Application Note

LentiBrite™ Lentiviral Biosensors for Fluorescent Cellular Imaging: Analysis of Autophagosome Formation

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Abstract

Expression of genetically-encoded fluorescently-tagged proteins has widely been employed for real-time visualization of cellular behavior and trafficking. Prepackaged, ready-to-use, high-titer lentiviral particles (which we have termed "lentiviral biosensors") encoding GFP- or RFP-tagged proteins are a convenient, robust solution for fluorescent imaging of transduced cells. Compared to other nonviral transfection methods, lentiviral transduction, in many cases, offers higher transfection efficiency and more homogeneous protein expression, particularly for traditionally hard-to-transfect primary cell types. Lentiviral biosensors are ideal for use with fixed and live cell fluorescent microscopy, and are non-disruptive towards cellular function. GFP- or RFP-protein localization matches well with antibody-based immunostaining and demonstrates altered patterns of expression upon treatment with modulators of cell function and phenotype. Lentiviral biosensors provide a broadly effective, convenient method for visualization of cell behavior under a variety of physiological and pathological treatment conditions, in both endpoint and real-time imaging modalities. In this study, we focus on lentiviral biosensors containing GFP-LC3 and RFP-LC3 for the study of autophagosome formation.

Introduction

Analysis of the dynamics of subcellular structures has been revolutionized in the past 15 years by the development and refinement of genetically-encoded fusions between fluorescent proteins and cellular structural proteins. Such fusion proteins, if designed properly, incorporate into the structure of interest without disturbing its function, and permit visualization of the structure in live cells and in real time by fluorescence microscopy[1]. The cDNAs encoding the fusion proteins have traditionally been delivered into cells by chemical transfection or electroporation. However, such transient transfection procedures have drawbacks, including highly variable expression levels and low efficiencies for transfecting primary cells. Selection of clonal cell lines stably expressing the construct of interest allows for optimized expression levels, but the process is time-consuming and is not feasible for primary cells.

Fortunately, recently developed viral gene delivery vectors, such as lentiviral and adenoviral vectors, permit transduction of virtually any cell type, at more tightly controlled expression levels. Although viral vectors have been utilized successfully for expressing genetically encoded subcellular markers, prepackaged viral vectors have not been widely available, and researchers have had to perform packaging procedures themselves in order to perform subcellular marker analyses.

The LentiBrite™ product family of prepackaged lentiviral particles encode fluorescent fusion proteins with subcellular markers for cell fate, cytoskeletal structures and adhesion. The lentivirus particles are packaged with 3rd generation lentiviral packaging plasmids, which produce pseudoviral particles that have vanishingly low probabilities of pathogenicity[2]. The fluorescent proteins employed are TagGFP2 and TagRFP, which have been demonstrated to be monomeric for minimal interference with the function of the fusion partner proteins and have quantum yields comparable to fluorescent proteins from other species[3,4].

In this article, we highlight biosensors for visualizing autophagy, in which TagGFP2 and TagRFP are fused at their C-termini to the autophagosome marker LC3. LC3 precursors, diffusely distributed in the cytosol, are proteolytically processed to form LC3-I. Upon initiation of autophagy, the C-terminal glycine is modified by addition of a phosphatidylethanolamine to form LC3-II, which translocates rapidly to nascent autophagosomes in a punctate distribution[5]. DNA constructs encoding fluorescent proteins fused to LC3 are widely employed for introduction into cells for monitoring autophagosome formation by fluorescence microscopy. Merck Millipore's LentiBrite™ GFP-LC3, GFP-LC3 Control Mutant, and RFP-LC3 lentiviral particles provide bright fluorescence and precise localization of LC3 to the autophagosome, enabling live cell analysis of autophagy even in difficult-to-transfect cell types.

Materials and Methods

Construction of lentiviral vectors encoding fluorescent protein fusions

LentiBrite™ GFP-LC3, RFP-LC3, and GFP-LC3 Control Mutant Lentiviral Biosensors were constructed as follows: the cDNAs encoding TagGFP2 and TagRFP were obtained (Evrogen) and the cDNA encoding human LC3A residues 1-120, which represents the proteolytically processed, mature form of LC3A, was cloned in-frame at the 3' end of the fluorescent protein cDNA. The resulting fusion proteins, TagGFP2-LC3 and TagRFP-LC3, leave the C-terminal glycine (Gly120) of LC3 available for lipidation upon induction of autophagy. To generate a control mutant that does not translocate upon induction of autophagy, site-directed mutagenesis was employed to mutate LC3 Gly120 to alanine, which renders the protein refractory to lipidation. Constructs were transferred to pCDH-EF1-MCS (System Biosciences Inc.), a lentiviral vector containing the constitutive, moderately expressing EF1α promoter. 3rd generation HIV-based VSV-G pseudotyped lentiviral particles were generated using the pPACKH1 Lentivector Packaging System from System Biosciences.

Cell Seeding and Lentiviral Transduction

Cells in growth medium were seeded onto 8-well glass chamber slides for fixed cell imaging, or chambered cover glasses for live cell imaging. Seeding densities were selected to provide for 50-70% confluency after overnight culture (e.g., 20,000-40,000 cells/cm^2). The next day after seeding, medium was replaced with fresh growth medium. High-titer lentiviral stock was diluted 1:40 with growth medium, and lentiviral volume was added to the seeded cells for the desired multiplicity of infection (MOI). MOI refers to the ratio of the number of infectious lentiviral particles to the number of cells being infected. Typical MOI values ranged from 10 to 40. Infected cells were then incubated at 37 °C, 5% CO_2 for 24 hours. 24 hours after lentiviral transduction, lentivirus-containing medium was removed and replaced with fresh growth medium. All lentivirus-containing medium and plasticware in direct viral contact were disinfected with 10% bleach before disposal. Cells were cultured for another 24-48 hours, changing medium every 24 hours. For autophagy experiments, cells were either left in growth medium or incubated in Earle's balanced salt solution (EBSS) containing a lysosomal inhibitor for 2-4 hours. In some cases, 5 mM 3-methyladenine (3-MA) was included to inhibit autophagosome formation.

Live Cell Imaging

For live cell visualization, the chambered cover glass was placed in a temperature-controlled microscope stage insert. Imaging was initiated as rapidly as possible following addition of modulator. LC3-expressing cells were imaged in EBSS containing a lysosomal inhibitor. Live cell imaging was performed upon a Leica DMI6000B inverted wide-field fluorescent microscope with a 63X oil-immersion objective lens and illumination/filters appropriate for GFP or RFP visualization.

Cell Fixation, Staining and Imaging

Cells were fixed for 30 minutes at room temperature with 3.7% formaldehyde in Dulbecco's phosphate-buffered saline (DPBS). During fixation and for all subsequent steps, cells were protected from light to minimize photobleaching. Samples were then rinsed twice with fluorescent staining buffer (DPBS with 2% blocking serum and 0.25% Triton X-100). For immunocolocalization studies, primary antibody in fluorescent staining buffer was added to each well for 1 hour incubation at room temperature. Samples were then rinsed three times with fluorescent staining buffer before proceeding on to 1 hour room temperature incubation with fluorescent secondary antibody and DAPI (1 µg/mL) in staining buffer. Finally, samples were rinsed twice with fluorescent staining buffer and DPBS, and slides were coverslipped with mounting media containing anti-fade reagent and No. 0 cover glasses (Ted Pella). Mounted specimens were imaged on inverted wide-field (as above) or Leica DMI4000B confocal fluorescent microscopes, utilizing illumination and filters appropriate for GFP, RFP, Cy5 (for immunocolocalization), or DAPI excitation and emission wavelengths. Imaging was performed with a 63X oil-immersion objective lens unless otherwise indicated.

Analysis of GFP-LC3 localization by flow cytometry

The LentiBrite™ Autophagosome Enrichment Kit was employed for analysis of autophagosome formation in primary cells. Human umbilical vein endothelial cells (HUVEC) were incubated with lentivirus encoding TagGFP2-LC3 or TagGFP2-LC3^{G120A} (control mutant) at an MOI of 40 for 24 hrs. After removal of the lentivirus, the cells were cultured for an additional 48 hrs. Cells were either left in complete growth medium or incubated in EBSS containing a lysosomal inhibitor, and were subsequently detached with Accutase™ and permeabilized. U2OS cells stably expressing TagGFP2-LC3 (FlowCellect™ GFP-LC3 Reporter Autophagy Assay Kit (U2OS), were treated in parallel as a positive control. Samples were analyzed immediately on a guava easyCyte™ 8HT flow cytometer. Data were analyzed with the InCyte™ Software Module.

Results and Discussion

Plasmid transfection vs. lentivirus transduction

Using LentiBrite™ lentiviral biosensors, we demonstrated the improved efficiency of gene delivery and homogeneity of gene expression achieved by lentiviral transduction. In Figure 1, easily transfectable HeLa cells were transfected with GFP-labeled tubulin via plasmid (with a chemical transfection reagent) or via lentivirus. Lentivirally transduced cells demonstrated higher transfection efficiency (percentage of cells positive for signal, compared to total number of cells) compared to chemically transfected cells, as well as more homogeneous expression (compared to the range of high and low expressers in the plasmid-transfected population). For a typically "hard-to-transfect" primary cell type such as human umbilical vein endothelial cells (HUVEC), lentiviral transduction produced homogeneously bright signal in a significant proportion of cells, in contrast to plasmid transfection, which resulted in minimal GFP-tubulin expression.

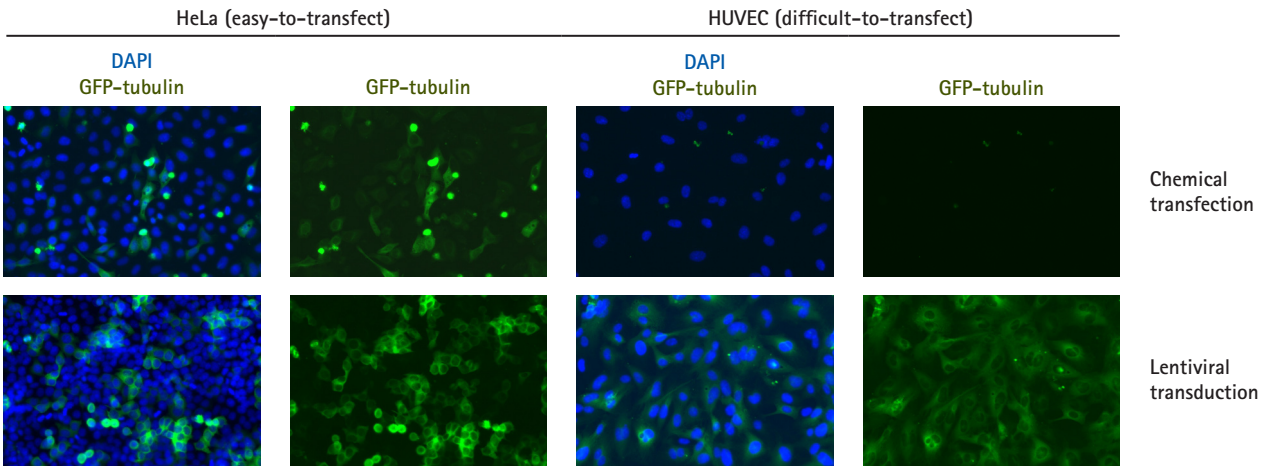

Figure 1. Plasmid vs. lentivirus transfection in easy- and hard-to-transfect cell types. HeLa cells and HUVECs were transfected with a TagGFP2-tubulin-encoding construct, either utilizing plasmid DNA in conjunction with a lipid-based chemical transfection reagent, or using LentiBrite™ lentiviral particles. Images were obtained via wide-field fluorescent imaging with a 20X objective lens (blue = DAPI nuclear counterstain, green = GFP-tubulin). Lentiviral transduction resulted in higher transfection efficiency (particularly for HUVEC, for which plasmid transfection was unsuccessful) and GFP-tubulin signal of more uniform fluorescence intensity.

Specificity of localization of lentivirally delivered GFP-LC3

Genetically-encoded biosensors for studying autophagy have become a widely employed technique since the first description of the use of GFP-tagged LC3 to detect autophagosome formation[5]. Although immunofluorescent detection of endogenous LC3 can be performed, genetically encoded biosensors achieve greater sensitivity for detecting changes in autophagosome formation. However, the use of transient transfection of plasmid DNA for expression of GFP-LC3 has been criticized for causing artifactual, autophagy-independent punctae, and the preferred approach is to use cell lines stably expressing the GFP-LC3 construct[6]. To determine whether lentiviral delivery of DNA encoding fluorescent proteins fused to LC3 avoids such artifacts, we produced lentivirus encoding TagGFP2-LC3 for transduction into a broad variety of cell types. We found that lentiviral delivery of fluorescent protein-tagged LC3 allowed for accurate detection of autophagosome formation, as determined by 1) immunofluorescent co-localization of LC3, 2) live cell imaging of autophagosome formation, 3) use of a mutant version of LC3 that is resistant to lipidation and fails to localize to autophagosomes, and 4) use of autophagy inhibitor.

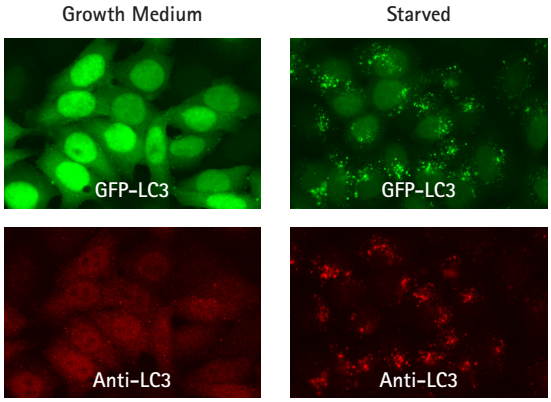

Figure 2. GFP-LC3 fluorescent signal (green) colocalizes with signal from fluorescent staining using LC3 antibody (red). HeLa cells were-transduced with TagGFP2-LC3, and 72 hrs later, either left in growth medium or starved for 4 hours in EBSS with a lysosomal inhibitor. Cells were subsequently fixed, immunostained, and imaged by wide-field microscopy. Starved, autophagic cells displayed punctate cytoplasmic LC3 distribution, in contrast to diffuse nuclear and cytoplasmic localization under fed conditions. Fluorescently tagged protein co-localized with staining obtained with anti-LC3.

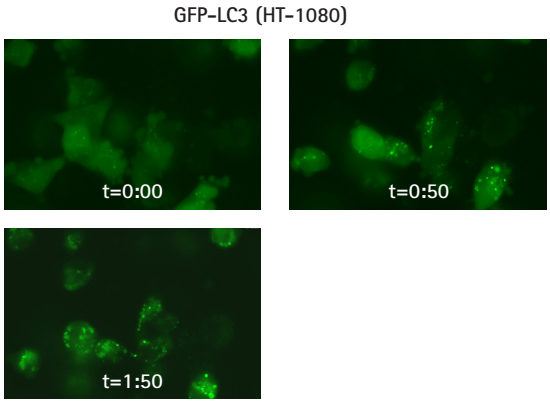

Figure 3. Live cell time-lapse imaging of lentivirally-transduced cells. HT-1080 cells were lentivirally transduced with TagGFP2-LC3, and imaged by oil-immersion wide-field microscopy in real-time. The cells were starved in the presence of a lysosome inhibitor, and imaging was immediately initiated, with images obtained every minute for 2 hours. Still-frame captures demonstrate formation of GFP-LC3-positive discrete cytoplasmic punctae.

To compare localization patterns of genetically-encoded fluorescent proteins with antibody-based immunofluorescence, HeLa cells were lentivirally transduced with GFP-LC3. GFP-LC3-expressing cells were either left untreated, or subjected to starvation conditions to induce autophagy by incubation in EBSS. A lysosomal inhibitor was also included to prevent degradation of LC3-containing autophagosomes. Both the fluorescent protein and anti-LC3 antibody displayed diffuse nuclear and cytoplasmic signal under fed conditions and a punctate distribution following starvation (Figure 2).

Next, we analyzed time-dependent LC3 translocation following autophagic induction in live cells. By wide-field microscopy, lentivirally-transduced cells were imaged every minute over the course of approximately 2 hours following treatment. Full length video is available at www.millipore.com/autophagyvideo. Autophagy was induced in GFP-LC3-transduced HT-1080 cells via EBSS/lysosomal inhibitor starvation, resulting in accumulation of punctate LC3 in newly formed autophagosomes. As shown in Figure 3, significant formation of autophagosomes was visible at 50 minutes. At 110 minutes, nearly all of the visible GFP signal was localized to autophagosomes.

For an additional assessment of the specificity of the GFP-LC3 biosensor, we employed two controls: a mutant LC3 that is resistant to lipidation, and an autophagy inhibitor. In Figure 4, cells were lentivirally transduced with TagGFP2-LC3 or a TagGFP2-LC3 non-translocating control mutant. Transduced cells were starved in EBSS with a lysosomal inhibitor, in the presence or absence of 3-methyladenine, an inhibitor of PI3 kinase that blocks autophagosome formation. The mutant LC3 fusion protein did not translocate to a punctate cytoplasmic distribution upon starvation. Also, when starved in the presence of 3-methyladenine, both the wild-type and mutant LC3 fusion proteins displayed a diffuse distribution throughout the nucleus and cytoplasm, as typical of fed cells.

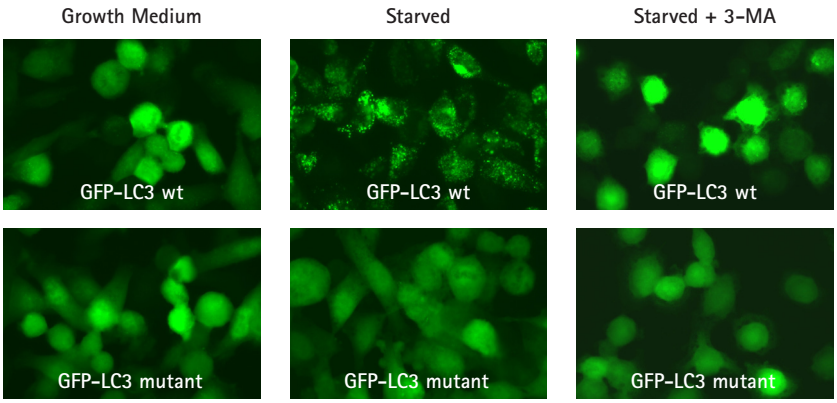

Figure 4. Autophagy inhibitor 3-MA prevents translocation of GFP-LC3. HT-1080 cells were lentivirally-transduced with TagGFP2-LC3 wild-type (GFP-LC3 wt) or TagGFP2-LC3^{G120A} (GFP-LC3 mutant) at MOI of 20. Transduced cells were either left in growth medium, or starved in EBSS with lysosome inhibitor in the presence or absence of 3-methyladenine (3-MA). Cells were fixed, mounted, and imaged by wide-field fluorescence microscopy. Cells transduced with wild-type GFP-LC3 no longer exhibited cytoplasmic punctae under starvation conditions in the presence of 3-methyladenine. In addition, cells transduced with a negative control mutant GFP-LC3 maintained diffuse nuclear and cytosolic distribution under all conditions.

Analysis of autophagy in difficult-to-transfect cell types by imaging and flow cytometry

Primary cell types, including HUVEC and human mesenchymal stem cells (HuMSC) are traditionally considered difficult-to-transfect cell types for plasmid DNA-based chemical transfection. LentiBrite™ lentiviral transduction is shown to be capable of inducing fluorescent protein expression in these cell types in Figure 5. Both HUVEC and HuMSC were successfully transduced at high efficiency with TagGFP2-LC3 and TagRFP-LC3. As seen with the lentivirally transduced HT-1080 cell line in the previous figures, the fluorescent protein fusions in the primary cells expressed diffusely when cultured in growth medium, and adopted a punctate distribution following starvation in the presence of a lysosome inhibitor.

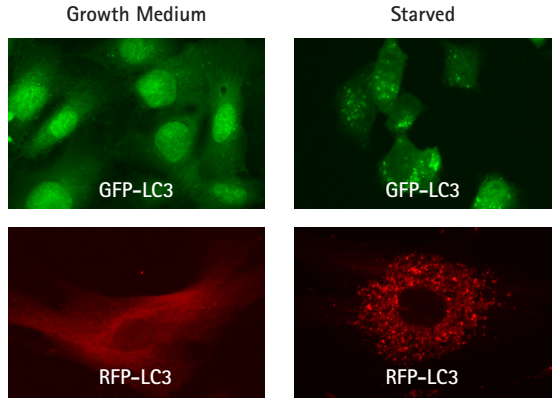

Figure 5. Lentiviral transduction enables analysis of autophagy in hard-to-transfect primary cell types. HUVEC and HuMSC were lentivirally transduced at an MOI of 40 with TagGFP2-LC3 or TagRFP-LC3, and fed or starved as in Figure 2. Cells were then fixed and imaged by wide-field microscopy. The transduced fluorescent proteins displayed diffuse distribution in growth media and a punctate distribution following starvation-induced autophagy.

To more accurately assess the extent of LC3 reporter redistribution in primary cells, we employed a flow cytometry assay in which the plasma membrane is selectively permeabilized such that free cytosolic fluorescent protein-tagged LC3 is released while autophagosome-bound LC3 fusion protein is retained. HUVECs were lentivirally transduced with TagGFP2-LC3 or TagGFP2-LC3^{G120A} (control mutant). The cells were subsequently starved of amino acids in the presence of a lysosome inhibitor or left untreated, then detached and either permeabilized (using the LentiBrite™ Autophagosome Enrichment Kit) or left intact.

Upon permeabilization, the GFP-LC3 in starved cells was almost completely retained, but was greatly depleted in fed cells (Figure 6). This result was similar to the pattern observed in U2OS cells stably expressing TagGFP2-LC3 (FlowCellect™ GFP-LC3 Reporter Autophagy Assay Kit (U2OS)). In contrast, permeabilization caused a large reduction in TagGFP2-LC3^{G120A} in both starved and fed cells. In U2OS cells transiently transfected with plasmid encoding TagGFP2-LC3, a very broad distribution of fluorescence was observed, and the shift upon permeabilization of fed cells was much less pronounced (data not shown).

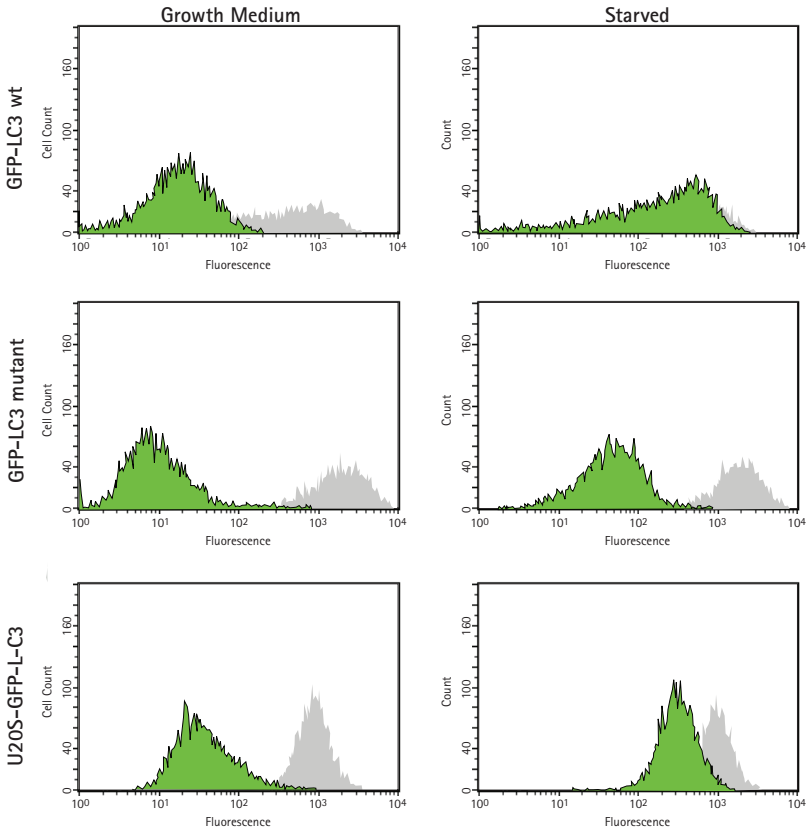

Figure 6. Analysis of GFP-LC3 localization in HUVEC by flow cytometry. HUVECs were lentivirally transduced with TagGFP2-LC3 wild-type (GFP-LC3 wt, top row) or TagGFP2-LC3^{G120A} control mutant (GFP-LC3 mutant, center row). U2OS cells stably expressing TagGFP2-LC3 wild-type were also analyzed (U2OS-GFP-LC3, bottom row). Transduced cells were detached and either permeabilized to release free, cytosolic LC3 (green peaks) or left intact (gray peaks). After processing, the cells were analyzed by flow cytometry on a guava easyCyte™ 8HT instrument. Upon permeabilization, only TagGFP2-LC3 wild-type-expressing cells under starvation conditions display retention of the fusion protein, indicative of tight association of LC3 with autophagosomes.

Conclusions

LentiBrite™ lentiviral biosensors enable convenient transduction of easy- and hard-to-transfect cell types with fluorescently-tagged proteins of interest. These pre-packaged lentiviral particles provided higher efficiency of gene delivery and more homogeneous expression of introduced proteins compared to non-viral transfection methods. The data presented here demonstrates the utility of a GFP- or RFP-tagged autophagy marker, LC3, in both fixed and live cell microscopy applications. The encoded proteins displayed co-localization with antibody staining and appropriate redistribution upon treatment with known modulators of autophagy. Other available constructs in the LentiBrite™ portfolio target foundational proteins associated with apoptosis, cell structure and adhesion: calreticulin, α-tubulin, β-actin, vimentin, α-actinin and paxillin. LentiBrite™ biosensors provide a ready-to-use solution for researchers seeking to fluorescently visualize the presence/absence or trafficking of a protein, under normal, abnormal, diseased, or induced cellular states.

References

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