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Fluorescence microscopy: A tool to study autophagy

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Autophagy is a cellular recycling process through which a cell degrades old and damaged cellular components such as organelles and proteins and the degradation products are reused to provide energy and building blocks. Dysfunctional autophagy is reported in several pathological situations. Hence, autophagy plays an important role in both cellular homeostasis and diseased conditions. Autophagy can be studied through various techniques including fluorescence based microscopy. With the advancements of newer technologies in fluorescence microscopy, several novel processes of autophagy have been discovered which makes it an essential tool for autophagy research. Moreover, ability to tag fluorescent proteins with sub cellular targets has enabled us to evaluate autophagy processes in real time under fluorescent microscope. In this article, we demonstrate different aspects of autophagy in two different model organisms i.e. yeast and mammalian cells, with the help of fluorescence microscopy. [http://dx.doi.org/10.1063/1.4928185]

INTRODUCTION

Over the years, fluorescence microscopy has become an invaluable tool to investigate cellular and molecular events precisely. Its applications includes: studying intracellular localization, biomolecular interactions (such as protein-protein) and protein/organelle turn-over. Towards this, labeling biomolecules of interest using fluorescent probes enable us to track the inter- and intra-cellular dynamics of cellular processes. One such intracellular process is autophagy which is conserved from yeast to humans.[1,2] Perturbation of autophagy has been implicated in several pathological and disease conditions like cancer, neurodegeneration and infections.[3–7] Hence, understanding the autophagy process is important.

Autophagy (a Greek word meaning *self-eating*) is a tightly regulated intracellular degradation process targeting damaged and redundant cellular contents to recycle them as building blocks for maintaining cellular homeostasis.[8] Apart from basal or constitutive levels of autophagy, this process can also be induced by nutrient starvation that suppresses TOR (Target Of Rapamycin) signaling and triggers autophagy.[2] Depending on the cellular contents (cargo) such as proteins and organelles it captures, autophagy can be selective or non-selective. The itinerary of autophagy involves entrapment of cargo within double membranous vesicles, called autophagosomes that eventually fuse with vacuole/lysosome wherein cargo gets degraded by various hydrolytic enzymes. The degraded materials, which generally include amino acids, are recycled back to the cytoplasm.[9] Autophagy process involves several steps: autophagosome formation, expansion, maturation and vacuolar/lysosomal fusion. These various steps of autophagy are carried out by several molecular players such as Autophagy related (Atg) proteins.[9–12] By employing fluorescence microscopy, molecular events of autophagy in a steady state and/or total flux, can be monitored by tagging these molecules and/or cargo with fluorescent probes.[13] Till now, lack of biochemical methods to investigate the initial events of autophagy such as Pre-Autophagosomal Structure (PAS) formation makes fluorescence

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microscopy an indispensable tool to study autophagy.[12] Indeed, fluorescence microscopy is one of the recommended methods in the Guidelines for use and interpretation of autophagy assays.[8,14]

Based on the cargo of choice, different autophagy and related processes have been studied employing fluorescent microscopy.[1,14–16] General autophagy (non-selective) is studied by monitoring the levels of fluorescent labeled autophagosome marker protein Light Chain 3 (LC3). Similarly, various selective autophagy processes like pexophagy (selective autophagy of peroxisomes), aggrephagy (selective autophagy of protein aggregates) and xenophagy (selective autophagy of intracellular pathogens) can be studied by following the degradation of fluorescent tagged cargos such as: peroxisomal thiolase-1 (Pot1), α-synuclein protein and bacterium *Salmonella typhimurium*, respectively.

To underline the essence of fluorescent microscopy in autophagy research, here we discuss some of its very important and prevalent applications. Additionally, the data presented here illustrates that fluorescent microscopy can be very instrumental to demonstrate in-depth study of cellular processes from most simple to most complex eukaryotic models.

MATERIALS AND METHODS

Materials

Strains and plasmids

All the strains of *Saccharomyces cerevisiae* used in this study were of BY4741 or BY4742 background and were purchased from EUROpean *Saccharomyces Cerevisiae* ARchive for Functional Analysis (EUROSCARF), Frankfurt, Germany. To study the degradation of peroxisomes, in BY4742 strain, the genomic copy of Peroxisomal thiolase-1 (POT1) gene was tagged with Green Fluorescent Protein (GFP). For protein aggregation study, BY4741strain, harboring genomic copy of GFP tagged α-synuclein gene, under the control of *gal* (galactose) promoter, was used.

For autophagy studies in mammalian cells, HeLa cells (cervical cancer cell line, gifted by Prof. P. Kondaiah, MRDG, IISc, Bengaluru, India) were transfected with pTF-LC3 (plasmid with Tandem Fluorescent tagged LC3) construct (Addgene Inc. Cambridge, USA, Plasmid ID # 21074). pTF-LC-3 construct was used as it has autophagosome marker protein, LC3 tagged with Red Fluorescent Protein and Green Fluorescent Protein in tandem (RFP-GFP-LC3). For Xenophagy assay, mCherry (a red fluorescent protein) tagged *S. typhimurium* strain SL1344 was used which was gifted by Dr. C.V. Srikanth, RCB, Faridabad, India.

Microscopy

For imaging, bright field fluorescence Delta-Vision Microscope (Applied Precision, GE) fitted with 60x (used for mammalian cells) and 100x (used for yeast cells) 1.4NA objectives and Cool-SNAP HQ2 camera was used. Excitation/ emission maxima of ~515/640 nm for FM4-64, ~482/505 for GFP, ~555/584 nm for RFP, 587/810 nm for mCharry, 345/455 nm for DAPI (4′,6-diamidino-2-phenylindole, a blue colored stain for nucleus) and 501/523 nm for Anti-rabbit IgG Atto 488 secondary antibody (used to label p62 primary antibody) were used to acquire fluorescence images. Images were processed using softWoRx® (Applied Precisions) and quantified using Image-J (NIH) softwares.

Reagents

Reagents for yeast culture

Following reagents were used: Yeast Extract, Peptone, Dextrose, Galactose, Yeast Nitrogen Base without amino acids and ammonium sulphate (YNB), Histidine, Leucine, Methionine and Uracil (HiMedia Laboratories, Mumbai, India). Lysine, Oleic acid and Tween® 40 (Sigma Alrich, St. Louis, USA). Ammonium sulphate and Di potassium hydrogen ortho phosphate (K_2HPO_4) (Merck Millipore, Darmstadt, Germany). Potassium di hydrogen ortho phosphate (KH_2PO_4) (Fisher Scientific, Hampton, USA). FM4-64 (Life Technologies, New York, USA).

Reagents for mammalian culture

Following reagents were used: Dulbecco's Modified Eagle's Medium (DMEM), Earle's Balanced Salt Solution (EBSS), Paraformaldehyde (PFA), Phosphate Buffered Saline (PBS), Gentamycin, Bovine Serum Albumin (BSA) and Anti-rabbit IgG Atto 488 secondary antibody (Sigma Aldrich, St. Louis, USA). VECTASHIELD® (Vector Laboratories Inc., Burlingame, USA). Anti p62 antibody (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan). Fetal Bovine Serum (FBS) and Lipofectamine-2000 (Life Technologies, New York, USA). Triton X-100 (HiMedia Laboratories, Mumbai, India).

Methods

Monitoring the autophagic degradation of organelles

BY4742 yeast strains (wild type, *atg1*Δ and *atg15*Δ) having GFP tagged genomic copy of POT1 gene were grown in growth medium (1% yeast extract, 2% peptone & 2% dextrose) overnight at 30°C, 250 rpm till it reached stationary phase. Next day the culture was diluted in growth medium to 0.2 A_{600nm}/ml and incubated at 30°C, 250 rpm till A_{600nm} reached 0.8 (early exponential phase). The cells were washed with autoclaved water, shifted to a fatty acid rich medium (0.066% K_2HPO_4, 0.434% KH_2PO_4, 0.3% yeast extract, 0.5% peptone, 0.1% oleate & 0.5% tween-40) containing 0.32 μM of vacuole marker FM4-64 and incubated overnight at 30°C, 250 rpm to induce peroxisome biogenesis. Next day, cells were washed with sterile water followed by inoculation in starvation medium (0.17% YNB, 2% dextrose) to induce autophagy. The cells before starvation and after 6 hours of starvation were used for microscopy to observe the fluorescence signals of FM4-64 and GFP. The acquired images were later processed and quantified.

Monitoring the clearance of mis-folded protein aggregates in yeast

BY4741 yeast strains, (wild type and *atg1*Δ) harboring genomic copy of GFP tagged α-synuclein, were grown in SD-Uracil (0.17% YNB, 2% dextrose, 0.25% ammonium sulphate, 0.1% histidine, 0.1% methionine, 0.6% leucine) medium for overnight at 30°C, 250 rpm till it reached stationary phase. Next day the cultures were diluted in SD-Uracil medium to 0.2 A_{600nm}/ml and incubated at 30°C, 250 rpm till A_{600nm} reached 0.8 (early exponential phase). The cells were then shifted to SG-Uracil (0.17% YNB, 2% galactose, 0.25% ammonium sulphate, 0.1% histidine, 0.1% methionine, 0.6% leucine) medium for 12 hours to induce the expression of α-synuclein-GFP protein. The cells were then observed under fluorescent microscope for GFP signal.

Monitoring autophagic flux in mammalian cells

Approximately 5 X 10^4 HeLa cells seeded on cover glasses were allowed to grow in growth medium (DMEM supplemented with 10% FBS) till they became 60-70% confluent. These cells were then transfected with pTF-LC3 construct using Lipofectamine-2000 in the ratio of 1:2 (2.5μg of pTF-LC3:5μl of lipofectamine). After 6 hours of transfection, fresh growth medium was added to recover the cells. Cells were allowed to express the protein for 48 hours. The cells were later treated for 2 hours either in growth medium or starvation medium (EBSS) to induce autophagy. After treatment, cells were fixed in 4% PFA for 20 minutes at room temperature. Fixed cells were then permeabilized with 0.25% Triton X-100 for 15 minutes at room temperature and then washed with PBS. The cover glasses were mounted on glass slides using VECTASHIELD® (anti-fade mounting media) without DAPI and sealed with nail paint. The images were acquired, processed and quantified.

Monitoring xenophagy in mammalian cells

Approximately 2 X 10^5 HeLa cells, seeded on cover glasses, were allowed to grow in growth medium till they became 60-70% confluent. These cells were then transfected with pTF-LC3 construct as explained in previous section. After two days, cover glasses were removed and placed in a 12 well plate. Cells were infected with mCherry tagged *S. typhimurium*, with a Multiplicity

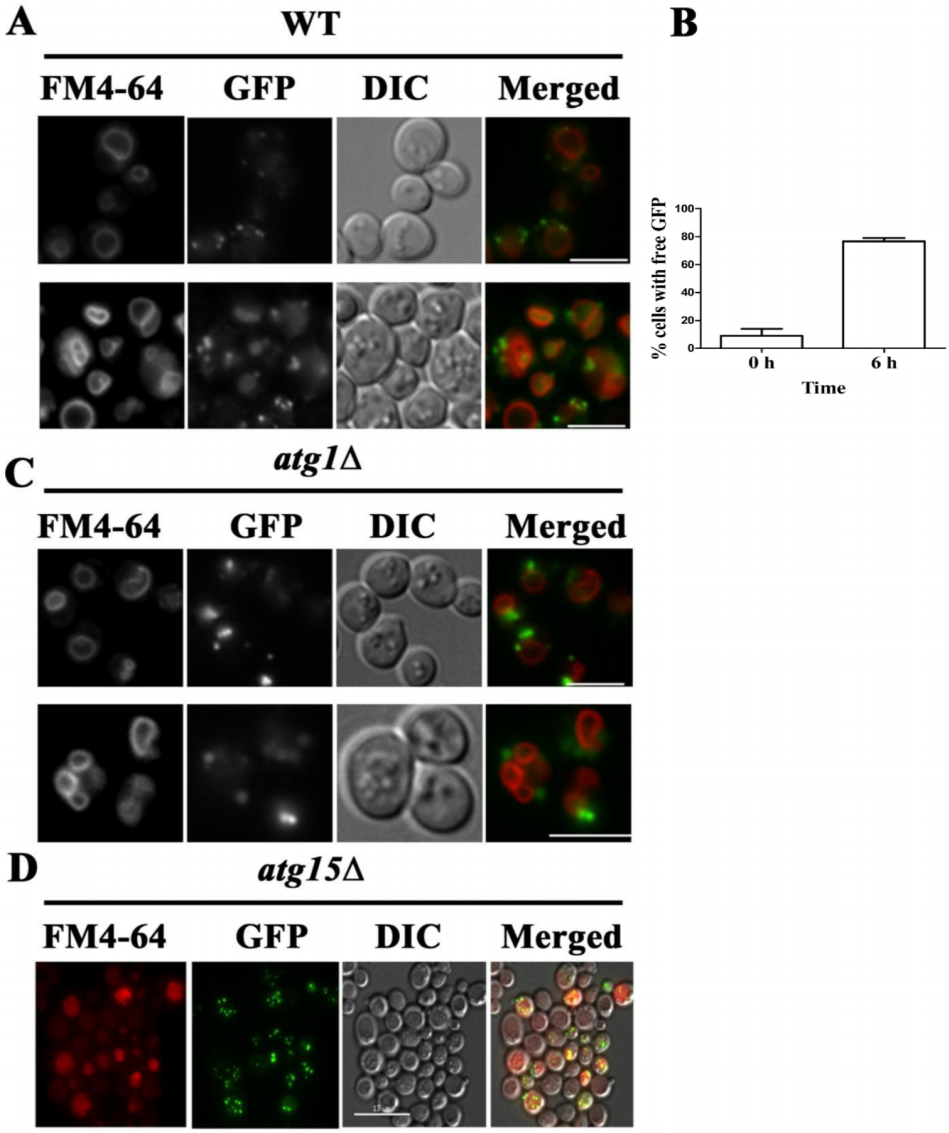

FIG. 1. A: Wild type yeast cells expressing Pot1-GFP were grown in a fatty acid rich medium, in the presence of the vacuolar stain FM4-64 (red), to induce peroxisome (cargo) biogenesis. The peroxisomes appeared as numerous green puncta in the cells at 0 hours (no starvation). Upon starvation (6 hours), the number of peroxisomes reduced with a concomitant increase in diffused GFP inside the vacuoles which is an indication of autophagic degradation of peroxisomes. Scale bar: 5μm. B: About 60 cells of each strain were examined and percentage of cells showing free GFP was plotted for only wild type (WT) strain. C: In the autophagy mutant strain, *atg1Δ*, the decrease in peroxisome numbers and accumulation of free GFP in the vacuole was not observed after 6 hours of starvation. Since *atg1Δ* strain did not show any free GFP inside vacuole, the values were not plotted. Scale bar: 5μm. D: In *atg15Δ* strain, in which the degradation of contents inside the vacuole is hampered after the autophagosomes fuse with the vacuole, the peroxisomes (cargo) accumulated inside the vacuole. Scale bar: 13 μm. All the experiments were done three times and the representative images are shown here.

of Infection (MOI) of 800, for an hour at 37°C. Post infection, gentamycin treatment (100μg/ml at 37°C for 2 hours) was given to kill the extracellular bacteria followed by PBS wash. Cells were then fixed, washed, permeabilized and again washed as explain in the previous section. Primary antibody for p62 (made in 0.2% BSA) at the dilution of 1:1000, was added to each cover glass and incubated overnight at 4°C. Next day, the cover glasses were washed three times (5 minutes each) with PBS and then secondary antibody, Anti-rabbit IgG Atto 488 at 1:1000 dilution (made in 1% BSA), was added followed by incubation for one hour at room temperature. The cover glasses were again washed three times for 5 minutes each with PBS and mounted on glass slides with

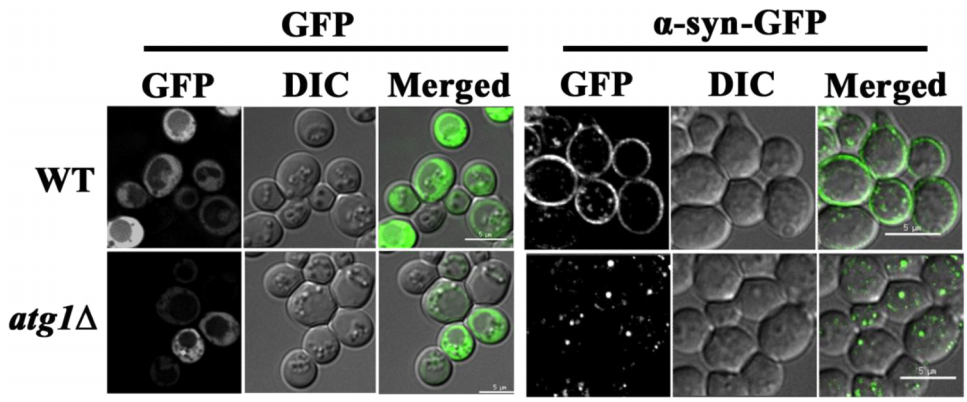

FIG. 2. : Majority of GFP tagged α-synuclein protein (α-syn-GFP) normally localizes to plasma membrane in wild type (WT) yeast. In *atg1Δ* strain, where autophagy is impaired, α-syn-GFP forms aggregates which can be seen in punctuate form. For both the strains (WT and *atg1Δ*), expression of only GFP was seen everywhere in the cytoplasm, which rules out the possibility of effect of GFP in aggregate formation. Scale bar is 5μm. The experiment was done three times and the representative images are shown here.

DAPI containing VECTASHIELD®. Images were acquired using respective excitation and emission maxima as mentioned in the microscopy section.

RESULTS AND DISCUSSION

Autophagy, as mentioned above is of both selective and non-selective nature. To understand this process completely, both the forms need to be studied. In this section fluorescent microscopy data are discussed to explain how both the forms of autophagy can be studied in yeast and mammalian cells. Based on advantages and limitations of both the model organisms, most appropriate assays were done to emphasize the importance of autophagy.

To measure selective autophagy, microscopy based pexophagy and aggrephagy assays were done in yeast whereas general autophagy and xenophagy assay were performed in mammalian cells. Pexophagy is relatively easy-to-measure, *via* fluorescent microscopy, in yeast compared to other forms of selective autophagy. The same is true for general autophagy in mammalian cells. Results for aggrephagy and xenophagy are shown to indicate the importance of autophagy research towards understanding of diseases.

Monitoring the autophagic degradation of organelles

In selective form of autophagy, specific damaged or superfluous cellular organelles are degraded.[17,18] In order to study this, specific proteins of the target organelle are labeled with fluorescent tag which can be monitored/chased using fluorescent microscopy.[15,16] The selective degradation of peroxisomes termed as pexophagy[19] is discussed here. Towards this, peroxisomal protein, Pot1 was tagged with GFP to monitor its degradation. Induction of peroxisomes was observed as multiple green puncta within the cytoplasm (Fig. 1(A) at 0h). Whereas, the degradation was indicated by decrease in the number of cytosolic green puncta with concomitant accumulation of free GFP inside vacuole (Fig. 1(A) at 6h and Fig. 1(B)). In the autophagy mutant, *atg1*Δ, in which autophagy is blocked, vacuolar free GFP was not observed (Fig. 1(C)). Similarly, another autophagy mutant, *atg15*Δ, in which vacuolar lipase is impaired, the cargo (peroxisomes) was not degraded after its vacuolar delivery. This was observed as increased number of puncta inside the vacuole (Fig. 1(D)).

Monitoring the clearance of mis-folded protein aggregates in yeast

Another selective form of autophagy studied here is aggrephagy, which involves degradation of toxic, mis-folded proteins like α-synuclein and β-amyloid to maintain proteostasis.[20] In our lab, we study the modulation of aggrephagy using small molecules in yeast model. One such protein

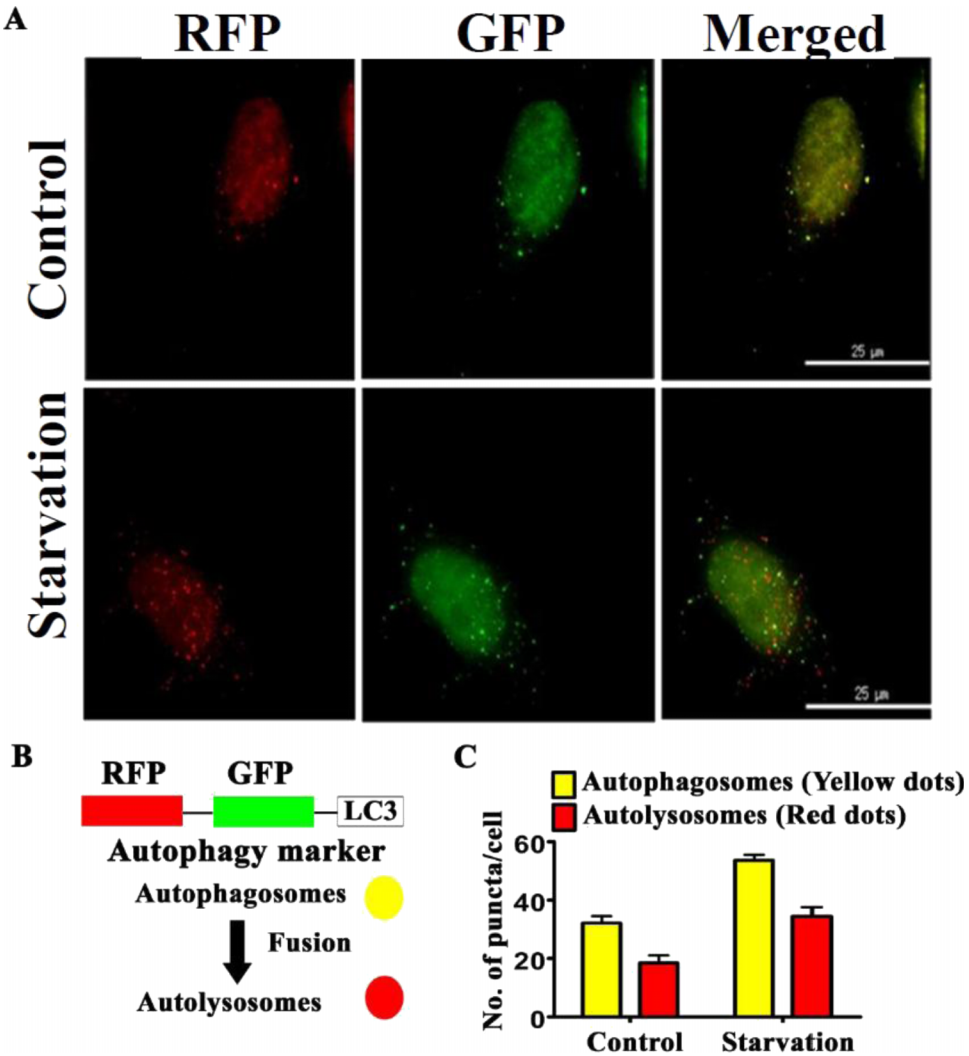

FIG. 3. A: The microscopy images of HeLa cells showed more number of yellow (autophagosomes) and red puncta (autolysosomes) after 2 hours of starvation, an indication of active autophagy. Scale bar is 25μm. B: The autophagosome marker protein, LC3 was tagged with RFP and GFP in tandem because of which autophagosomes appeared yellow whereas autolysosomes appeared red. C: The autophagy induction was confirmed by quantification. Increase in the number of autophagosomes (yellow) and autolysosomes (red) was observed. n=20 cells. The error bars indicate SEM. The experiment was done three times and the representative images are shown here.

is α-synuclein which is intrinsically disordered and involved in Parkinson's disease pathogenesis.[5] Upon overexpression of GFP-tagged α-synuclein, its plasma membrane localization and large clumps of cytosolic aggregates were observed (Fig. 2 WT). In an autophagy mutant (*atg1*Δ), the cytosolic aggregate formation was more prominent than wild-type (Fig. 2 *atg1*Δ) indicating the importance of autophagy. Utilizing α-synuclein overexpression model along with fluorescence based high content imaging we can decipher molecular players and novel drug targets involved in this process.

Monitoring autophagic flux in mammalian cells

Similar to yeast, autophagy process can be monitored in mammalian cells.[2,21] Indeed autophagy perturbation has been implicated in several human pathological conditions and its modulation using pharmacological agents can be of therapeutic importance.[22] Here we discussed a fluorescence microscopy based method, known as Traffic Light Assay, to study the autophagic flux, in mammalian cells.[23] In this method, LC3, which is an autophagosomal membrane protein, is tagged

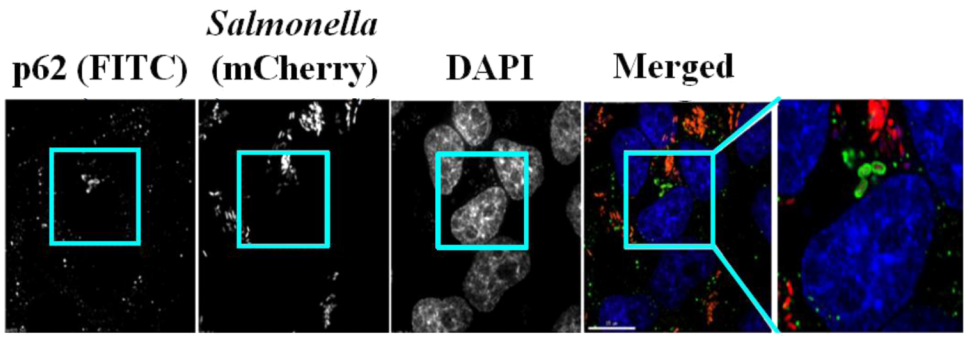

FIG. 4. The microscopy image shows autophagy protein (p62, FITC) interacts with bacteria (*Salmonella typhimurium*) tagged with mCherry (shown as magnified image), while the HeLa cell nuclei and the bacterial DNA are stained with DAPI (blue). The scale bar is 15μm. The experiment was done three times and the representative images are shown here.

with GFP and RFP in tandem (Fig. 3(B)). When autophagosome fuses with lysosome forming autolysosome, GFP signal gets quenched due to its sensitivity towards lysosomal pH (Fig. 3(B)). Hence autophagosomes are observed as yellow puncta whereas autolysosomes are red in color[9] (Fig. 3(A) and 3(B)). Counting the number of yellow and red puncta enables us to monitor the autophagic flux. Upon autophagy induction by starvation, we observed increase in total number of autophagosome and autolysosomes (Fig. 3(A) and 3(C)).

Monitoring xenophagy in mammalian cells

Autophagy is also involved in the selective clearance of pathogenic micro-organisms like bacteria and viruses termed as xenophagy.[24] Xenophagy can be monitored by tagging the protein specific to micro-organisms as well as host. Here we discuss a method for clearing the pathogenic bacteria *S. typhimurium* in HeLa cells. One of the autophagy adaptor proteins, p62, which is known to be involved in xenophagy[18,21] was immunostained whereas *S. typhimurium* was tagged with mCherry protein (Fig. 4). We observed *S. typhimurium* decorated with p62 indicating its target for autophagic degradation[25,26] (Fig. 4). This result clearly indicates the active involvement of autophagy in clearing a foreign pathogen from cells to maintain its health.

CONCLUSION

Based on assays described here, it is evident that, fluorescence microscopy contributes significantly in understanding different forms of autophagy (pexophagy in yeast and general autophagy in mammalian cells) across two different model organism. It is also instrumental in illuminating disease related processes (like protein aggregate formation and xenophagy) in mammalian cells. Hence, fluorescence microscopy is an indispensable tool which, aided with recent advancements, will take the autophagy research to new heights.

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