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Supporting Information

Direct Visualizing Mitochondrial Shrinkage and Lysosomal Expansion During Mitophagy using Super Resolution Microscopy

Farhan Anjum[a, b,c] ¶, Chethana Rao[a, b] ¶, Aditya Yadav[a, b] ¶, Kush Kaushik[a, b], Pushpendra Mani Mishra[a, b,c] and Chayan Kanti Nandi[a, b,c] *

[a]School of Basic Sciences, Indian Institute of Technology Mandi, Mandi, HP-175005, India

[b]Advanced Materials Research Centre, Indian Institute of Technology, Mandi, HP-175005, India

[c] BioX centre, Indian Institute of Technology Mandi, Mandi, HP-175005, India

¶ *Farhan Anjum, Chethana Rao and Aditya Yadav have contributed equally to the work*

Materials

All glassware were washed with aqua regia, followed by rinsing several times with double distilled water. BSA was purchased from Sigma Aldrich life sciences (Cat no. A2153-10G), having purification ≥ 96 %. Hela cells were obtained from NCCS Pune, and all the cell culture media, media supplements were purchased from Gibco, Invitrogen Corporation. Cy3 and Cy5 dyes were purchased from Lumiprobe Life Science Solution. All chemicals were used without further purification. Double-distilled (18.3 mΩ) deionized water (ELGA PURELAB Ultra) was used throughout the entire process.

Synthesis and Purification of BSA-Dye bioconjugate:

The BSA-dye bioconjugates were synthesized by following the NHS-ester crosslinking reactions. In brief, 4:0.1 mg/mL of BSA:dye were taken according to the volume of the reaction mixture, mixed in DI water (maintain the pH at pH 7-8.4). The reaction mixture was vortexed well and allowed to conjugate for 12-24 hours on a spinner at 22-25 °C in dark conditions. The NHS esters act as crosslinkers and the leaving group after labeling the primary amine of protein to yield stable amide bonds. Purification and free dye separation were achieved by dialysis in a 20 kDa dialysis bag for 24 to 48 h with sterile DI water, followed by 2-3 times buffer (sterile water) change at room temperature. Before cell staining, the conjugation condition was maintained sterile to prevent contamination load and stored at 4 °C for the short term and at -20 °C for long-term storage.

Photophysical characterization of BSA-Dye bioconjugate:

UV-Vis Absorption and steady-state fluorescence spectroscopy

The UV-Vis absorption spectra were recorded using Shimadzu UV-Vis 2450 spectrophotometer. The spectra were collected using a quartz cuvette having a 10 mm path length and 1 ml volume. All the measurements were repeated at least three times. Steady-state fluorescence was measured using a Horiba Fluorolog-3 spectrofluorometer. The fluorescence was measured in a 1 ml quartz cuvette.

Fluorescence lifetime and SDS-PAGE

The fluorescence lifetime was measured using the Horiba Scientific Delta Flex TCSPC system with Pulsed LED Sources. The acquired data were fitted using an n-exponential reconvolution function. Ludox has been used to calculate IRF for de-convolution of the spectral value. SDS-PAGE was performed to confirm the conjugation with 15% polyacrylamide gels and running buffer (25 mM TRIS base, 192 mM glycine, and 0.1% SDS). Electrophoresis was carried out at 100 V voltages and 20 mA current for 3 hrs. Staining was achieved using a Coomassie Brilliant Blue R-250 solution. Furthermore, fluorescent bands were visualized with Gel Doc by exciting the bands with required lamps.

Confocal and Super-resolution Imaging of HeLa cells labeled with Bioconjugates

Cell Culture, staining, and fixation: HeLa cells were cultured and maintained in T-25 tissue culture flask with DMEM tissue culture medium (Gibco, Invitrogen Corporation) supplemented with 2 mM of glutamine,10% fetal bovine serum (FBS- Gibco, Invitrogen Corporation), 1% HEPES, 1% Anti-Anti (Penicillium-Streptomysine) and incubated in a humidified incubator (at 37 ^{o}C with 5% CO_2). Further healthy cells were taken from the T-25 flask and grown in a 6-well plate on the coverslips. Each well was supplemented with 2 ml of growth medium and the cells were allowed to grow overnight for the proper confluence. The growth and the attachment of the cells to the coverslips were examined by an optical microscope. Once the cells properly adhered, they were incubated with optimized concentrations of bioconjugates (BSA-Cy3 and BSA-Cy5: 200 nM) for 2h (30 min was enough to stain the lysosomes) to achieve an appropriate labeling density for imaging in the confocal microscope. The cells were then washed with PBS buffer several times to remove any unbound bioconjugates and fixed by incubating with 4% paraformaldehyde solution

in 1x PBS buffer for 10 min at room temperature. Cells were washed again 3 times with 1x PBS then coverslips were fixed on a glass slide by using a drop of glycerol and the edges were sealed before imaging. Finally, the stained cell lines were examined through the confocal microscope.

Confocal microscopy

Nikon Eclipse Ti inverted microscope was used for the confocal microscopy, and images were acquired using Nikon Nis-Element software. To image the Lysotracker and BSA-Cy3 a 561 nm excitation laser and a 595/50 nm emission filter were used. On the other hand, for BSA-Cy5, a 639 nm excitation laser and a 700/75 nm emission filter were used. The data clearly shows that the BSA-dye bioconjugates could be used as a multicolor imaging Lysotracker in real cellular systems.

Colocalization analysis with Lysotracker Red DND-99

A double stain, colocalization with bioconjugates (BSA-Cy5) and Lysotracker Red DND-99 were undertaken in HeLa cells. The colocalization coefficient analysis was performed by Nikon confocal microscope software, NIS element. The Lysotracker Red DND-99 & bioconjugates showed Pearson's coefficient and Mander's overlap value ~0.8 for both the conjugates.

Photobleaching study using confocal microscopy

For the photobleaching study, stained cells were imaged through a confocal microscope. The same laser power i.e. 8.06 µW was used to image lysosomes stained with Lysotracker (561 nm laser) and BSA-Cy5 (639 nm laser). A 90 min timelapse movie was captured with a time interval of 10 s each frame, 540 frames were captured during this time interval.

Super-resolution Imaging

Super-resolution radial fluctuation (SRRF)

To obtain a high-resolution image of lysosomes after being stained with BSA-Cy3 and BSA-Cy5 super resolution radial fluctuations (SRRF) microscopy was carried out. In this method, point spread functions (PSFs) was created by single molecule that contained a higher degree of local geometrical symmetry than the background. Radiality distribution is independent of the PSF intensity and the full width at half maximum (FWHM) of this distribution and was adjusted by the gradient convergence radius. To perform single molecule localization, we recorded a time series with a 50 ms acquisition time per image under the wide field illumination with corresponding filter

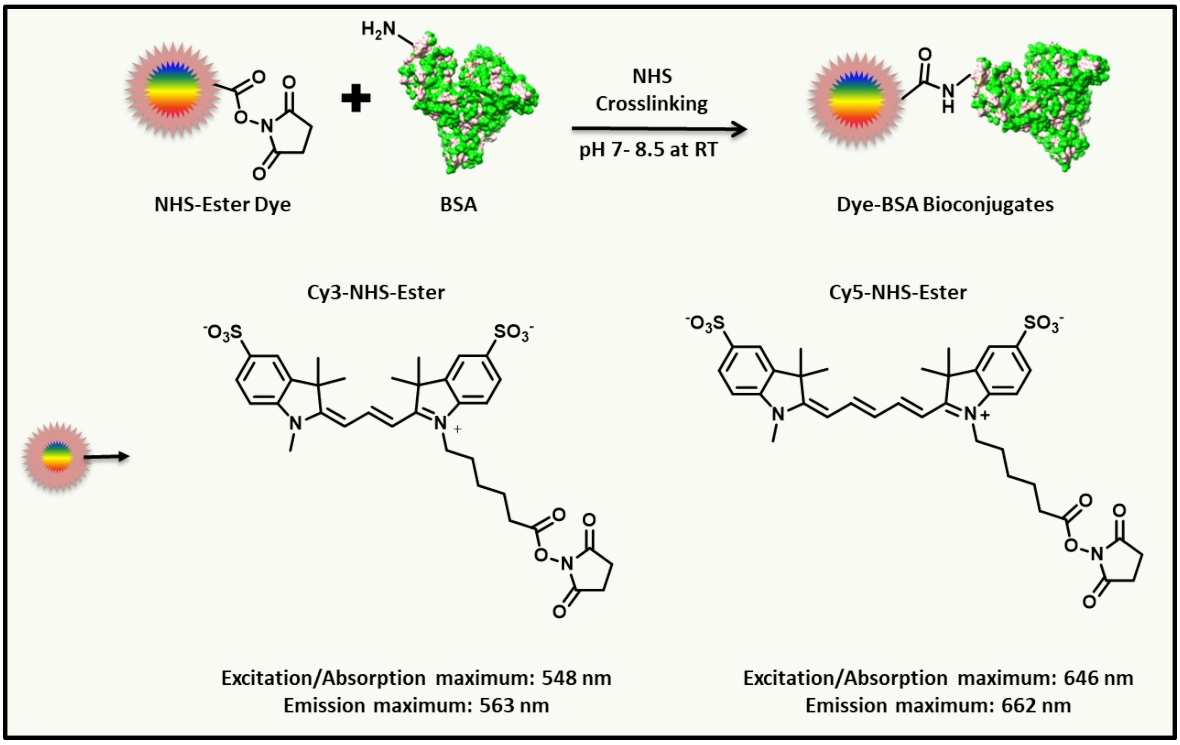
sets. The movie contained 5000 stacked images was analyzed with an open-source version of NanoJ-SRRF on a high performance NVIDIA GeForce1050TiGPU. For the Mitophagy study, BSA-Cy5 and Mitotracker green treated HeLa cells were prepared. A movie containing 2000 frames was recorded under appropriate illumination conditions.

Stochastic optical reconstruction microscopy (STORM)

The stack of video analyzed for STORM was the same as obtained for SRRF. Similar to SRRF, 5000 frames of the video were taken for analysis using the ThunderSTORM plugin of ImageJ using default settings. Default settings of the plugin were used with Filter: Wavelet filter (B-Spline order 3 and scale 2.0), localization of molecule method used was Local maximum method and 8 neighborhood connectivity was used, PSF: Integrated Gaussian method was used for sub pixel localization with fitting radius 3pixels and initial sigma of 1.6 pixels was used with weighted least square fitting method, multi emitter fitting was turned OFF during all analysis. Cross-correlation method was used for drift correction with 10 steps and 5 magnifications. Drift correction on all images was done using cross correlation method.

Application to trace the Mitophagy event

To elucidate the ability of BSA-dye conjugate CCCP induced mitophagy event was studied in HeLa cells. Cells were grown in DMEM tissue culture medium supplemented with 2 mM/L of glutamine,10% fetal bovine serum, 1% HEPES, 1% Anti-Anti (Penicillium-Streptomysine) and incubated in a humidified incubator (at 37 ^{o}C with 5% CO_2). Further cells (at a concentration of 1 $\times$ 10^4 cells/coverslip in 500 µL culture medium) were cultured in 6-well plates. Cells were treated with 10 uM of CCCP (carbonyl cyanide m-chlorophenylhydrazone) to induce mitophagy in Hela cells for 12 h. Finally, cells were incubated with Mitotracker green FM-Invitrogen and BSA-Cy5. Then the cells were washed with PBS and fixed with 4% paraformaldehyde prior to confocal and super resolution imaging.



Scheme 1. A schematic representation of the conjugation of cyanine dye with BSA.

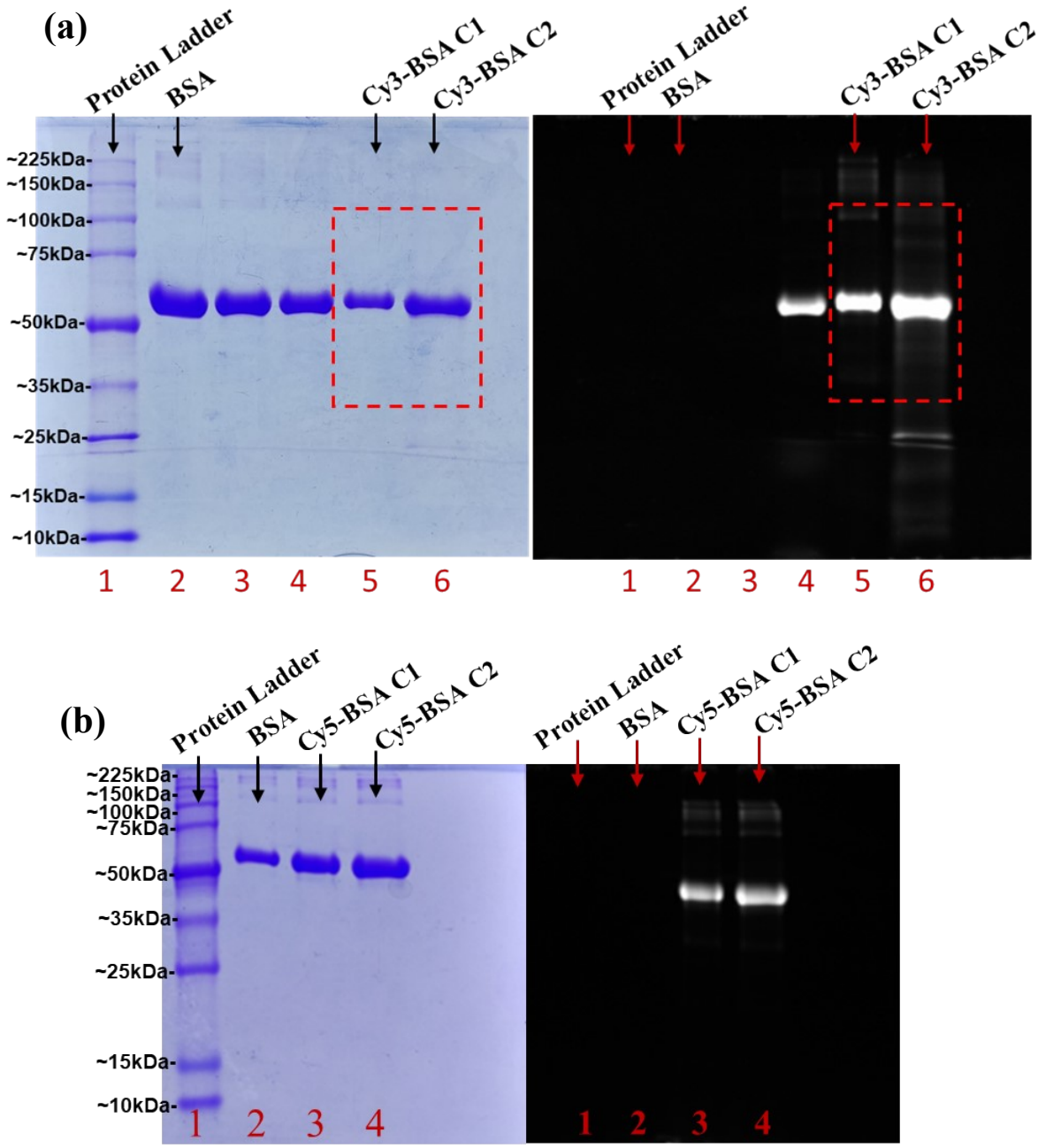


Figure S1. The SDS PAGE for protein ladder, BSA, BSA-Cy3 and BSA-Cy5, the fluorescent bands were observed for BSA-dye conjugates under appropriate lamp illumination due to covalent binding with BSA and the corresponding dye, coomassie brilliant blue staining showing the BSA protein (MW ~66 kDa) bands coinciding with fluorescent bands. SDS PAGE was run at different concentrations of Cy3 and Cy5 with different conjugates, (a) Lane 1 corresponds to protein ladder, Lane 2 for BSA, 3 & 4 for other conjugates, 5 & 6 for Cy3-BSA conjugate, (b) Lane 1 for protein ladder, Lane 2 for BSA, Lane 3 & 4 for Cy5-BSA conjugate at two concentrations.

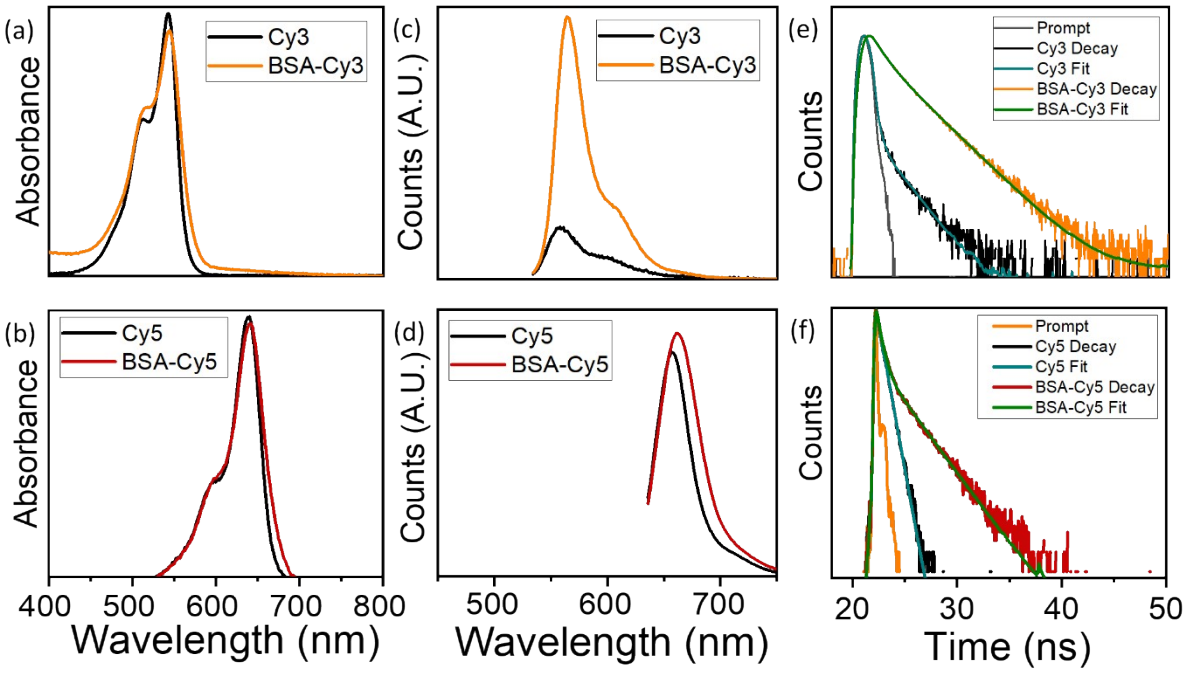


Figure S2. The absorbance spectra of dye and the BSA-dye conjugates, (a) Cy3, BSA-Cy3 and (b) Cy5, BSA-Cy5, the fluorescence emission spectra of (c) Cy3, BSA-Cy3 and (d) Cy5, BSA-Cy5, show enhanced emission characteristics of each dye upon conjugation with BSA, the enhancement of lifetime are presented in (e) Cy3, BSA-Cy3 and (f) Cy5, BSA-Cy5.

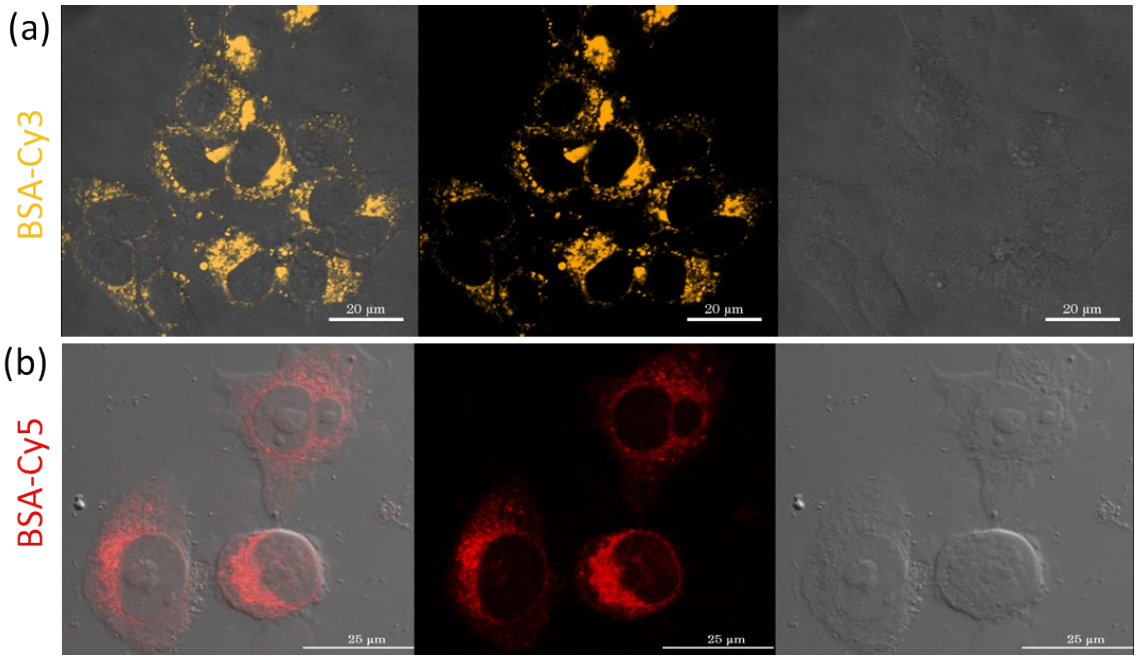


Figure S3. Confocal images of HeLa cells treated with (a) BSA-Cy3 and (b) BSA-Cy5 showed sufficient lysosomal accumulation of BSA-dye conjugates.

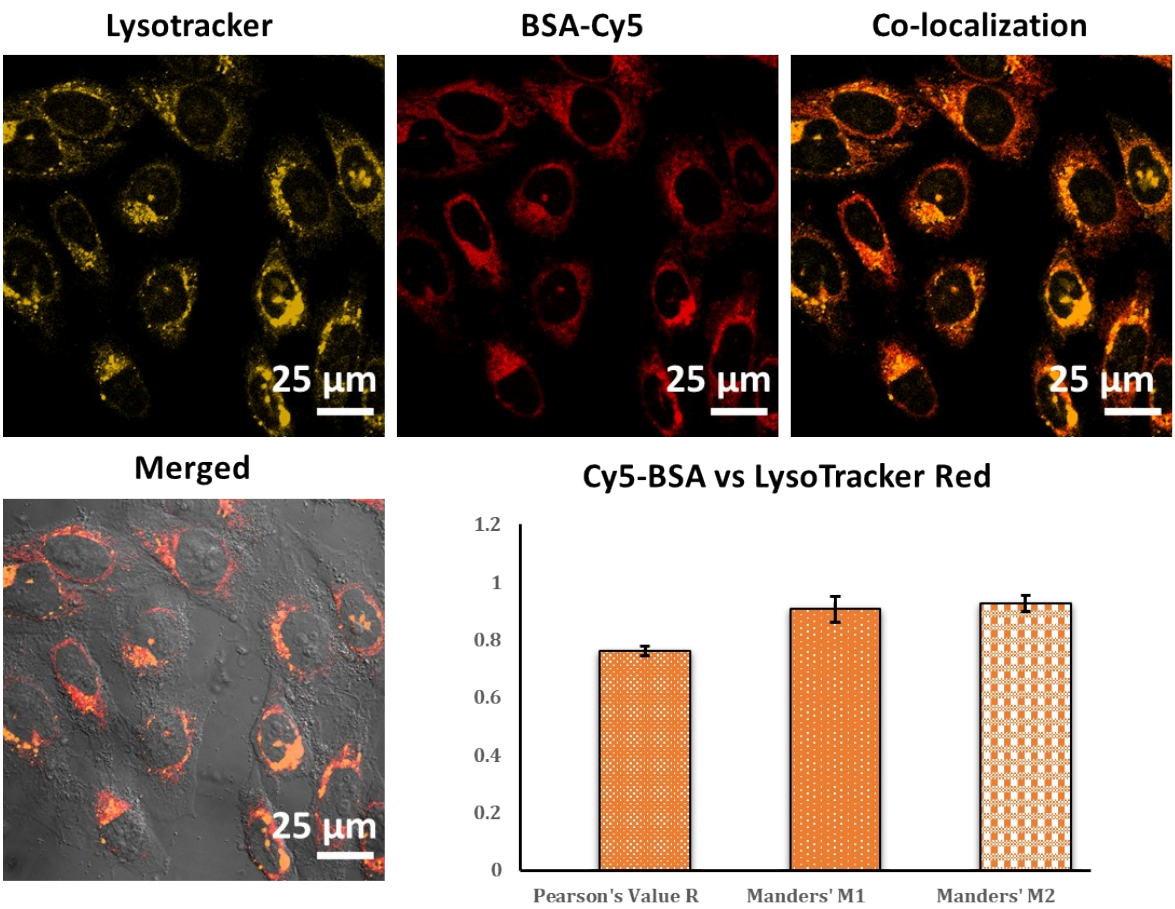


Figure S4. The representative confocal images show colocalization of commercial Lysotracker Red DND-99 with BSA-Cy5, inset represents the Pearson's and Mander's overlap values, which fall between ~0.7-0.8.

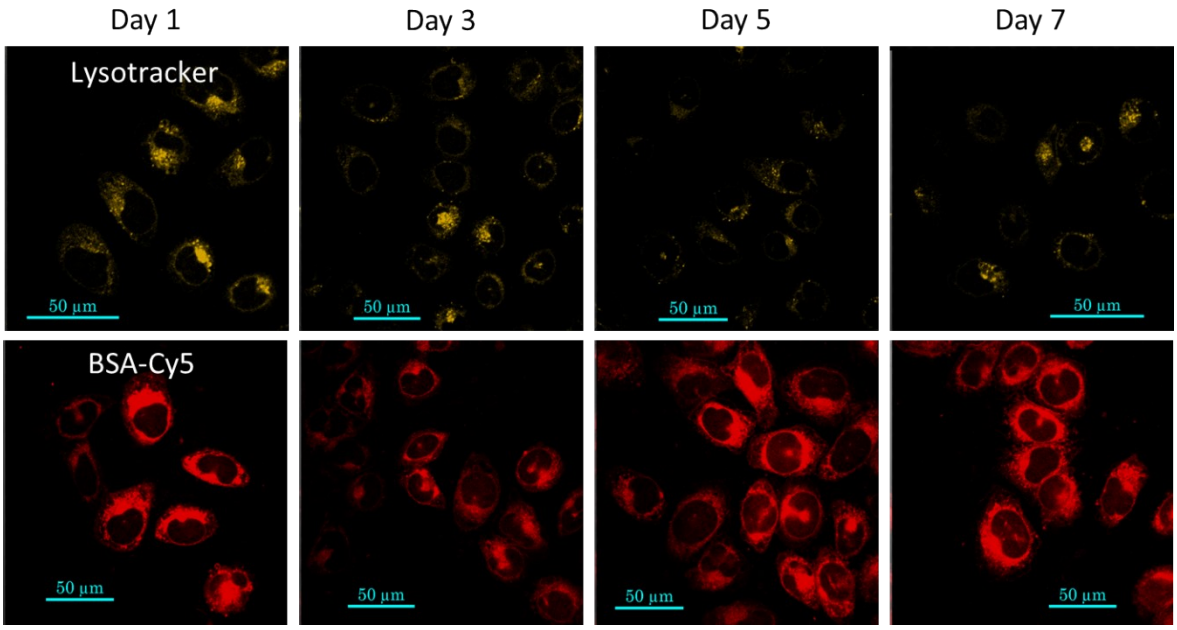


Figure S5. Comparison of retention time of BSA-Cy5 with commercial lysotracker in lysosomes. The BSA-Cy5 was stable even up to the 7 days.

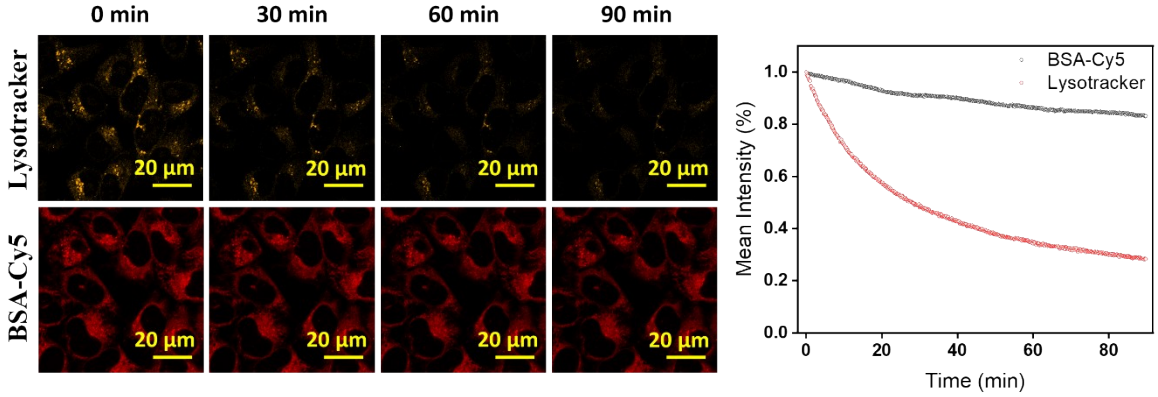


Figure S6. The confocal images show the effects of time dependent laser exposure of same power on the lysotracker and BSA-Cy5.

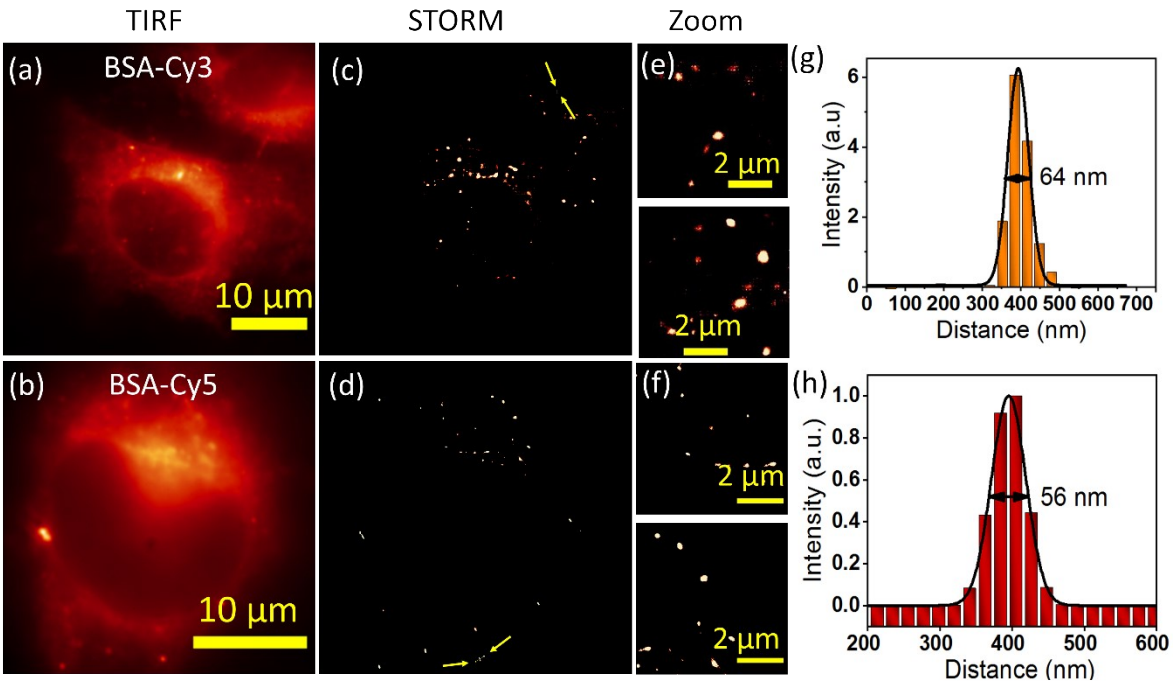


Figure S7. The (a, b) TIRF and the (c, d) STORM images show lysosomes of HeLa cells treated with BSA-Cy3 and BSA-Cy5 respectively, the zoomed in images (e, f) also show super-resolved individual lysosomes for the same, the line profile (shown by arrows) plots fitted with gaussian, representing the smallest lysosome structures (g, h) for BSA-Cy3 and BSA-Cy5 respectively.

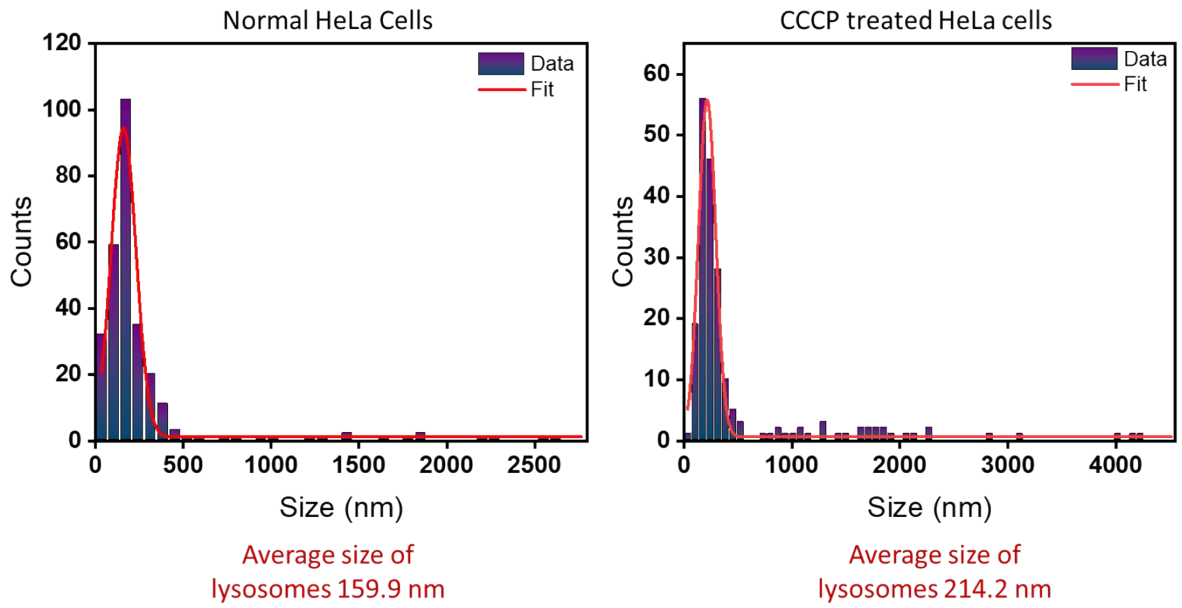


Figure S8. CCCP treated cells showed lysosomal expansion during mitophagy.

Sample	Photons	Photons per cycle	No. of cycles
Cy3	7949±1913	396±56	22±5
BSA-Cy3	26659±5111	2394±174	16±1
Cy5	6588±926	263±12	15±1
BSA-Cy5	13798±1857	1453±155	15±0.4

Table S1. Table of photophysical parameters of dye and their conjugates with BSA.

Sample	Pearson's Coefficient	Mander's Coefficient M1 (ch1 vs ch2 or BSA-Cy5 vs Mitotracker)	Mander's Coefficient M2 (ch2 vs ch1 or Mitotracker vs BSA-Cy5)
Untreated	0.12	0.092	0.379
Untreated Zoom	0.01	0.153	0.026
CCCP treated	0.37	0.427	0.64
CCCP treated Zoom	0.35	0.478	0.578

Table S2. Pearson's and Mander's coefficients for images in figure 3.

Sample	M value (y-intercept FWHM/ Smallest FWHM among lysosome and mitochondria)
Untreated (i)	0
(ii)	0
(iii)	0
(iv)	0
CCCP treated (i)	1
(ii)	1
(iii)	0.79
(iv)	0.98

Table S3. The M value for the images in Figure 3.

S.No.		FWHM (nm)	Distance (nm)
Untreated (i)	Cy5-BSA	107	330
	MTG	267	
(ii)	Cy5-BSA	159	275
	MTG	180	
(iii)	Cy5-BSA	98	208
	MTG	141	
(iv)	Cy5-BSA	107	486
	MTG	233	
CCCP Treated (i)	Cy5-BSA	252	Overlapping
	MTG	112	
(ii)	Cy5-BSA	228	Overlapping
	MTG	109	
(iii)	Cy5-BSA	134	41
	MTG	126	
(iv)	Cy5-BSA	169	59
	MTG	111	

Table S4. FWHM of lysosome and mitochondria and the relative distance between them in untreated and CCCP treated cells.