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GRADUATE RESEARCH

An In-Gel Fluorescence Technique to Study Autophagy in Human Embryonic Kidney 293 Cells

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ABSTRACT

Autophagy is a recycling pathway used by cells to maintain homeostasis during times of stress. Common methods for the analysis of autophagy include western blotting and fluorescent imaging, but these methods are time consuming, expensive, and complex, making them inherently difficult for research laboratories at primarily undergraduate research institutions. We propose an in-gel fluorescence method for the analysis of autophagy in cells transfected with a dual-reporter plasmid. We verify that this method allows users to detect autophagic stimulation and is a cost effective, less complex method that sidesteps the challenges inherent with existing methods.

Keywords: autophagy, in-gel fluorescence, LC3I/II

1 Introduction

Macroautophagy is a conserved catabolic process that is essential for maintenance of cellular homeostasis. During autophagy, cells break down materials such as protein complexes, aggregates, and organelles to provide energy, recycle metabolic building blocks, and to ameliorate damage due to reactive oxidant species or toxic protein aggregates [1]-[4]. Cells are capable of performing three types of autophagy: macroautophagy, chaperone-mediated autophagy, and microautophagy. During macroautophagy (referred to simply as autophagy in this study), double membraned vesicles called autophagosomes engulf structures that have been marked by the cell for destruction. The autophagosomes then fuse with lysosomes, and the low pH of the lysosomal lumen degrades the contents of the autophagolysosome [3].

One common approach to track autophagic activity in cells involves monitoring the expression of an autophagy marker protein called LC3. When autophagic activity is low, LC3 is distributed throughout the cytosol as LC3-I. When autophagy begins, LC3-I is conjugated to a phosphatidylethanolamine and joins

the membrane of autophagosomes as LC3-II [3][5][6]. LC3-II serves as a docking site for cargo receptors in the cell that bring ubiquitinated targets to the autophagosome. Researchers can track this change in LC3 by using western blotting or fluorescent cell imaging of the LC3 protein [7]. Western blotting can be used to separate and identify the LC3-I and -II protein bands and semi-quantitative measurements of relative band intensity can be used to determine the ratio between LC3-II and LC3-I. A higher LC3-II ratio often indicates autophagic activity in a cell (although it should be noted that LC3 ratios and expected outcomes can vary by cell lines and the type of stressor applied) [7]. Alternatively, two types of fluorescent imaging can be used: immunocytochemistry using an anti-LC3 antibody [7] or transfection of cells with a plasmid encoding for LC3 conjugated to two fluorescent proteins, such as EGFP and mCherry [7]-[9]. Tracking of LC3 via immunocytochemistry or transfection relies on visualization of puncta (small intensely fluorescing regions in the cell where LC3 has been aggregated into autophagosomes) as an indicator of autophagic activation.

Western blotting and immunocytochemistry rely on endogenous LC3, which is of obvious benefit. Yet, these methods cannot directly track flux through the autophagy pathway, which means that they are prone to misinterpretation if the pathway is inhibited or blocked at a midpoint such as during autophagosome-lysosome fusion [7]. Transfection of cells with a plasmid containing LC3 fused to fluorescent probes can provide evidence of autophagic stimulation and is a reliable indicator of pathway inhibition [8][9]. This system relies on pH fluctuations at different stages of autophagy to distinguish between early versus late autophagy. In early autophagy, prior to lysosome fusion, the autophagosome is at near physiological pH and transfected exogenous LC3 containing the two fluorescent proteins, will be aggregated into puncta visible in both fluorescent spectra. Upon lysosome fusion, the autophagosome is subjected to low pH that ameliorates the fluorescence emitted by the EGFP. Tracking of puncta that fluoresce in both colors and subsequently in only one color, allows for tracking of cells through the entire autophagy pathway [8][9].

Although these methods are commonly used to study autophagy, they each have drawbacks for many labs. Western blotting is one of the most common methods of tracking autophagy. Yet, the method does not distinguish between autophagy activation and inhibition of autophagosome-lysosome fusion, which warrants use of comparative controls that demonstrate expected outcomes for each scenario [7]. Additionally, western blotting is time consuming, often requires troubleshooting, and LC3 western blotting can be highly technical in nature. These drawbacks can make western blotting prohibitive, particularly for labs at primarily undergraduate institutions where students have less continuity of time to commit to a multiday, prolonged process and where technical skills are still being refined and inevitable mistakes in the learning process can overwhelm small lab budgets. The cost of antibodies for western blotting and immunocytochemistry can also be prohibitive to these same labs. Immunocytochemistry, like western blotting, has limitations in distinguishing between autophagy activation and inhibition of autophagosome-lysosome fusion and can be subject to bias depending on the field of view imaged [7]. Semi- or quantitative studies are difficult as image analysis requires either manual counting of cells with puncta or software-programmed counting, both of which require user-defined intensity, size, and background parameters and demonstrate low reliability [7].

Fluorescent imaging of transfected cells with a dual reporter LC3 plasmid, on the other hand, can distinguish between autophagy activation and inhibition of autophagosome-lysosome fusion (through probing fluorescent intensity changes); however, some limitations of immunocytochemistry that are noted above remain. For example, semi- or quantitative comparisons are difficult to achieve and image analyses are particularly prone to user bias. Manual or programmed counting of fluorescent puncta is time consuming, prone to subjectivity, and prone to error/bias [6]. To address these shortcomings, while maintaining the enhanced utility of the dual reporter LC3 system to track stimulation of the autophagy pathway, we developed and validated an in-gel fluorescence method to be used in conjunction with dual reporter LC3 transfection imaging experiments. With this technique, cells are first subject to transfection and subsequent imaging to track autophagic stimulation and puncta formation with the dual reporter LC3

system. After image acquisition, cells are lysed and separated by semi-native gel electrophoresis. Gels are subsequently imaged using an imaging system with filters appropriate for the transfected dual reporters: EGFP and mCherry. Fluorescence protein bands, rather than antibody probes, can then be used to compare the ratio of LC3-1 to LC3-II. Here, we provide evidence that this method can be used to track autophagy activation and is comparable to results expected of western blotting for endogenous LC3. Our method, to be used in combination with dual reporter transfection presents a cheaper and easier autophagy tracking option for labs.

2 Research Methodology

Human Embryonic Kidney 293 (HEK293) Cell Plasmid Transfection

HEK 293 cells were seeded into a 6-well dish at a density of 150,000 cells/mL and allowed to attach overnight in an incubator at 37℃ and 5% CO_2. The next day, cells were transfected with an mCherry-EFGP-LC3B plasmid, a gift from Jayanta Debnath; Addgene, plasmid # 22418; http://n2t.net/addgene:22418; RRID: Addgene 22418. Each well was incubated with 1000 ng of plasmid encapsulated in lipid carriers created with TurboFect transfection reagent (ThermoScientific, R0533) in 100 µL of Opti-MEM reduced serum media (ThermoScientific, 31985062). The cells were incubated for 24 hours to allow for uptake and expression of the plasmid DNA. After incubation, transfection was verified via imaging with an EVOS FL Auto epifluorescence microscope.

Chemical Induction of Transfected Cells, Imaging, and Lysis

Following verification of transfection, media was changed in all wells to avoid nutrient-depletion of control samples, which could induce autophagy. Wells were divided into untreated groups (3 wells) and treatment groups (3 wells). Treatment groups received 0.3 µM rapamycin, a potent autophagy inducer [7]. Cells were incubated for 24hrs. After incubation, images were obtained of each well (3 images per well each field of view containing between 23-60 cells per image) using the EVOS FL Auto epifluorescence microscope equipped with Texas Red and GFP light cubes. Each well was subsequently lysed using 200 µL of protease inhibitor/RIPA lysis buffer (Sigma,36 S8820-2TAB, Thermo Scientific, J63306.AK). Samples were kept on ice for 20 minutes. After 20 minutes, the cell suspension was centrifuged at 12,000 rpm on an Eppendorf 5452 Minispin centrifuge for 10 minutes at 4°C. The resulting supernatant was removed and aliquoted into Eppendorf tubes. The samples were flash frozen using liquid nitrogen and were subsequently stored at -80°C. This experiment was performed in triplicate to ensure reproducibility (3 control wells & 3 rapamycin-treated wells = 1 experiment).

Quantification of Total Fluorescence in Lysates and Standardization of Sample Loading

To ensure uniform loading of samples, lysate fluorescence was quantified on a Molecular Devices SpectraMax iD5 Multi-Mode Plate Reader and samples were prepared so they contained equal amounts of fluorescent proteins loaded into each well. This step was important because total protein quantification of transfected samples would measure protein from both transfected and nontransfected cells in each lysate and in this scenario gel loading could erroneously inflate or reduce fluorescent protein quantities for each sample. As a secondary measure of loading standardization, total lane fluorescence was quantified in the gel images used to compare banding post electrophoresis. Total lane fluorescence was similar between lanes and normalization with and without total lane fluorescence preserved the ratios observed in LC3 protein banding and quantification.

In-Gel Fluorescence Protocol and Subsequent Western Blotting for LC3

Transfected cell lysates (untreated and rapamycin-dosed) were mixed with 2X Laemmli buffer (Bio-Rad, 161-0737) without beta-mercaptoethanol. Samples were not heated before loading onto the gel. Running buffer stock (15.1 g Tris base, 94 g electrophoresis grade glycine, 50 mL 10% SDS, pH 8.3) was diluted to 5% in distilled water and poured over a 12% SDS-PAGE gel. Samples were added to the gel, and

the gel was run at 120 volts for 25 minutes. After 25 minutes, the voltage was increased to 200 volts for 1 hour. After the protein ladder reached the bottom of the gel, the gel was imaged with a BIORAD ChemiDoc Imaging System. The following filters were used to acquire images: Alexa488 (corresponding to the EGFP probe), Alexa546 (corresponding to the Texas Red probe), and Alexa647 (corresponding with the molecular weight marker). Following imaging with the ChemiDoc Imaging System, protein gels were transferred to a PVDF membrane and probed with an anti-LC3 antibody (Novus Biologicals, NB100-2220) for confirmation that fluorescing protein bands contain the transfected LC3 protein. The full western blotting protocol is provided below.

Preparation of Cells for Endogenous LC3 Western Blotting

HEK 293 cells were seeded into a 6-well dish at a density of 150,000 cells/mL and allowed to attach overnight in an incubator at 37℃ and 5% CO_2. To mirror time points used in the transfection experiment, cells were allowed to grow an additional day (accounting for the time in culture required of transfection). Media was then changed to ensure a nutrient-rich state, and 3 wells were left untreated while another 3 wells were treated with 0.3 μM rapamycin to induce autophagy. After 24-hr incubation, cells were lysed as described above and stored at -80°C until further use. These experiments were performed in triplicate (3 untreated and 3 rapamycin-treated wells = 1 experiment).

Western Blotting of Endogenous LC3

Lysates were removed from the -80°C freezer and thawed on ice. Lysates were combined 1:3 with reducing buffer (12% SDS w/v, 6% beta-mercaptoethanol v/v (MP Biomedicals, 194834), 30% glycerol w/v, 0.05% Coomassie Brilliant Blue 6-250 (Thermo Scientific, 20278), and 150 mM Tris-HCl pH 6.8). Samples were heated to 95°C for five minutes. Samples were cooled for five minutes and briefly centrifuged to collect evaporated moisture. Samples were loaded on a 12% SDS-PAGE gel alongside the Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific, 26634) and inserted into the BIORAD Mini Tetracell gel electrophoresis apparatus. The gel was run at 120 volts for 25 minutes. After 25 minutes, the voltage was increased to 200 volts for 1 hour. After SDS-PAGE was complete, a blotting sandwich was assembled with the 12% gel in transfer buffer stock (30.385 g Tris Base, 144.134 g electrophoresis grade glycine, 1 liter of water) diluted to 10% in distilled water. A 0.45 μm pore size PVDF (Millipore IPVH20200) membrane was used for the transfer. Transfer was run at 100 volts for 70 minutes. The membrane was then blocked overnight in the cold room with 50 mL of 3% nonfat milk in PBST. Detection of LC3 and GAPDH proteins was performed by incubating the blot in 15 mL of 2.5% Bovine Serum Albumin (BSA) (Thermo Scientific, J65731.22) supplemented with primary antibody titers of 1:10,000 for transfected LC3 (Novus Biologicals, NB100-2220) and 1:10,000 for GAPDH (Cell Signaling Technology, D16H11). Membranes were incubated with primary antibodies for 1 hour at room temperature with rocking. Membranes were then washed four times for five minutes and one time for 5 seconds in PBST. 15 mL of 2.5% BSA supplemented with secondary antibodies (BIORAD, 170-6515) at a titer of 1:5000 were applied to the membrane for one hour at room temperature with rocking. Membranes were then washed four times for five minutes and one time for 5 seconds in PBST. 1 mL SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, 34577) was then applied to each membrane and incubated for five minutes. The membrane was imaged using the BIORAD ChemiDoc Imaging System.

In Gel Fluorescence and Western Blot Densitometry Analysis

In gel fluorescence and western blot band densitometry was performed using FIJI (ImageJ) software. ChemiDoc images were opened in ImageJ and inverted. Regions of interest (ROIs) were generated for each protein type. The size of each ROI was adjusted to accommodate the largest band of each protein type. The intensity of each band in the ROI was measured. A background measurement for each band was also taken using the same ROI. The background measurement was subtracted from its corresponding band intensity value. The ratio of band intensities between LC3-II/LC3-I was calculated by

dividing the intensity of the LC3-II band by that of the LC3-I band. Ratios were graphed using GraphPad Prism software.

Statistical Analysis

To obtain protein quantities sufficient for western blotting of LC3, lysed samples were pooled. All lysates used for western blotting and in gel fluorescence were pooled from 3 identically treated wells of a six-well dish. Each separate experiment therefore consisted of 3 control wells lysed and pooled and 3 treatment wells lysed and pooled. Three separate experiments were performed in this manner to provide three total replicates for statistical comparison (3 pooled wells = 1 replicate (n)). For fluorescent image analysis, multiple images were acquired per well to ensure that the selected field of view did not bias data analyses. The multiple image counts performed for a single well were averaged before statistical analysis occurred to avoid pseudo-replication. T-tests were performed in GraphPad Prism software to determine statistical significance. A p-value of less than 0.05 was considered statistically significant.

3 Results and Discussion

HEK 293 cells transfected with the dual reporter mCherry-EGFP-LC3 plasmid construct were imaged to confirm usability of the system in our HEK 293 cells and its ability to distinguish between early and late autophagy by fluorescent color changes (as demonstrated by N'Diaye et al., 2022 [9]). Figure 1A shows the putative dual reporter LC3 construct with expected molecular weights labeled for each component. Figure 1B shows a representative image of HEK 293 cells transfected with the plasmid. The same field of view is shown in all images. Filters optimized for EGFP and mCherry allow for determination of overlapping and non-overlapping regions. Localized intensely fluorescing puncta that are visible under both filters, overlay as yellow and are indicative of early autophagy when LC3 is aggregated into autophagosomes. Puncta visible only under the red filter will remain red in the overlay and are indicative of late autophagy when the lysosome fuses with the autophagosome. At this stage, the EGFP reporter in the LC3 construct is incapable of fluorescing in the resulting low pH environment. Therefore, this system can distinguish between early and late autophagy and can be used to determine whether autophagosome-lysosome fusion is inhibited, under which scenario, all puncta will be visible in green and red, or yellow in overlay.

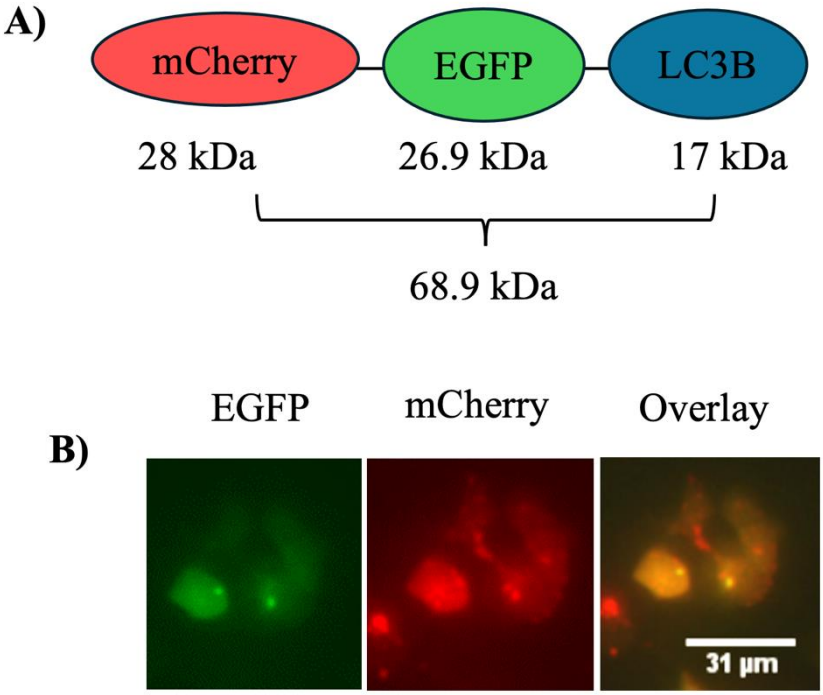

Figure 1. *A dual-reporter plasmid construct containing the gene sequence for LC3 fused to two fluorescent probes can be used to visualize autophagic stimulation via fluorescent microscopy.* ***(A)*** *The Illustration shows the putative transfected LC3 protein construct with expected molecular weights labeled for each component. The total molecular weight of the protein construct is expected to be 68.9 kDa.* ***(B)*** *The images show a representative region of HEK 293 cells transfected with the LC3-containing plasmid. Diffuse fluorescence and localized fluorescent puncta are observable under filters optimized for excitation and emission of EGFP and mCherry. Overlaid images demonstrate regions of overlapping fluorescent activity from both probes. Intensely fluorescing puncta that overlay as yellow (meaning both probes are observable) represent autophagosomes. Red only puncta in the overlay are suggestive of downstream autophagolysosome formation (EGFP is prevented from fluorescing in the acidic environment of the lysosome).*

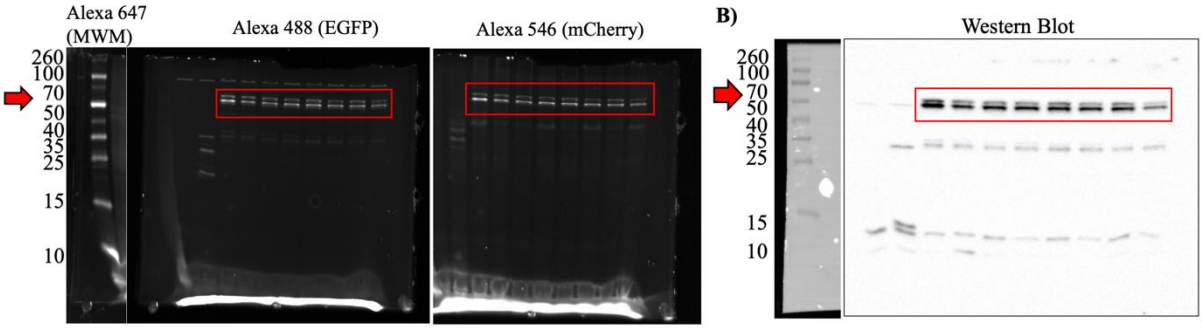

Figure 2. *In-gel fluorescence can be used to visualize EGFP-mCherry-LC3 protein constructs in transfected HEK 293 cell lysates.* ***(A)*** *HEK 293 cells transfected with the dual-reporter LC3 plasmid were lysed and protein lysates separated via semi-native SDS-PAGE. Protein bands were visible under Alexa 488 (optimized to the EGFP probe) and Alexa 546 (optimized to the mCherry probe) filters (highlighted in the red box). The fluorescing bands are approximately 70 kDa, which correlates with the expected molecular weight of the EGFP-mCherry-LC3 construct (68.9 kDa).* ***(B)*** *Probing of the semi-native gels with an anti-LC3 antibody via western blot confirmed LC3 bands corresponding to the expected 70 kDa range (highlighted in the red box).*

To determine whether in-gel fluorescence can be used to visualize fluorescently tagged LC3 and to resolve the two forms of LC3 (LC3-I and LC3-II), transfected cell lysates were separated under non-reducing, semi-native SDS-PAGE conditions similar to those proposed by prior studies [11][12][13]. A few very recent studies suggest that certain fluorophores may refold under denaturing gel conditions [14][15]; however, these observations were secondary to the reported research goals for both studies, and further controlled studies are necessary to understand whether denaturing conditions are compatible with in-gel fluorescence, particularly for multiple fluorophores. To this end, this study used semi-native PAGE, to preserve the fluorescence of the EGFP and mCherry proteins [11][12][13]. Imagining of the gel under filters overlapping with excitation/emission spectra of EGFP and mCherry (Alexa 488 & Alexa 546, respectively), demonstrates banding patterns consistent with that expected of LC3-I and LC3-II at the molecular weight range expected for the transfected LC3 construct (Figure 2A). The LC3 construct's expected molecular weight is 68.9 kDa. Bands were visible, under each filter at ~70 kDa. Subsequent transfer to a membrane and probing of the membrane with an anti-LC3 antibody confirmed the bands to be LC3 (Figure 2B).

After confirming that in-gel fluorescence could be used to resolve fluorescently tagged LC3, we then investigated whether the system could be used to quantify autophagy changes occurring in cells and whether it provides results comparable to standard western blotting of endogenous LC3-I and LC3-II. We first ran a standard western blot of non-transfected cells treated with 0.3 μM rapamycin to assess changes in LC3 when an autophagy stimulator is used. As would be expected, analysis with western blotting for endogenous LC3 demonstrated an increase in the LC3-II/LC3-I ratios in rapamycin treated cells compared to untreated controls (Figure 3A).

Next, we tested whether this same change was observable in transfected cells using the traditional fluorescent imaging method and our newly developed in-gel fluorescence method. To this end, we used transfected HEK 293 cells with the LC3 dual reporter plasmid and dosed with rapamycin as described in the methods. Post-dosing, fluorescent images were obtained and the percentage of cells with puncta (in any color) were counted as an indicator of autophagy activation (at either an early or late stage). As expected, counts of cells with puncta suggested a higher percentage of cells undergoing autophagy in the rapamycin-treated group (Figure 3B).

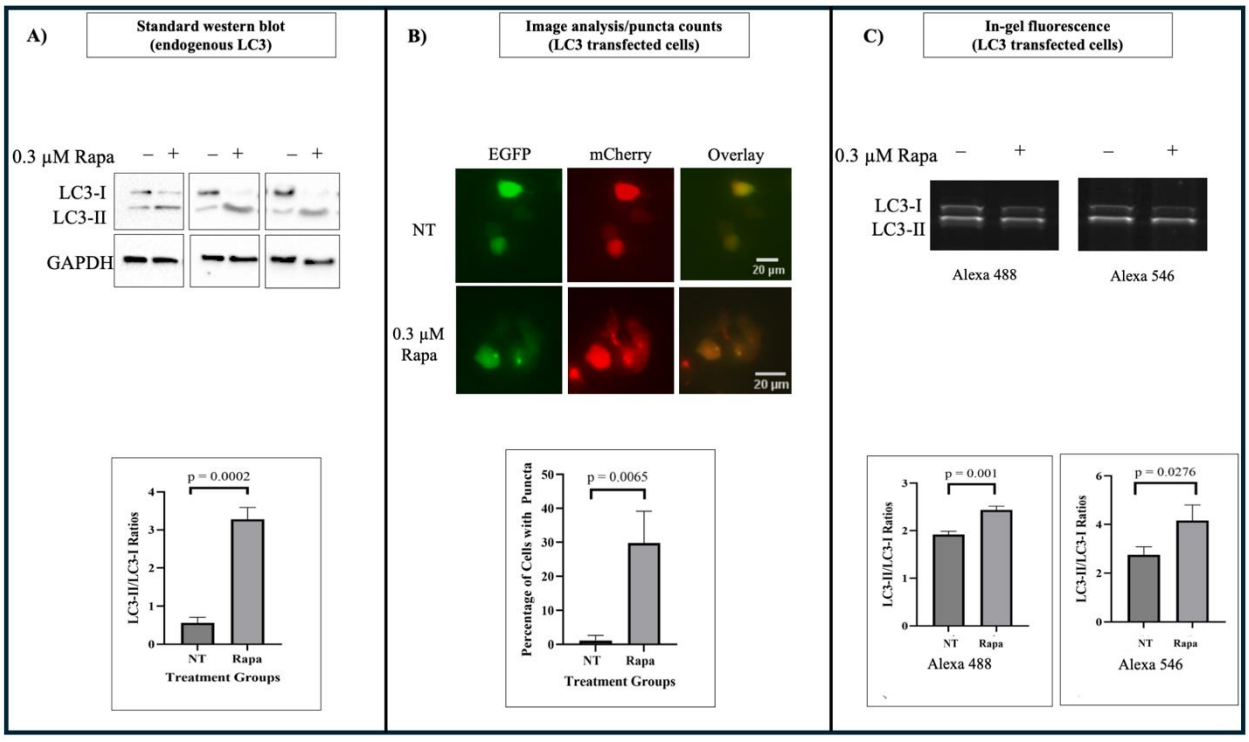

Figure 3. *In-gel fluorescence can reliably identify variation in LC3-I vs LC3-II protein levels and differences between non treated and rapamycin-treated cells.* ***(A)*** *Standard western blotting of endogenous LC3 in HEK 293 cells treated with 0.3 µM rapamycin for 24 hours shows an expected increase in the ratio of LC3-II/LC3-I. Endogenous LC3-I, LC3-II, and GAPDH bands were analyzed via western blotting. LC3-II/LC3-I ratios for no treatment and rapamycin-treated groups were calculated via densitometry analysis in ImageJ. Statistical significance was determined using a T-Test. A p value of $p < 0.05$ indicates statistical significance. $p = 0.0002$; error bars depict standard deviation. Western blots were performed on lysates obtained from three separate experiments. Each experimental group used lysates pooled from 3 wells of a 6-well dish.* ***(B)*** *Dual-reporter LC3 transfected cells display increased puncta (brightly fluorescing regions indicative of phagophore or autolysosome formation) in cells treated with 0.3 µM rapamycin for 24 hours vs untreated controls. Images shown are cropped representative regions from images of treated and non-treated culture wells. Manual counts of cells with and without puncta were performed using ImageJ by two separate individuals to control for manual counting bias. Rapamycin-treated cells displayed a higher percentage of puncta vs the no treatment control group. Statistical significance was determined using a T-Test. $p = 0.0065$; error bars= standard deviation. Experiments were performed in three separate iterations with 3-wells per treatment group in each experiment and 3 images obtained per well. Counts were obtained for each image and the three images were averaged for individual wells to obtain a percentage of cells with puncta per well. Graphs were generated by each separate user and displayed continuity of results (both comparisons demonstrated statistical significance between rapa and NT groups); a representative graph is shown for one individual's manual counts of the combined three experiments.* ***(C)*** *In-gel fluorescence can distinguish LC3 II/I ratio differences in cells treated with rapamycin vs untreated controls. Dual-reporter LC3 transfected cells were lysed and separated using semi-native gel electrophoresis. The gels were imaged using Alexa 488 and Alexa 546 filters to distinguish between the EGFP and mCherry signals (respectively) on the transfected LC3 protein construct. LC3-II/LC3-I ratios were calculated via densitometry analysis in ImageJ. Statistical significance was determined using a T-Test. $p = 0.001$ (Alexa 488) and $p = 0.0276$ (Alexa 546). Experiments were performed in triplicate with three pooled wells per treatment group.*

Cells were then lysed, and lysates separated via semi-native SDS-PAGE. Imaging and subsequent densitometry analysis confirmed an expected statistically significant increase in LC3-II in the rapamycin-treated group (Figure 3C), consistent with results of manual cell counting and those shown for standard

western blotting. Increases were observed under both filters. The comparison of LC3-II/LC3-I ratios obtained from in-gel fluorescence to western blotting suggests that activation of the autophagy pathway is similarly distinguishable using either platform. However, quantitative comparisons of total LC3 in either sample are not possible. This is because the LC3 that is visualized by in-gel fluorescence is exogenously supplied through plasmid transfection. Production of this construct is expected to be variable in different cells, even in the same culture dish population, but also much higher than typical endogenous expression of LC3. Additionally, lysate volume was standardized in gels intended for western blot by measuring total protein while lysate volume in gels intended for in-gel fluorescence was standardized by measuring total fluorescence. This limits the ability to study quantitative differences between endogenous and exogenous LC3 using our method.

4 Conclusions

Our experimental results suggest that in-gel fluorescence can serve as a complementary approach to western blotting and provides a semi-quantitative complement to be used in tandem with fluorescent imaging of cells transfected with a dual-reporter LC3 plasmid. Future experiments will investigate whether the utility of the dual reporter imaging system to recognize autophagosome-lysosome fusion inhibition can be retained through pH modulation of the lysis buffer. Importantly, in the present form, the system presents a cheap, easier alternative for the investigation of autophagic stimulation compared to western blotting.

5 Declarations

5.1 Study Limitations

This study was performed on a limited budget. The outcomes presented were thoroughly validated through numerous experimental replicates over a two-year timespan; however, additional budget would be necessary to test further cell lines and controls.

5.2 Acknowledgements

The authors wish to thank the Biology Department and Provost's Office at Western Carolina University for funds to support this project. HEK 293 cells were a gift from Dr. Jennifer Pluznick (Johns Hopkins University).

5.3 Competing Interests

The authors have no conflicts of interest to declare.

5.4 Human and Animal Related Study

No human, human-derived materials, or animal subjects were used in this study. Only established human cell lines were employed.

5.5 Ethical Approval

Experiments involving human cells and recombinant DNA were approved by the university Institutional Biosafety Committee (IBC) (protocol #2022-03-22-02).)

5.6 Informed Consent

This study did not require informed consent.

5.7 Hazard Warnings

There were no unusual risks related to chemicals or equipment used in this study.

5.8 Use of AI tools

No AI assisted tools were used in this study.

5.9 Publisher's Note

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