Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

Rational engineering and synthetic application of high specificity BiFC probe derived from Springgreen-M

Yuao Sun[1], Yao Wang[1], keyang Chen[2], Yujie Sun[1, 3,*] and Sheng Wang[4, 5,*]

[1] State Key Laboratory of Membrane Biology, Biomedical Pioneering Innovation Center (BIOPIC), School of Life Sciences, Peking University, Beijing 100871, China.

[2] Yuanpei College, Peking University, Beijing 100871, China.

[3] National Biomedical Imaging Center, School of Future Technology, Peking University, Beijing 100871, China.

[4] School of Biopharmacy, China Pharmaceutical University, Nanjing 211198, China.

[5] Changzhou High-Tech Research Institute of Nanjing University, Changzhou 213164, China.

**Corresponding Author, sun_yujie@pku.edu.cn, wangsheng9411@sina.com*

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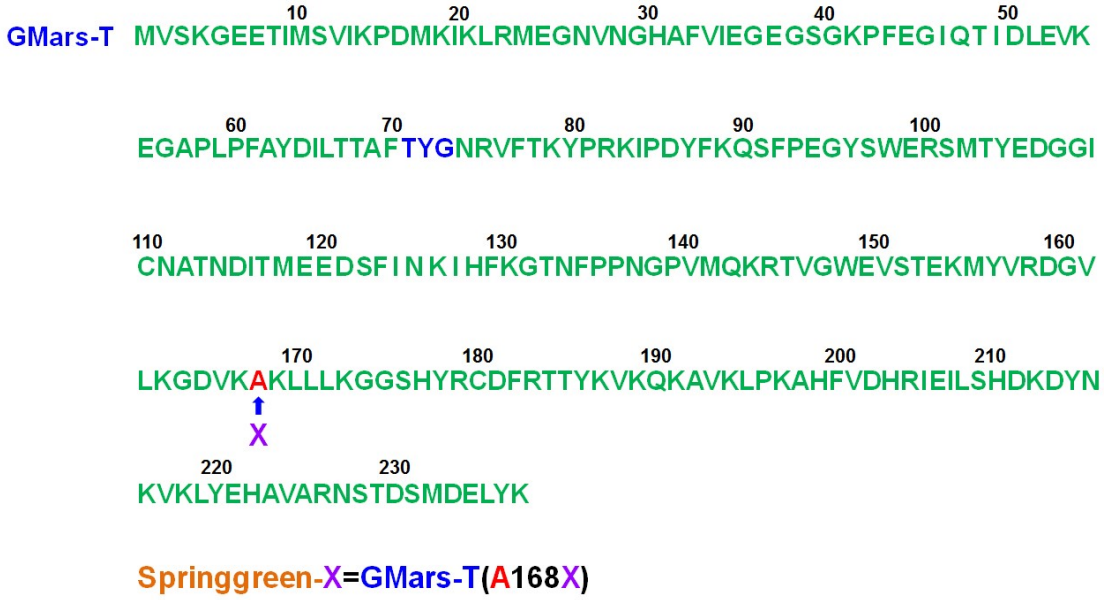


Fig. S1 Generation of Springgreen-X variants based on GMars-T.

Amino acids sequence of GMars-T (green) with chromophore TYG (blue) and the 168Ala residue (red) highlighted. X (purple) represents other 19 natural amino acids used to substitute Ala at the 168 residue (indicated by blue arrow) of GMars-T for Springgreen-X variants generation.

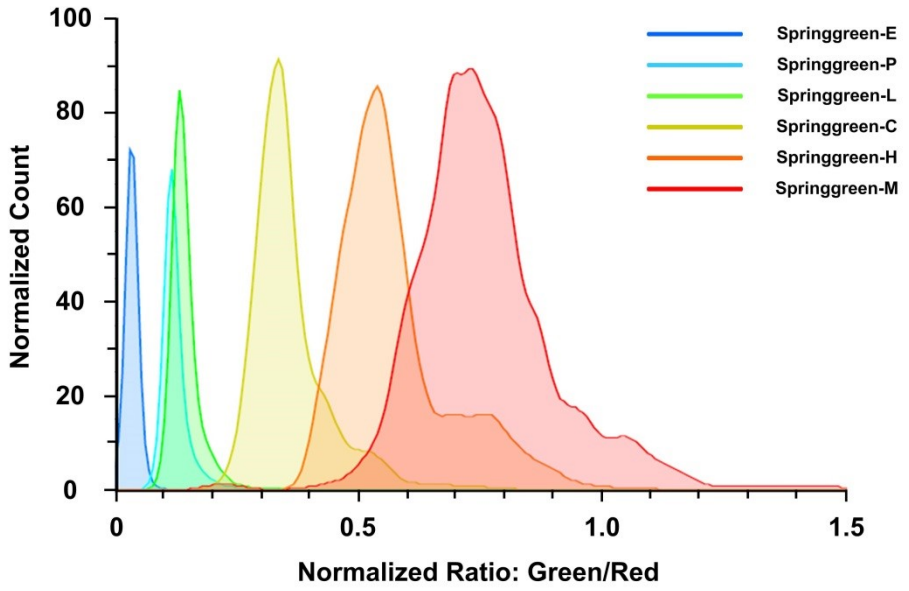


Fig. S2 In-cell brightness comparison of Springgreen variants.

HeLa cells with mCherry-P2A-Springgreen-X expression vectors transfection were analyzed 24 hours after plasmid transient transfection. Where X stand for substituting amino acids as indicated to the 168Ala of GMars-T. In such constructs design, live HeLa cells produce mCherry and a Springgreen variant of interest separately in a 1:1 ratio. Green and red fluorescence in live cells were measured by flow cytometry and the normalized green/red fluorescence intensity ratio was determined from individual live HeLa cell.

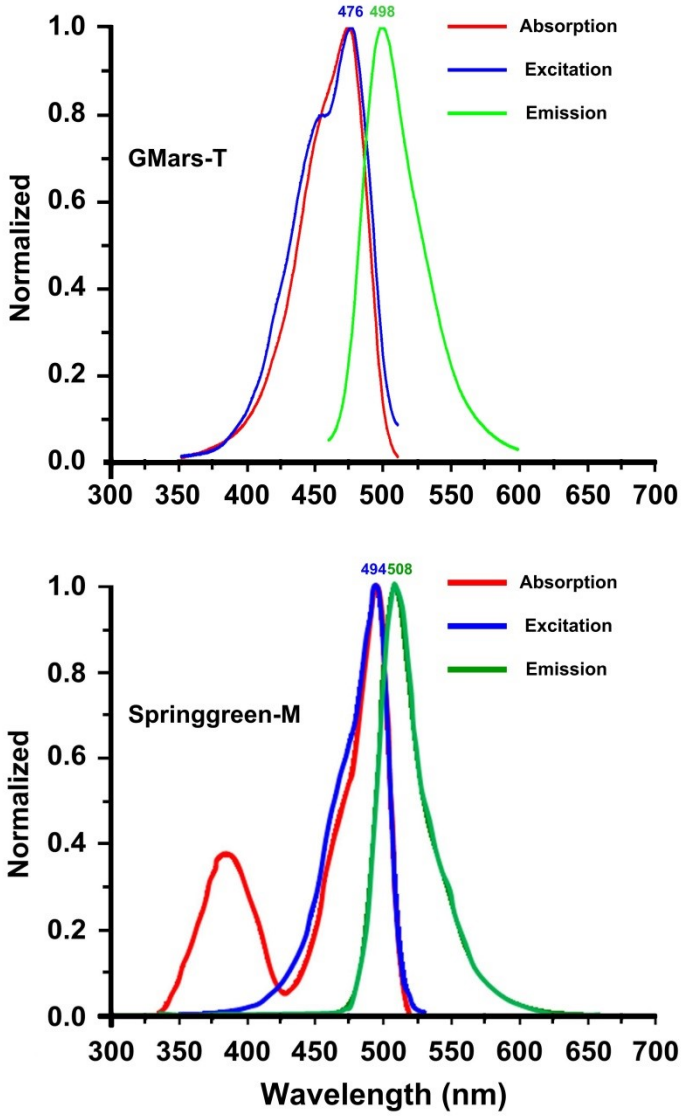


Fig. S3 Spectra of GMars-T and Springgreen-M at physiological pH.

Absorption (red), excitation (blue, maximum indicated above), and emission (green, maximum indicated above) spectra of GMars-T and Springgreen-M in its equilibrium state at pH7.5; the absorption bands at 380 nm and 494 nm of Springgreen-M presumably correspond to the protonated *trans* and the deprotonated *cis*-chromophore, respectively. The spectra of GMars-T are from reference.[1]

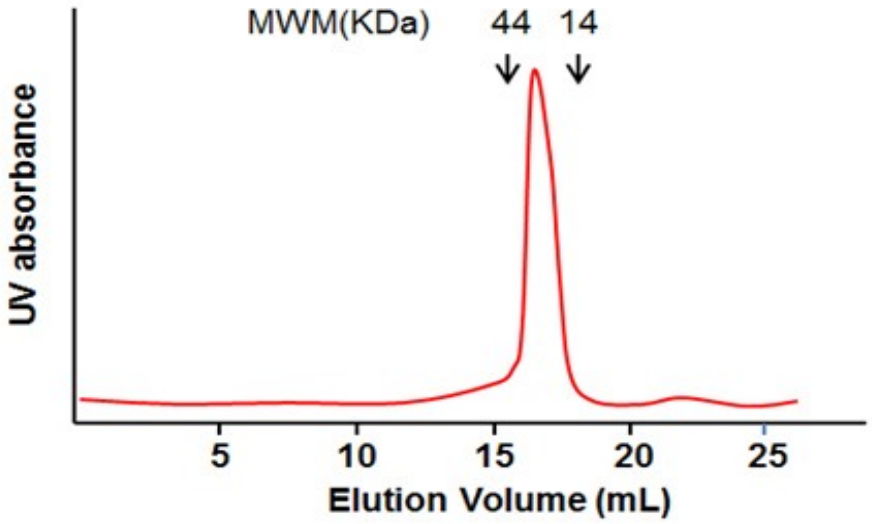


Fig. S4 Gel filtration analysis of Springgreen-M.

Springgreen-M and marker proteins were analyzed by gel filtration. The single UV absorbance peak indicated that Springgreen-M protein were monomer in solution.

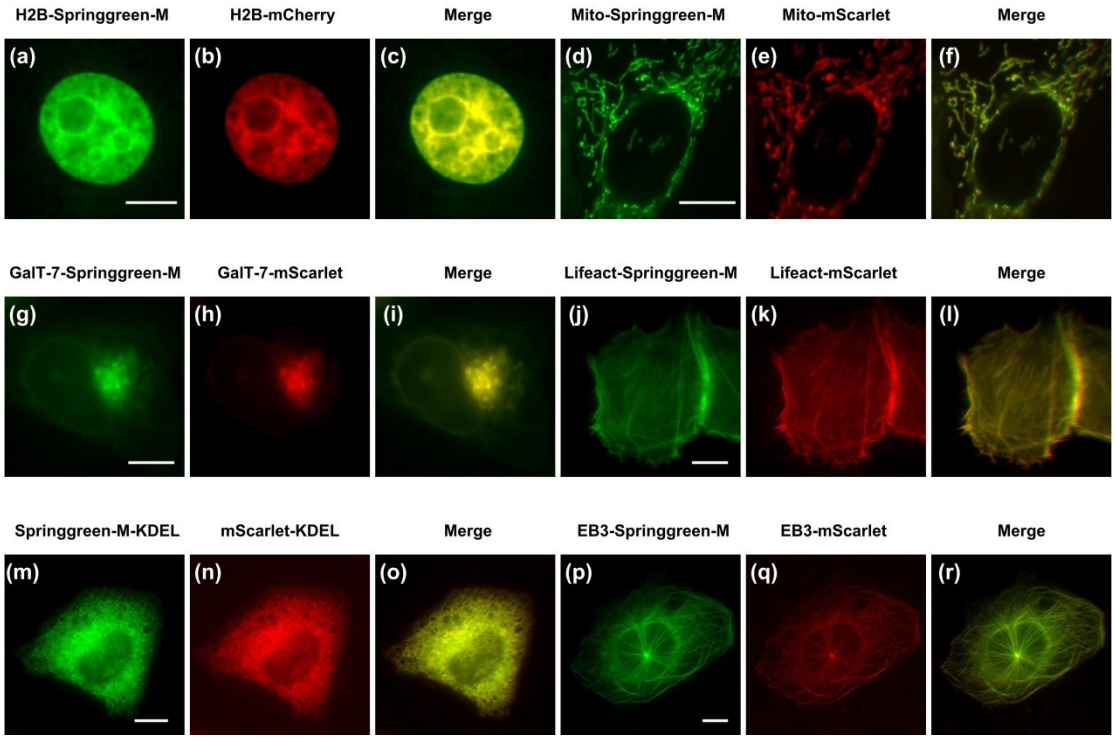


Fig. S5 Colocalization of subcellular structures by Springgreen-M and mCherry or mScarlet fusion proteins.

Live HeLa cells co-expression of H2B-Springgreen-M and H2B-mCherry (a, b) or Mito-Springgreen-M and Mito-mScarlet (targeting to mitochondria) (d, e) or GalT-7-Springgreen-M(targeting to Golgi apparatus) and GalT-7mScarlet (g, h) or Lifeact-Springgreen-M and Lifeact-mScarlet (targeting to F-actin) (j, k) or Springgreen-M-KDEL(targeting to endoplasmic reticulum) and mScarlet-KDEL (m, n) or U2OS cells co-expression of EB3-Springgreen-M (targeting to Microtubule) and EB3-mScarlet (p, q) were imaged under a wide-field fluorescence microscope. Merged images (c, f, i, l, o, r) shown the colocalization of subcellular structures by Springgreen-M and mCherry or mScarlet fusion proteins in individual live cell. Scale bar: 10 μm.

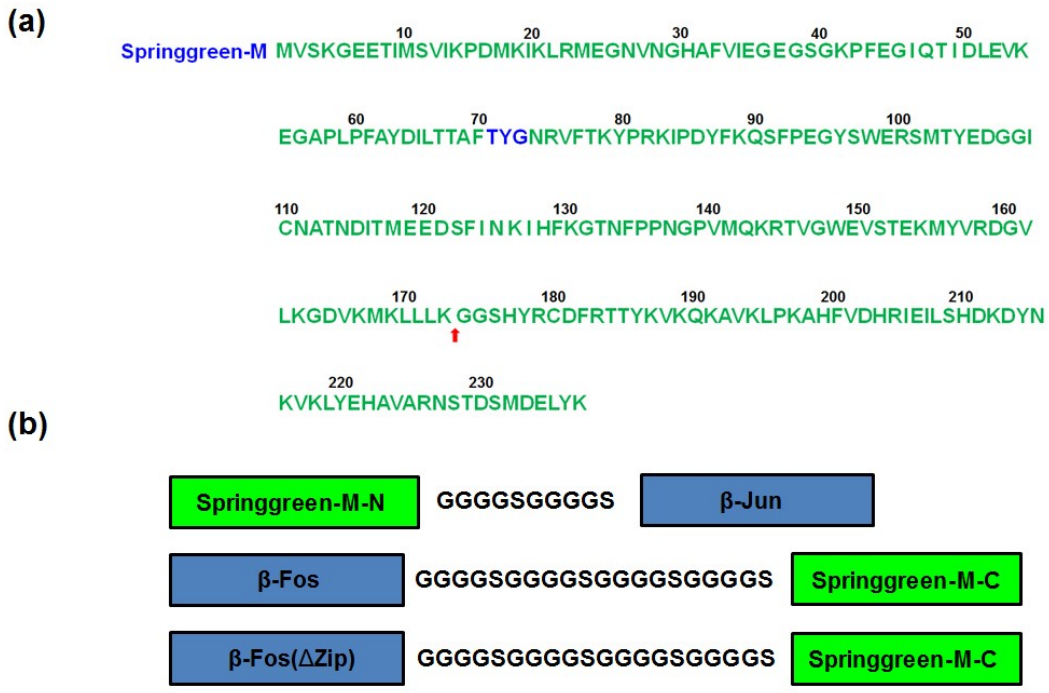


Fig. S6 Sequence of Springgreen-M and constructs design for BiFC.

(a) Amino acids sequence of Springgreen-M (green), the chromophore amino acids TYG of Springgreen-M are highlighted with blue color. The splitting site between 173K and 174G of Springgreen-M for BiFC assay is indicated by red arrow. (b) β-Fos/β-Jun was used in establishing Springgreen-M-based BiFC assay. β-Jun was fused to the Springgreen-M-N with 2×GGGGS linker while β-Fos or β-Fos(ΔZip) was fused to the Springgreen-M-C with 4×GGGGS linker.

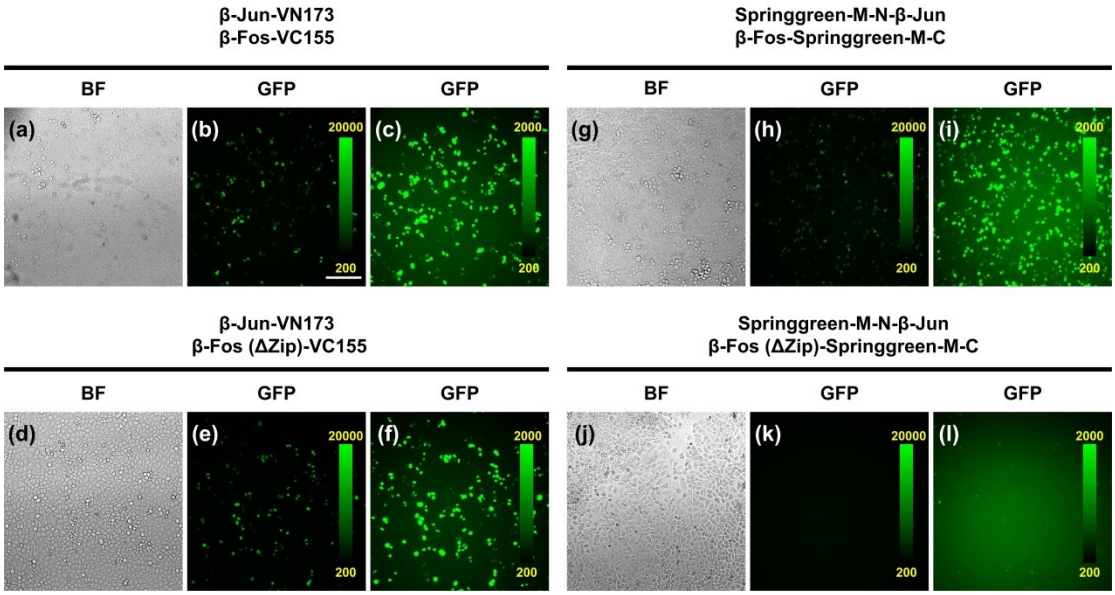


Fig. S7 Typical images of live HeLa cells detecting and visualizing β-Fos/β-Jun heterodimer protein interaction by VN173/VC155 Venus-based BiFC assay or Springgreen-M-based BiFC assay.

(a-c) cells cotransfected with β-Jun-VN173 and β-Fos-VC155 (0.5μg+0.5μg) or (d-f) β-Jun-VN173 and β-Fos(ΔZip)-VC155 (0.5μg+0.5μg) or (g-i) Springgreen-M-N-β-Jun and β-Fos-Springgreen-M-C (0.5μg+0.5μg) or (j-l) Springgreen-M-N-β-Jun and β-Fos(ΔZip)-Springgreen-M-C (0.5μg+0.5μg) plasmids were imaged under a wide-field fluorescence microscope 24 hours later after transfection, all the images were taken with the same illumination power, same exposure times, 10× objective lens and displayed with the intensity scale assigned from 200 to 20000 (16 bit image) for (b),(e),(h),(k). The intensity scale was also assigned from 200 to 2000 for the (b), (e), (h), (k) as (c), (f), (i), (l). The lower false-positive signal of Springgreen-M-based BiFC assay in responding to β-Fos(ΔZip)/β-Jun is only visible in (l) as the intensity scale range was turned down 10-fold from 20000 in (k) to 2000 in (l). BF: bright field channel, GFP: GFP channel. Scale bar: 200μm.

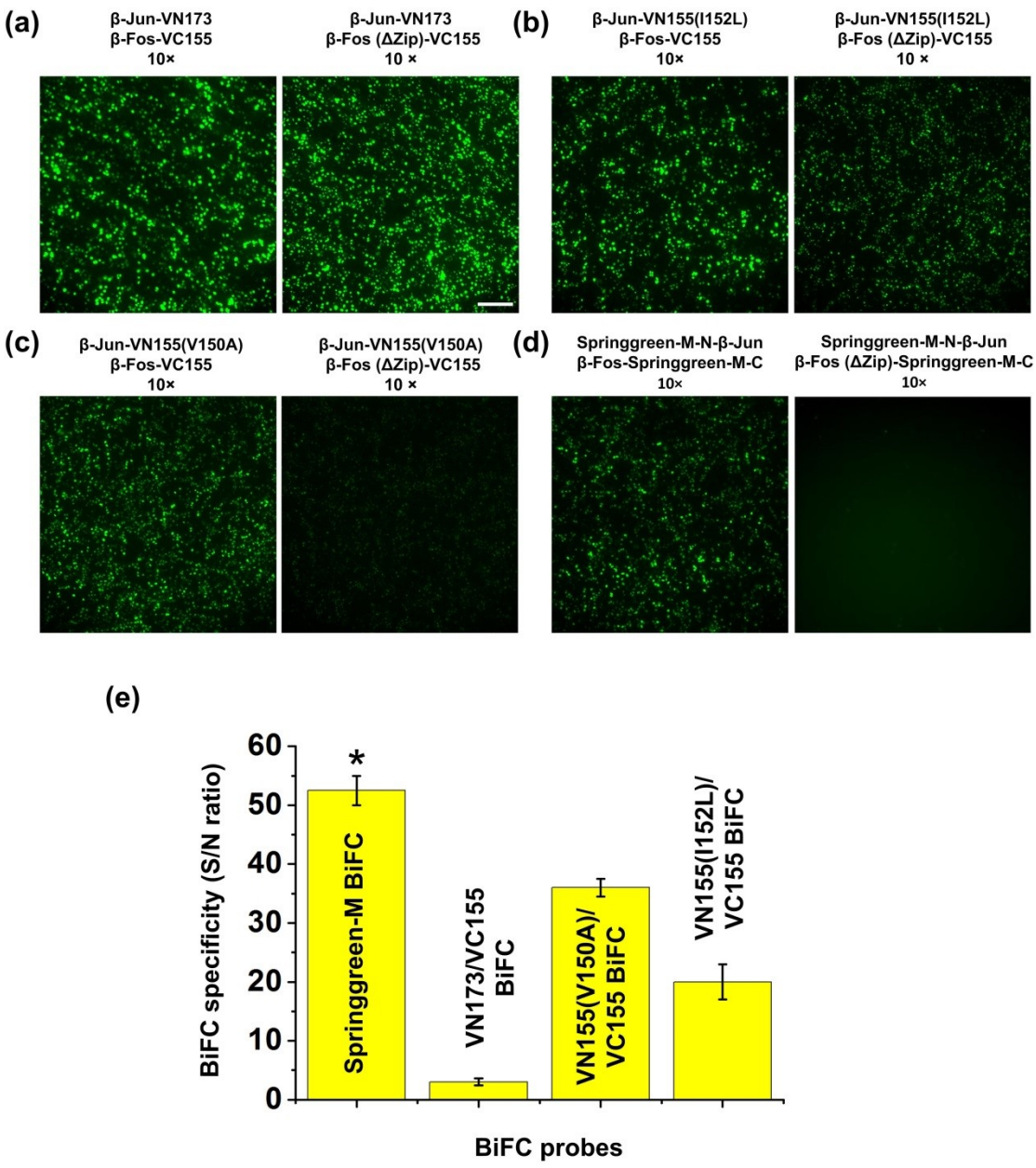


Fig. S8 Visualizing and detecting β-Fos/β-Jun heterodimer protein interaction in live HeLa cell by Springgreen-M-based BiFC assay and Venus or Venus variants-based BiFC assays.

(a) Live HeLa cells cotransfected with β-Jun-VN173 and β-Fos-VC155 (0.5μg+0.5μg) or β-Jun-VN173 and β-Fos(ΔZip)-VC155 (0.5μg+0.5μg) or (b) β-Jun-VN155(I152L) and β-Fos-VC155 (0.5μg+0.5μg) or β-Jun-VN155(I152L)

and β-Fos(ΔZip)-VC155 (0.5μg+0.5μg) or (c) β-Jun-VN155(V150A) and β-Fos-VC155 (0.5μg+0.5μg) or β-Jun-VN155(V150A) and β-Fos(ΔZip)-VC155 (0.5μg+0.5μg) or (d) Springgreen-M-N-β-Jun and β-Fos-Springgreen-M-C (0.5μg+0.5μg) or Springgreen-M-N-β-Jun and β-Fos(ΔZip)-Springgreen-M-C (0.5μg+0.5μg) plasmids were imaged under a wide-field fluorescence microscope with 10× objective lens. Red fluorescent protein mCherry (0.2μg) as an internal control to normalize expression levels of individual experimental group was also cotransfected. 24 hours later after transfection, all the images were taken with the same illumination power, same exposure times, 10×objective lens and displayed with the same intensity scale for individual experimental group. (e) BiFC specificity or S/N ratios of each BiFC assay detecting and visualizing β-Fos/β-Jun heterodimer protein interaction were calculated following standard protocol[2] previously reported. *$p<0.01$ compared with VN173/VC155, VN155(I152L)/VC155 and VN155(V150A)/VC155 groups. Scale bar: 300μm.

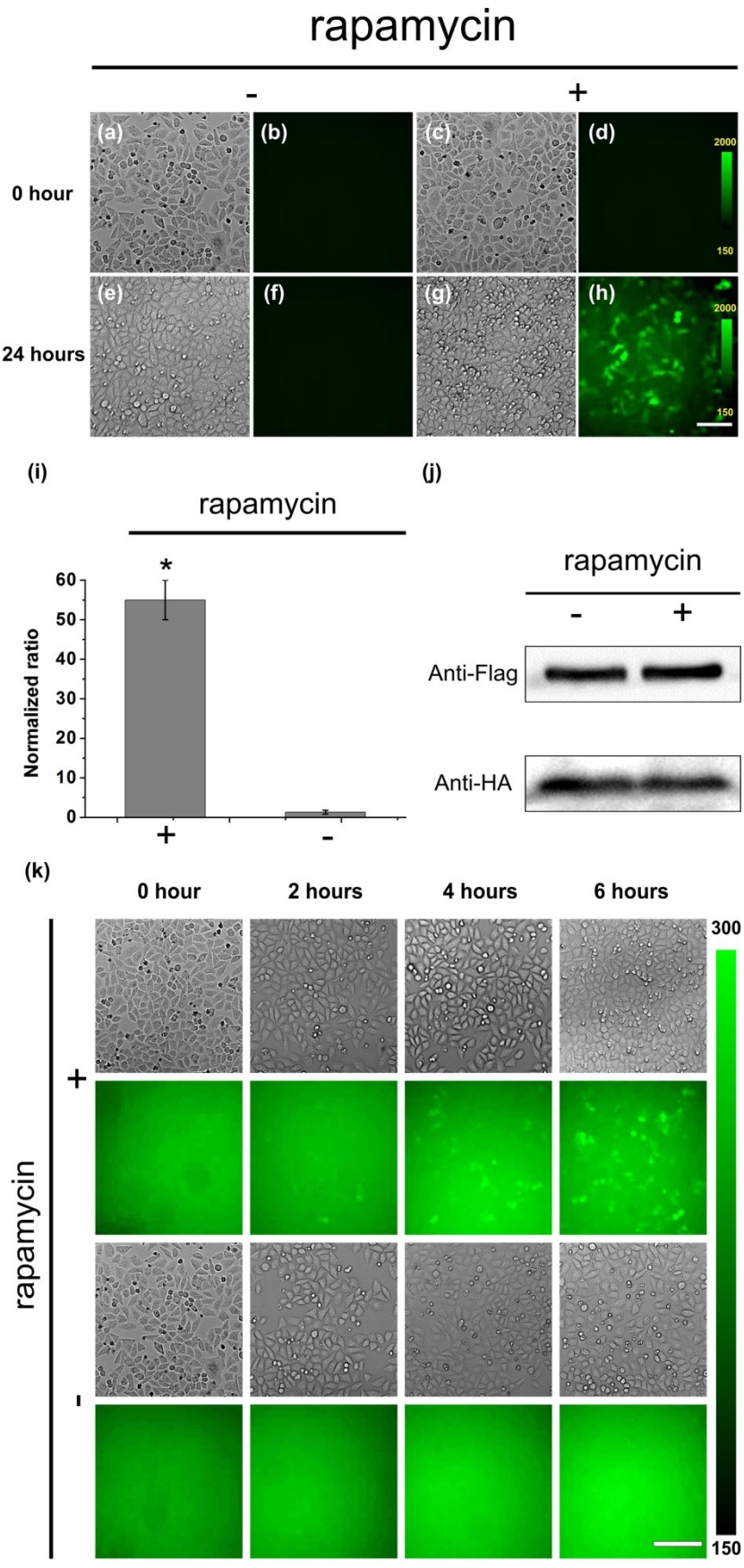

rapamycin
-
+
(a)
(b)
(c)
(d)
2000
150
0 hour
(e)
(f)
(g)
(h)
2000
150
24 hours
(i)
rapamycin
*
60
50
40
30
20
10
0
Normalized ratio
+
-
(j)
rapamycin
-
+
Anti-Flag
Anti-HA
(k)
0 hour
2 hours
4 hours
6 hours
300
150
rapamycin
+
-

Fig.S9 Characterization of Springgreen-M-based BiFC assay in live HeLa cells by rapamycin-inducible FRB/FKBP interaction system.

Two dish of HeLa cells cotransfected parallelly with the same amount of Springgreen-M-N-FKBP, FRB-Springgreen-M-C and mCherry plasmids (internal control) were incubated at 37°C for 24 hours, and then the cells were imaged with the same imaging conditions (a, b, c, d). (a, c) DIC channel, (b, d) GFP channel. After taking images of (a, b, c, d), 100 nM rapamycin was added to only one dish (c, d) and then the two dish of HeLa cells were maintained at 37°C for the next 24 hours and imaged with the same imaging conditions as previously (e, f, g, h). (e, g) DIC channel, (f, h) GFP channel, the intensity scale was set the same from 150 to 2000 for (b, d, f, h). Scale bar: 200μm. (i) After 24 hours rapamycin induction, the ensemble green and red fluorescence from cells were measured by fluorescence spectrophotometer and the normalized green-to-red ratios were calculated and expressed as means±SD values. *$p<0.01$* compared with cells without rapamycin induction. The data were from three independent measurements. (j) Comparable expression levels of the fusion proteins in (f) and (h) were determined by western blotting with anti-Flag and anti-HA antibodies. (k) Time-lapse imaging of live HeLa cells coexpressing Springgreen-M-N-FKBP and FRB-Springgreen-M-C with or without 100 nM rapamycin induction. In order to avoid severe photobleaching probably caused by excitation light exposure, we used the same short exposure times for every fluorescence images during time-lapse recording. The intensity scale was set to the same for all fluorescence images from 100 to 300. Scale bar: 200μm.

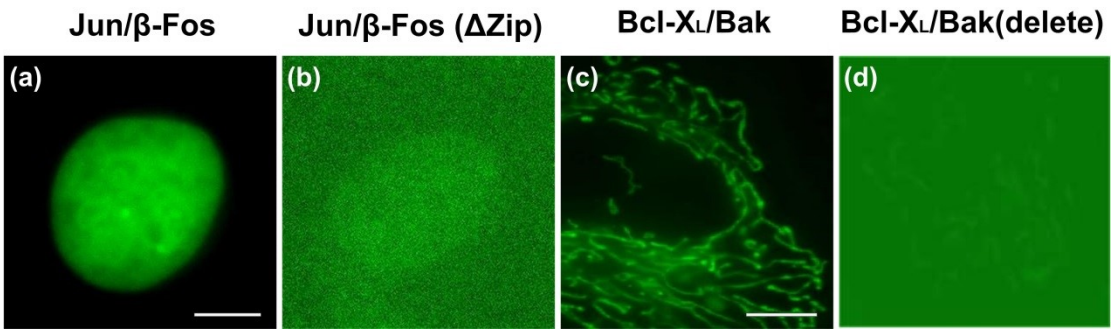
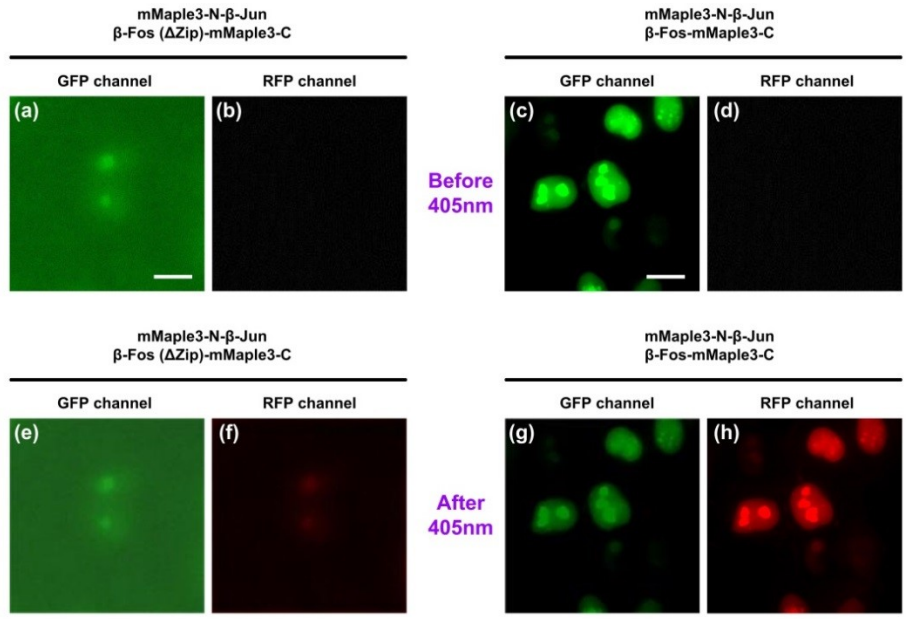
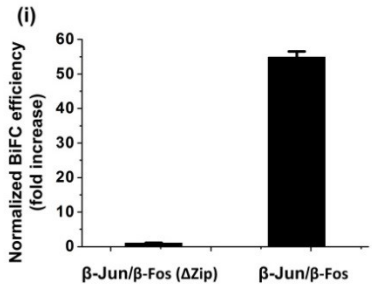
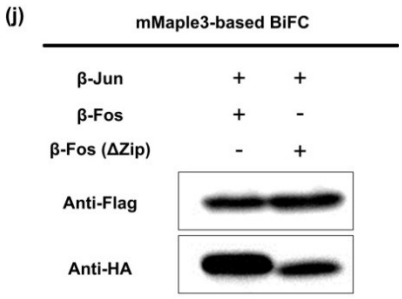


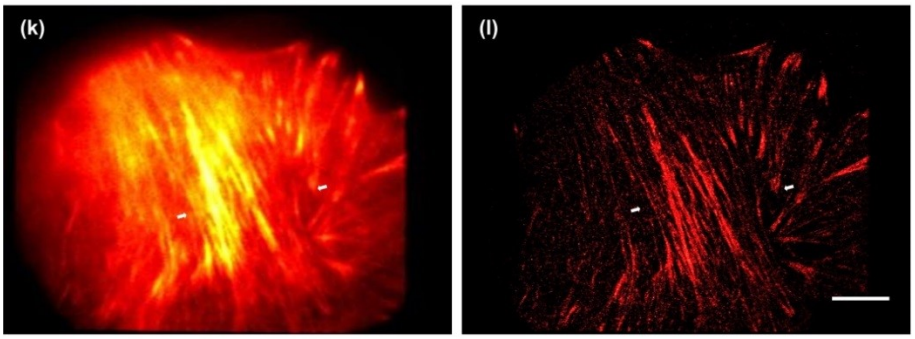
Fig. S10 High specificity of Springgreen-M-based BiFC assay in detecting and visualizing protein-protein interactions in live HeLa cells.

(a) Typical fluorescence image of Jun/β-Fos heterodimerization visualized by Springgreen-M-based BiFC; (b) Typical fluorescence image and false-positive signal of Springgreen-M-based BiFC assay in responding to Jun/β-Fos(ΔZip); (c) Typical fluorescence image of Springgreen-M-based BiFC assay in detecting and visualizing Bcl-X_L/Bak heterodimerization in live intact HeLa cell; (d)Typical image and false-positive signal of Springgreen-M-based BiFC assay in responding to Bcl-X_L/Bak(delete). Bak (delete) is a mutant of Bak with Bcl-X_L interaction residues (GQVGR) deleted. The intensity scale was set to very low range to display the extremely low level of false-positive fluorescence signals in (b) and (d).Scale bar: 5μm.


mMaple3-N-β-Jun
β-Fos (ΔZip)-mMaple3-C
GFP channel
RFP channel
(a)
(b)
mMaple3-N-β-Jun
β-Fos-mMaple3-C
GFP channel
RFP channel
(c)
(d)
Before 405nm
mMaple3-N-β-Jun
β-Fos (ΔZip)-mMaple3-C
GFP channel
RFP channel
(e)
(f)
mMaple3-N-β-Jun
β-Fos-mMaple3-C
GFP channel
RFP channel
(g)
(h)
After 405nm


(i)
Normalized BiFC efficiency (fold increase)
60
50
40
30
20
10
0
β-Jun/β-Fos (ΔZip)
β-Jun/β-Fos


(j)
mMaple3-based BiFC
β-Jun + +
β-Fos + -
β-Fos (ΔZip) - +
Anti-Flag
Anti-HA


(k)
(l)

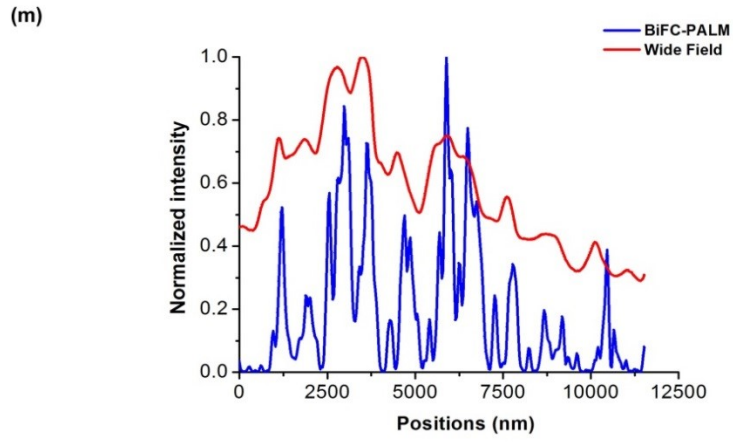

(m)
BiFC-PALM
Wide Field
Normalized intensity
1.0
0.8
0.6
0.4
0.2
0.0
0
2500
5000
7500
10000
12500
Positions (nm)

Fig. S11 Generation and application of mMaple3-based BiFC assay.

(a-h) Generation of mMaple3-based BiFC assay; live HeLa cells cotransfected with mMaple3-N-β-Jun and β-Fos(ΔZip)-mMaple3-C (0.5 μg+0.5 μg) (a-b) or mMaple3-N-β-Jun and β-Fos-mMaple3-C (0.5 μg+0.5 μg) plasmids (c-d) were imaged under a wide-field fluorescence microscope with100× objective lens before 405nm light activation in both GFP channel (a, c) and RFP channel(b, d) or after 405nm light activation in both GFP channel (e, g) and RFP channel(f, h). Scale bar: 10μm. mCherry plasmids (0.25 μg) were also cotransfected as internal control to determine BiFC efficiency of individual experimental group in (i). Comparable expression levels of the fusion proteins in (a) and (c) were determined by western blotting with anti-Flag and anti-HA antibodies (j). (k-m) Detecting and super-resolution imaging Lifeact/Lifeact homodimerization targeting to polymerized G-actin with mMaple3-based BiFC assay; (k) conventional wide-field BiFC fluorescence image of a live HeLa cell expressing Lifeact-mMaple3-N and Lifeact-mMaple3-C, (l) BiFC-PALM image of (k); (m) intensity profiles measured of white arrowed regions in (k) and (l) indicating the improved resolution. Scale bar: 5μm.

Table S1. Properties of BiFC probes derived from Venus (variants) and Springgreen-M

Fluorescent protein	Fragments	Interaction pair	Organelle	BiFC specificity(S/N)
Venus	VN173 VC155	β-Fos β-Jun	Nucles	~3[a,c]
	VN155 VC155	β-Fos β-Jun	Nucles	~5[a]
	VN173 VC173	β-Fos β-Jun	Nucles	~5[a]
Venus (I152L)	VN155(I152L) VC155	β-Fos β-Jun	Nucles	~20[a,c]
Venus (V150A)	VN155(V150A) VC155	β-Fos β-Jun	Nucles	~43[b] ~36[c]
Springgreen-M	Springgreen-M-N Springgreen-M-C	β-Fos β-Jun	Nucles	~52[c]

[a] Data from reference 3 and the data were reported in live COS-1 cells.

[b] Data from reference 4 and the data were reported in fixed Mouse C3H10T1/2 cells.

[c] Data were measured and calculated in live HeLa S6 cells by us.

References

1. Wang, S.; Chen, X.; Chang, L.; Ding, M.; Xue, R.; Duan, H.; Sun, Y., GMars-T Enabling Multimodal Subdiffraction Structural and Functional Fluorescence Imaging in Live Cells. *Anal. Chem.* **2018**, *90*, 11, 6626-6634.

2. Kodama, Y.; Hu, C. D., Bimolecular fluorescence complementation (BiFC) analysis of protein-protein interaction: how to calculate signal-to-noise ratio. *Methods Cell Biol*. **2013**, *113*, 107-121.

3. Kodama, Y.; Hu, C. D., An improved bimolecular fluorescence complementation assay with a high signal-to-noise ratio. *Biotechniques* **2010**, *49*, 793-805.

4. Nakagawa, C.; Inahata, K.; Nishimura, S.; Sugimoto, K., Improvement of a Venus-based bimolecular fluorescence complementation assay to visualize bFos-bJun interaction in living cells. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 1399-1401.