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Flux-Based Assay for the Identification of Autophagy Modulators for Osteoarthritis

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Dear Editor,

We are really pleased to submit our manuscript entitled "**Autophagic Flux-Based Screening Assay for the Identification of Autophagy Modulators for Osteoarthritis**" as an Article.

Rheumatology diseases, in particular Osteoarthritis have been the focus of our research for many years. Our multidisciplinary team has generated knowledge not only about molecular basis underlying disease but also on novel mechanistic and therapeutic hypothesis with translational potential.

Autophagy is a central mechanism to regulate homeostasis. Alterations of autophagy contribute to aging-related diseases. Phenotypic methods to identify regulators of autophagy could be used for the identification of novel therapeutics. Here, we develop a cell-based imaging screening workflow for monitoring autophagic flux by using LC3 as a reporter of autophagic flux (mCherry-EGFP-LC3B) in human chondrocytes. Physiologically relevant phenotypic approaches targeting hallmarks of aging can facilitate more effective drug discovery and drug development strategies for age-related musculoskeletal diseases.

On behalf of our research team, we thank you in advance and look forward to your editorial decision.

We declare that the material is original research that has not been previously published and has not been submitted for publication elsewhere while under consideration. We also declare no conflict of interest.

With our best regards,

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TITLE:

Flux-Based Assay for the Identification of Autophagy Modulators for Osteoarthritis

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KEYWORDS:

aging, osteoarthritis, homeostasis mechanism, autophagy flux, high throughput screening, therapeutics

SUMMARY:

This paper details monitoring autophagy flux to identify new molecules by cell-based imaging screening.

ABSTRACT:

Autophagy is a central mechanism to regulate homeostasis. Alterations of autophagy contribute to aging-related diseases. Phenotypic methods to identify regulators of autophagy could be used for the identification of novel therapeutics. This article describes a cell-based imaging screening workflow developed to monitor autophagic flux using LC3 as a reporter of autophagic flux (mCherry-EGFP-LC3B) in human chondrocytes. Data acquisition is performed using an automated High Content Imaging Screening System microscope. An algorithm-based automated image analysis protocol was developed and validated to identify molecules activating autophagic flux. Critical steps, explanatory notes, and improvements over current autophagy monitoring protocols are reported. Physiologically relevant phenotypic screening approaches to target hallmarks of aging can facilitate more effective drug discovery strategies for age-related musculoskeletal diseases.

INTRODUCTION:

Many chronic diseases are associated with the hallmarks of aging, including autophagy[1]. Osteoarthritis (OA) is the most prevalent joint disease and has a major impact in

restricting everyday activities in the aging population, but neither preventive measures nor disease-modifying treatments are yet available[2].

Joint aging and OA are associated with hallmarks that define the progression of cartilage degeneration, including defective autophagy and senescence[3,4]. Targeting autophagy in musculoskeletal tissues can help find therapeutic innovations for rheumatologic diseases[5,6]. Pharmacological modulation of autophagy is a promising, relevant mechanism for intervention in preclinical disease models[7]. In OA, autophagy activation has been used to prevent joint dysfunction[8]. Methods to monitor autophagy based on robust, reproducible protocols that allow quantitative analysis can be used to identify novel agents and facilitate the pharmacological targeting of disease-relevant hallmarks of aging.

Autophagy flux reflects degradation activity and is a relevant measurement to identify new molecules activating autophagy[9]. This study describes a method developed to determine autophagic flux by measuring autophagic degradation activity using an autophagy reporter cell line in human chondrocytes (TC28a2). mCherry-EGFP-LC3-transient expression allows simultaneous monitoring of autolysosome formation and degradation events by quantifying the differences in the pH sensitivity between GFP and mCherry LC3 signals in the lysosomes of live cells[10].

Adapting this flow cytometry reported method to a stable expression imaging monitoring system in live chondrocytes may allow the identification of molecules activating autophagic flux in the context of cartilage biology.

PROTOCOL:

1. Generation of autophagy reporter cell line of immortalized human chondrocytes

NOTE: The generation of an autophagy reporter cell line by stable transfection of pBABE-mCherry-GFP-LC3 by establishing a flow cytometry quantitative readout was described previously[11]. Two cell lines were used in the retroviral transfection: HEK 293-T17 during the cotransfection process and T/C28a2 in the infection step.

1.1. Cotransfection process

1.1.1. Prepare the growth medium for HEK 293-T17 cells: 500 mL of Eagle's minimum essential medium (EMEM), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (P/S). Store the growth medium at 4 °C and warm to 37 °C before each use.

1.1.2. Seed 1 x 10^6 HEK 293-T17 cells per well into 100 mm cell culture plates, incubate at 37 °C and 5% CO_2, and make sure that they are at ~75% confluence before starting the cotransfection process.

1.1.3. Remove the culture medium, rinse with Hank's balanced salt solution (HBSS), and add 8 mL of EMEM with 2% FBS and 1% P/S.

1.1.4. In a conical tube, add 10 µg, 3 µg, and 7 µg of pBABE-puro mCherry-EGFP-LC3B, VSV.G, and pCL-Eco plasmids, respectively. Then add 200 µL of 1x reduced serum medium (**Table of Materials**). Add transfection reagent (**Table of Materials**) as a nonliposomal mixture of lipids to have a 1:3 ratio (1 µg DNA : 3 µg transfection reagent; final volume = 60 µL).

NOTE: A total of 20 µg of mix is prepared for each 10 cm cell plate to be transfected.

1.1.5. Incubate the mixture for 20 min at room temperature. Carefully transfer the transfection mix dropwise to HEK 293-T17 packaging cells. Incubate the cells at 37 °C and 5% CO_2 at least 48 h before checking for green and red protein expression by fluorescent microscopy.

NOTE: Upon these conditions, more than 75% of the cells should be positive for fluorescence. Ensure that transfection efficiency is high (e.g., more than 75% of the cells are positive). Use high-quality plasmid DNA (e.g., predominantly supercoiled and free of genomic DNA, RNA, and protein; highly concentrated; free of endotoxins and salts) as well as high-quality cell cultures (e.g., homogeneous, low passage, monolayer, transfected in the exponential growth phase, free of mycoplasma).

1.2. On the same day of cotransfection, prepare T/C28a2 chondrocytes.

1.2.1. Prepare T/C28a2 cell growth medium: 500 mL of Dulbecco's modified Eagle medium (DMEM), 10% fetal calf serum (FCS), and 1% P/S. Store growth medium at 4 °C and warm to 37 °C before each use.

1.2.2. Seed 2 x 10^5 T/C28a2 chondrocytes in two 6 well multiplates and incubate for 48 h at 37 °C and 5% CO_2.

1.3. Retroviral infection process

1.3.1. Aspirate culture medium from HEK 293-T17 retroviral packaging cells obtained from step 1.1.5. Rinse 1x with 4 mL of HBSS and aspirate HBSS.

1.3.2. Add 2 mL of trypsin and wait 2 min at 37 °C. Neutralize trypsin by adding 6 mL of HEK 293-T17 culture medium and tranfer cells and medium into 15 mL conical tubes.

1.3.3. Centrifuge cells at 400 x *g* for 5 min. Tranfer supernatant to a sterile filter through a 0.45 µm membrane.

1.3.4. Aspirate the T/C28a2 chondrocyte culture medium from the 6 well multiplates. Infect T/C28a2 cells by adding 2 mL of viral suspension (step 1.3.3) per well into 6 well multiplates. Use one well for control (i.e., untransfected cells), adding 2 mL of T/C28a2 cell growth medium instead of viral suspension.

1.3.5. Incubate for 48 h at 37 °C and 5% CO_2.

1.4. To perform the selection process, use an antibiotic selection on transfected cells to eliminate untransfected cells and to obtain a homogenous cell population.

NOTE: The pBABE-puro mCherry-EGFP-LC3B retroviral vector provides mammalian antibiotic resistance to puromycin, which enables selection of a stable cell culture after viral transfection. Kill curves for optimal puromycin concentration of T/C28a2 chondrocytes should be set up in advance to establish the appropriate conditions to select the cell clones carrying the transgene with high expression levels.

1.4.1. Prepare T/C28a2-pBABE-puro mCherry-EGFP-LC3B stable cell line growth medium: 500 mL of DMEM, 10% FCS, 1% P/S, and 2.5 µL/mL puromycin. Store medium at 4 °C and warm to 37 °C before each use.

1.4.2. Change the medium. For each 6 well multiplate, aspirate culture medium, rinse 1x with 2 mL of HBSS and aspirate it. Add 2 mL of medium and incubate at 37 °C and 5% CO_2. This is the beginning of the selection process.

1.4.3. Replace the medium every 2 days and observe dead cells and puromycin resistant cell populations. Compare results to the untransfected control cells, which are not resistant to puromycin and die.

1.5. To start clonal selection, ensure that the monoclonal populations grew. For each 6 well multiplate:

1.5.1. Add 200 µL of medium per well into a 96 well multiplate.

NOTE: Each single cell selected from the 6 well multiplate will be plated in individual wells of the 96 well multiplate.

1.5.2. Use a microscope to identify each monoclonal population in the 6 well multiplates. Colonies formed from single cells can be observed.

1.5.3. Aspirate culture medium from 6 well multiplates, rinse 1x with 2 mL of HBSS, and aspirate HBSS.

1.5.4. Add 10 µL of trypsin to each monoclonal population to tranfer single cells into each well of the 96 well multiplates. Incubate for 48 h at 37 °C and 5% CO_2.

1.6. After 48 h, replace the medium with 100 µL of fresh medium per well. Repeat this procedure until colonies form due to expansion of single cells.

1.7. Once a single cell has expanded well, plate each monoclonal population in individual wells of 24 well, 6 well, and 100 mm cell culture plates.

1.8. Generate a stable monoclonal cell line using a single cell sorting method.

1.8.1. Aspirate culture medium from 100 mm cell culture plates. Rinse 1x with 8 mL of HBSS and aspirate it.

1.8.2. Add 2 mL of trypsin on each plate and incubate for 2 min at 37 °C. Neutralize trypsin by adding 8 mL of culture medium.

1.8.3. Centrifuge cells at 252 x *g* for 5 min and discard the supernatant. Add 4 mL of HBSS to each cell pellet, resuspend it carefully, and transfer it to cytometry tubes.

1.8.4. Inject each cell sample into a flow cytometer (**Table of Materials**) with cell sorting technology and separate cells with high green and red fluorescence.

1.8.5. Tranfer selected cells to 15 mL conical tubes and centrifuge cells at 252 x *g* for 5 min. Add cell pellet to a T-25 flask with 9 mL of stable cell line growth medium. Incubate at 37 °C and 5% CO_2.

NOTE: After cell sorting, three monoclonal populations (**Table 1**) should be obtained.

1.9. After 24 h, remove the medium and add 8 mL of fresh medium. Repeat this procedure every 2 days until the cells reach confluence.

NOTE: To study autophagic flux, two controls can be used: rapamycin as a positive control and chloroquine as a negative control. Prepare a 10 mM rapamycin stock solution in dimethyl sulfoxide (DMSO) and a 30 mM chloroquine stock solution in H_2O and store at -20 °C. To select the best monoclonal population by testing the expression mCherry and GFP, two methods can be used: flow cytometry (step 1.10) or high content fluorescent image analysis (step 1.11).

1.10. Flow cytometry

1.10.1. Seed 2.5 x 10^5 cells per well of each monoclonal population in 12 well plates at least 24 h prior to treatment in 1 mL of stable cell line growth medium and incubate at 37 °C and 5% CO_2.

1.10.2. On the day of the treatment, remove the medium, add 1 mL of stable cell line growth medium supplemented with 2% FCS per well. Add 10 µM rapamycin and 30 µM chloroquine to the stable cell line growth medium with 2% FCS and incubate for 16 h at 37 °C and 5% CO_2.

1.10.3. Remove media, rinse with 1 mL of HBSS per well, and aspirate it.

1.10.4. Add 500 µL of trypsin in each well, incubate 1 min at 37 °C and neutralize the reaction by adding 500 µL of culture medium. Scrape cells and transfer them to conical tubes.

1.10.5. Centrifuge the cells at 252 x *g* for 5 min and aspirate the supernatant. Rinse the cell pellets with 500 µL of phosphate buffered saline (PBS). Centrifuge at 252 x *g* for 5

min and discard the supernatant.

1.10.6. Add 500 µL of PBS, tranfer cells to cytometry tubes, and inject each sample into the cytometry equipment. For each condition, collect 10,000 events.

1.10.7. To select the monoclonal population by flow cytometry, use a 488 nm laser to excite EGFP and a 633 nm laser for mCherry. Then, establish a ratio of mCherry and EGFP fluorescence to select the cell population with high and low autophagic flux.

1.11. High content fluorescent image analysis

1.11.1. Seed 4 x 10^3 cells of each monoclonal population per well in 384 well plates (black wall/clear bottom) in growth medium supplemented with 10% FCS, 1% P/S, and 2.5 µL/mL puromycin. Incubate at 37 °C and 5% CO_2 for 24 h prior to treatment.

1.11.2. Remove the medium and add 10 µM rapamycin and 30 µM chloroquine in 50 µL of stable cell line growth medium supplemented with 2% FCS per well. Incubate for 16 h at 37 °C and 5% CO_2.

1.11.3. Remove media, rinse with 50 µL of HBSS per well, and aspirate it. Fix cells with 4% paraformaldehyde (PFA) for 10 min at 37 °C. Remove PFA and rinse with 50 µL of HBSS per well.

1.11.4. To identify the cell nuclei, stain with Hoechst 33342 (2.5 µg/mL) for 10 min at 37 °C. Aspirate Hoechst 33342 dye, rinse with 50 µL of HBSS per well, and add 50 µL of PBS per well.

1.11.5. Perform data acquisition and analysis of autophagic flux obtained by HTS platform as described in section 3.

NOTE: The criteria to select the monoclonal population is based on whether autophagic flux increased or decreased with rapamycin or chloroquine, respectively (**Table 1**). Clones with high levels of mCherry and GFP expression are more appropiate for accurate high content fluorescent image analysis quantitation of autophagy flux.

2. Image-based autophagic flux assay in live chondrocytes

NOTE: After selecting a clone, start the assay to quantify autophagic flux.

2.1. Using a handheld electronic 384 channel pipette, seed 4 x 10^3 cells of the selected clone (step 1.11) per well into 384 well plates (black wall/clear bottom) in 50 µL of stable cell line growth medium supplemented with 10% FCS, 1% P/S, and 2.5 µL/mL puromycin per well. Incubate at 37 °C and 5% CO_2 for 24 h prior to treatment.

2.2. On the day of the experiment, prepare an interval plate for compound addition.

2.2.1. Add a volume of each compound/controls and stable cell line growth medium to the interval plate using acoustic liquid handling technology and an electronic 384 channel pipette, respectively, to get an interval concentration.

NOTE: The controls in this assay included stable cell line growth medium supplemented with 2% FCS, 30 µM chloroquine, and 10 µM rapamycin. Interval concentrations will depend on the desired final concentration. For example, in this assay, chloroquine and rapamycin were added in 50 µL of stable cell line growth medium per well to obtain an interval concentration of 300 µM and 100 µM, respectively.

2.2.2. Using a microplate washer robot, aspirate medium from cell plate in an automated fashion and add 45 µL of stable cell line growth medium supplemented with 2% FCS per well.

2.2.3. Then transfer 5 µL per well from the interval plate to the assay plate to get a final concentration using aother automated liquid handler workstation.

2.4. Incubate at 37 °C and 5% CO_2 for 16 h. Using a microplate washer robot, aspirate the medium.

2.5. Fix cells with 50 µL of 4% PFA per well for 10 min at 37 °C. Wash with 50 µL of HBSS per well.

2.6. Stain the nuclei with Hoechst 33342 (2.5 µg/mL) for 10 min at 37 °C. Aspirate Hoechst 33342 and rinse with 50 µL of HBSS per well.

2.7. Add 50 µL of PBS per well. The plate is now ready for reading, data collection, and subsequent analysis.

NOTE: This protocol is described for high throughput screening (HTS). However, if few compounds are used, they can be added manually, without automatic instruments. Minimal manipulation of cells and supernatant using calibrated liquid handling instruments and/or electronic pipettes facilitate successful preparation of cell culture monolayers for quantitation of autophagy flux by high content fluorescent image analysis.

3. Data acquisition and analysis of autophagic flux

3.1. For data acquisition, use a high content screening system (HCS) and an automated microscope, which allows automated image acquisition and analysis by image processing algorithm.

3.2. To develop an acquisition protocol, select several channels and exposure times (**Table 2**) to identify the nuclei and the formation of autophagosomes and autolysosomes during autophagic flux events.

3.3. For each well, collect images from four fields covering the well surface and in four stacks starting from 8 µm separated by a working distance of 0.2 µm. Capture the images using a 60x objective for detailed cell imaging.

3.4. Analyze the data using the image analysis software provided by the HCS microscope. To develop a robust analysis protocol algorithm for autophagy flux monitoring, select the most appropriate method for automated cell image segmentation and for specifically detecting the speckles corresponding to LC3 activation. Perform the final analysis sequence by adding methods, cell segmentation steps, and quantification instructions as described below:

3.4.1. Click the **Find Nuclei** building block and choose the nuclear dye channel **Hoechst 33342**. Select **Method** and choose the output population **Nuclei**.

3.4.2. Click the **Find Cytoplasm** building block and choose the cytoplasmic dye channel **Fluorescein**. Select **Method** | **Individual Threshold**.

3.4.3. Click the **Find Cytoplasm (2)** building block and choose the cytoplasmic dye channel **RFP**. Select **Method** | **Individual Threshold**.

3.4.4. Click the **Population** building block and select the study population **Nuclei.** Select the method **Common filters (Remove border objects)** and choose the output population **Nuclei Inside Borders**.

3.4.5. Click the **Find Spots** building block and choose the specific marker channel **Fluorescein**. Select the study population **Nuclei Inside borders** and select the study region **Cytoplasm**. Select **Method** and choose the output population **Spots Inside Borders**.

3.4.6. Click the **Find Spots (2)** building block and choose the specific marker channel **RFP**. Select the study population **Nuclei Inside borders** and select the study region **Cytoplasm (2)**. Select **Method** and choose the output population **Spots (2) Inside Borders**.

3.4.7. Click the **Calculate Intensity Properties** building block and choose the specific marker channel **Fluorescein**. Select the study population **Nuclei Inside Borders** and select the study region **Spot**. Select **Method** and choose the output properties **Intensity Spot Fluorescein Inside Borders**.

3.4.8. Click the **Calculate Intensity Properties** building block and choose the specific marker channel **RFP**. Select the study population **Nuclei Inside Borders** and select the study region **Spot (2)**. Select **Method** and choose the output properties **Intensity Spot (2) RFP Inside Borders**.

3.4.9. Click the **Define Results** building block, select population **Nuclei Inside Borders**, select **Number of Objects of Nuclei (Mean)**, select **Relative Intensity Spots Fluorescein – mean per well**, and select **Relative Intensity Spots (2) – RFP – mean per well**.

3.5. To quantify autophagic flux, use the same method described previously for flow cytometry analysis[11]. Establish the mCherry/EGFP ratio of relative intensity spots.

NOTE: Cells with higher flux are less green, which increases the mCherry/EGFP ratio in the cell. Identifying and accurately quantifying a meaningful number of the relevant signals coming from the autophagy flux events in the cells is very important to establish statistically significant differences.

REPRESENTATIVE RESULTS:

Autophagic flux can be monitorized in cells by pharmacological modulation or by cell stress response. Inmortalized Human Chondrocytes (T/C28a2) were employed to develop an autophagy reporter cell line using LC3 as a fluorescent reporter (mCherry-EGFP-LC3B) for autophagy. **Figure 1** shows the schematic workflow of the screening assay starting with the development of the autophagy reporter cell line in human chondrocytes (mCherry-EGFP-LC3-T/C28a2), to the induction or inhibition of autophagic flux by pharmacological modulation, and then the data analysis performed with a High Content Imaging Microscope. **Figure 2** shows the schematic representation of the cell-based imaging assay to identify autophagy modulators by monitoring autophagic flux. This basis of the system lies in the higher sensitivity of EGFP fluorescence to the acidic environment of the autolysosome relative to mCherry. **Figure 3** shows the sequence of analysis to quantify autophagic flux from cell-based images. **Figure 4** shows representative images of low and high autophagic flux represented by the ratio of mCherry/GFP of chloroquine and rapamycin, respectively.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic overview of the workflow used to determine autophagic flux by cell-based imaging assay. To create an autophagy reporter cell line, two cell lines were used in the retroviral transfection, the HEK 293-T17 cell line during the cotransfection process and the T/C28a2 immortalized human chondrocytes in the infection step. The pBABE-puro-mCherry-EGFP-LC3B plasmid was introduced into the cells as described previously[10] and puromycin was used to select a stable cell culture after viral transfection. Cells were seeded into 384 well plates (8×10^3 cells per well) in growth medium and incubated at 37 °C and 5% CO_2 for 24 h prior to treatment. Then, medium was removed and growth medium supplemented with 2% FCS and 1% P/S, and 2.5 µL/mL puromycin. Cells were treated with 30 µM chloroquine and 10 µM rapamycin. A high content screening system was used for data acquisition. Several channels and exposure times were selected to develop a reading protocol. Data were analysed using image analysis software and an analysis protocol was selected. Finally, the mCherry/EGFP ratio of relative intesity spots was confirmed.

Figure 2: Representation of cell-based imaging assay to identify autophagy modulators. The pBABE-puro-mCherry-EGFP-LC3B plasmid was introduced into the cells. When autophagy was activated, the cargo was engulfed by the phagophores and autophagosomes were created. LC3 was lipidated and incorporated into autophagosomal membranes where LC3 could interact with cargo receptors. Due to the basic environment in the autophagosomes and EGFP pKa ≥ 6.0, green fluorescence was emited. When autophagy flux continued, autophagosomes fused with lysosomes,

autolysosomes were created, the pH decreased, and red-orange fluorescence was emited.

Figure 3: Sequence of analysis to quantify autophagic flux from cellular images using different building blocks to create a protocol. Nuclei were identified using the Hoechst channel. Then, the cytoplasm was identified using two channels, fluorescein (EGFP) and RFP. Next, border objects were removed to select the cell population. The software identifies whole cells in green and incomplete cells in red. In each cytoplasm identified, accumulation of speckels (called spots) was seen. Finally, relative intensity spots from each cytoplasm were calculated and autophagy flux was quantified based on the ratio of mCherry/EGFP associated to LC3 activation. Scale bar = 50 µm.

Figure 4: Quantification of mCherry-EGFP-LC3 T/C28a2 chondrocytes treated with chloroquine and rapamycin by imaging. Culture medium supplemented with 2% FCS was used as a control. Chloroquine was used as autophagic flux inhibitor. When the autophagic flux was activated, autophagosomes were created, but the fusion between autophagosomes and lysosomes did not occur. Therefore, autophagosomes accumulated, green fluorescence was emitted, and the ratio of mCherry/EGFP decreased. Additionally, rapamycin was used as an autophagic flux activator. Autophagosomes fused with lysosomes, pH decreased, and red-orange fluorescence was emitted, resulting in an increase in the ratio of mCherry/EGFP. Scale bar = 50 µm.

Table 1: Data obtained by flow cytometry and HTS to quantify autophagic flux and select the best monoclonal population.

Table 2: Reading conditions in the autophagic flux process. Emission and excitation wavelength and exposure time of each channel.

DISCUSSION:

Defective autophagy is an important hallmark of musculoskeletal system aging, but neither preventive nor disease-modifying treatments targeting autophagy are yet available for cartilage degeneration[2]. Given its relevance and clinical implications, autophagy has become a target of interest for drug discovery and development, although methods to directly monitor changes in this key homeostasis mechanism has proved challenging.

Here, a cell-based phenotypic assay to determine the autophagic flux in live human chondrocytes is described. A plasmid containing a dual reporter of autophagy activation, mCherry-EGFP-LC3, is used to simultaneously monitor autolysosome formation and degradation events due to differences in the pH sensitivity of GFP and mCherry signals in live cells by flow cytometry[11]. Human chondrocytes with stable, high expression of mCherry-EGFP-LC3B can be used to accurately monitor the dynamics of autophagosome flux. A cell-based imaging assay using chondrocytes can be used to identify autophagy modulators, because the dynamic pH changes due to autolysosome formation can be observed in real-time. When autophagy is activated, phagophore and autophagosomes are created and catch disposable cargo. LC3 lipidation, inclusion into autophagosomal membranes, and interaction with cargo receptors provide a basic environment of pKa ≥

6.0, corresponding to green fluorescence emission. If autophagy flux is sustained, autophagosomes fuse with lysosomes to generate autolysosomes, decreasing pH and emitting red-orange fluorescence.

Critical steps for setting up the protocol, include getting high transfection efficiency by using high-quality cell cultures and DNA to generate viral particles, the eficient selection of the cell clones carrying high levels of the transgene by optimal antibiotic treatment and flow cytrometry sorting, the gentle manipulation of cell monolayers to make sure that high-quality images allow quantitative analysis of autophagy flux, as well as the accurate identification of a meaningful number of relevant signals coming from the autophagy flux events.

Current methods rely heavily on flow cytometry, which requires cells to be in suspension, making information on cell–cell communication and intracellular events difficult to obtain. Although accurate and suitable to purify small or complex subpopulations, sorting can be too slow and provide the data only in terms of the average of surface receptor densities. Therefore, cell-based imaging presents an advantage in monitoring and measuring autophagy by detection of markers by static analyses, which cannot distinguish between upregulation and degradation inhibition, for example[12]. Also, high sensitivity for image acquisition and automated methods allowing the capture and the analysis of the images are only possible by using High Content Screening Systems. Automatic quantitative analyses show consistent low autophagy signals (green) obtained by exposing chondrocytes to chloroquine, while high autophagy activation signals are obtained by exposing chondrocytes to rapamycin (red-orange). These opposite effects allow the systematic identification of agents (e.g., libraries of small molecules, genomic loss-of-function or gain-of-function screening libraries) that modulate autophagy in large scale experiments. Indeed, by using this cell-based assay in a drug repurposing approach, the PPARα fibrate used as a lipid lowering drug was identified as a candidate disease-modifying therapeutic for osteoarthritis[13].

This method adapts flow cytometry to a stable expression imaging monitoring system in live chondrocytes. This protocol may allow the identification of molecules activating autophagy flux in the context of cartilage biology, as well as a method to screen agents affecting these mechanisms.

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DISCLOSURES:

The authors have nothing to disclose.

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13. Nogueira-Recalde, U., et al. Fibrates as drugs with senolytic and autophagic activity for osteoarthritis therapy. *EBioMedicine*. **45**, 588–605 (2019).

Figure 1

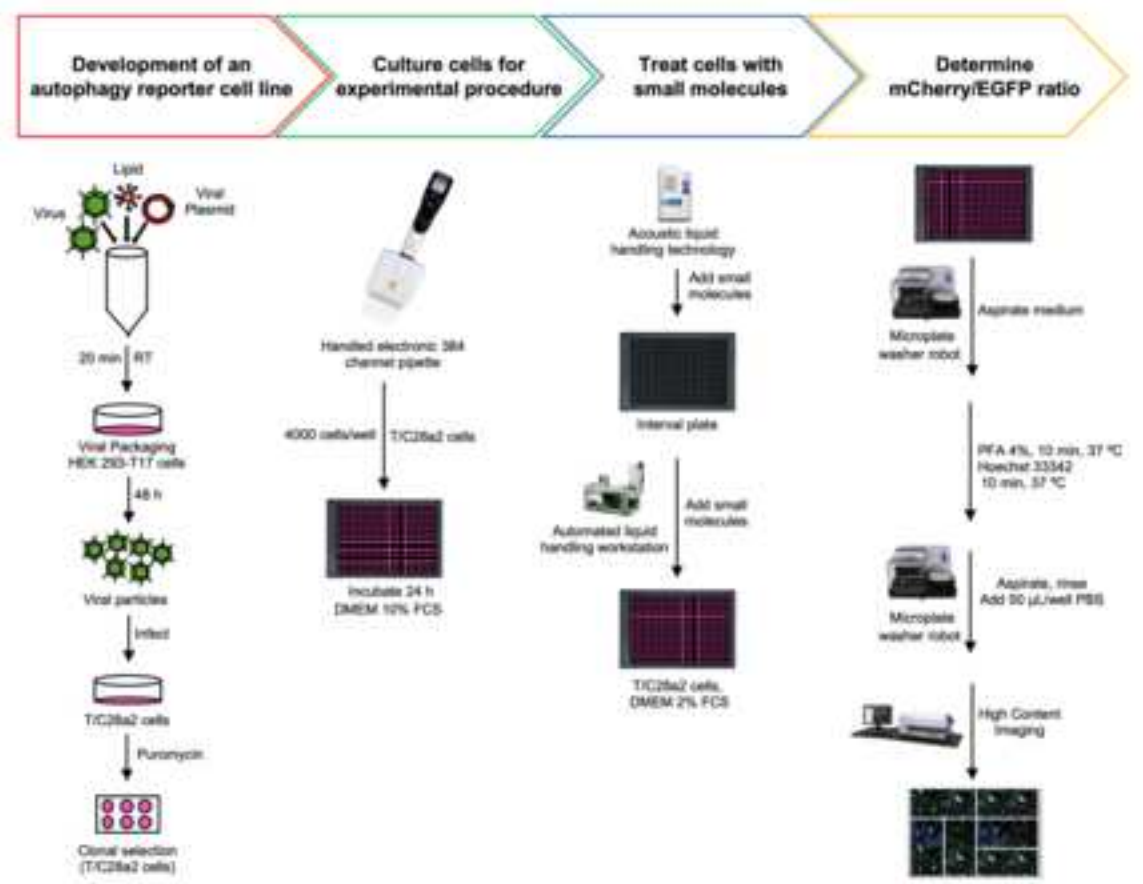

Figure 2

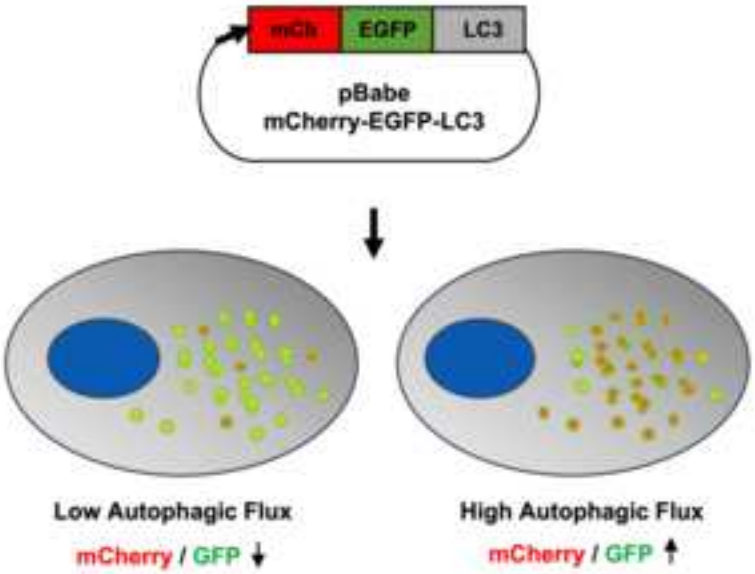

Figure 3

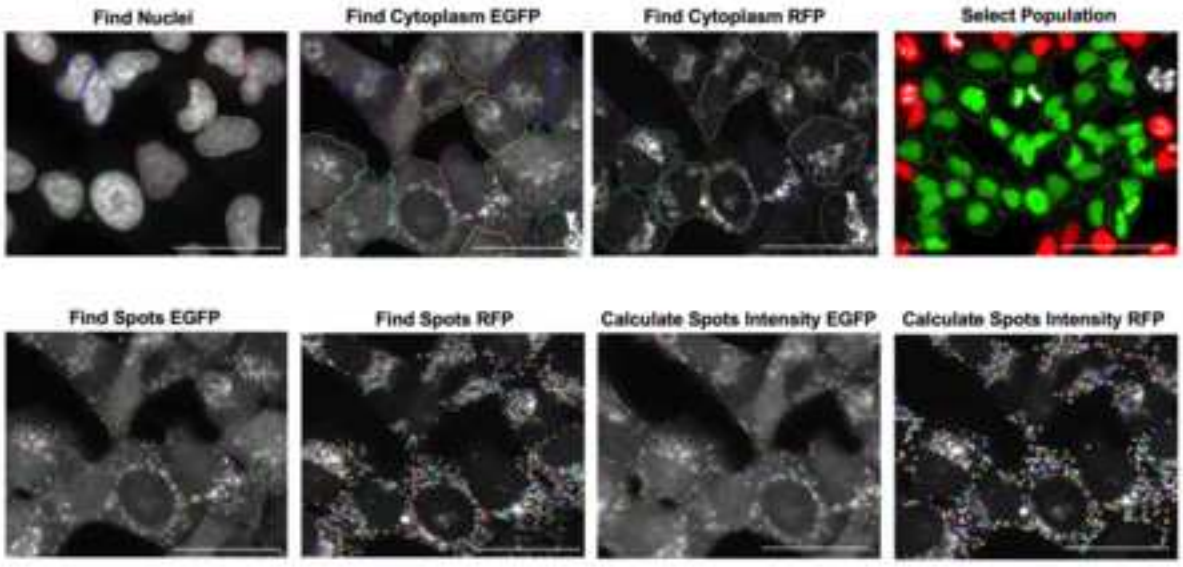

Figure 4

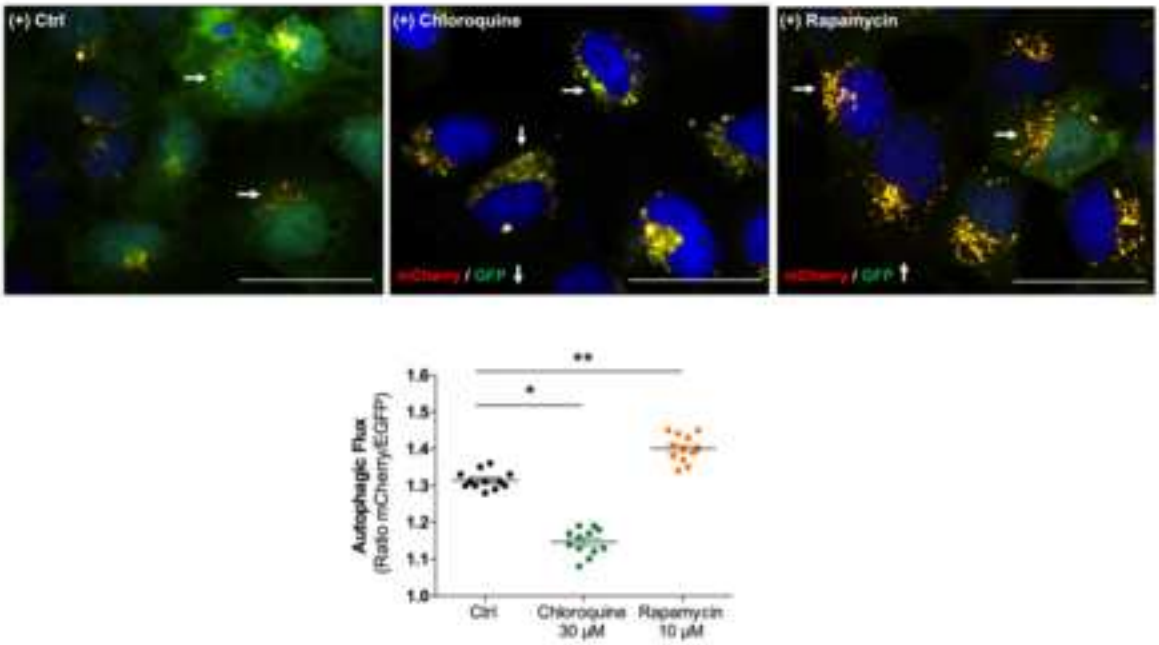

Table 1

Monoclonal population	Conditions	Ratio mCherry/EGFP flow cytometry	Ratio mCherry/EGFP HTS
Clon B	DMEM 2% FCS	1	1
	Rapamycin 5 µM	1.5	1.13
	Chloroquine 30 µM	0.96	0.76
Clon D	DMEM 2% FCS	1	1
	Rapamycin 5 µM	1.32	1.22
	Chloroquine 30 µM	1.05	0.76
Clon H	DMEM 2% FCS	1	1
	Rapamycin 5 µM	1.64	1.12
	Chloroquine 30 µM	0.88	0.87

Table 2

Channels	Emission (nm)	Excitation (nm)	Exposure (ms)
Brightfield	652–760	Transmission	20
Hoechst	410–480	360–400	100
Fluorescein	500–550	460–490	700
RFP	560–630	520–550	1000

Name of Material/Equipment	Company	Catalog Number
100 mm cell culture plate	Corning	430167
12-well multiplate	Corning	353043
15 mL Centrifuge conical tube	Falcon-Corning	352095
24-well multiplate	Corning	351147
25 cm2 Cell Culture Flask	Falcon-Corning	353014
384-well multiplate Cell Carrier	Perkin Elmer	6007550
6-well multiplate	Corning	351146
96-well multiplate	Corning	353077
Acoustic liquid handling technology	Labcyte	–
Chloroquine	Sigma-Aldrich	C6628
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO	D2650
Dulbecco's Modified Eagle's Medium (DMEM)	Lonza, Basel, Switzerland	BE-604F
Eagle's Minimum Essential Medium (EMEM)	ATCC	30–2003
FACScalibur cytometer	Becton Dickinson, CA	–
Fetal Bovine Serum (FBS)	Sigma-Aldrich, St. Louis, MO	F9665
Fetal Calf Serum (FCS)	Gibco by Life Technologies, CA	26010–074
FuGene	Promega	E2691
Handheld electronic 384 channel pipette	Integra	–
Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich	H6648
HEK 293-T17	ATCC	CRL-11268
High Content Screening System	Perkin Elmer	–
Hoechst 33342	Thermo Fisher Scientific	62249
Image Analysis Software	Perkin Elmer	–
Liquid Handler workstation	Perkin Elmer	–
Microplate washer robot	Biotek	–
Opti-MEM® (1x)	Thermo Fisher Scientific	11058
Paraformaldehyde (PFA)	Sigma-Aldrich	158127
pBABE-puro mCherry-EGFP-LC3B	Addgene, Cambridge, MA	22418
pCL-Eco	Addgene, Cambridge, MA	12371
Penicillin-Streptomycin (P/S)	Sigma-Aldrich	P0781
Phosphate-buffered saline (PBS)	MP Biomedicals	2810305

Puromycin	Sigma-Aldrich, St. Louis, MO	P8833
Rapamycin	Calbiochem, Germany	5053210
Software CellQuestPro	Becton Dickinson	–
Syringe filters 0.45 µm	Corning	CLS431220
T/C28a2	–	–
Trypsin	Gibco by Life Technologies, CA	15400054
Trypsin	Sigma-Aldrich, St. Louis, MO	SM-2002-C
VSV.G	Addgene, Cambridge, MA	14888

Comments/Description

Cell culture plate
Cell culture plate
Centrifuge conical tube
Cell culture plate
Cell culture flask
Cell culture plate
Cell culture plate
Cell culture plate
https://www.labcyte.com
autophagic flux inhibitor
Disolvent
T/C28a2 growth medium
HEK 293-T17 growth medium
https://www.bdbiosciences.com/en-eu
HEK 293-T17 serum
Serum
A nonliposomal mixture of lipids as a plasmid delivery method to create autophagy reported cell line
https://www.integra-biosciences.com/united-states/en/electronic-pipettes/viaflo-96384#downloads
Buffer
Kidney cell line. Cells were used to facilitate retroviral packaging
https://www.perkinelmer.com/es/product/operetta-cls-system-hh16000000
DNA staining
https://www.perkinelmer.com/es/product/harmony-4-9-office-license-hh17000010
https://www.perkinelmer.com/es/category/janus-liquid-handler-workstations
https://go.biotek.com/405tradein
Transfection medium
Fixer
Plasmid
Plasmid
Antibiotic
Buffer

Antibiotic
autophagic flux activator
https://www.bdbiosciences.com/en-eu
Sterile filter
human chondrocytes cell line
Trypsin used with T/C28a2 cells
Trypsin used with HEK 293-T17 cells
Plasmid

MANUSCRIPT ID: JOVE61078

TITLE: Autophagic Flux-Based Screening Assay for the Identification of Autophagy Modulators for Osteoarthritis

AUTHORS: Uxía Nogueira-Recalde, Francisco Triana-Martínez , Eduardo Domínguez, Beatriz Caramés

EDITOR´S COMMENTS

Thank you for your comments. We addressed point-by-point the formatting issues in order to follow the JoVE Editorial requirements. The revised manuscript with highlighted changes is resubmitted for your assessment.

Following please find our responses to the editorial and reviewer´s comments.

Editorial comments:

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise lines 31-43 to avoid textual overlap with previous publications.
3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts (without brackets) after the appropriate statements.
4. Please define all abbreviations before use, e.g., HBSS, HTS.
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Operetta®, Viaflo, Biotek 405 Select TS, JANUS, Harmony®

RESPONSE: Thank you for your comments. We revised the manuscript to fullfil the editorial requirements:

1. The manuscript was carefully reviewed to address spelling and grammar issues.
2. The introduction was revised to avoid potential text overlap with previous publications, specifically lines 31-43.
3. Text-formatting for references was changed as indicated.
4. Abbreviations were defined before use as follow: Osteoarthritis (OA) (Pag 2); Eagle´s Minimum Essential Medium (EMEM) (Pag 3); Fetal Bovine Serum (FBS) (Pag 3); Penicillin-Streptomycin (P/S) (Pag 3); Dulbecco´s Modified Eagle Medium (DMEM) (Pag 3); Hank's Balanced Salt Solution (HBSS) (Pag 3); Deoxyribonucleic acid (DNA) (Pag 3); hours (h) (Pag 3); Fetal Calf Serum (FCS) (Pag 4); Dimethyl Sulfoxide (DMSO) (Pag 5); Phosphate buffer saline (PBS) (Pag 5); High Content Screening System (HCS) (Pag 6); long Working Distance (WD) (Pag 6).
5. Commercial language was avoid as follow: Operetta was replaced by high-content fluorescent image acquisition and “a High Content Screening System (HCS), an automated microscope” (Pag 5 and 6); Biotek was replaced by “A microplate washer robot” (Pag 6); FACSCalibur and CellQuestPro software were replaced by flow cytometer instrument and flow cytometer software (Pag 5); Viaflo was replaced by “A

handheld electronic 384 channel pipette" (Pag 6); Echo was replaced by "An acoustic liquid handling technology" (Pag 6); JANUS was replaced by Automated liquid handler workstation (Pag 6); Harmony was replaced by "Image Analysis Software" (Pag 7).

Protocol:
1. Please consistently use the imperative (e.g., 'do this', 'ensure that') in the Protocol section.
2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.
3. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

RESPONSE: Thank you for your comments. We revised the manuscript to follow the journal requirements for the protocol, including the use of imperative tense, answering how the action step is performed, and identifying the pages with the essential steps of the protocol for filmable content.

Specific Protocol steps:
1. 1.1, 1.3, etc.: Please give volumes for all media.
RESPONSE: The volume amounts were added for all media used.

2. 2.3, 2.4, 2.6: Please give more information about how to operate these instruments and/or provide citations (note that links to product manuals should not be in the references but rather the Table of Materials or inline).

RESPONSE: The detailed information about each instrument is now included in the Table of Materials.

3. 3.4: Please provide more specific directions ('click', 'select', etc.) for software steps if they are to be filmed.

RESPONSE: The software analysis steps are now described as "Add" sequence on Protocol section 3. 3. Data analysis of autophagic flux (pages 7-8)

4. 3.5: Which steps do you mean by 'previously'?

RESPONSE: "Previously" means the specific relation between the mCherry/EGFP ratio of relative intensity spots (cells with higher flux are less green, which increases the mCherry/EGFP ratio in the cell) described in reference number 11.

Figures and Tables:
1. Please split the Figure file into one file per figure (6 in total). Please remove 'Figure 1' etc. and remove unnecessary white space.
2. Please remove the embedded table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
3. Figure 1: Please be consistent with tense (i.e., 'Develop' instead of 'Development of').

RESPONSE: Thank you for your comments. The Figures and Tables were revised and changed according to the editorial suggestions.

Discussion:
1. Please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods

RESPONSE: Thank you for your comments. The Discussion section was revised to cover the points suggested. Please refer to lines 413-432 for these information.

References:
1. Please ensure that the references appear as the following: [Lastname, F. I., LastName, F. I., LastName, F. I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
2. Please do not abbreviate journal titles.

RESPONSE: Thank you for your comments. The References section was revised to follow the journal requirements.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
2. Please revise the Table of Materials to more closely reflect JoVE format (in particular, one column each for the name, company, catalog number, and any notes). See attached Materials Table template.

RESPONSE: Thank you for your comments. The table of materials was revised to follow the journal requirements.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
In their manuscript, Nogueira-Recalde et al. describe an assay in order to monitor autophagic flux to perform screenings assays to find new autophagic modulators. Autophagy is a central mechanism for cell homeostasis, and in the last years it has been shown that its downregulation is involved in several human pathologies. Therefore, the discovery and development of new drugs able to modulate autophagy is of great interest. In their manuscript, the authors describe a cell-based imaging screening assay based on the use of human chondrocytes stably expressing the genetic reporter mCherry-EGFP-LC3B. Upon induction or autophagy, LC3 is lipidated and incorporated into the nascent autophagosome membrane, where it plays an essential role in autophagosome formation. Once autophagosome fuses with lysosome, LC3 is degraded inside the autophagolysosome. Therefore, LC3 degradation is used as a marker of autophagic flux. By using the mCherry-EGFP-LC3B reporter, LC3 fluorescence can be followed in live cells. Since GFP tag is more sensitive to acidic pH, once in the lysosome will be degraded and then red fluorescence coming from mCherry will predominate. The ratio between green and red fluorescence is then used as a measurement of autophagy activation. The manuscript

is well written and structured, and address a very relevant topic, which is the development of high-throughput assays for the discovery of modulators of autophagy. However, some parts of the method should be explained with more detail and addition of some results will help to fully characterize and validate the method described.

Major Concerns:

1. The first part of the manuscript describe the generation of a new stable cell line expressing mCherry-EGFP-LC3B (human chondrocytes). The authors should explain in more detail this part (how the transfection has been performed, % of transfection, how the selection of clones has been done…).

RESPONSE: A more detailed explanation about the generation of the autophagy reporter cell line of immortalized human chondrocytes was included in the protocol section (Pages 3 and 4).

2. In point 1.7, the authors describe the use of an HTS platform to quantify autophagic flux and select the best clone, comparing the results with those obtained by flow cytometry. It would be very helpful to include these comparative data (HTS vs. flow cytometry) and which is the autophagic flux for the clone selected.

RESPONSE: The quantitative data from Flow Cytometry and HTS analysis was included in Table 1. This comparative data was used to choose the clone providing the more robust and less variable autophagy signals.

3. In Figure 3 it is not clear what the authors wanted to show. Is it the same than in figures 4 and 5? Moreover, the images in figure 3 do not correspond to the same population of cells. Could the authors provide more information about that?.

RESPONSE: Figure 3 is an example on how to quantify autophagic flux from cellular images and Figure 4 and 5 showed the sequence analysis from the positive and negative controls, respectively.

4. What the colors red and green in the panel "Select Population" in figures 3,4 and 5 come from? What do they mean?

RESPONSE: We removed border objects in order to select for analysis whole cells (nucleus and cytoplasm). The software recognized whole cells in green (used for the analysis) and incomplete cells that do not fit within the scope of the objective in red (discarded for the analysis).

5. In figure 7, the authors show a representative image of cells with activated autophagy (+ rapamycin) or reduced autophagy (+ chloroquine). A control cell should be included in order to compare basal vs. activated/inhibited autophagy conditions. In addition, a quantification of the mCherry/GFP signal should be included in order to have a quantitative data for the validation of the method.

RESPONSE: A control corresponding to 2% FCS was now included in Figure 4.

Minor Concerns:

1. In figure to legend 6, it is stated that chloroquine inhibits autophagic flux, and then autophagic flux is "activated" (line 233). It should say "inhibited". Also in line 233, "autophagosomes were created" instead of "was created".

RESPONSE: Changes in the legend were made as the reviewer suggested.

2. Is it necessary to show 6 figures? The authors could discard figures 4 and 5, presenting the cell-based imaging method in figure 3, and then the application of this method to control, and autophagy-activated/inhibited conditions with their respective quantification in figure 6.

RESPONSE: We agree with the reviewer. Changes in the figures displayed were made as the reviewer suggested.

Reviewer #2:

Manuscript Summary:

The manuscript from Uxía Nogueira-Recalde et al. describes a methodology to visualize autophagic flux from a chondrocyte cell line in vitro. The authors use the pBabe-mCherry-GFP-LC3 packaged in adenovirus generated in HEK293 cells to infect cells of the T/C28a2 human chondrocyte cell line. The authors high mCherry-GFP-LC3-expressing clones and validate their response to Rapamycin and Chloroquine, two small molecules known to promote and inhibit autophagic flux, respectively. The authors then describe using an automated system to evaluate small molecules in high throughput. Overall, the protocol for generation of the cell line and automated process for drug testing is of importance to the field of cartilage biology, given that autophagy regulates chondrocyte homeostasis. There is, however, a lack of information given by the authors in how clones were selected from their populations for further analysis. Some clarification on experimental procedures are also needed in order for others to appropriately replicate the procedures described herein. Specific concerns are listed below.

Major Concerns:

1) It would be helpful for the authors to provide a brief protocol for the generation of the virus as well as the infected cell line. Currently they refer to reference 11 for this, however, it would benefit readers to know how this was completed in the authors hands.

RESPONSE: The explanation about the generation of the autophagy reporter cell line of immortalized human chondrocytes was included in the protocol section (Pages 3 and 4).

2) There is a lack of information as to how the authors collected "monoclones" from the infection step. It is necessary to know their exclusion/inclusion criteria to select monoclones, how many clones were assayed and how many were grown for validation and future studies using small molecule inhibitors. Was there a selection criteria for knowing which monoclones responded to rapamycin/chloroquine?

RESPONSE: To select the monoclones we used two methods: Flow cytometry and High-content fluorescent image adquisition. Both methods are thoroughly explained in the Protocol Section. Moreover, to select monoclones we used Rapamycin as an autophagic flux activator and Chloroquine as an autophagic flux inhibitor. Comparative quantitative data between both methods and between two monoclones using Rapamycin and Chloroquines are showed in Table 1.

3) Can the authors clarify what the compound/interval plate is? Is this just the small molecule tested at various concentrations?

RESPONSE: An interval plate was performed in order to dispense the proper amount of compound to achieve a final concentration of 10 µM.

4) On line 113, the authors use the acronym "long WD." Can the authors please describe what WD stands for?

RESPONSE: Long WD means “long Working Distance”. Long working distance provides a significant space between the lens surface and the object.

5) Lines 71-84 appear to be very repetitive to the protocol described in lines 87-93. Can the authors please clarify why the explanation of of lines 71-84 are needed?

RESPONSE:

6) In Figure 1, having images of the various different components of the protocol pathway would be visually appealing, especially for JOVE.

RESPONSE: We included images to Figure 1 as the reviewer suggested,

7) A description of the red and green nuclei should be given, at minimum in the figure legends in figures 3-5. It is assumed that these are selected vs boarder (unselected) objects, but this should be described.

RESPONSE: The description of this step is now included in the Figure Legends.

8) A graph of results from the addition of chloroquine vs Rapamycin vs untreated cells would be needed to show the validity of the approach for autophagic flux quantification (could be associated with Figure 6).

RESPONSE: A graph with the analysis results was included in Figure 6.

MANUSCRIPT ID: JOVE61078

TITLE: Autophagic Flux-Based Screening Assay for the Identification of Autophagy Modulators for Osteoarthritis

AUTHORS: Uxía Nogueira-Recalde, Francisco Triana-Martínez , Eduardo Domínguez, Beatriz Caramés

EDITOR´S COMMENTS

Thank you for your comments. We addressed point-by-point the formatting issues in order to follow the JoVE Editorial requirements. The revised manuscript with highlighted changes is resubmitted for your assessment.

Following please find our responses to the editorial and reviewer´s comments.

Editorial comments:

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.
2. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please revise lines 51-53 and 56-59 to avoid textual overlap with previously published work.
3. For each protocol step, please ensure you answer the “how” question, i.e., how is the step performed? Please address specific comments marked in the attached manuscript.
4. After you have made all the recommended changes to your protocol section, please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.
5. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
6. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
7. Figure 1: Please use the time unit (min) instead of “” symbol as in “20’, 10’”, etc. Please include a space between all numbers and the corresponding unit: 37 °C, 60 s, 24 h, etc. Please abbreviate liters to L (µL) to avoid confusion. Please include a space before FCS (i.e., 10% FCS, 2% FCS). Please change “Clon selection” to “Clonal selection”.
8. Figure 3 and Figure 4: Please include a scale bar, ideally at the lower right corner, for all microscopic images to provide context to the magnification used. Define the scale in the appropriate figure Legend.
9. Figure 4: Please include a space between all numbers and the corresponding unit: 30 µM, 10 µM.

RESPONSE: Thank you for your comments. We revised the manuscript to fullfil all the editorial requirements.