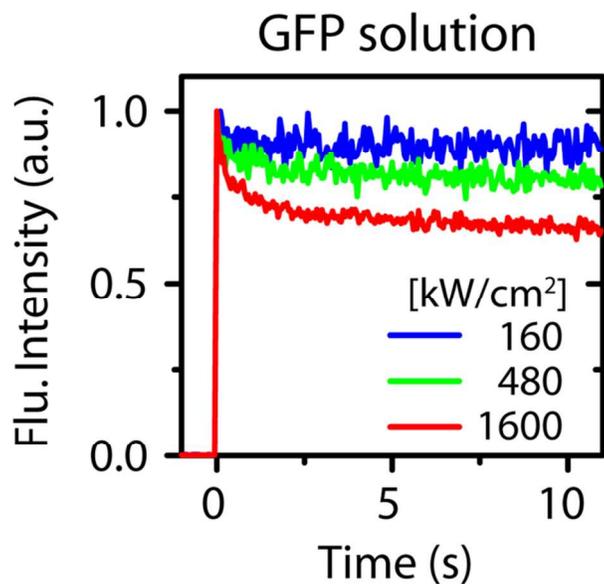


Figure S1. Fluorescence time courses of GFP in freely diffusing solutions at different irradiances by confocal microscopy.

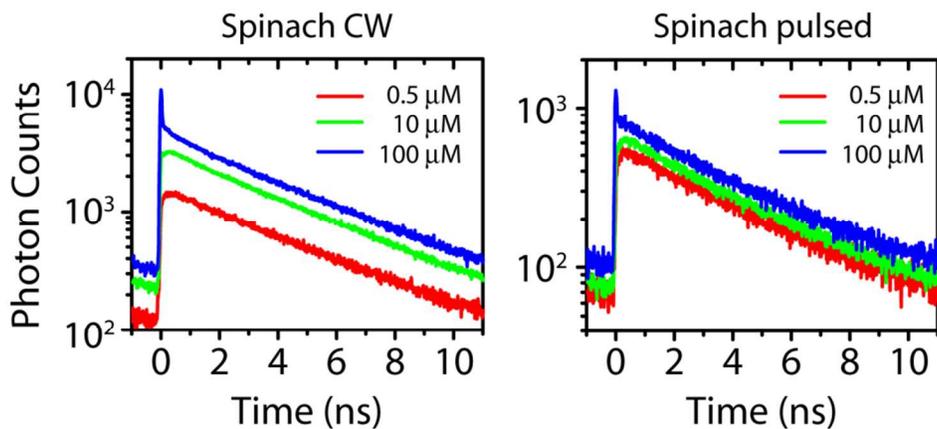


Figure S2. Fluorescence lifetime of Spinach is not greatly changed at higher concentrations of DFHBI. At [DFHBI] = 100 μM, the fluorescence lifetime gets slightly shorter ($\tau_f = 3.9 \pm 0.1$ ns) probably due to the aggregation of DFHBI. The peaks at 100 μM DFHBI originate from free DFHBI whose lifetime is shorter than 80 ps.

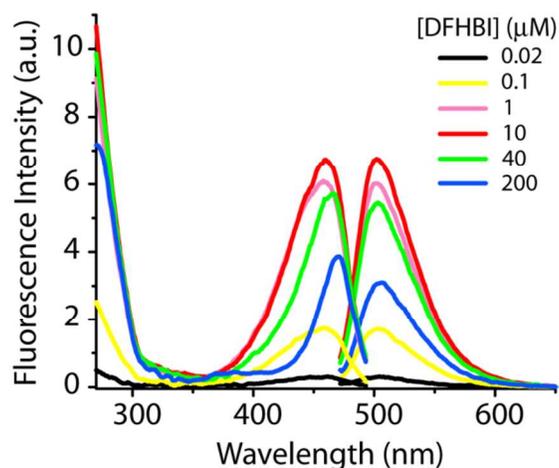


Figure S3. Fluorescence excitation and emission spectra of the Spinach-DFHBI complex at different concentrations of DFHBI. The excitation maximum is red-shifted at higher [DFHBI]. A similar change can be seen in DFHBI-only solution, implying that it is inherited from DFHBI itself. The fluorescence intensity was measured at $\lambda_{em} = 505 \pm 5$ nm for excitation spectra and $\lambda_{exc} = 460 \pm 5$ nm for emission spectra, respectively.

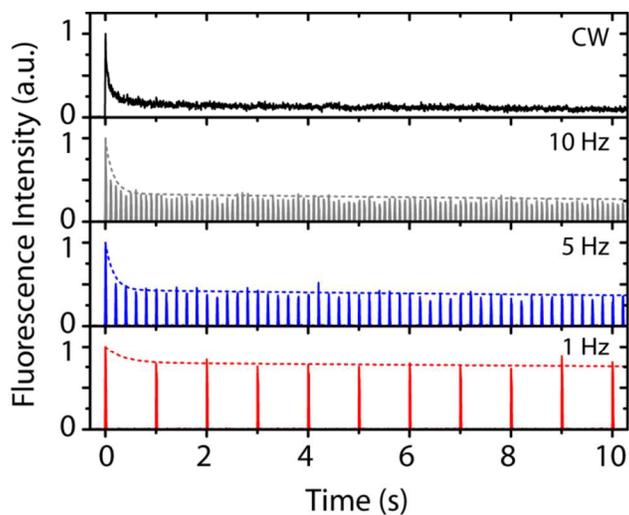


Figure S4. Repetition rate dependent fluorescence yield of the surface immobilized Spinach-RNA complex in the presence of $20 \mu\text{M}$ DFHBI. A pulse width was 20 ms and pulse-peak intensity was $1 \text{ kW}/\text{cm}^2$.

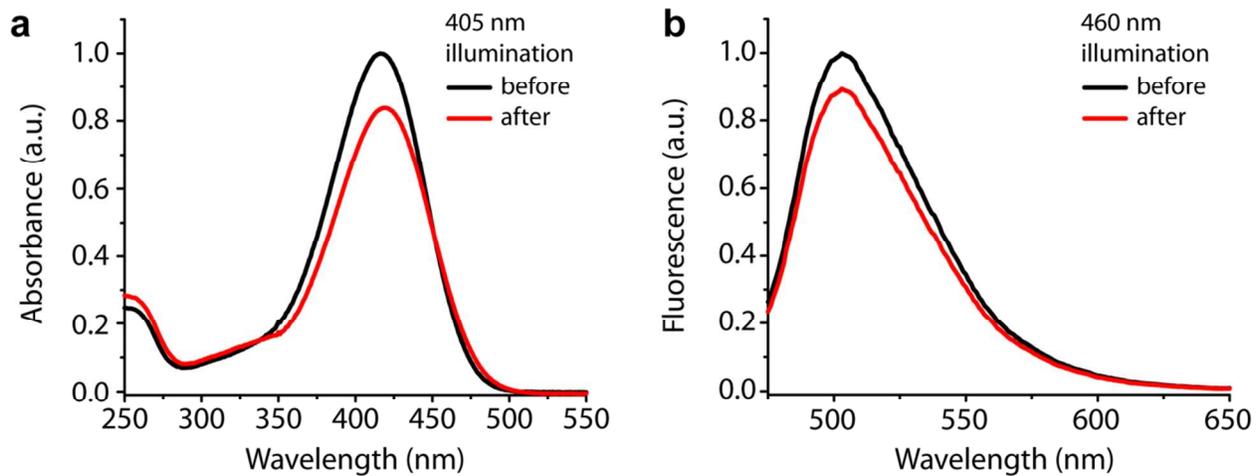


Figure S5. (a) Absorption spectrum of DFHBI before and after 405 nm illumination. (b) Fluorescence excitation spectrum of Spinach-DFHBI before and after 460 nm illumination.