

## Highly sensitive fluorescent protein FRET detection using optofluidic lasers

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**We achieved optofluidic protein lasing using genetically encoded fluorescent protein FRET pairs linked by length-tunable peptides. Up to 25-fold reduction in the donor laser emission was observed when the donor and the acceptor were brought to close proximity, as compared to only 17% reduction in the donor emission using the conventional FRET detection. Our work opens a door to a broad range of applications in studying protein–protein interactions and protein–drug interactions.**

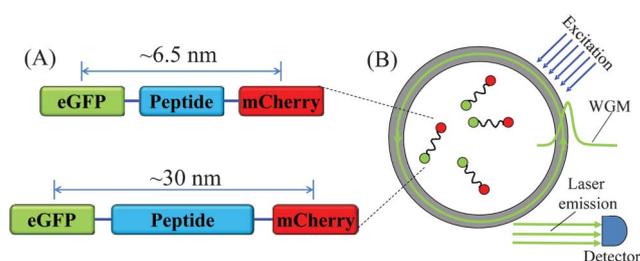
Protein–protein interactions play a central role in cellular signaling.<sup>1,2</sup> Monitoring and modulating protein interactions provides important mechanistic insight into cellular processes and is essential for developing pharmacological intervention in disease states. Currently, investigations of protein interactions commonly employ fluorescence resonance energy transfer (FRET) between two genetically encoded fluorescent proteins to extract information about intermolecular and intramolecular changes in proteins.<sup>3</sup> However, conventional FRET detection suffers severely from small changes in the FRET signal (usually only a few percent) in the donor and acceptor emission brought about by weak protein–protein or protein–drug interactions. Enhancement of the FRET response is highly desirable, as it would provide much more detailed information about protein interactions that otherwise cannot be resolved using conventional FRET detection.

Here we developed an optofluidic protein laser for highly sensitive intra-cavity detection of protein interactions. The optofluidic laser employs stimulated laser emission (rather than fluorescence normally used in the conventional FRET) as the sensing signal.<sup>4,5</sup> When FRET takes place inside the laser cavity, a small change in FRET induced by protein interactions will be optically amplified by the optofluidic laser, thus resulting in a

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drastic increase in the FRET signal. As the first step towards fully implementing intra-cavity detection of protein interaction, in this work we achieved optofluidic laser emission from genetically encoded fluorescent proteins. The gene transfection method allows for synthesis of a protein FRET pair linked by a length-tunable peptide, thus providing a highly versatile platform to precisely control the FRET efficiency for systematical investigation of protein interactions.<sup>6</sup> Detailed studies show that the optofluidic laser characteristics could be drastically modulated by the energy transfer between the protein pair, thus significantly enhancing the FRET signal.

Two kinds of genetically encoded proteins were used to demonstrate the FRET-modified laser characteristics in this study (Fig. 1(A)). The short-linked FRET pair was connected by a short peptide (6-residue protease site and Gly-Ser-Gly linker), making the distance between eGFP (with A206K mutation to minimize dimerization) and mCherry approximately 6.5 nm. The long-linked pair was connected by a length-tunable ER/K  $\alpha$ -helix.<sup>6</sup> For the current work, the length was 30 nm.



**Fig. 1** (A) Two types of genetically encoded fluorescent protein pairs (eGFP as the donor and mCherry as the acceptor) used in the experiment. eGFP and mCherry were linked by a peptide, whose length could be adjusted during synthesis. The Förster distance between eGFP and mCherry is about 4.7 nm. Fluorescence resonance energy transfer (FRET) occurs for the short-linked protein pair when the distance between eGFP and mCherry is approximately 6.5 nm. For the long-linked protein pair ( $\sim 30$  nm), FRET diminishes. (B) The optofluidic ring resonator (OFRR) is a piece of thin-walled capillary whose circular cross section supports a high-Q whispering gallery mode (WGM) that provides the optical feedback for fluorescent proteins to lase upon external optical excitation with a 5 ns pulsed optical parametric oscillator.

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We used insect cell line sf9 (a clone originally established from ovarian tissue of the insect *Spodoptera frugiperda*) to produce the genetically encoded fluorescent protein pairs. Sf9 cells were transiently transfected with the pBiex1 vector with insertion of a genetic construct encoding either one of the two types of the FRET pairs. Both constructs had a FLAG tag. Cells were lysed three days post-transfection in HEPES Lysis Buffer (0.5% IGEPAL, 4 mM MgCl<sub>2</sub>, 200 mM NaCl, 7% Sucrose, 20 mM HEPES, pH 7.5, 5 mM DTT, 50 μg mL<sup>-1</sup> PMSF, 5 μg mL<sup>-1</sup> aprotinin, 5 μg mL<sup>-1</sup> leupeptin). Lysates were clarified by ultracentrifugation (45 000 × g, 4 °C, 45 min) and bound to Anti-FLAG M2 Affinity gel (Sigma-Aldrich). The gel was washed with HEPES Wash Buffer (150 mM KCl, 20 mM HEPES, pH 7.5, 5 mM MgCl<sub>2</sub>) and eluted using FLAG peptide. Evaporation was utilized to condense the resulting protein solution to the desirable concentration. A FluoroMax-4 spectrofluorometer (Horiba Scientific) was used to characterize the protein pairs. 100 μL 20 nM protein pair was placed in a cuvette. 484 nm and 587 nm light were used to excite the eGFP and mCherry, respectively. The emission of eGFP and mCherry was then collected and analyzed.

To achieve protein lasers, the optofluidic ring resonator (OFRR) was used (Fig. 1(B)).<sup>4,5,7</sup> The OFRR is a piece of thin-walled glass capillary. The circular-shaped OFRR cross section supports the high-Q whispering gallery mode, which has an evanescent field inside the core to provide the optical feedback for proteins to lase. The fabrication process of the OFRR was the same as that in our earlier work.<sup>4</sup> The OFRR had an outer diameter of 90 μm and a wall thickness below 2 μm. During the experiment, ~10 μM solution of the protein pair was withdrawn into the capillary by a syringe pump at a flow rate of 1 μL min<sup>-1</sup>. The solution was excited by a pulsed optical parametric oscillator (OPO) with a pulse duration of about 5 ns and a repetition rate of 20 Hz. Emission of the solution was collected by a multimode optical fiber and sent to a spectrometer for analysis.

We first characterized the protein pairs used in the experiments. Fig. 2(A) shows the mCherry emission when the protein pairs were excited within the mCherry absorption band (587 nm). Virtually identical spectra between the long- and short-linked protein pairs suggest that mCherry emission is not affected by its

distance from the eGFP (donor). In contrast, when the protein pairs were excited within the eGFP absorption band (484 nm), a decrease in the emission of eGFP and concomitant increase in mCherry emission are observed for the short-linked protein pair, as compared to the long-linked protein pair. The energy transfer efficiency of approximately 17% between eGFP and mCherry is obtained according to:

$$E = 1 - \frac{I_{D,\text{FRET}}}{I_{D,0}} \quad (1)$$

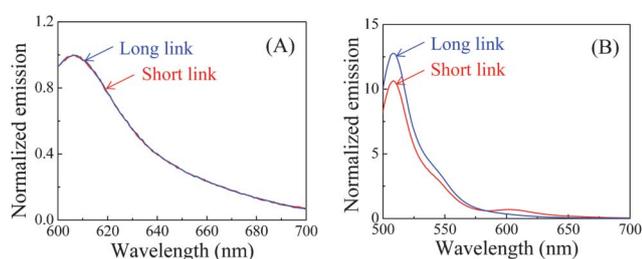
where  $E$  is the energy transfer efficiency.  $I_{D,\text{FRET}}$  and  $I_{D,0}$  are the donor emission in the presence and absence of FRET, respectively. The experimentally measured energy transfer efficiency agrees well with the estimation obtained by using

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (2)$$

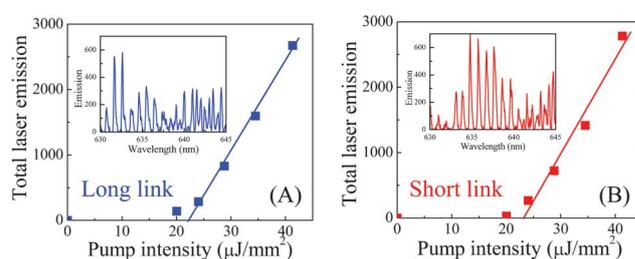
where  $R_0$  (= 4.7 nm) is the Förster distance and  $R$  (= 6.5 nm) is the distance between eGFP and mCherry.

Before we moved to investigate the FRET effect on the protein laser emission, we carried out a control experiment. Previously, in Fig. 2(A) we have shown that the fluorescence from mCherry was not affected by its distance from eGFP when the protein pairs were excited directly within the mCherry absorption band (which is well below that of eGFP). Likewise, Fig. 3 compares the laser emission from mCherry for both long- and short-linked protein pairs when they were excited with the OPO at 588 nm, at which wavelength only mCherry was excited. Nearly identical lasing threshold curves with the same lasing thresholds and laser efficiencies (the slope above the lasing threshold) are obtained, suggesting that both long- and short-linked protein samples were identical with respect to mCherry. Additionally, since eGFP and mCherry in each sample had an equal molar ratio (1 : 1), the result in Fig. 3 further indicates that both long- and short-linked samples were identical, except for the distance between eGFP and mCherry.

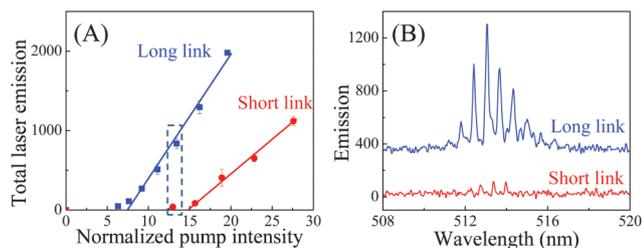
Fig. 4 investigates the FRET effect on the eGFP lasing characteristics. Fig. 4(A) compares the eGFP laser emission threshold curve between the long- and short-linked protein pairs.



**Fig. 2** (A) The mCherry fluorescence emission spectra for the long- and short-linked protein pairs normalized to their respective emission peaks. Excitation wavelength was 587 nm so that only the acceptor was excited. (B) The donor and acceptor spectra for the long- and short-linked protein pairs normalized to their respective acceptor emission peaks in (A). Excitation wavelength was 484 nm so that only the donor was excited. FRET efficiency is approximately 17% based on eqn (1). The spectra were obtained using FluoroMax-4 spectrofluorometer (Horiba Scientific).



**Fig. 3** Spectrally integrated mCherry lasing emission vs. pump intensity for (A) long- and (B) short-linked protein pairs. Spectral integration takes place between 621 nm and 655 nm. The excitation wavelength was 588 nm from the OPO so that only mCherry was excited. The lasing threshold is approximately 22.4 and 23.2 μJ mm<sup>-2</sup> for long- and short-linked protein pairs, respectively. Solid curves are the linear fit above the respective lasing threshold. The insets show part of the mCherry lasing spectra for long- and short-linked protein pairs when pumped at 41 μJ mm<sup>-2</sup>.



**Fig. 4** (A) Spectrally integrated eGFP lasing emission vs. pump intensity normalized to their respective mCherry lasing threshold for long- and short-linked protein pairs. Spectral integration takes place between 508 nm and 542 nm. Error bars are obtained by five measurements. (B) eGFP laser emission spectra for long- and short-linked protein pairs when pumped at  $300 \mu\text{J mm}^{-2}$  (corresponding to the boxed datapoints in (A)). Curves are vertically shifted for clarity. The excitation wavelength was 488 nm from the OPO.

For the long-linked pair, the distance between eGFP and mCherry ( $\sim 30$  nm) is much larger than their Förster distance (4.7 nm). Therefore, the FRET effect of mCherry on eGFP is negligible and strong eGFP laser emission emerges. In contrast, for the short-linked protein pair ( $\sim 6.5$  nm), the energy transfer efficiency is about 17%. Though small, this is sufficient to cause drastic changes in the eGFP laser characteristics with  $2 \times$  increase in the lasing threshold and  $2 \times$  reduction in the lasing efficiency. In a typical FRET measurement, the donor emission reduction can be used as the sensing signal. Fig. 4(A) shows that a  $5\text{--}25 \times$  reduction in the eGFP laser emission can be obtained when the pump ranges from 12.5 to 22.5. One example with  $25 \times$  reduction is shown in Fig. 4(B). Note that an even larger reduction ratio can be achieved when the pump is between 7.5 and 12.5. In this case, the eGFP lasing is completely quenched for the short-linked protein pair and only fluorescence exists, which is usually over  $100 \times$  smaller than the laser emission.<sup>4</sup>

In summary, we have demonstrated the laser emission from eGFP and mCherry using optofluidic lasers. Our results show that the donor (eGFP) laser emission can be strongly affected by the presence of the acceptor in proximity. Up to  $25 \times$  reduction in the donor emission was observed using the optofluidic laser intracavity detection method, as compared to only 17% in the conventional FRET.

Our work provides two new approaches to quantifying the protein interaction sensitively. The first one is through analyzing the FRET signal typically used in protein interaction analysis but amplified by the optofluidic laser. The second one utilizes the lasing threshold unique to the optofluidic laser and unavailable in the conventional FRET analysis. In both approaches, a series of protein donor and acceptor pairs with well-defined distances (such as those used in our current work) can be used as the calibrator. Meanwhile, a rigorous theoretical model can be established to aid quantitative analysis of protein interactions.

There are a few drawbacks of our approach that need to be overcome. While the analysis time for the first approach is as fast as the conventional FRET analysis, the second approach is time-consuming, as it requires acquisition of emission spectra at different levels of excitation. This issue can be mitigated with robotic systems for autonomous measurement. The other

challenge relates to the protein concentration. In our current work,  $\sim 10 \mu\text{M}$  of protein is needed, which is  $10\text{--}100$  higher than that for conventional FRET analysis. However, due to the excellent microfluidic design, the total protein mass used in our method is the same as or even lower than that in the conventional FRET analysis. In addition, it needs to be emphasized that in protein–protein or protein–drug interaction studies, what is of paramount importance is not to detect protein of trace quantities (like in typical biosensing) but to discern the small difference or change in those interactions.

Our future work will use protein FRET pairs whose distance can be modulated by the presence of drug molecules. The significantly enhanced FRET signal will allow us to differentiate the efficacy of drug molecules, which is difficult to accomplish with the current FRET technology due to the small FRET signal caused by drug molecules. In addition, we will translate the optofluidic laser intracavity detection technology to living cells. Recently, eGFP lasing was achieved in a cell.<sup>8</sup> Combination of biofunctional protein FRET pairs, which undergo conformational change upon bio/chemical activation, and the cell laser, will open a door to a broad range of applications in protein interaction studies, cellular signaling, and drug discovery.

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