

Ferritinophagy: Assessing the Selective Degradation of Iron by Autophagy in Human Fibroblasts

Carmen J. Pastor-Maldonado¹, Tassula Proikas-Cezanne¹

¹ Interfaculty Institute of Cell Biology, Eberhard Karls University Tübingen

Corresponding Author

Tassula Proikas-Cezanne

tassula.proikas-cezanne@uni-tuebingen.de

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Abstract

Mutations in the autophagy gene *WDR45/WIP14* are the cause of beta-propeller-associated neurodegeneration (BPAN), a subtype of human diseases known as neurodegeneration with brain iron accumulation (NBIA) due to the presence of iron deposits in the brains of patients. Intracellular iron levels are tightly regulated by a number of cellular mechanisms, including the critical mechanism of ferritinophagy. This paper describes how ferritinophagy can be assessed in primary, skin-derived human fibroblasts. In this protocol, we use iron-modulating conditions for inducing or inhibiting ferritinophagy at the cellular level, such as the administration of bafilomycin A1 to inhibit lysosome function and ferric ammonium citrate (FAC) or deferasiox (DFX) treatments to overload or deplete iron, respectively. Such treated fibroblasts are then subjected to high-throughput imaging and CellProfiler-based quantitative localization analysis of endogenous ferritin and autophagosomal/lysosomal markers, here LAMP2. Based on the level of autophagosomal/lysosomal ferritin, conclusions can be drawn regarding the level of ferritinophagy. This protocol can be used to assess ferritinophagy in BPAN patient-derived primary fibroblasts or other types of mammalian cells.

Introduction

The WIP1 (WD-repeat interacting with phosphoinositides) proteins are evolutionarily conserved PI3P effectors with distinct roles in autophagy¹. The four human WIP1 proteins (WIP11 through WIP14) fold into seven-bladed β -propellers with two evolutionarily conserved regions in which homologous and invariant amino acids cluster on opposite sites of the propeller. One site is aligned with the phosphoinositide-binding region in propeller blade 5

and blade 6. The other site is aligned with the protein-protein interaction region where WIP1s associate with distinct members of the autophagy machinery or autophagy regulatory factors².

WIP14 functions in controlling energy-driven autophagy regulation and at the level of controlling the size of the growing nascent autophagosome³. A mutation in the *WIP14* gene, referred to as *WDR45*, is causative of beta-propeller-

associated neurodegeneration (BPAN), a neurodegeneration with brain iron accumulation (NBIA) subtype in human patients that is characterized by a hypointense iron halo in the substantia nigra and globus pallidus⁴. The presence of abnormal iron deposits in BPAN patients provokes neuronal damage that translates into encephalopathy, global developmental delay, seizures, and ultimately, parkinsonism, dementia, and spasticity⁵.

Iron homeostasis is tightly regulated by multiple cellular mechanisms, with the concentration of cytosolic iron being controlled by the expression levels and functionality of iron carriers, iron transporters, and iron storage proteins⁶. The concentration of cytosolic iron, in turn, determines whether degradation pathways such as ferritinophagy⁷ or the proteasomal clearance of oxidized ferritin⁸ are involved.

During ferritinophagy, ferritin is selectively recruited to autophagosomes by the cargo receptor NCOA4 and targeted for lysosomal degradation in response to low intracellular iron levels⁷. Given the role of WIPI4 in regulating the size of autophagosomal membranes³, it is reasonable to predict that the loss of this specific function may affect the selective sequestration of ferritin in autophagosomes and, thus, ferritinophagy. Studying this key step in iron metabolism may open doors to therapeutic options for BPAN patients; however, commonly used protocols to study ferritinophagy in human cells are only now being developed.

This paper describes a high-throughput, fluorescence-based method to assess ferritinophagy in human cells. The application of this assay to patient-derived BPAN cells can provide valuable insight into how ferritinophagy differs compared to healthy human cells and may serve as a

benchmark for understanding the molecular mechanisms underlying this rare human disease.

Protocol

1. Culturing and seeding primary cells

1. Fetch the vial with frozen cells from the liquid nitrogen tank, and keep it on ice until reaching the cell culture room. In a previously sterilized hood, hold the vial in the hand so the cell suspension thaws. Resuspend the cells, and transfer them to a 10 cm cell culture plate with 9 mL of prewarmed DMEM with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (P/S). Shake gently so that the cells distribute evenly on the plate, and incubate overnight at 37 °C and 5% CO₂.
2. The following morning, look at the cells to confirm that they are attached. Change the medium to fresh DMEM + 10% FCS + 1%P/S, and let the cells grow until they reach 80% confluency.
3. Remove the culture medium, wash 2x with DPBS, add 1 mL of trypsin to the plate, and incubate it at 37 °C and 5% CO₂ for 10 min. Add 9 mL of DMEM + 10% FCS to the plate, and resuspend the cells.
4. Transfer the cell suspension to a 15 mL centrifuge tube. Determine the cell count manually using a Neubauer chamber, and dilute the cell suspension in DMEM + 10% FCS until a cell concentration of 40,000 cells/mL is achieved.
5. In a glass-bottom 96-well plate, seed 100 µL/well of the cell suspension. Incubate at 37 °C and 5% CO₂ for 16 h.

2. Treatments

1. Prepare the following treatments according to the **Table of Materials**: control medium, control medium plus deferasiox (DFX) for iron chelation, or control medium plus ferric ammonium citrate (FAC) for iron loading. Prepare all the treatments in both the presence and absence of a lysosomal inhibitor (bafilomycin A1) for the assessment of the ferritinophagy flux.
2. Remove the culture medium from the plate, and replace it with the corresponding treatment (100 μL /well).
3. Incubate at 37 °C and 5% CO_2 for 6 h.

3. Fixation and staining

1. Aspirate the treatment medium, and wash 2x with DPBS (**Table of Materials**).
2. Add 100 μL of 3.7% paraformaldehyde (PFA, **Table of Materials**) per well. Fix the cells for 20 min at room temperature in darkness.
3. Remove the PFA, and wash the cells 2x with PBS/T (**Table of Materials**).
4. Block in PBS/T containing 1% bovine serum albumin (BSA) for 1 h at 4 °C.
5. Prepare the primary antibody (**Table of Materials**) mix in PBS/T (ferritin antibody 1:50, LAMP2 antibody 1:50).
6. Wash the cells 2x with PBS/T, and apply 50 μL of the antibody mix per well. Wrap the plates with parafilm, and incubate overnight at 4 °C.
7. The following morning, prepare the secondary antibody (**Table of Materials**) mix (1:200) in PBS/T.

8. Wash the cells 2x with PBS/T, and apply 50 μL of the secondary antibody mix per well. Incubate at 4 °C for 1 h in the dark.
9. Prepare 5 $\mu\text{g}/\mu\text{L}$ 4',6-diamidino-2-phenylindole (DAPI, **Table of Materials**) solution in room-temperature PBS.
10. Wash the cells 2x with PBS/T, and add 100 μL /well of DAPI stain. Incubate for 20 min at room temperature in the dark.
11. Wash the cells 2x with PBS, and add 50 μL of PBS to each well. Wrap the plates with parafilm, and store at 4 °C in the dark.

4. Automated imaging using confocal laser-scanning microscopy

1. Exchange the PBS in the plate for 50 μL of fresh PBS at room temperature.
2. Cover the plate with aluminum foil and take it to the microscopy room.
3. Turn the confocal laser-scanning microscope on, set a sample holder for 96-well plates on the microscope table, and select an appropriate objective (here: 40x). Refer to **Table 1** for details.
4. Adjust the imaging settings (**Table 1**).
 1. In **Smart Setup**, select the **lasers and filters**, and assign them to tracks (Tracks 1-3) for optimal image quality and speed (**Table 1**).
 2. When selecting the **type of experiment**, click on **Tiles** to enable the option of selecting and saving the determined **X,Y positions on the plate**.
 3. In the **Imaging Setup** module, visualize the **acquisition channels**, the **tracks** assigned to them,

and their **excitation and emission wavelengths** (Table 1).

4. In the **Acquisition Mode** module, select the following: Scan Speed: 6; Direction: bidirectional; Averaging: 2x; Bits per pixel: 8.
5. In the **Channels** module, adjust the **laser power**, **pinhole**, and **master gain** for each channel individually (Table 2). Fine-tune these parameters to achieve properly exposed images without oversaturating them.
6. Click on the **Focus strategy** module.
 1. Select **Combine Software Autofocus** and **Definite Focus**.
 2. Under **Reference Channel and Offsets**, select the **most stable/brightest channel** as a **reference** (normally DAPI or 488 nm).
 3. Under **Stabilization Event Repetitions and Frequency**, select **Standard**.
7. In the **Software Autofocus** module, select the following: **Mode | Intensity; Search | Smart; Sampling | Fine; Relative Range**.
8. Click on the **Tiles** module.
 1. Select **Positions | single positions**.
 2. Under **Advanced Setup**, navigate through the plate, identify a position for imaging, and click on the **+** button under the **Position Setup** tab.
 3. Label each position with additional information that will be recorded on the image metadata. To do so, open the **Properties** tab, click on the **wheel icon** next to the **Category** dropdown menu, and click on **New** to open a new dialog box where the **new category name**

is to be entered. To assign a category to a certain position, select the **position**, click on the **Properties** tab, go to the **Categories** dropdown menu, and select the corresponding **label**.

NOTE: It is recommended to create a category for each treatment to label each position with the treatment that the cells were subjected to.

4. Under **Sample Carrier**, select **Multiwell 96-Cellvis glass bottom #0**. Click on **Calibrate** to calibrate the microscope to the plate surface. Choose between **1 point** or **7 point calibration** depending on the system's specifications.
5. Under **Options**, select a **tile overlap** of **10%**. Under **Travel in Tile Regions**, select **Comb**. Tick the following: **use stage speed from stage control**, **use stage acceleration from stage control**, and **image pyramid during acquisition**.
5. Run **image acquisition**, and save the image results (czi files) and the image metadata (csv files) on a portable hard drive.
6. Export the images as TIFF files.

5. High-throughput image analysis

1. Create a separate metadata spreadsheet file with the following columns: Position, Well, Condition, Series, and Set. The appropriate data to fill these columns are retrieved from the original metadata file that comes from confocal laser-scanning microscopy.
2. Download the open-source software CellProfiler 4.2.4 (<https://cellprofiler.org/previous-releases>).

3. Launch CellProfiler 4.2.4 by double-clicking, and import the CellProfiler pipeline (**Supplemental File 1**) by drag and drop.

NOTE: Find below the steps to compile the CellProfiler pipeline (**Supplemental File 1**) for the automated recognition of fibroblasts using DAPI staining (**Module 1, nuclei recognition**, see **Table 2**) and background fluorescence in single cells derived from AF488 (**Module 2, cell recognition**, see **Table 2**). Puncta (termed objects) recognition is determined by the puncta size and the puncta fluorescence intensity over background (**Module 3, puncta recognition**, see **Table 2**). Finally, according to the cell boundaries, the puncta recognized by the software are assigned to the corresponding cells (**Assign relationships**). A detailed description of how to implement a CellProfiler pipeline for autophagy studies has been published previously⁹. In addition, **Table 2** contains the CellProfiler settings used in this study. Moreover, **Supplemental File 2** displays the CellProfiler pipeline (**Supplemental File 1**) with screenshots taken in consecutive order and referring to the modules detailed in **Table 2**.

4. Before adapting the CellProfiler pipeline (**Supplemental File 1**), **drag and drop** individual .czi image files or a folder containing multiple .czi image files to the **Image** module.
5. Customize the image recognition pipeline. In the **Names and Types** module, replace the **c1-c3 code** with a code that allows the software to identify and sort the single-channel images to be analyzed.
6. Tune the pipeline settings for the **IdentifyPrimaryObjects** and **IdentifySecondaryObjects** modules by optimizing the

numeric entries in the modules. The values used in this study are presented in **Table 2**.

7. To track the adjustments, select the **Test Mode** option by clicking on the **Start Test Mode** button. Then click the **Step** button between the modules. Wait for pop-up windows (the original input image, the recognition outlines drawn over the original image, and the analysis result) showing how an image would be analyzed with the settings that were just entered.
8. Once the pipeline is optimized, end the **test mode**. Execute the analysis by activating the **Exit Test Mode**, followed by clicking on the **Analyze Images** button.
9. After the analysis is complete, verify the accuracy of the cell and puncta detection by comparing the input images with the overlay outputs (cell boundaries, puncta) generated by CellProfiler. If not satisfactory, adjust the numerical values in the different modules (**Table 2**) until the analysis result is sufficiently accurate.

6. Data analysis

1. After running the analysis, wait for the software to create a set of tabular text files with the results. If using the provided CellProfiler pipeline (**Supplemental File 1**), look for the following output files:
 1. The **CellProfilerOutputCells** file, which lists the image number assigned by the software, the cell number assigned to single cells, the original file name, and the path indicating where the files are stored, the number of puncta (ferritin, LAMP2) per cell, and the mean puncta area (ferritin, LAMP2) per cell.
 2. The **CellProfilerOutputExperiment** file, which provides information on the technical details related

to the experiment (e.g., the software version, the image channels, the metadata tags).

2. Open these files as spreadsheets.
3. Proceed to conduct the statistical analysis of the puncta counts and puncta co-localization numbers using the preferred statistics software (**Table of Materials**).

Representative Results

Human skin-derived primary fibroblasts (F-CO-60) from healthy donors (**Figure 1A**) were prepared for ferritinophagy assessments using bafilomycin A1 treatment followed by immunostaining of endogenous ferritin and LAMP2, automated image analysis, and CellProfiler-based analysis (**Figure 1B**).

The colocalization of endogenous ferritin and LAMP2 increased after the lysosomal degradation of ferritin was blocked by the addition of bafilomycin A1, consistent with its ability to inhibit lysosomal V-ATPase enzymes and, thus, block ferritinophagy⁷ (**Figure 2**). In addition, the software-based cell recognition, as well as the ferritin and LAMP2 recognition, are shown as an example (**Figure 2**).

In this regard, it should be noted that the CellProfiler pipeline outlined here is extremely versatile and can be adapted to additional and/or alternative readouts, such as to assess the effects of certain BPAN mutations on cell growth, mitochondria, or other organelles in the context of functional single-cell marker systems.

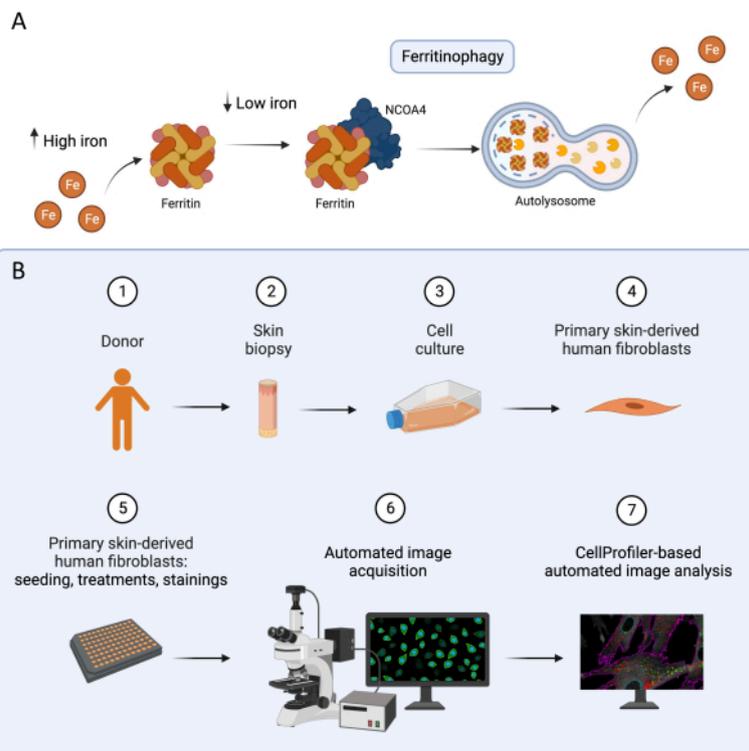


Figure 1: Experimental overview. Graphical representation of **(A)** the process of ferritinophagy and **(B)** the experimental workflow to assess ferritinophagy in primary skin-derived human fibroblasts, as described in this protocol. [Please click here to view a larger version of this figure.](#)

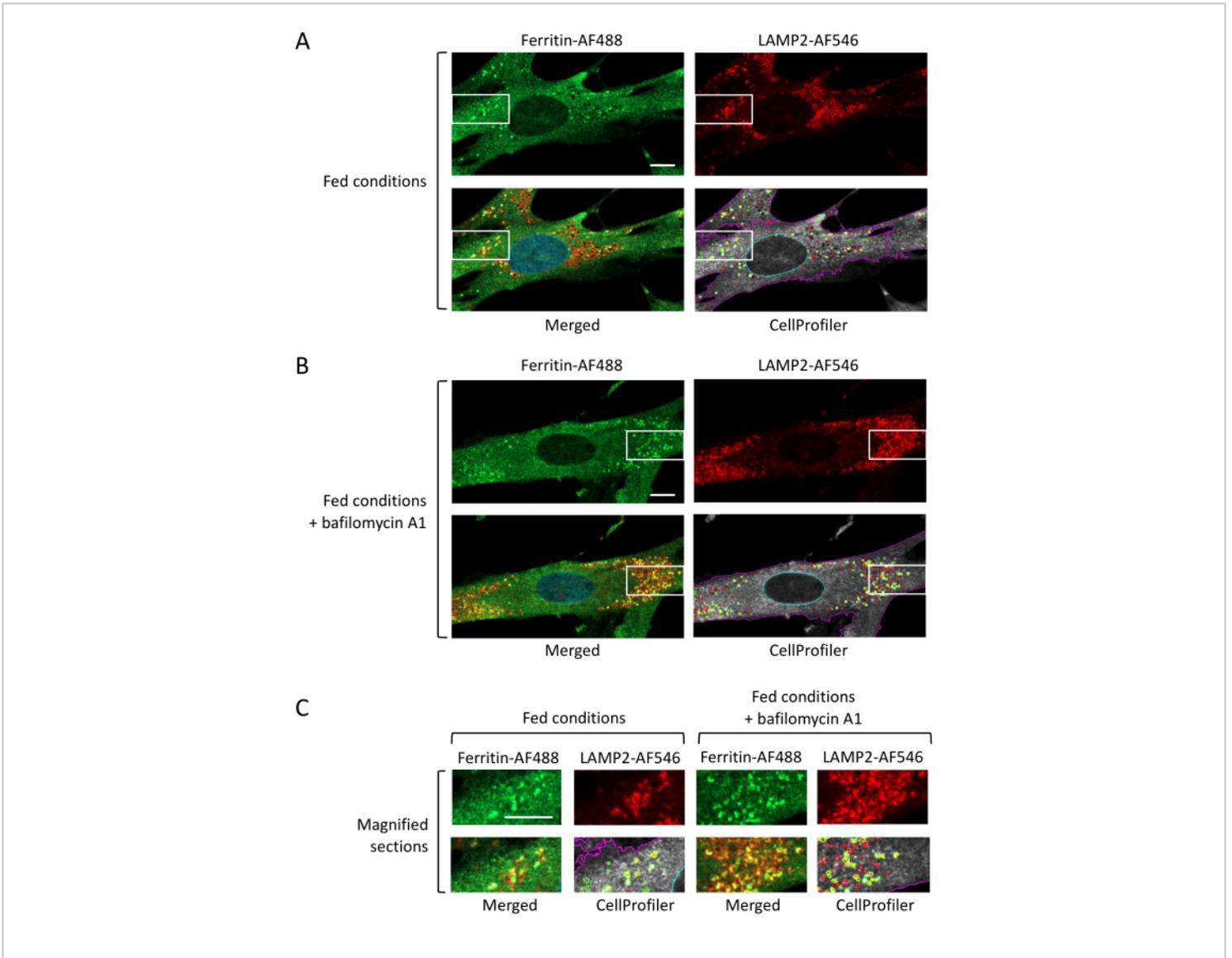


Figure 2: Representative images. Primary skin-derived human fibroblasts in control medium (fed conditions) in the (A) absence or (B) presence of bafilomycin A1 were subjected to an indirect immunofluorescence analysis using anti-ferritin and anti-LAMP2 antibodies, while the cell nuclei were stained with DAPI. Exemplary fluorescence images acquired with confocal laser-scanning microscopy automation are presented (scale bars: 10 μ m). Changes in ferritin and LAMP2 abundance and colocalization could be observed upon lysosomal inhibition upon bafilomycin A1 administration. The CellProfiler-based analysis is also displayed, showing the software-derived image overlays of cell recognition (purple), nuclei (blue), ferritin (green), LAMP2 (red), and co-localizing ferritin/LAMP2 (yellow) puncta. (C) Magnified image sections (scale bar = 10 μ m).

[Please click here to view a larger version of this figure.](#)

Table 1: The confocal laser-scanning microscopy settings used in this study. [Please click here to download this Table.](#)

Table 2: The numerical CellProfiler settings used in this study. [Please click here to download this Table.](#)

Supplemental File 1: The CellProfiler pipeline used in this study. [Please click here to download this File.](#)

Supplemental File 2: Display of the CellProfiler pipeline (see Supplemental File 1) with screenshots taken in consecutive order (see Table 2). [Please click here to download this File.](#)

Discussion

This method, which uses automated fluorescence microscopy combined with open-access CellProfiler-based image analysis⁹ to assess the intracellular localization of key ferritinophagy factors, was designed to provide valuable information about the efficacy of lysosomal ferritin clearance *via* selective autophagy, referred to as ferritinophagy⁷. This protocol enables the handling of big sample sets with multiple cell lines and treatments simultaneously and the assessment of desired readouts, such as ferritin puncta numbers and ferritin puncta numbers colocalizing with LAMP2, which is a typical lysosomal marker for assessing the ferritinophagy flux. However, it should be emphasized here that accurate, automated image analysis depends on the image quality, which, in turn, depends on the antibody specificity and optimal imaging. Therefore, software-based image analysis should only be performed with high-quality images.

The modulation of iron levels in cells can provide valuable information about the mechanisms that are activated to regulate iron homeostasis in the context of disease. This paper describes a method to study BPAN diseases in patient-

derived cells treated with iron-loading and iron-chelating agents. Deferasiox (DFX) is a known iron chelator used extensively in the field¹⁰. Upon application, it traps the free iron in the cytoplasm and triggers a cellular response to iron starvation. Since the aim of this study was to assess ferritinophagy, depriving the cells of iron was a straightforward way to promote ferritin degradation *via* autophagy as a compensation mechanism to replenish the cellular iron pool. Ferric ammonium citrate (FAC), on the contrary, is used to load cells with iron. Cells activate ferritin synthesis as a response to excessive and, thus, toxic cytosolic iron concentrations¹¹. Ferric ammonium citrate, hence, promotes ferritin synthesis and the entrapment of iron within the ferritin heteropolymers, which can, in turn, activate ferritinophagy.

To study the ferritinophagic flux, in this protocol, we selectively stain the key players in the process-ferritin and the lysosomes (here marked by LAMP2)-and assess their colocalization. A high percentage of colocalizing puncta is indicative of effective lysosomal degradation of ferritin. To maximize the information retrieved from such experiments, additional dyes or marker antibodies can be used.

Of note, there are technical challenges inherent in this method. Primary fibroblasts derived from different human donors may proliferate and grow differently *in vitro*. Hence, for comparable results, different cells should be seeded at the same passage. Therefore, it is advisable to perform a test experiment before proceeding with this protocol to examine the doubling times of the cells or cell lines that are to be used.

This method is not restricted to the study of ferritinophagy; by simply changing the treatments and antibodies used, it can be repurposed to assess various other selective autophagy pathways; for example, mitophagy can be examined by using

CCCP as a mitochondrial stress inducer and optineurin, LC3, and LAMP2 antibodies for the staining¹².

Overall, the combination of automated image acquisition and CellProfiler analysis constitutes a powerful tool for the high-throughput functional assessment of single human fibroblasts. This is especially relevant for studying human diseases, for which the analysis and comparison of large cohorts of patient-derived cells and healthy donor-derived cells are of great interest.

Disclosures

The authors declare no conflicts of interest.

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Figure 1 was created using BioRender.

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