Chapter 8

Studying Selective Autophagy of Protein Aggregates Using Particles Induced by Multimerization (PIMs)

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Abstract

Selective autophagy of protein aggregates, called aggrephagy, is vital for maintaining cellular homeostasis. Classically, studying aggrephagy has been challenging due to the infrequent occurrence of autophagic events and the lack of control over the specificity and timing of protein aggregation. We previously reported two variants of a PIM (particles induced by multimerization) assay that enable the formation of chemically induced, fluorescently labeled protein aggregates in cells. PIMs are recognized by the selective autophagy machinery and are subsequently degraded in the lysosome. By making use of pH-sensitive fluorescent proteins, such as GFP or mKeima, the PIM assay allows for direct visualization of aggregate clearance in cells. Here, we describe a protocol for the use of the PIM assay to study aggrephagy in live and fixed cells.

Key words Aggrephagy, Live-cell imaging, Inducible dimerization

1 Introduction

The formation of potentially toxic protein aggregates containing damaged and mutated proteins is a hallmark of neurodegenerative diseases and aging [1]. In order to combat the presence of protein aggregates, cells employ a selective autophagy process called aggrephagy. Aggrephagy is initiated by ubiquitination of the aggregated protein and subsequent accumulation of selective autophagy receptors (SARs). Then, an isolation membrane, or omegasome, forms around the aggregate. This membrane expands and ultimately seals, forming an autophagosome around the protein aggregate. The autophagosome then fuses with late endosomes (LE) and lysosomes (LY), resulting in the acidification and clearance of the protein aggregate [2].

Studying selective autophagy is challenging since the process occurs infrequently under basal conditions. To study the aggrephagy pathway, previous research has therefore focused on the induction of protein aggregates using either chemical induction

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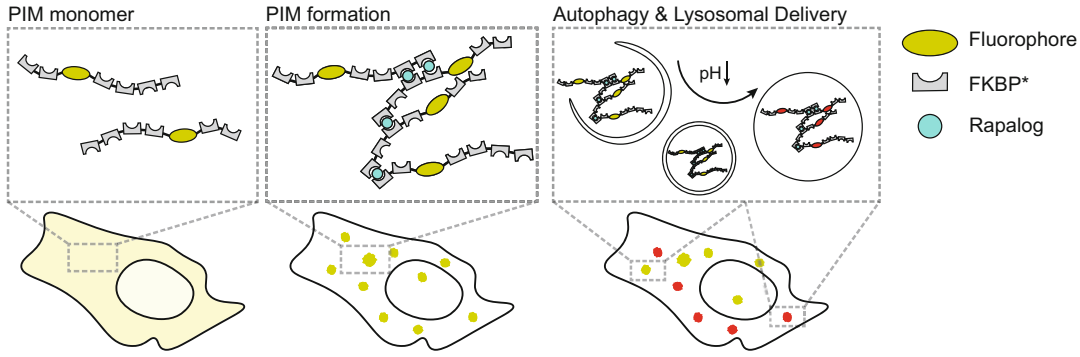
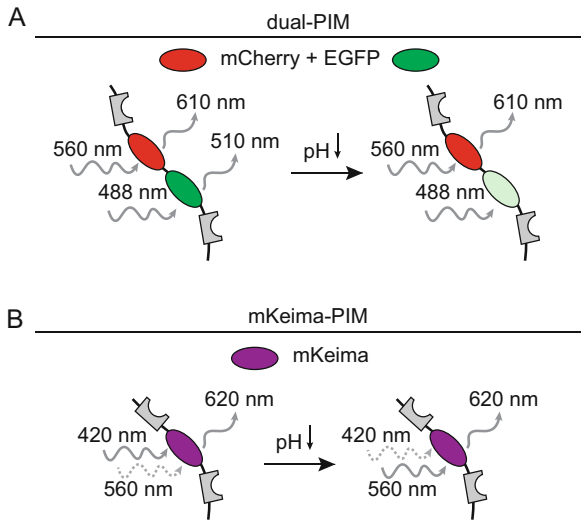


Fig. 1 Schematic representation of the PIM assay. Dual-PIM or mKeima-PIM constructs are soluble and homogeneously distributed throughout the cytosol prior to rapalog addition. The addition of rapalog results in rapid multimerization and aggregate formation of PIM monomers. PIM aggregates are recognized by the aggrephagy machinery and undergo selective lysosomal degradation. The presence of mCherry/EGFP or mKeima fluorophores allows monitoring of acidification, indicating entry into lysosomes

of protein misfolding or the expression of aggregation-prone proteins [3, 4]. These approaches have provided valuable insight into the aggrephagy process, but they suffer from several drawbacks. While the expression of aggregation-prone proteins often yields aggregation in cells, this approach does not provide temporal control over aggregation [3–5]. Furthermore, the expression of mutant proteins, such as mutant huntingtin, can impair autophagy and preclude analysis of the physiological aggrephagy pathway [5–7]. Conversely, the formation of aggregates by inducing proteotoxic stress can be precisely timed [3], but it affects a large part of the proteome and likely also impairs the autophagy pathway.

In order to mitigate these issues, we previously introduced the PIM (particles induced by multimerization) assay as a tool to study aggrephagy in live and fixed cells [8]. The PIM construct is comprised of multiple multimerization domains, including four FKBP* homodimerizing domains. The addition of the rapamycin analog AP20187 (hereafter rapalog) enables the rapid formation of protein aggregates (Fig. 1). To monitor the autophagic clearance of these inducible aggregates, an mCherry-EGFP tag was added to the PIM construct (termed dual-PIM). This dual-tag approach was previously used to study the acidification of p62 inclusions and autophagosomes [3, 9]. In short, monitoring dual-PIMs relies on the difference in resistance to lysosomal acidification and breakdown between GFP and mCherry fluorophores. Upon lysosomal acidification, GFP rapidly and irreversibly quenches while mCherry fluorescence remains functional (Fig. 2a). Therefore, ratiometric imaging of the GFP/mCherry signal serves as a direct readout for lysosomal delivery of aggregates. This approach yields a robust tool to visualize aggrephagy of individual aggregates in cells. Notably, because mCherry, unlike GFP, resists lysosomal degradation, dual-PIMs can be used in both live- and fixed-cell assays [9, 10].



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	dual-PIM	mKeima-PIM
Green emitting fluorophore compatibility	-	+
Live-cell imaging	+	+
Fixed-cell imaging	+	-
Ease of use; e.g. visibility by eye	High	Medium (at neutral pH)

Fig. 2 Characteristics of the dual- and mKeima-PIM tools. (**a**) Schematic representation of the dual-PIM tool. At neutral pH, dual-PIM fluorophores mCherry and EGFP are both functional and show emission at 610 nm and 510 nm, respectively. Acidification results in the loss of EGFP emission at 510 nm. (**b**) Representation of mKeima-PIM construct containing the mKeima fluorophore. Emission of mKeima occurs consistently at 620 nm, while efficient excitation of mKeima depends on pH. At neutral pH, the mKeima excitation spectrum peaks at 420 nm, while excitation using 560 nm light is most efficient at low pH. (**c**) Schematic depicting the experimental applicability of dual-PIM and mKeima-PIM

However, since dual-PIMs rely on continuous imaging of GFP to determine the acidification status of PIMs, they cannot be used in combination with other green-emitting fluorophores. This limits the applicability of dual-PIMs for multicolor live-cell imaging. Therefore, we developed mKeima-tagged PIM (mKeima-PIM) to facilitate live-cell imaging of aggrephagy in combination with (endogenous) GFP-tagged proteins [11]. In the mKeima-PIM constructs, the mCherry-EGFP tag is exchanged for an mKeima fluorophore [12]. mKeima has a pH-sensitive bimodal excitation spectrum, peaking at 440 nm in neutral pH environments and at 568 nm at low pH. Moreover, like mCherry, mKeima is resistant to lysosomal degradation and has an emission spectrum peaking around 620 nm (Fig. 2b). These characteristics allow for simultaneous imaging of green-emitting fluorophores. We showed that mKeima-PIMs enable the visualization of aggregate clearance in combination with markers for various stages of the aggrephagy pathway, such as endogenously tagged LE/LY marker Rab7 [12]. Together, our previous reports established PIMs as a novel inducible aggrephagy probe and provided valuable insight into the spatiotemporal regulation of aggregate clearance.

Here, we describe an in-depth protocol for probing the selective autophagy of protein aggregates using dual-PIM and mKeima-PIM constructs, highlighting the relative strengths of each tool.

This protocol will enable researchers to study aggrephagy using fixed- and live-cell approaches, providing an easy-to-use template for developing (high-throughput) light microscopy analysis of aggrephagy without the side effects of expressing toxic proteins or inducing general protein stress.

2 Materials

2.1 Constructs

1. The constructs used in this protocol are all available on Addgene.
   (a) Dual-PIM: Addgene plasmid #111758; http://n2t.net/addgene:111758; RRID:Addgene_111758.
   (b) mKeima-PIM: Addgene plasmid #174650; http://n2t.net/addgene:174650; RRID:Addgene_174650.
2. The constructs were amplified using regular DH5alpha bacteria and prepared using the Qiagen Plasmid Plus Midi Sample Kit (Qiagen, cat. 12941). DNA was eluted in Milli-Q and stored at −20 °C.
3. *See* **Note 4.1**. for recommendations on cloning strategies.

2.2 Cell Culture

1. In this protocol, we used HeLa-WT (ATCC) and previously described HeLa-Rab7 endogenous knock-in cells [12]. *See* **Note 4.2.1**. for additional information on cell line compatibility.
2. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, penicillin, and streptomycin.

2.3 Sample Preparation: Transfection and PIM Formation

Cell Seeding

1. Cells.
2. Round micro cover glasses, No. 1, 18 or 25 mm (VWR, cat. 48380-046 and cat. 48380-080).
3. Tweezers.
4. 12-well or 6-well plastic cell culture plates.

Transfection

1. Optimem (Gibco, Thermo Fisher Scientific, cat. 15392402).
2. Fugene 6 (Promega, cat. E2692).
3. DNA constructs, as described above.

PIM Formation

1. Rapalog (Takara, B/B homodimerizer, cat. 635059). Rapalog is aliquoted upon arrival in order to avoid multiple freeze-thaw cycles. The stock concentration is 0.5 mM.

Live Cell: Sample Mounting

1. Attofluor Cell Chamber (Thermo Fisher Scientific, cat. A7816) or Ludin chamber, depending on the coverslip size.
2. Imaging medium (regular culturing medium or DMEM without phenol red; Thermo Fisher Scientific, cat. 21063029).

Fixed Cell: Sample Fixation

1. 4% prewarmed PFA.
2. PBS 1×.
3. Prolong Diamond Antifade Mountant (Fisher Scientific, cat. P36970).
4. Microscope slides.

2.4 Microscopy

Microscopes

1. For the mKeima-PIM assay, we used a Nikon Eclipse Ti Microscope equipped with:
   (a) ASI motorized stage (MS-2000-XYZ) with Piezo top plate and on-stage incubation chamber (Tokai Hit, INUBG2E-ZILCS).
   (b) Perfect Focus System (Nikon).
   (c) CSU-X1-A1 confocal head (Yokagawa).
   (d) Plan Apo VC 60×, N.A. 1.4, oil immersion objective (Nikon).
   (e) Evolve 512 EMCCD (Photometrics).
   (f) Excitation lasers used for excitation; Vortran Stradus 445 nm (mKeima-PIM neutral pH), Cobolt Jive 561 nm (mKeima-PIM low pH), and Cobolt Calypso 491 nm (GFP).
   (g) Filter cubes; ET-mCherry (49008, Chroma) and ET-GFP (49002, Chroma).
   (h) MetaMorph 7.7 software (Molecular Devices).
2. For imaging of the dual-PIM assay, we used a Nikon Eclipse TE2000E equipped with:
   (a) Motorized stage (Prior) equipped with a Tokai Hit incubation chamber (INUBG2E-ZILCS).
   (b) Perfect Focus System (Nikon).
   (c) Plan Fluor 40×, NA 1.3, or Plan APO 60×, NA 1.4 oil immersion objectives (both Nikon).
   (d) pco.edge 4.2 CLHS sCMOS camera (Excelitas Technologies).
   (e) CoolLED pE4000 (CoolLED) setup for illumination.

(f) Filter cubes; ET-EGFP (49002, Chroma), ET-mCherry (49008, Chroma).

(g) μManager software [13].

2.5 Data Analysis

1. Fiji (Fiji is just ImageJ, v1.53t).
2. Comdet (v. 0.5.5.) plugin for Fiji.
   (a) For a guide on installation, see (https://github.com/UU-cellbiology/ComDet). There, you will also find extensive information on the Comdet particle-detection pipeline.
3. Microsoft Excel.
4. GraphPad Prism v. 9.4.0.

3 Methods

3.1 . Constructs

1. In Fig. 2, we provide a brief overview of the fluorophore characteristics of dual-PIM (Fig. 2a) and mKeima-PIM (Fig. 2b), as well as the applicability and general strengths of each tool (Fig. 2c).

3.2 Cell Culture

1. Maintain cells in full DMEM prior to splitting and seeding.
2. Seed cells on glass coverslips 1 day prior to transfection.
   (a) Note that the optimal density and amount of cells per coverslip might depend on the specific experimental setup. In general, we recommend carefully titrating densities to prevent overcrowding, since this will hamper both sample acquisition and downstream analysis.

3.3 Sample Preparation: Transfection and PIM Formation

1. Transfect seeded cells ±24 h prior to the start of the experiment. For this, we transfect 1 μg of plasmid DNA per 18 mm coverslip or 2 μg of plasmid DNA for a coverslip of 23 mm in diameter. For additional information on expression using transient transfection, (*see* **Notes 4.2.2**. and **4.2.3**).
   (a) First transfer the appropriate amount of plasmid DNA to a sterile Eppendorf tube. Add Optimem (100 μL per well) and resuspend well by pipetting.
   (b) Add Fusegene6 to the mixture (3 μL Fusegene6 per 1 μg of DNA).
   (c) Mix well by resuspending vigorously or by briefly vortexing (3–5 s). Briefly spin down using a tabletop centrifuge. Let the mixture incubate for 10–15 min.
   (d) Add the transfection mix drop by drop to the well.

2. After ±24 h of transfection, samples can be used for protein aggregation induction. For an illustrated overview of the PIM formation protocol used here, see Fig. 1. Rapalog addition and washout can be performed in culturing plates (for fixed-cell imaging or prior to mounting and transfer to the microscope) but can also be done successfully on stage.
   (a) Replace the culturing medium with medium (≤ 250 μL for 12-well plate, ≤ 500 μL for 6-well plate) containing rapalog (500 nM).
   (b) After 45 min, remove unbound rapalog by washing once and then adding culturing medium.
   (c) Depending on the experimental timeline, cells can now be mounted or fixed at the desired time points. *See* **Note 4.3**. for further recommendations on fixations.

3.4 Microscopy

In Fig. 3, we provide some example images of dual-PIM fixed-cell (Fig. 3a, b) and mKeima-PIM live-cell (Fig. 3c, e) experiments.

Fixed-Cell Microscopy

1. Remove the culturing medium and wash cells with 1× PBS.
2. Add prewarmed 4% PFA and incubate for 10 min.
3. Wash three times using 1× PBS.
   (a) If no further immunofluorescent labeling is required, proceed to **step 4**. Alternatively, samples can be permeabilized and probed with antibodies.
4. Stain nuclei with DAPI (1:1000 diluted in 1× PBS) for 2 min.
5. Wash three times using 1× PBS.
6. Dip the coverslip in Milli-Q and let dry prior to mounting.
7. Cells can now be mounted using Prolong Diamond. Apply a small drop of Prolong Diamond mounting medium on a microscopy slide and place the coverslip face-down on the drop. Press gently and dry overnight at RT.

Live-Cell Microscopy for Dual-PIM and mKeima-PIM

1. Mount cells in the appropriate imaging ring using sterile tweezers.
2. Add imaging medium. Use a minimum of 500 μL for long-term imaging (> 6 h).
   (a) Alternatively, to avoid evaporation and gas exchange, imaging rings can be sealed by placing a coverslip on top and removing excess medium. When sealing imaging rings, make sure to use imaging medium that contains

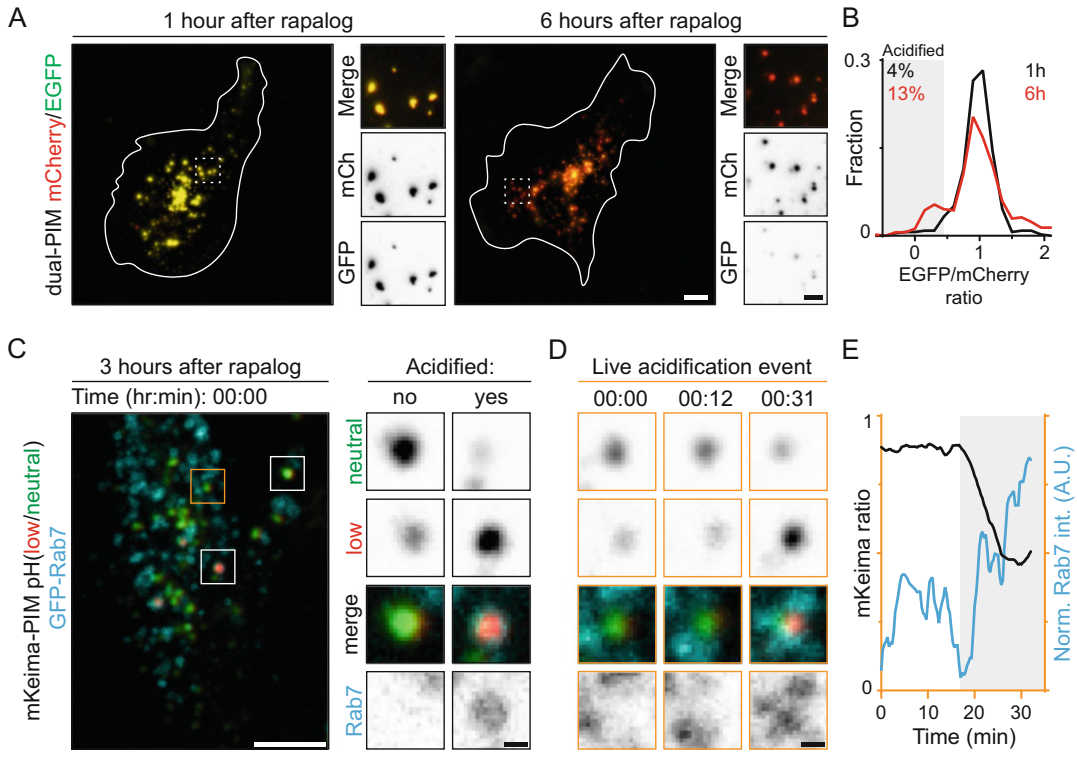

Fig. 3 Application of dual-PIM and mKeima PIM assays to monitor aggrephagy. (**a**) Representative images of HeLa-WT cells expressing dual-PIM after 1 and 6 h of rapalog addition. Zooms (dashed white boxes) show a number of dual-PIM aggregates in neutral (dual positive, yellow) or acidic compartments (mCherry positive, red). (**b**) Frequency distribution of EGFP/mCherry signal intensity of individual aggregates from cells shown in (**a**). The emergence of a peak at low EGFP/mCherry ratios indicates the increased clearance of dual-PIM aggregates (13% vs. 4%). (**c**) Representative image of HeLa-KI cells expressing endogenously tagged GFP-Rab7 and mKeima-PIM 3 h after rapalog addition. Zooms (solid white boxes) show examples of mKeima-PIMs at neutral- and low pH (Acidified no/yes). Only mKeima-PIMs in low-pH environments colocalize with high levels of Rab7. (**d**, **e**) Zooms (orange boxes) show time-lapse imaging of the acidification of a single mKeima-PIM aggregate coinciding with the accumulation of GFP-Rab7. (**e**) Graph showing the quantification of the acidification event shown in D. Scale bars represent 10 μm (**a**, **c**), 2 μm (zooms of **a**), and 1 μm (zooms of **c** and **d**)

CO_2. For this, place imaging medium in an incubator supplemented with 5% CO_2 overnight (e.g., a 15 mL tube with the cap unscrewed).

3. Locate PIM-expressing cells.
   (a) When using dual-PIM, we generally locate cells expressing PIMs using the mCherry signal. Be aware that dual-PIM-expressing cells can be difficult to locate prior to rapalog addition due to low expression levels. Aggregated dual-PIMs should be readily visible in the mCherry channel at all stages post rapalog addition (1–24 h).

(b) When using mKeima-PIM, we generally locate expressing cells by excitation with a 561 nm laser due to a better signal-to-noise ratio. At the very early stage of aggregation, visualization using a 445 nm laser might be favored due to the lack of cleared PIMs.

4. For long-term imaging of aggregate turnover in whole cells, use 5–10 min intervals. To limit phototoxicity, this interval can be increased.
5. Imaging settings for fast, high-resolution live-cell imaging of single acidification events and aggregate dynamics:
   (a) Time intervals of ≤20 s between frames.
   (b) Z-range of ±5–8 μm depending on cell type with z-steps of ≤0.5 μm.
   (c) Exposure time at approximately 100 ms per frame, but this can be changed depending on laser power and the required speed of acquisition.

3.5 Data Analysis

For an overview of the data analysis pipeline and example images, see Figs. 3 and 4.

1. For the analysis of whole cell aggregate clearance, use the Comdet plugin to perform automated detection and measurements of single particles and colocalization analysis.
   (a) First, ensure the image contains only the channels that you want to be included into the Comdet detection.
   (b) Draw an ROI around the object of interest (e.g., delineating one cell). Note that the plugin can also run without a prespecified ROI. It will then do particle detection on the entire image. *See* **Note 4.4.1**. for additional information on ROI selection.
   (c) Run Comdet v. 0.5.5. (Plugins→Comdet v.0.5.5.). See Fig. 4 for a summary of the Comdet pipeline and detection/colocalization parameters. A link to a comprehensive explanation of the plugin can be found in (*see* **Note 4.4.2**).
   (d) Ensure the "Calculate colocalization?" box is marked and set the threshold for colocalization, called "Max Distance," at ±3 pixels. For visualization and further analysis, ROIs detected can be visualized and added to the "ROI manager" tab. Comdet also produces a "Summary" table. If you want to use this table for data collection of multiple ROIs, select "Append" from the dropdown menu.
   (e) Per channel, specify the detection parameters. For the data presented in this protocol, we selected "Include larger particles?" and "Segment larger particles?," and

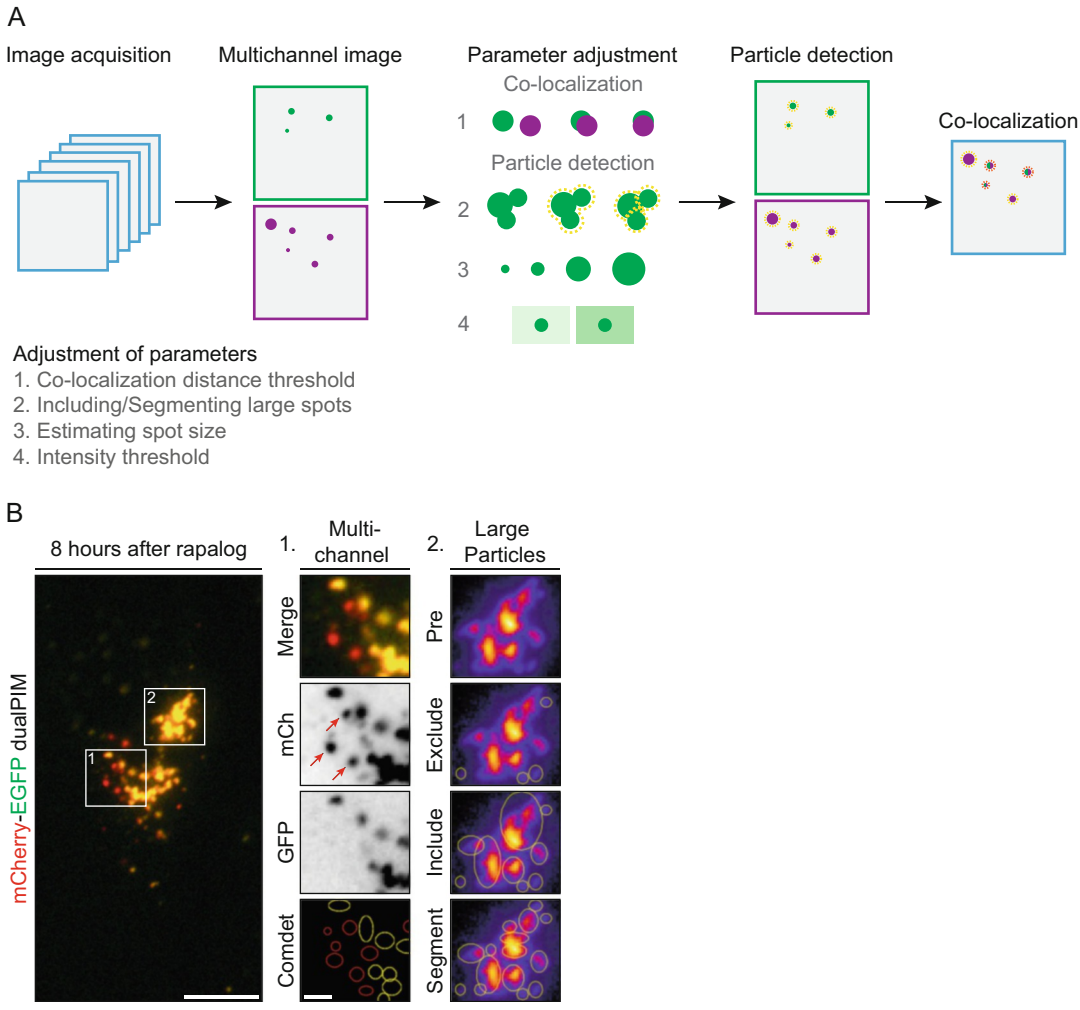

Fig. 4 Automated particle detection and colocalization analysis using Comdet. (**a**) Schematic representation of Comdet detection and colocalization pipeline. For colocalization of detections in multichannel images, first select the colocalization distance threshold (1). Next, per channel, select particle detection parameters: (2) Determine whether Comdet should include or exclude larger particles from the detection and whether larger particles should be segmented. (3) Set the estimated particle size in pixels. (4) Finally, adjust the intensity threshold value. These values can be checked using the "preview" function and adjusted to facilitate detection of particles in all channels of the image. (**b**) Representative image of HeLa-WT cell expressing the dualPIM construct 8 h after rapalog addition. Zooms on the left show mCherry-EGFP positive aggregates at neutral pH and acidified aggregates that are only positive for mCherry (red arrows). Annotation of the Comdet detection shows particles assigned as mCherry positive (red) or dual positive (yellow) puncta. Zooms on the right show Comdet detection of larger particles. Scale bar represents 10 μm and 2 μm (zooms)

set "Approximate particle size" at 3–4 pixels and the intensity threshold at 8–10 depending on the channel.

(f) Comdet now generates "Summary" and "Results" tables containing all detections, intensities, and colocalization

analysis. Save the "Log" window with the parameters used during detection as a reference. *See* **Note 4.4.3**. for notes on Comdet application.

(g) Next, transfer the data to Excel and calculate acidification ratios. Since Comdet measurements are already background corrected, the intensity measurement (Integrated density) can be used directly to calculate the ratios.

- To determine the mKeima ratio r_{mKeima}, divide intensity at neutral pH by the total intensity of the mKeima signal in both channels.

$$r_{\text{mKeima}} = \frac{I_{\text{neutral pH}}}{I_{\text{neutral pH}} + I_{\text{low pH}}}$$

- To determine the dual-PIM ratio $r_{\text{dual-PIM}}$, simply divide the intensity of the EGFP signal by the mCherry signal intensity.

$$r_{\text{dual-PIM}} = \frac{I_{\text{EGFP}}}{I_{\text{mCherry}}}$$

- This provides an acidification ratio between 0 and 1, indicating cleared and non-cleared aggregates, respectively.

(h) It is possible that the value of mKeima-PIM intensities is slightly negative, due to the Comdet background subtraction of aggregates in highly acidic (low $I_{\text{neutral pH}}$) or neutral (low $I_{\text{low pH}}$) environments. We set these values to 0. The resulting ratios still accurately reflect the acidification status of the aggregates and are either 0 or 1.

(i) After calculating ratios of dual-PIM intensities, we perform normalization by fitting a single (cells at early time points) or double Gaussian (cells at later time points) distribution in GraphPad PRISM. We then use the peak of this Gaussian to normalize individual ratios. In the case of a double Gaussian fit, we use the peak associated with the higher EGFP/mCherry ratio, since this peak represents the aggregates in neutral environments.

(j) The (normalized) dual- and mKeima-PIM ratios can then be visualized using a frequency distribution plot (Fig. 3b). Another convenient estimation of aggrephagy activity can be made by calculating the amount of aggregates with low dual-PIM ratios (e.g., <0.4).

2. For analysis of single events over time (example shown in Fig. 3f), an aggregate can be manually traced to measure acidification (for example data, see Fig. 3c, e).

(a) For each frame, using Fiji, draw a small ROI around a single aggregate and measure all channels (mCherry/EGFP or mKeima-neutral/mKeima-low including third channel, e.g., Rab7-GFP).

(b) Obtain background measurements by performing multiple measurements per channel for each frame around the aggregate, where background signal in the cytosol is homogeneously distributed.

(c) Subtract the average background intensity from each channel to calculate the background-corrected intensity values.

(d) The acidification ratio can then be calculated as described above.

(e) Normalize the acidification ratio by dividing the EGFP/mCherry or mKeima ratios by the average ratio of the first ±5 frames (Fig. 3e).

(f) The acidification of single aggregates can then be plotted as a single curve. Overlaying the intensities of GFP-tagged proteins in the aggregate ROI can then be used to visualize the dynamic association and dissociation of these factors during the aggrephagy process.

(g) Multiple acidification events can then be averaged by synchronization based on the onset of acidification (ratio falling <0.9).

4 Notes

4.1 Cloning and Viral Production of PIM Constructs

1. Naturally, the PIM module can be appended with other fluorophores or other sequences. Be aware that cloning using the PIM module can be challenging. The construct contains repetitive sequences due to the multiple FKBP domains. Especially, PCR amplification can yield unexpected, unwanted results. We recommend avoiding PCR amplification of the construct if possible. If alternatives are unavailable, purification and excision of single bands of PCR product on a DNA gel prior to ligation, together with whole plasmid sequencing of the finalized construct, are recommended.
2. Note that the production of lentiviral particles containing a correct copy of the original PIM construct and its successful integration in target cells is likely challenging. We speculate this is a consequence of the highly repetitive sequences found in the PIMs.

4.2 Expression of PIM Constructs

1. In this protocol, we used WT and Rab7-KI HeLa cells, but we previously successfully transfected PIMs into multiple other cell lines (including HEK293, RPE-1, and U2OS).
2. For the data provided in this protocol we used PIM constructs that used a crippled β-actin promoter (Addgene plasmid # 111758 or Addgene plasmid # 174650) to ensure moderate expression levels. In our hands, the level of protein expression driven by this promoter is sufficient to form PIMs in most transfected cells without creating overexpression artifacts, such as aggregation prior to rapalog addition. In some instances, higher expression levels might be desirable. For this purpose, we also deposited a pGW1-dual-PIM variant under a strong CMV promoter. Alternatively, one could suggest varying the amount of DNA transfected per well.
3. In our hands, transfection of PIM constructs using Fusegene6 is well tolerated by cultured cells. To optimize cell viability; (1) make sure to use DNA preps that are kept no longer than 1 year at 4 °C, (2) consider replacing the medium with full DMEM after 6–8 h of transfection.

4.3 Fixation and Antibody Labeling

1. Fluorescence of non-degraded mCherry and EGFP is maintained after fixation, and the remaining fluorescent signal allows for robust imaging and quantification of mCherry/EGFP ratios. Therefore, we opt not to probe the PIMs with secondary antibodies. We recommend keeping fixed samples at 4 °C for short-term storage and suggest imaging within 3 days after fixation.

4.4 Comdet: Aggregate Detection and Analysis

1. When automating larger batches of particle detection, make sure to perform the Comdet detection on the entire image. Since the threshold for detection of spots relies on the background values, it is critical that these are included. Therefore, do not crop images to precise cellular or subcellular scales. Instead, a restrictive ROI can be placed. Comdet will then only detect particles inside the ROI but will determine the threshold based on the entire image.
2. An elaborate explanation of the installation, particle detection, and use of the plugin can be found here; https://github.com/UU-cellbiology/ComDet/wiki.
3. The optimal settings for Comdet detection of aggregates will depend on the imaging setup, sample, and research question. As a convenient starting point for optimizing detection, we recommend the following: a particle size of >3 pixels is sufficient to detect PIMs without introducing noise. The desired signal-to-noise ratio (SNR) depends heavily on the imaging

setup and will vary between 4 and 20. Comdet offers a convenient "preview" function that allows for easy optimization.

References

1. Hipp MS, Kasturi P, Hartl FU (2019) The proteostasis network and its decline in ageing. Nat Rev Mol Cell Biol 20:421–435
2. Jahreiss L, Menzies FM, Rubinsztein DC (2008) The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes. Traffic 9:574–587
3. Pankiv S, Clausen TH, Lamark T et al (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282: 24131–24145
4. Bjørkøy G, Lamark T, Brech A et al (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol 171:603–614
5. Ashkenazi A, Bento CF, Ricketts T et al (2017) Polyglutamine tracts regulate beclin 1-dependent autophagy. Nature 545:108–111
6. Martin DDO, Ladha S, Ehrnhoefer DE, Hayden MR (2015) Autophagy in Huntington disease and huntingtin in autophagy. Trends Neurosci 38:26–35
7. Wong YC, Holzbaur ELF (2014) The regulation of autophagosome dynamics by huntingtin and HAP1 is disrupted by expression of mutant huntingtin, leading to defective cargo degradation. J Neurosci 34:1293–1305
8. Janssen AFJ, Katrukha EA, van Straaten W et al (2018) Probing aggrephagy using chemically-induced protein aggregates. Nat Commun 9: 4245
9. Kimura S, Noda T, Yoshimori T (2007) Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 3:452–460
10. Katayama H, Yamamoto A, Mizushima N et al (2008) GFP-like proteins stably accumulate in lysosomes. Cell Struct Funct 33:1–12
11. Katayama H, Kogure T, Mizushima N et al (2011) A sensitive and quantitative technique for detecting autophagic events based on lysosomal delivery. Chem Biol 18:1042–1052
12. Janssen AFJ, Korsten G, Nijenhuis W et al (2021) Direct observation of aggregate-triggered selective autophagy in human cells. J Cell Sci 134:jcs258824
13. Edelstein AD, Tsuchida MA, Amodaj N et al (2014) Advanced methods of microscope control using μManager software. J Biol Methods 1:e10