A sensitive FRET biosensor reveals Fyn kinase regulation by sub-membrane localization

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The list of supporting materials:

1. Supporting experimental procedures
2. Supporting figures S1-S8
3. Supporting movie legends

Supporting Information

Supporting Experimental Procedures

Reagents and cell culture

MEF cells were cultured in Advanced DMEM (Gibco) supplemented with 3% fetal bovine serum (FBS), 2 mM L-Glutamine, 100 I.U./ml penicillin and 100 μg/ml streptomycin, and maintained at 37°C in 5% CO_2 incubator. The DNA constructs were transfected into MEF cells by Lipofectamine2000 or Lipofectamine3000 reagent (Invitrogen) 2-3 days before imaging experiments. For the comparison of the FRET responses to different kinases or Fyn mutants in cells, the biosensor construct was co-transfected with the same amount of plasmids encoding different kinases to control the expression levels of proteins within the similar ranges. After transfection for 8-16 hours, cells were maintained in Advanced DMEM without serum, then seeded on 1 μg/ml (for membrane-targeted biosensor) or 10 μg/ml fibronectin-coated glass-bottom dishes (Cell E&G) in Advanced DMEM without serum for 6-24 hr before imaging on microscope.

The active tyrosine kinases Fyn, Src, Yes were purchased from Upstate Biotechnology, PDGFR from Millipore, Abl from Calbiochem, Lck and EGFR from Sigma. Fibronectin was purchased from Thermo Fisher Scientific. Rat PDGF-BB and Src family inhibitor PP1 were purchased from Sigma.

Purification of the biosensor and *in vitro* kinase assay

The recombinant biosensor proteins with an N-terminal 6xHis tag in pRSETb vector were expressed in *E. Coli* (BL21) by culturing with 0.4 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside, Sigma) at 25°C for 16-24 hours, and purified by nickel chelation chromatography, as described previously[1]. *In vitro* characterization of the biosensors by the tyrosine kinase assay was performed at 37°C on a fluorescence plate reader (TECAN, Sapphire

II), as described in previous work[2]. In brief, purified biosensor protein was diluted at a final concentration of 1 μM in kinase assay buffer (50mM Tris-HCl, PH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 2mM DTT) containing 1 μg/ml active kinase in a 96-well plate (100 μl of total volume per well). The emission spectra of the biosensor solutions between 450nm and 550nm were read out every 3 min under 427nm excitation before and after addition of 1 mM ATP (Sigma). The emission ratio (ECFP/FRET) was calculated as emission 478nm/emission 528nm according to the fluorescence intensity readouts at 478nm and 528nm.

Microscope, and image acquisitions

Cells expressing biosensors and other molecules were seeded on glass-bottom dishes and mounted in a customized 5% CO_2 imaging chamber on a Nikon microscope system which is equipped with a cooled charge-coupled device camera (Cascade 512B; Photometrics), and an XYZ-stage controller with multiple-position and auto-focus functions. The parameters of dichroic mirrors, excitation and emission filters for imaging of FRET and mCherry fluorescent protein were described previously [3]. Images were acquired at 37°C by using the software MetaMorph (Universal Imaging). Twenty to thirty positions from each sample dish were selected and imaged under the same condition.

FRET quantification

The ECFP and FRET images were processed and quantified by our image analysis software package *Fluocell*, which is developed in MATLAB. The *Fluocell* source code and documents for Windows and Mac systems can be downloaded from Github (http://github.com/lu6007/fluocell). All the ECFP and FRET images were background-subtracted and smoothed with a median filter defined by a 3 × 3 pixel size window. Then the pixelwise ECFP/FRET ratio images were calculated

and visualized with the intensity modified display (IMD) method. To quantify the time courses of ECFP/FRET ratio for each single cell, *Fluocell* calculated the cell mask with global image threshold using Otsu's method [4]. To quantify the ECFP/FRET ratio signal outside the nucleus, the cell mask was divided into three layers to allow only the outermost layer to be quantified. For comparison among different experimental groups, ECFP/FRET ratio time courses from multiple cells in the same group were plotted together. Alternatively, the average time course within one group was plotted with error bars (SEM).

Supporting Figures

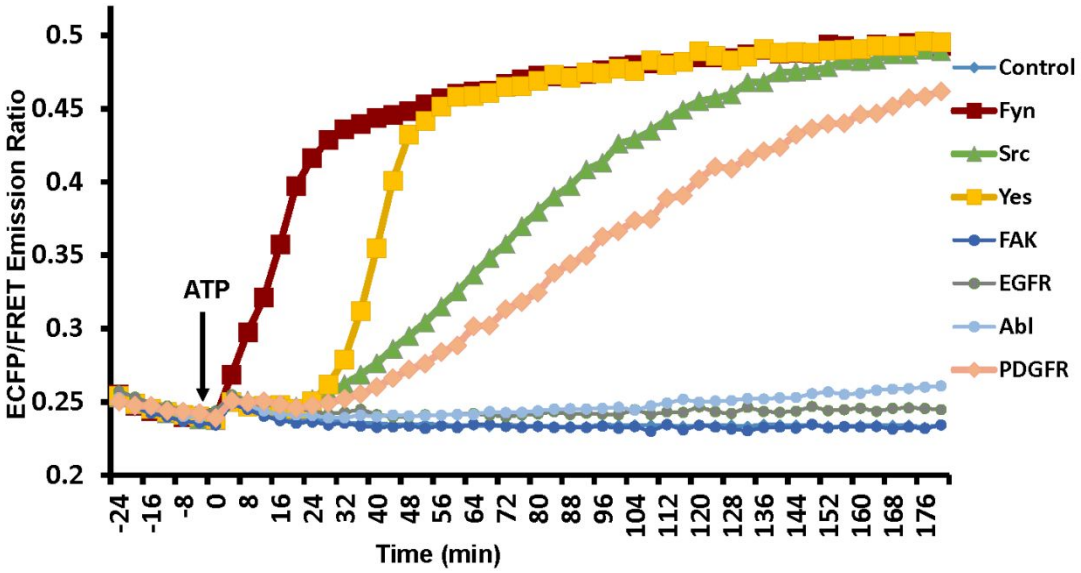

Figure S1. *In vitro* assay of the biosensor with wt-SH2. The *in vitro* ECFP/FRET emission ratio time courses of the Fyn biosensor with wt-SH2 domain in response to different active tyrosine kinases and ATP.

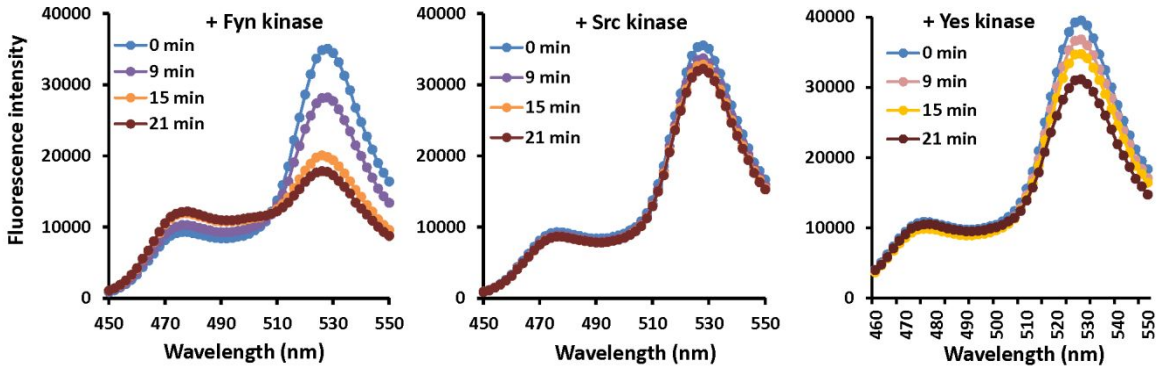

Figure S2. *In vitro* characterization of the specificity of the biosensor with C185A-SH2. The emission spectrum changes of Fyn biosensor with C185A-SH2 in response to active Fyn, Src, or Yes kinases from the indicated time points after addition of ATP to initiate the reactions during in vitro assay.

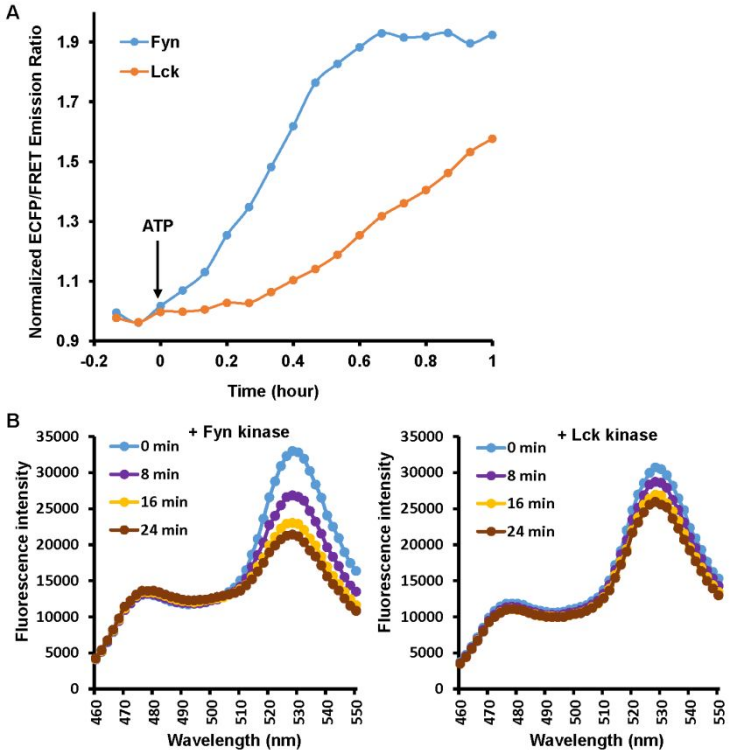

Figure S3. Sensitivity comparison of the biosensor to Fyn and Lck kinases. (A) The time courses for the FRET change of Fyn biosensor with C185A-SH2 in response to active Fyn or Lck kinase. (B) The emission spectrum changes of Fyn biosensor with C185A-SH2 in response to active Fyn, or Lck kinases after addition of ATP to initiate the reactions during in vitro assay.

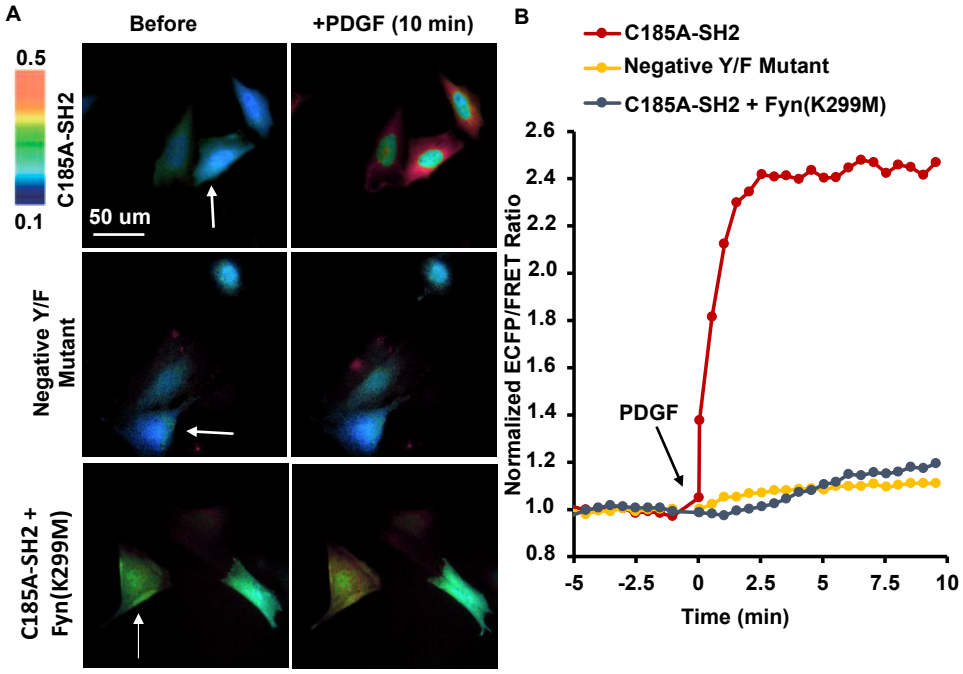

Figure S4. PDGF-induced FRET response of Fyn biosensor, its Y/F negative mutant, or of Fyn biosensor with co-expression of kinase-dead Fyn(K299M) in MEF cells. The time courses of FRET change in Graph (**B**) were quantified respectively from the cells indicated by the arrows in the ratiometric FRET images of (**A**).

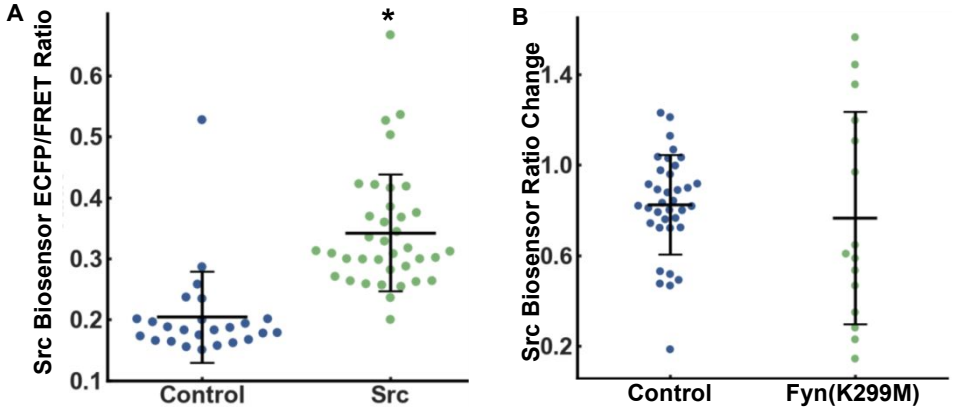

Figure S5. The FRET responses of Src biosensor by co-expressing wild-type Src kinase in SYF(-/-) cells, or Fyn(K299M) in MEF cells. (**A**) The emission ratio was quantified (scatter plots with mean±S.D.) from SYF(-/-) cells co-transfected with Src (0.34+/-0.096) or with control vector

(0.20+/-0.075). The cells were seeded on glass-bottom dishes coated with 10 μg/ml fibronectin. * indicates statistically significant difference by Student's t-test ($n_1 = 22$, $n_2 = 32$, p-value < 10^{-7}). (**B**) The ECFP/FRET emission ratio change was quantified (scatter plots with mean±S.D.) from PDGF-induced MEF cells co-transfected with Fyn(K299M) (0.77+/-0.47, n = 15) or with control vector (0.82+/-0.22, n = 36).

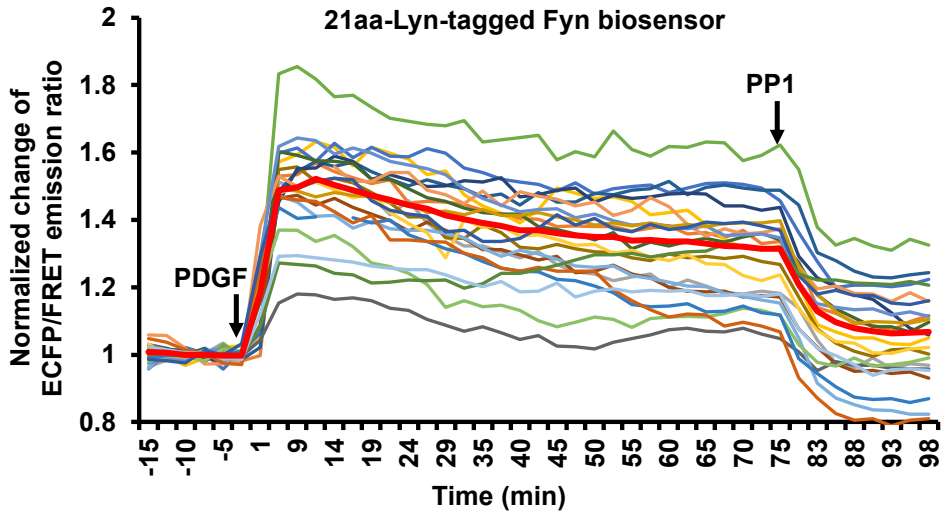

Figure S6. Detection of Fyn activity by 21aa-Lyn-tagged biosensor. The normalized time-course curves show PDGF-induced FRET response of the 21aa-Lyn-tagged biosensor from a group of MEF cells, followed by addition of Src family inhibitor PP1 (10 μM). Each colored line was quantified from one single cell. The bold red line represents the averaged value of the group at each corresponding time point.

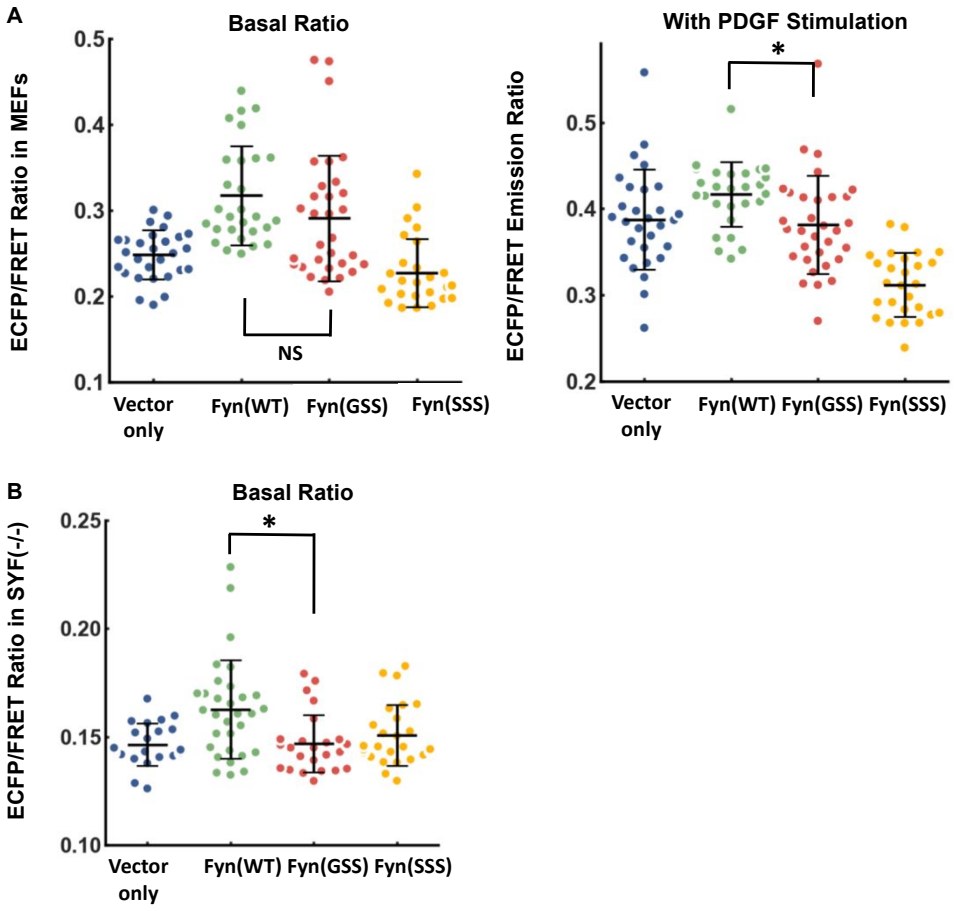

Figure S7. The FRET responses of Lyn-tagged Fyn biosensor to Fyn mutants. (**A**) The scatter plots with mean±S.D. show the ECFP/FRET emission ratio (mean ± SEM) of Lyn-tagged biosensor at the basal level (left) or after PDGF stimulation (right) in MEF cells co-expressing vector only, wild-type Fyn(wt), Fyn(GSS) or Fyn(SSS) constructs. (**B**) The scatter plots with mean±S.D. show the basal ECFP/FRET emission ratio of Lyn-tagged biosensor in SYF(-/-) cells co-expressing vector only, wild-type Fyn (wt), Fyn(GSS) or Fyn(SSS) constructs. SYF(-/-) cells were seeded on fibronectin-coated glass-bottom dishes overnight, followed by FRET measurement. (* indicates a statistically significant difference between groups by Student's t-test, $p<0.02$, $23<n<33$. NS: not significantly different.)

Supporting Movie Legends

Supporting Movie S1. PDGF-induced FRET response of cytosolic Fyn biosensor in MEF cells.

Supporting Movie S2. PDGF-induced FRET response of Lyn-tagged Fyn biosensor in MEF cell.

Supporting Movie S3. PDGF-induced FRET response of Fyn-tagged Fyn biosensor in MEF cell.

References:

1. Miyawaki, A.; Tsien, R. Y., Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol* **2000,** *327*, 472-500.
2. (a) Wang, Y.; Botvinick, E. L.; Zhao, Y.; Berns, M. W.; Usami, S.; Tsien, R. Y.; Chien, S., Visualizing the mechanical activation of Src. *Nature* **2005,** *434* (7036), 1040-5; (b) Ouyang, M.; Sun, J.; Chien, S.; Wang, Y., Determination of hierarchical relationship of Src and Rac at subcellular locations with FRET biosensors. *Proc Natl Acad Sci U S A* **2008,** *105* (38), 14353-8.
3. Sun, J.; Lu, S.; Ouyang, M.; Lin, L. J.; Zhuo, Y.; Liu, B.; Chien, S.; Neel, B. G.; Wang, Y., Antagonism between binding site affinity and conformational dynamics tunes alternative cis-interactions within Shp2. *Nature communications* **2013,** *4*, 2037.
4. Otsu, N., A threshold selection method from gray-level histograms. *IEEE Transactions on Systems, Man, and Cybernetics* **1979,** *SMC-9* (1).