Establishment and Application of a Dual-Fluorescent Multifunctional Autophagic Flux Monitoring System Based on a Lentiviral System: Postprint

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Abstract

Objective To construct a red fluorescent protein-green fluorescent protein-rat LC3 fusion lentiviral multifunctional expression vector (PCDH-Duo-mRFP-eGFPph-LC3rat, PCDH-Duo) capable of stably and dynamically monitoring autophagic flux changes and overexpressing genes, and to establish a stably transfected mouse peritoneal macrophage Raw264.7 cell line for observing autophagic flux changes. Methods The mRFP-eGFPph-LC3rat fusion full-length gene was accurately synthesized by PCR and cloned into the lentiviral expression vector PCDH-CMV-MCS-EF1a-GFP. The recombinant plasmid was verified by colony PCR, restriction enzyme digestion, and sequencing. Lentivirus was then packaged and used to transfect Raw264.7 cells. Stable transfectants were obtained by fluorescence-activated cell sorting, and their reliability was confirmed through a chloroquine autophagy inhibition model and Western blotting analysis of eGFP protein expression. Results The PCDH-Duo recombinant lentiviral plasmid was successfully constructed, lentivirus was packaged, and a Raw264.7 stable cell line (Raw264.7-PCDH-Duo) was obtained that could stably express dual fluorescent proteins. After treatment with 3 mM chloroquine for 6 h, the system could stably and accurately indicate autophagic flux changes. Conclusion A dual-fluorescent labeled multifunctional autophagic flux monitoring system based on the lentiviral system was successfully constructed, providing a convenient and powerful tool for studying the relationship between autophagy and both coding and non-coding genes.

Full Text

Establishment and Application of a Dual-Fluorescent Labeling Multifunctional Autophagy Flux Monitoring System Based on Lentiviral System

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Abstract

Objective: To construct a red fluorescent protein-green fluorescent protein-murine LC3 fusion lentiviral multifunctional expression vector (PCDH-Duo-mRFP-eGFPph-LC3rat, abbreviated as PCDH-Duo) capable of stably and dynamically monitoring autophagy flux changes and overexpressing exogenous genes, and to establish a stable Raw264.7 mouse peritoneal macrophage cell line for observing autophagy flux dynamics.

Methods: The mRFP-eGFPph-LC3rat fusion gene was precisely synthesized using PCR-based accurate synthesis (PAS) and cloned into the lentiviral expression vector PCDH-CMV-MCS-EF1a-GFP. The recombinant plasmid was validated through colony PCR, restriction enzyme digestion, and sequencing analysis. Lentivirus was then packaged and used to transduce Raw264.7 cells, with stable transductants obtained via fluorescence-activated cell sorting (FACS). The system' s reliability was confirmed using a chloroquine-mediated autophagy inhibition model and Western blotting for eGFP protein expression.

Results: The PCDH-Duo recombinant lentiviral plasmid was successfully constructed, and lentivirus was packaged to obtain a stable Raw264.7 cell line (Raw264.7-PCDH-Duo) that stably expressed dual fluorescent proteins. Following treatment with 3 mM chloroquine for 6 hours, the system reliably and accurately indicated autophagy flux changes.

Conclusion: We successfully established a dual-fluorescent labeling multifunctional autophagy flux monitoring system based on lentiviral technology, providing a convenient and powerful tool for investigating the relationship between autophagy and both coding and non-coding genes.

Keywords: Autophagy flux; Vector construction; Lentivirus; mRFP-eGFP-LC3

Autophagy is a membrane-mediated metabolic process in eukaryotic cells whereby autophagosomes engulf macromolecular substances and deliver them to lysosomes, forming autolysosomes to degrade contents for recycling. Research has demonstrated that autophagy plays crucial roles in maintaining intracellular homeostasis, proliferation and differentiation, immunity, and detoxification of abnormally aggregated proteins [1]. As a rapid and continuous dynamic process comprising initiation, elongation, and fusion stages, autophagy cannot be fully characterized by single-point analyses [2]. Therefore, dynamic observation of autophagy flux is essential.

Current methods for detecting autophagy include transmission electron microscopy to observe autophagosome and autolysosome quantity and structure [3], immunofluorescence of endogenous LC3 puncta [4], exogenous GFP-LC3 puncta [5], colocalization analysis of endogenous LC3 with lysosomal membrane proteins (Lamp1/Lamp2) [6], and Western blot analysis of autophagy marker proteins including the substrate p62 and initiation complex components BECN1, ATG5, ATG16L1, etc. [7]. However, these approaches suffer from several limitations: First, they cannot monitor autophagy dynamics in real-time, and establishing stable cell lines is challenging for stem cells, primary cells, immune cells, and other difficult-to-transfect cell types, constraining related research. Second, these systems cannot reflect the sequential stages of autophagy or quantitatively analyze autophagy intensity. Third, they cannot achieve spatiotemporal synchronization of exogenous gene overexpression and autophagy detection, compromising experimental accuracy.

LC3, the mammalian homologue of yeast ATG8, is a widely used intracellular protein marker that undergoes ubiquitin-like post-translational modification [8]. The cytosolic LC3 precursor is first cleaved at its C-terminus by Atg4b to form soluble LC3-I (14 kDa), which is then modified by ATG7 and ATG3 in a ubiquitin-like manner. LC3-I is conjugated with phosphatidylethanolamine (PE) to form membrane-bound lipidated LC3-II (16 kDa) anchored on both inner and outer autophagosomal membranes, enabling punctate aggregation [9]. LC3-II serves as an excellent marker for monitoring autophagosome formation, and autophagy can be tracked using GFP- or RFP-tagged LC3 combined with live-cell imaging or laser confocal microscopy [10].

Dual-fluorescent autophagy tracing systems have been developed, primarily including mRFP-LC3-GFP and mRFP-EGFP-LC3 formats [11]. GFP fluorescence is sensitive to pH changes and readily quenched in the acidic lysosomal lumen, whereas red fluorescent proteins like mRFP remain relatively stable. This principle enables design of mRFP-GFP-LC3 fusion proteins to monitor the critical process of autophagosome-lysosome fusion and track autophagy flux. Colocalization of GFP and RFP signals (non-overlapping) indicates structures not yet fused with lysosomes, such as autophagosomes and phagophores. Conversely, mRFP signals without GFP colocalization represent structures that have fused with lysosomes, such as autolysosomes and amphisomes. Combined with appropriate image analysis algorithms, these systems enable accurate quantification of acidified cellular components [12].

In this study, we retrieved published mRFP, EGFP, and LC3 sequences, performed homology alignment, optimized EGFP base sequences for acid resistance [13], and synthesized the fusion gene using PAS. The construct was subcloned into the lentiviral expression vector PCDH-CMV-MCS-EF1a-GFP to create a dual-fluorescent lentiviral autophagy flux vector. Lentivirus was packaged and used to transduce Raw264.7 cells, with stable transductants established via FACS sorting. Western blot confirmed fusion protein expression, and a chloroquine-mediated autophagy inhibition model validated system effectiveness, laying the foundation for subsequent studies on gene and non-coding RNA regulation of autophagy.

1.1 Materials

The endotoxin-free plasmid extraction kit (DP-100) was purchased from Tiangen. The total protein extraction kit (KGP1050-KGP10100) was from KeyGen Biotech. GFP rabbit anti-mouse tag antibody (66002-1-Ig) was from Proteintech. FITC-conjugated goat anti-rabbit IgG was from Zhongshan Golden Bridge. Restriction enzymes Xba I and Sal I, ligation Mix, and PCR Mix were from Takara. DMEM high-glucose medium, phosphate-buffered saline (PBS), and fetal bovine serum (FBS) were from BI. Chloroquine (CQ) was from Sigma. Sodium pyruvate, GlutaMax, and Lip3000 were from Thermo Fisher. Yeast extract, glucose, NaCl, and PEG-8000 were from Sigma. Mouse peritoneal macrophage cell line RAW264.7, 293T cells, bacterial strains Top10 and Stbl3, lentiviral expression plasmid PCDH-CMV-MCS-EF1a-CopGFP, and helper packaging plasmids pLp.1, pLp.2, and VSV.G were preserved in our laboratory. Primary bioinformatics software included Tbtools, Vector NTI 11, Chromas, and FV1000-ASW. Major equipment included a fluorescence inverted microscope (Olympus IX71, Japan), laser confocal microscope (Olympus FV-1000, Japan), and FACS flow cytometer (BD Calibur, USA).

1.2 Methods

1.2.1 Design of mRFP-EGFP-LC3rat Lentiviral Expression Plasmid

Published mRFP and EGFP sequences were retrieved from PubMed, and murine LC3 sequence was obtained from Addgene. Multiple sequence alignment was performed to determine each gene’ s coding sequence (CDS) region. Following reference [13], 11 amino acid sites in EGFP were optimized and the multiple cloning site (MCS) was modified to introduce an AgeI restriction site. The fusion gene sequence arrangement was: 5’ -XbaI-MCS-mRFP-Linker1-EF1a-eGFP-Linker2-LC3rat-SalI-3’ , with a total length of 2559 bp encoding a protein of approximately 71 kDa.

1.2.2 PAS-Based Gene Synthesis, Restriction Digestion, Ligation, Transformation, and Sequencing Validation The designed sequence was submitted to Nanjing Zhongding Biotechnology for full-length gene synthesis using PCR-based Accurate Synthesis (PAS). The fragment was subcloned into PCDH-MCS-EF1a-CopGFP vector via Xba I and Sal I double digestion. Recombinant plasmids were transformed and screened, with positive clones identified by PCR and restriction analysis. Sanger sequencing confirmed the clone sequence matched the design exactly. The successfully constructed vector was named PCDH-Duo, and recombinant plasmid was extracted, purified, and stored at -20°C. Sequencing primers are listed in .

1.2.3 Lentivirus Packaging and Titer Determination High-purity expression plasmid PCDH-Duo and helper packaging plasmids pLp1, pLp2, and VSV.G were obtained using the Tiangen endotoxin-free plasmid maxiprep kit. After concentration measurement, plasmids were mixed at a mass ratio of pLp1:pLp2:VSV.G = 1:2:1 to prepare ViralMix. The expression plasmid and ViralMix were combined at equal proportions for packaging following the Lip3000 lentivirus production protocol. Virus was concentrated using the PEG-8000 method: filtered viral supernatant (0.45 m filter) was mixed with 7.5 mL of 5× PEG-8000-NaCl master solution per 30 mL of original supernatant, gently mixed every 20 minutes for 3-5 rounds, incubated overnight at 4°C, then centrifuged at 4,000g for 20 minutes at 4°C. The pellet was resuspended in PBS containing 5% FBS, aliquoted, and stored at -80°C. Viral titer was determined by limiting dilution assay [14].

1.2.4 Establishment of Dual-Fluorescent Raw264.7 Macrophage Stable Cell Line Based on the measured titer, appropriate MOI was calculated for cell infection. Infected Raw264.7 cells in 6-well plates were pooled, expanded to T-25 flasks, and grown to 90% confluence. After observing cell morphology and fluorescence density under fluorescence microscopy, cells were digested, completely resuspended in DMEM medium, filtered through 400-mesh sterile gauze to prepare cell suspension for flow sorting. The total volume of digested cell suspension did not exceed 2 mL. FACS was performed using DMEM medium as blank control with excitation wavelength set at 470 nm. Sorted cells were cultured in 6-well plates and expanded. Multiple rounds of FACS sorting were performed to complete stable cell line establishment.

1.2.5 Detection of EGFP Protein Expression in Stable Cell Line To confirm lentiviral integration of the fusion gene into the Raw264.7 genome, Raw264.7-PCDH-Duo cells were seeded in 6-well plates at 10 cells per well, with mock (non-transfected) group as control. After 24 hours of culture in complete DMEM medium, cell samples were collected, total protein was extracted and quantified by BCA assay, and Western blot was performed to detect mRFP-eGFP-LC3rat fusion protein expression.

1.2.6 Chloroquine-Mediated Autophagy Inhibition Model in Raw264.7 Cells To validate whether the cell line could correctly indicate autophagy progression through dual fluorescence and monitor autophagy flux, Raw264.7-PCDH-Duo cells were plated in 24-well plates at 5×10 cells per well. After 12 hours in complete medium, the experimental group (n=3) was treated with DMEM containing 3 mM CQ, while the control group received complete DMEM medium. After 6 hours of autophagy inhibition, cells were fixed with 4% paraformaldehyde and fluorescence aggregation was examined by laser confocal microscopy.

1.2.7 Statistical Analysis Data were analyzed using SPSS 21 software. Fluorescent puncta counts were calculated using ImageJ direct counting. Intergroup comparisons were performed using t-test or ANOVA based on data characteristics. $P<0.05$ was considered statistically significant.

2.1 Design of mRFP-EGFP-LC3rat Lentiviral Plasmid

The recombinant lentiviral vector map is shown in [Figure 1: see original paper]. The total vector length is 8692 bp, with an AgeI restriction site introduced in the MCS region and an XhoI site (at position 609) at the EF1a promoter terminus to facilitate replacement of the indicator element. The CMV promoter independently drives exogenous gene expression for full-length expression of miRNA precursors, lncRNAs, and coding genes, while EF1a independently drives the autophagy flux monitoring system. Homology modeling confirmed correct expression of all elements.

2.2 PAS-Based Gene Synthesis, Colony PCR Identification, Restriction Digestion, and Sequencing Validation

PAS synthesis yielded a 2559 bp fusion gene fragment. Colony PCR identified clones #1, #2, and #4 as positive. Plasmids extracted from expanded cultures were digested with SpeI and SalI, producing correctly sized fragments and backbone. Sanger sequencing confirmed the clone sequence matched the design exactly, enabling downstream virus packaging experiments.

2.3 Lentivirus Packaging and Titer Determination

At 48 hours post-packaging, fluorescence microscopy revealed approximately 70% double-fluorescence positivity in 293T cells ([Figure 3: see original paper]). After 72 hours, supernatants were pooled, filtered, and concentrated using the PEG-8000 method. Limiting dilution assay determined a viral titer of 2×10 TU/mL, suitable for downstream experiments.

2.4 Establishment of Dual-Fluorescent Raw264.7 Macrophage Stable Cell Line

Following flow sorting sample preparation requirements, cell suspensions were prepared and subjected to fluorescence-activated sorting. After culture and multiple rounds of sorting, a stable cell line was obtained. Fluorescence examination ([Figure 4: see original paper]) showed that 72 hours post-infection at MOI=10, approximately 60% of Raw264.7 cells expressed both fluorescent proteins, with transduction efficiency significantly higher than conventional methods (~15%). FACS results ([Figure 5: see original paper]) demonstrated ~55% positivity at 470 nm excitation. The sorted positive cells were designated Raw264.7-PCDH-Duo.

2.5 Detection of EGFP Protein Expression

Western blot results demonstrated mRFP-EGFP-LC3 fusion protein expression in Raw264.7-PCDH-Duo cells, while the negative control group showed no expression ([Figure 6: see original paper]), confirming successful stable integration of the fusion gene into the Raw264.7 genome.

2.6 Validation of Chloroquine-Mediated Autophagy Inhibition Model

In control cells where autophagy was not inhibited, red and green fluorescent proteins were diffusely distributed in the cytoplasm with only minimal fluorescent puncta due to basal autophagy. In contrast, CQ treatment in the experimental group inhibited lysosome-autophagosome fusion, causing fluorescent puncta accumulation (increased red puncta). Since autophagosomes failed to fuse with lysosomes to form autolysosomes, the absence of an acidic environment resulted in mRFP and EGFP colocalization, producing numerous yellow fluorescent puncta in the merged image ([Figure 7A: see original paper]). The number of fluorescent puncta was statistically significant compared to the control group ([Figure 7B: see original paper], $P<0.05$). Western blot detection of LC3-I/II conversion indicated LC3-II maturation, and CQ significantly increased LC3 expression levels, demonstrating that the Raw264.7 stable cell line effectively indicates autophagy status at the live-cell level.

Discussion

Autophagy comprises four stages and is a rapid, dynamic process. Consequently, autophagy detection techniques should be categorized into static and dynamic approaches. Static techniques depend on autophagy induction and measure outcomes, including transmission electron microscopy, immunoblotting for ATG5/ATG12/BECN1/P62/LC3, LC3 immunofluorescence, TOR/ATG1 kinase activity assays, transcriptional mRNA detection (Northern blot or RT-qPCR), and monodansylcadaverine (MDC) staining. Notably, antibodies for LC3 Western blot are critical, as some have limited sensitivity for LC3-I, potentially yielding false results where LC3-I appears significantly lower than LC3-II; therefore, multi-indicator composite detection is recommended.

Dynamic techniques are more suitable for observing autophagy progression and include the p62 luciferase reporter system [15], fluorescent small molecule tracers [16], radiolabeled amino acid incorporation assays [17], LC3-II turnover analysis [18], GFP-LC3 lysosomal transport and degradation analysis [19], autophagosome-organelle colocalization analysis (including Lamp2-labeled lysosomes [20], LysoTracker [21], pDsRed2-mito [22], JC-1 probe [23]), multispectral imaging flow cytometry measuring LC3, p62, and LAMP1 colocalization [24], and combined green-red fluorescent protein laser confocal [25] or live-cell imaging analysis [26]. Specifically, in the RFP-GFP-LC3 dual-fluorescent system, RFP is more stable than GFP in the acidic and/or proteolytic lysosomal environment. Colocalization of GFP and RFP signals indicates components not yet fused with lysosomes (e.g., autophagosomes, phagophores), while mRFP signals without GFP indicate fused structures (e.g., autolysosomes). Combined with a third organelle marker like Lamp2, these systems enable comprehensive autophagy process detection and quantification (fluorescent puncta), though optimization of GFP acid sensitivity, background autophagy levels, and automated analysis remains challenging [27].

Primary and immune cells are difficult to transfect, significantly impacting experimental reproducibility. Commercial adenoviral and baculoviral systems carrying dual-fluorescent markers are available but cannot establish stable cell lines. mRFP-EGFP-LC3 stable cell lines can continuously express marker molecules, effectively overcoming limitations of transient transfection including low efficiency, long cycles, cumbersome operation, and instability, thereby improving research result reliability.

This study leveraged lentiviral technology advantages to construct a dual-fluorescent labeling system and Raw264.7 stable cell line that incorporates exogenous gene overexpression, enabling simultaneous overexpression and autophagy monitoring in a single transduction event. This eliminates spatiotemporal asynchrony and variable factors from multiple transfections, enhancing experimental accuracy and reliability for autophagy research. Autophagy flux smoothness is critical for stable cellular physiological function. The mRFP-GFP-LC3 dual-fluorescent lentiviral autophagy indicator system, combined with live-cell imaging and laser confocal microscopy, enables dynamic real-time monitoring of autophagy flux in living cells. Future adaptation to luciferase-based reporter systems may enable high-throughput screening of autophagy-modulating genes, ncRNAs, and small molecules through exogenous gene overexpression (lncRNA), siRNA, or mimics, advancing autophagy research.

In summary, we successfully established a lentivirus-based dual-fluorescent labeling multifunctional autophagy flux research system that simultaneously enables exogenous gene expression and autophagy flux monitoring, facilitating convenient establishment of Raw264.7 stable cell lines. This represents an optimization of existing RFP-GFP-LC3 systems and Raw264.7 stable cell lines [28], providing an improved technical platform for high-throughput autophagy flux detection.

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Note: Figure translations are in progress. See original paper for figures.