Electronic Supplementary Information

In Vivo Measuring Autophagic Flux by Fluorescence Correlation Spectroscopy

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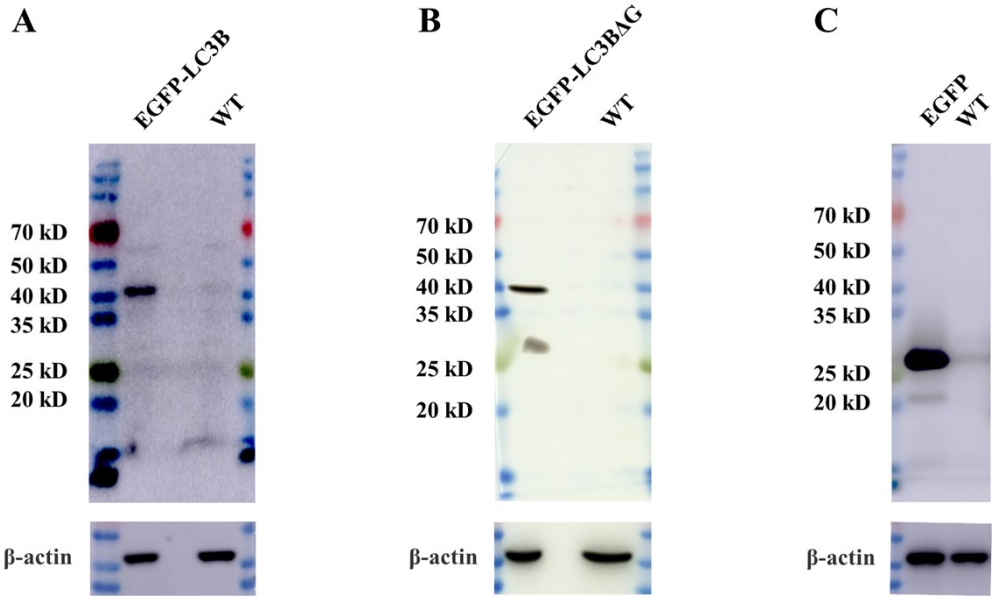

Fig. S1. Western blot analysis. HeLa cells stably expressing EGFP-LC3B (A) and EGFP (C) were detected by anti-His tag antibody. HeLa cells stably expressing EGFP-LC3BΔG (B) was detected by anti-GFP antibody. β-actin was used as an internal control. Wild-type HeLa cells was used as a negative control.

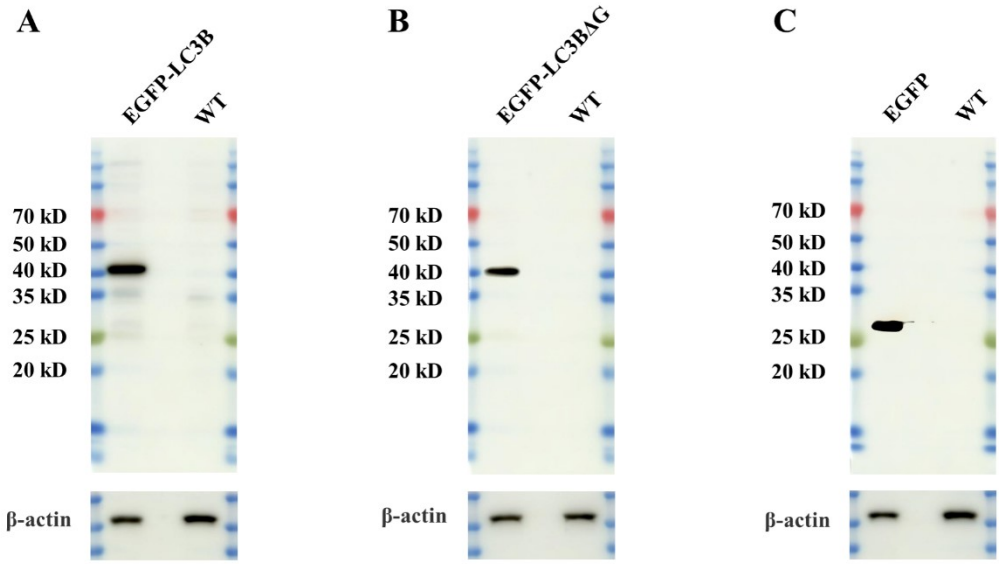

Fig. S2. Western blot analysis. H1299 cells stably expressing EGFP-LC3B (A), EGFP-LC3BΔG (B) and EGFP (C) were detected by anti-GFP antibody. β-actin was used as an internal control. Wild-type H1299 cells was used as a negative control.

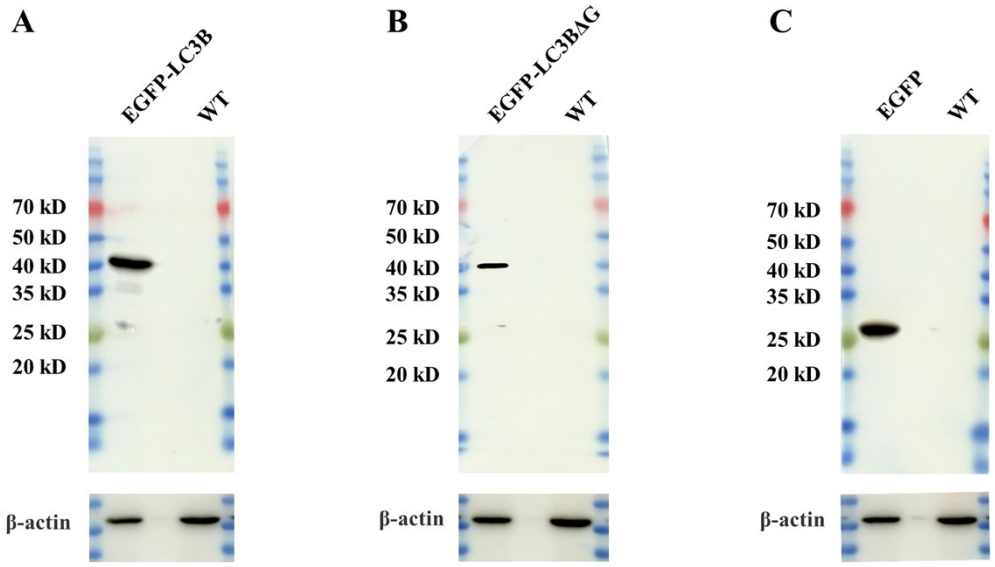

Fig. S3. Western blot analysis. HEK293T cells stably expressing EGFP-LC3B (A), EGFP-LC3BΔG (B) and EGFP (C) were detected by anti-GFP antibody. β-actin was used as an internal control. Wild-type HEK293T cells was used as a negative control.

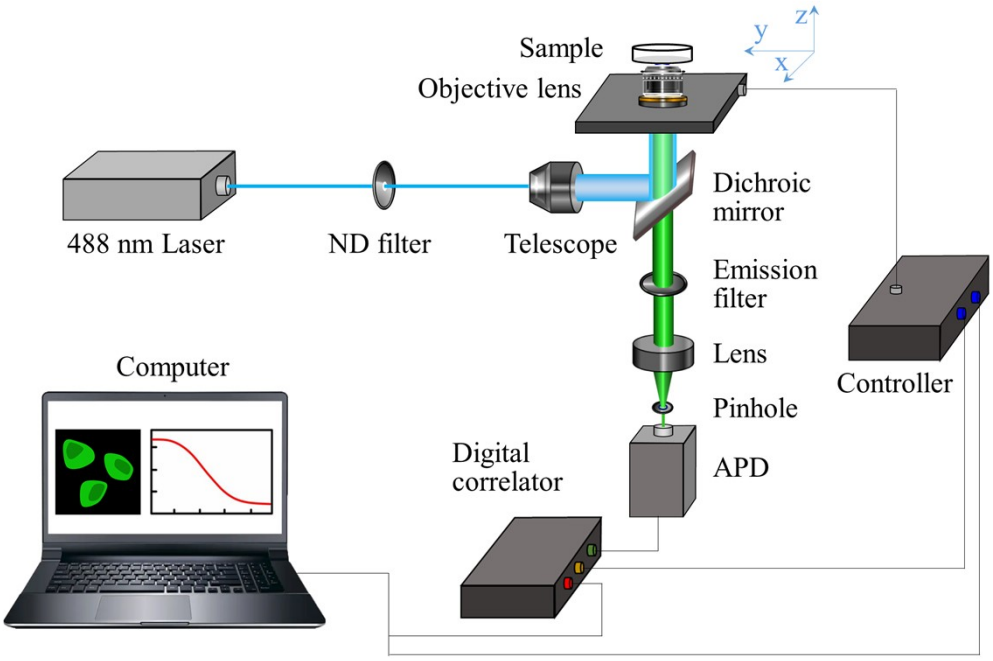

Fig. S4. Schematic diagram of the homemade FCS system.

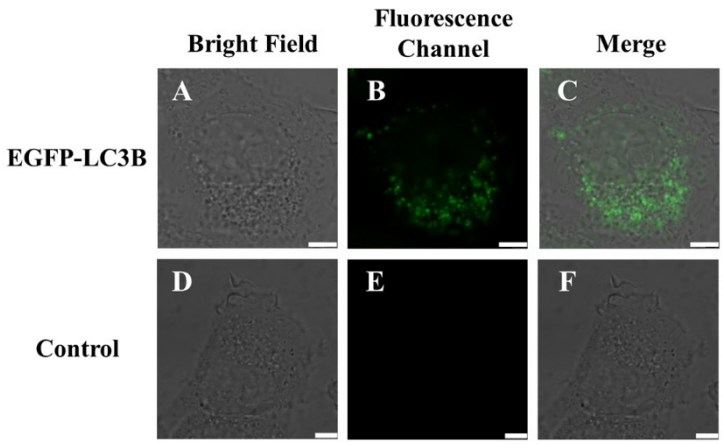

Fig. S5. The bright field image (A), confocal fluorescence image (B) and merged image (C) of HeLa cells stably expressing EGFP-LC3B, and the bright field image (D), confocal fluorescence image (E) and merged image (F) of wild-type HeLa cells. Scale bar = 5 μm.

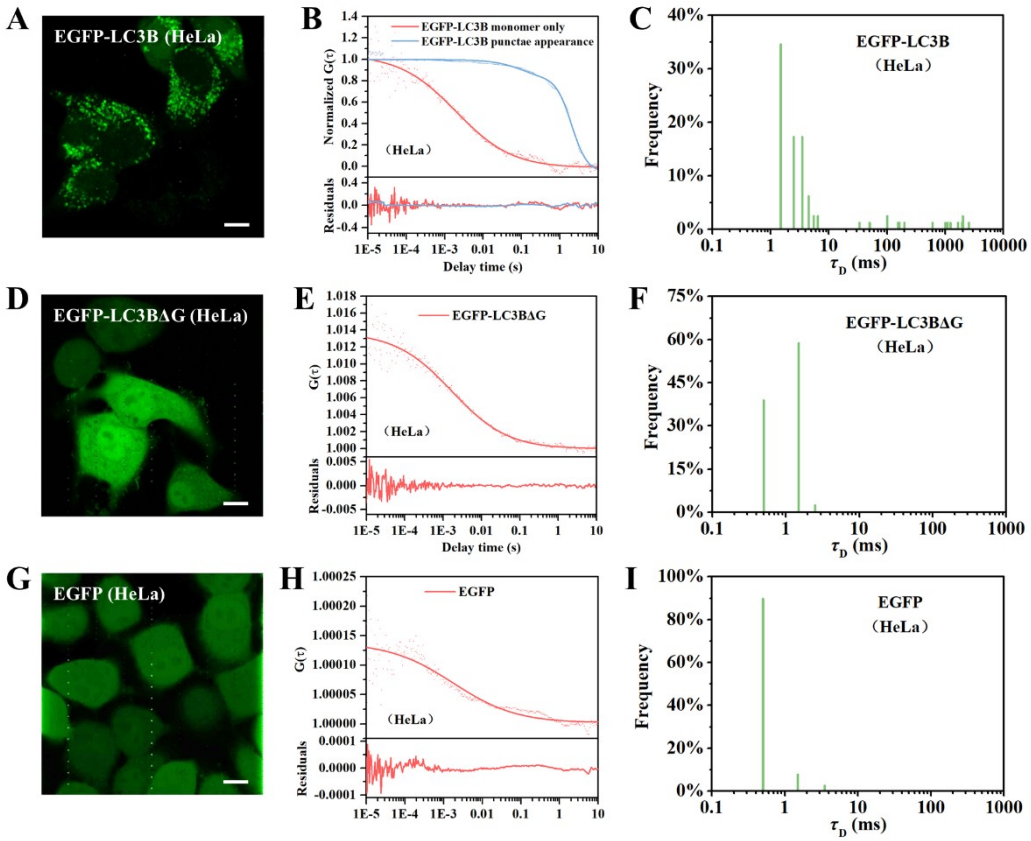

Fig. S6. The confocal fluorescence images, FCS curves and distribution frequency of the τ_D values of HeLa cells stably expressing EGFP-LC3B, EGFP-LC3BΔG and EGFP. EGFP-LC3B punctae was monitored by FCS in HeLa cells stably expressing EGFP-LC3B. (A) The confocal fluorescence image of HeLa cells stably expressing EGFP-LC3B. (B) Two typical kinds of FCS curves and fitting residuals in HeLa cells stably expressing EGFP-LC3B. (C) The distribution frequency of the τ_D values in HeLa cells stably expressing EGFP-LC3B. (D) The confocal fluorescence image of HeLa cells stably expressing EGFP-LC3BΔG. (E) One kind of FCS curve and fitting residuals in HeLa cells stably expressing EGFP-LC3BΔG. (F) The distribution frequency of the τ_D values in HeLa cells stably expressing EGFP-LC3BΔG. (G) The confocal fluorescence image of HeLa cells stably expressing EGFP. (H) One kind of FCS curve and fitting residuals in HeLa cells stably expressing EGFP. (I) The distribution frequency of the τ_D values in HeLa cells stably expressing EGFP. Scale bar = 10 μm.

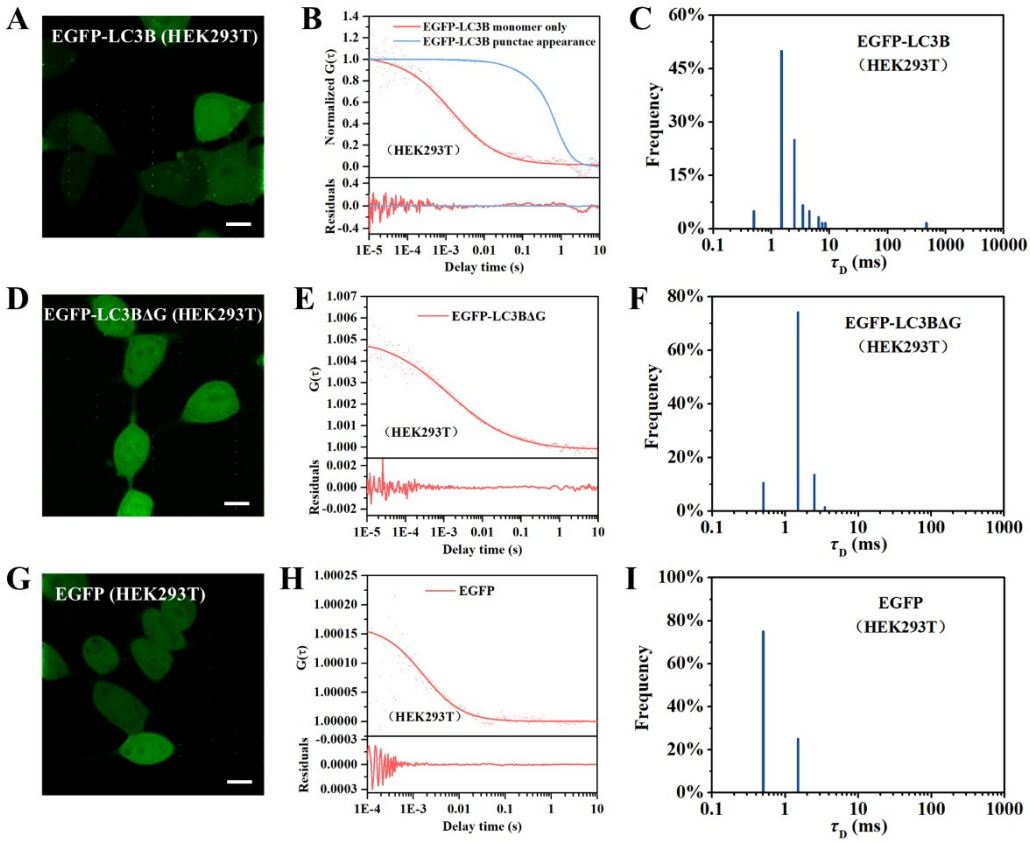

Fig. S7. The confocal fluorescence images, FCS curves and distribution frequency of the τ_D values of HEK293T cells stably expressing EGFP-LC3B, EGFP-LC3BΔG and EGFP. EGFP-LC3B punctae was monitored by FCS in HEK293T cells stably expressing EGFP-LC3B. (A) The confocal fluorescence image of HEK293T cells stably expressing EGFP-LC3B. (B) Two typical kinds of FCS curves and fitting residuals in HEK293T cells stably expressing EGFP-LC3B. (C) The distribution frequency of the τ_D values in HEK293T cells stably expressing EGFP-LC3B. (D) The confocal fluorescence image of HEK293T cells stably expressing EGFP-LC3BΔG. (E) One kind of FCS curve and fitting residuals in HEK293T cells stably expressing EGFP-LC3BΔG. (F) The distribution frequency of the τ_D values in HEK293T cells stably expressing EGFP-LC3BΔG. (G) The confocal fluorescence image of HEK293T cells stably expressing EGFP. (H) One kind of FCS curve and fitting residuals in HEK293T cells stably expressing EGFP. (I) The distribution frequency of the τ_D values in HEK293T cells stably expressing EGFP. Scale bar = 10 μm.

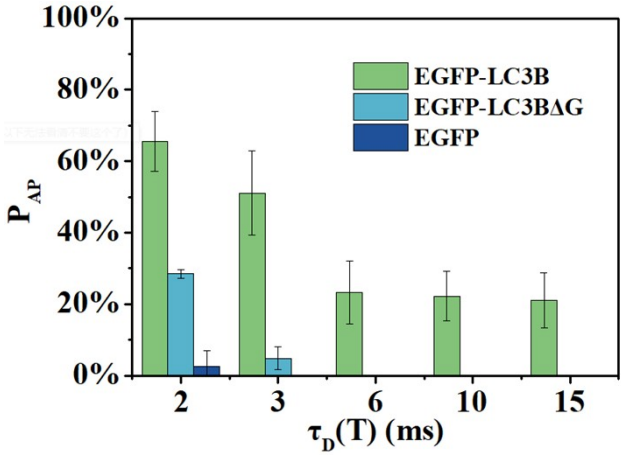

Fig. S8. The effect of τ_D(T) values on P_{AP} values. The P_{AP} values of HeLa cells stably expressing EGFP-LC3B, EGFP-LC3BΔG and EGFP at different τ_D(T) values.

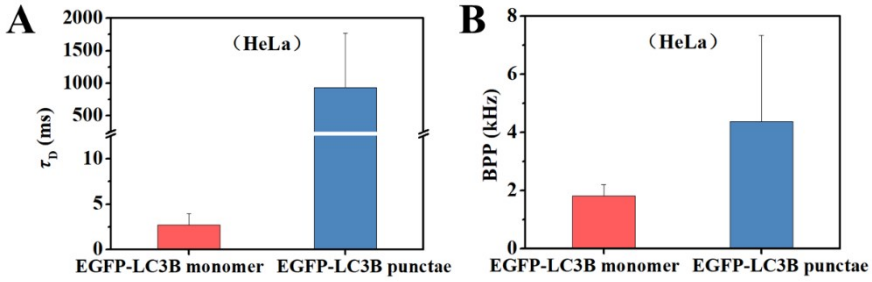

Fig. S9. The τ_D values and BPP values in HeLa cells stably expressing EGFP-LC3B. (A) The τ_D values of two typical kinds of FCS curves in HeLa cells stably expressing EGFP-LC3B. (B) The BPP values of two typical kinds of FCS curves in HeLa cells stably expressing EGFP-LC3B.

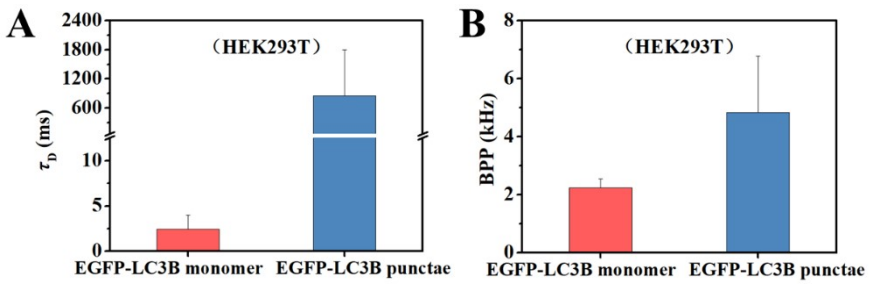

Fig. S10. The τ_D values and BPP values in HEK293T cells stably expressing EGFP-LC3B. (A) The τ_D values of two typical kinds of FCS curves in HEK293T cells stably expressing EGFP-LC3B. (B) The BPP values of two typical kinds of FCS curves in HEK293T cells stably expressing EGFP-LC3B.

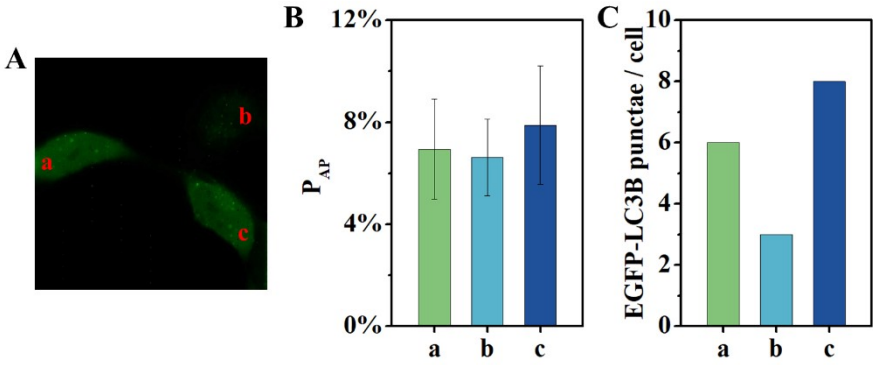

Fig. S11. The assessment of basal autophagy in H1299 cells with different expression levels of EGFP-LC3B based on confocal fluorescence imaging technique and FCS. Monitoring basal autophagy in H1299 cells with different expression levels of EGFP-LC3B (cell a, cell b and cell c) using confocal fluorescence imaging technique (A) and FCS (B), and the number of EGFP-LC3B punctae per cell was measured using ImageJ software (C).

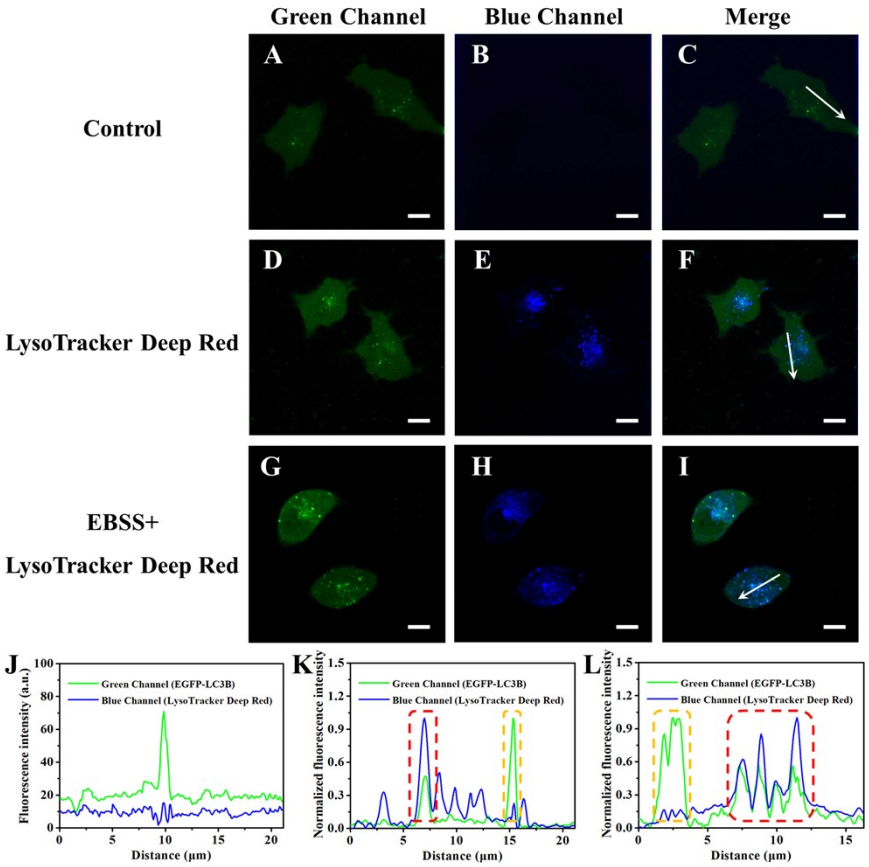

Fig. S12. Co-localization analysis of EGFP-LC3B (green channel) and the commercially available lysosomal probe LysoTracker Deep Red (blue channel, Thermo Fisher Scientific, L12492, 50 μM) in H1299 cells stably expressing EGFP-LC3B. H1299 cells stably expressing EGFP-LC3B were treated with fully supplemented complete medium (control, A-C), LysoTracker Deep Red (D-F) or EBSS plus LysoTracker Deep Red (G-I) for 1 h. In green/blue merged images (F and I), cyan puncta indicate the fusion of autophagosomes and lysosomes. (J-L) Fluorescence intensity profiles of EGFP-LC3B and LysoTracker Deep Red in the interest linear region (white line) across cells (J for Control, K for LysoTracker Deep Red, and L for EBSS plus LysoTracker Deep Red). The overlapping fluorescent regions of green channel and blue channel (red circle) represent the fusion of autophagosomes with lysosomes, while the fluorescent regions of green channel only (yellow circle) represent autophagosomes that are not fused with lysosomes. Confocal fluorescence images were obtained from the home-constructed multi-channel FCS system. EGFP-LC3B: λ_{ex} = 488 nm, emission filter: 530DF30; LysoTracker Deep Red: λ_{ex} = 640 nm, emission filter: 660DF50. Scale bar = 10 μm. The fluorescence intensity profiles of

cells was measured using “Plot Profile” function of ImageJ software (National Institutes of Health, Bethesda, MD, USA).

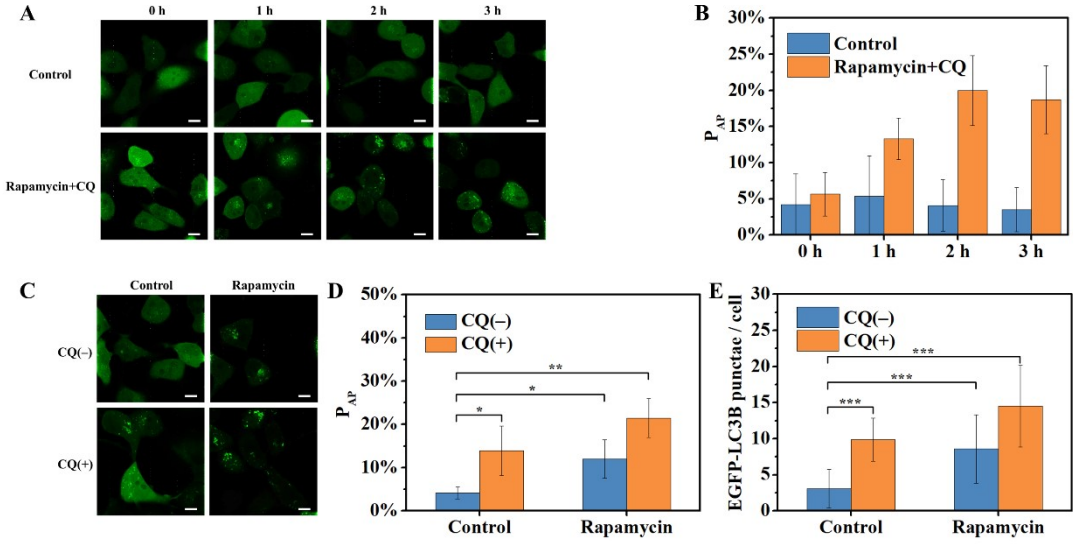

Fig. S13. Monitoring rapamycin-induced autophagic flux in H1299 cells stably expressing EGFP-LC3B using confocal fluorescence imaging technique and FCS. Monitoring rapamycin-induced autophagic flux at different times in H1299 cells stably expressing EGFP-LC3B using confocal fluorescence imaging technique (A) and FCS (B) with the help of CQ. And H1299 cells stably expressing EGFP-LC3B were treated with fully supplemented complete medium (Control) or CQ (10 μM) plus rapamycin (1 μM) for 0 h, 1 h, 2 h and 3 h. Monitoring rapamycin-induced autophagic flux at 2 h in H1299 cells stably expressing EGFP-LC3B using confocal fluorescence imaging technique (C) and FCS (D). And H1299 cells stably expressing EGFP-LC3B were treated with fully supplemented complete medium (Control), CQ (10 μM), rapamycin (1 μM), or CQ (10 μM) plus rapamycin (1 μM) for 2 h. (E) The number of EGFP-LC3B punctae per cell was measured using ImageJ software. And H1299 cells stably expressing EGFP-LC3B were treated with fully supplemented complete medium (Control), CQ (10 μM), rapamycin (1 μM), or CQ (10 μM) plus rapamycin (1 μM) for 2 h. *t* test: ns denoted non-significant; *P < 0.05; **P < 0.01; ***P < 0.001. Scale bar = 10 μm.

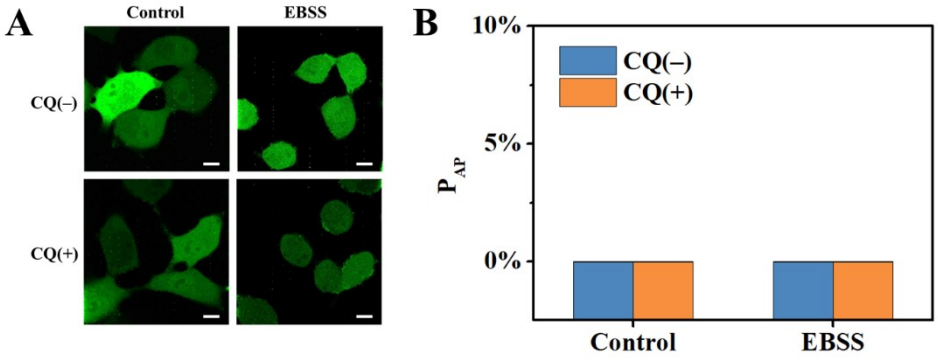

Fig. S14. Monitoring EBSS-induced autophagic flux in H1299 cells stably expressing EGFP-LC3BΔG using confocal fluorescence imaging technique (A) and FCS (B). And H1299 cells stably expressing EGFP-LC3BΔG were treated with fully supplemented complete medium (Control), CQ (10 μM), EBSS, or CQ (10 μM) plus EBSS for 1 h. Scale bar = 10 μm.

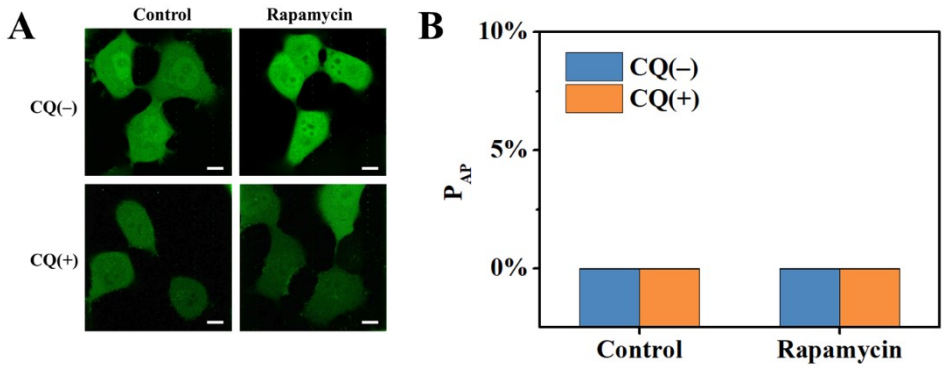

Fig. S15. Monitoring rapamycin-induced autophagic flux in H1299 cells stably expressing EGFP-LC3BΔG using confocal fluorescence imaging technique (A) and FCS (B). And H1299 cells stably expressing EGFP-LC3BΔG were treated with fully supplemented complete medium (Control), CQ (10 μM), rapamycin (1 μM), or CQ (10 μM) plus rapamycin (1 μM) for 2 h. Scale bar = 10 μm.

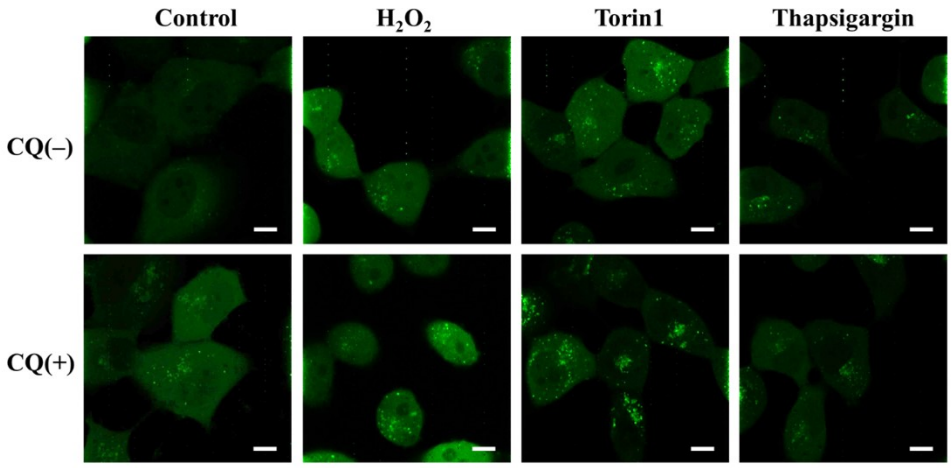

Fig. S16. Monitoring the effect of different autophagy inducers on autophagic flux in H1299 cells stably expressing EGFP-LC3B using confocal fluorescence imaging technique. And H1299 cells stably expressing EGFP-LC3B were treated with fully supplemented complete medium (Control), H_2O_2 (1 μM), Torin 1(10 nM) or thapsogargin (0.5 μM) for 0.5 h with and without the help of CQ (10 μM). Scale bar = 10 μm.

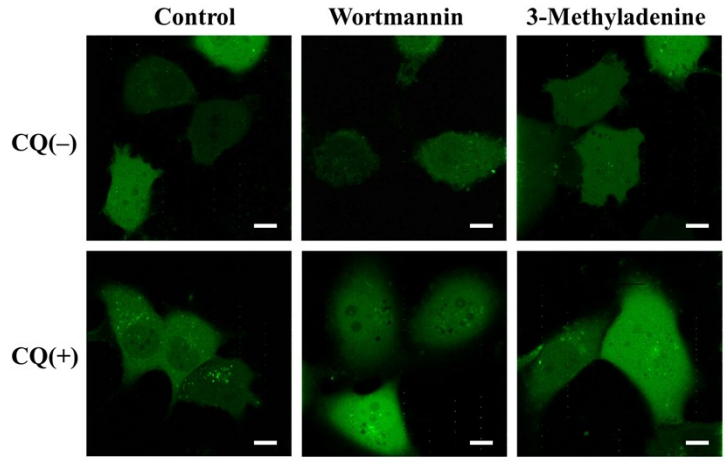

Fig. S17. Monitoring the effect of different early-stage autophagy inhibitors on autophagic flux in H1299 cells stably expressing EGFP-LC3B using confocal fluorescence imaging technique. And H1299 cells stably expressing EGFP-LC3B were treated with fully supplemented complete medium (Control), wortmannin (0.5 μM) or 3-methyladenine (5 mM) for 0.5 h with and without the help of CQ (10 μM). Scale bar = 10 μm.

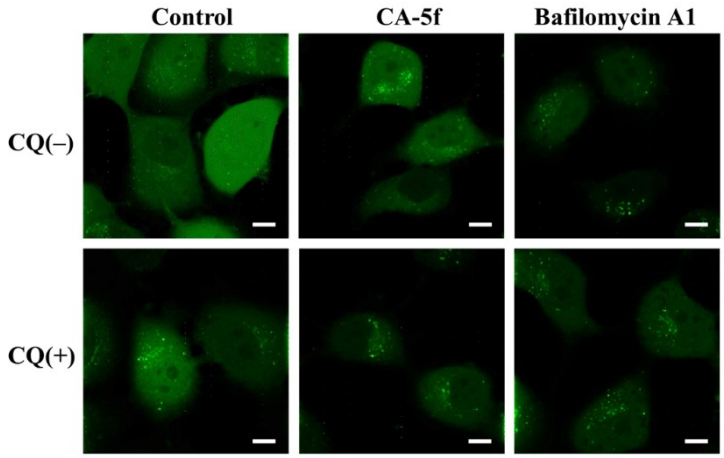

Fig. S18. Monitoring the effect of different late-stage autophagy inhibitors on autophagic flux in H1299 cells stably expressing EGFP-LC3B using confocal fluorescence imaging technique. And H1299 cells stably expressing EGFP-LC3B were treated with fully supplemented complete medium (Control), CA-5f (5 μM) or bafilomycin A1 (50 nM) for 0.5 h with and without the help of CQ (10 μM). Scale bar = 10 μm.

Table S1. Detection techniques for autophagy/autophagic flux and their advantages or limitations

Techniques	Detection type	Revealing the heterogeneity of cellular autophagy	Sensitivity of autophagosomes detection	Resolution	Other advantages or limitations
Electron microscopy	Thin cell-section[1, 2]	Yes	Difficulty in distinguishing autophagosomes from different types of vesicles[2]	High	● Label-free identification of autophagy-related structures (including phagophore, autophagosome and autolysosome).[3] ● Difficulty to evaluate autophagic flux in living cells. ● Time consuming and requiring complex sample preparation and fixation.
Western blot analysis	Cell lysate	No	Can't capture information about autophagosomes	/	● Indirectly assessing autophagic flux by measuring the expression of certain proteins.[4] ● The specificity of western blot analysis depends on the type of antibody used, but most commercially available antibodies may cross-react with several protein isoforms.[4, 5] ● Time consuming and requireing complex experimental procedures.
Flow cytometry	Living cells	Yes	Can't capture information about autophagosomes	Low	● Rapid detection of autophagy in a large number of living cells. ● Difficulty to *in situ* study of living cells. ● Cell samples need to be pre-treated before the assay.[6]
Fluorescence	Living cells	Yes	High	Low	● Can visually observe the punctate structure of autophagosomes.

microscopy					● Quantification of autophagosomes requires the help of computer software such as ImageJ software.[7] ● The identification of fluorescently labeled autophagosomes will be disturbed by fluorescence in the cytoplasm.[8]
FCS	Living cells	Yes	High	High	● Can quantitatively monitor autophagosomes and assess autophagic flux. ● Suitable for low-expressing cells. ● Can study the diffusion behaviors from molecules to nanoparticles. ● Compared with two color fluorescence cross-correlation spectroscopy (FCCS) technique, FCS can't get the information on the different detection channels.[9]

Notes and references

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