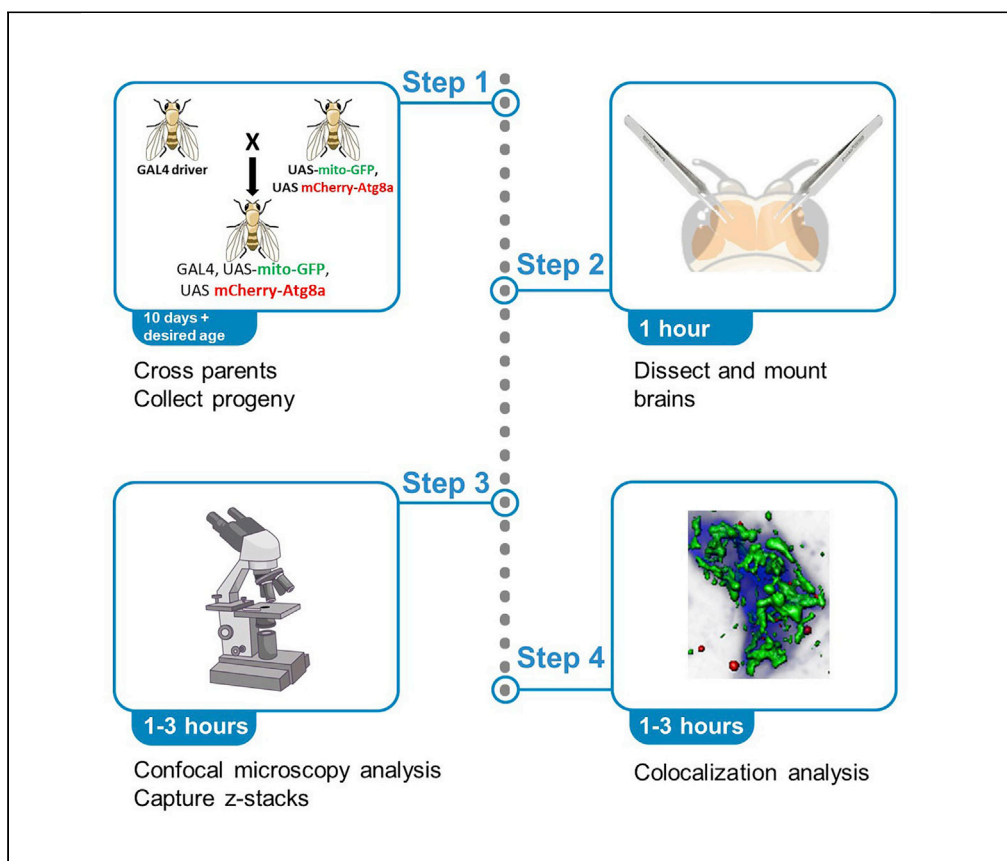


## Protocol

# 3D quantification of autophagy activation and autophagosome-to-mitochondria recruitment in a *Drosophila* model of Parkinson's disease



Here, we describe a protocol for comprehensive quantification of autophagosome recruitment to mitochondria as an early step in mitophagy. Data collected using this protocol can be useful in the study of neurodegenerative disease, cancer, and metabolism-related disorders using models in which co-expression of mito-GFP and mCherry-Atg8a is feasible. This protocol has the advantage of assessment in an *in vivo* model organism (*Drosophila melanogaster*), where tissue-specific mitophagy can be investigated.

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### Highlights

Protocol to quantify early mitophagy in the adult *Drosophila* brain

Mitochondria and autophagosomes are visualized in 3D with mitoGFP and mCherry-Atg8a

Brains are dissected, and confocal images of dopaminergic neurons are acquired

Colocalization analyses of Atg8a with mitochondria determine ongoing mitophagy

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## Protocol

3D quantification of autophagy activation and autophagosome-to-mitochondria recruitment in a *Drosophila* model of Parkinson's diseaseSusana J. Gutierrez-Luke,<sup>1</sup> Amber N. Juba,<sup>2,4</sup> Giulia Bertolin,<sup>3,5,\*</sup> and Lori M. Buhlman<sup>2,\*</sup><sup>1</sup>Arizona College of Osteopathic Medicine, Midwestern University, Glendale, AZ 85308 USA<sup>2</sup>College of Graduate Studies, Midwestern University, Glendale, AZ 85308 USA<sup>3</sup>Univ Rennes, CNRS, IGDR (Genetics and Development Institute of Rennes), UMR 6290, 35000 Rennes, France<sup>4</sup>Technical contact<sup>5</sup>Lead contact\*Correspondence: [giulia.bertolin@univ-rennes1.fr](mailto:giulia.bertolin@univ-rennes1.fr) (G.B.), [lbuhlman@midwestern.edu](mailto:lbuhlman@midwestern.edu) (L.M.B.)  
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## SUMMARY

Here, we describe a protocol for comprehensive quantification of autophagosome recruitment to mitochondria as an early step in mitophagy. Data collected using this protocol can be useful in the study of neurodegenerative disease, cancer, and metabolism-related disorders using models in which co-expression of mito-GFP and mCherry-Atg8a is feasible. This protocol has the advantage of assessment in an *in vivo* model organism (*Drosophila melanogaster*), where tissue-specific mitophagy can be investigated.

For complete details on the use and execution of this protocol, please refer to (Cackovic et al., 2018).

## BEFORE YOU BEGIN

Mitochondrial autophagy (mitophagy) is a process in which damaged mitochondria are selectively degraded to prevent potentially harmful consequences to cell and tissue homeostasis (Youle and Narendra, 2011). This gatekeeping mechanism is particularly relevant in postmitotic cells like neurons, where global mitochondrial fitness is required to meet the high energetic demands of these cells. A broad wave of reports have identified two Parkinson's disease (PD)-related proteins, parkin and PINK1, as key mediators of mitophagy (Pickrell and Youle, 2015). *D. melanogaster* is a convenient model for following PINK1/parkin-mediated mitophagy, as *park*-null flies (*park* is the fly orthologue of human *PARK2/PARKIN*) display severe mitochondrial abnormalities leading to altered neuronal morphology, reduced lifespan, and male sterility (Greene et al., 2003; Pesah et al., 2004). However, growing evidence also identifies PINK1/parkin-independent mitophagy programs (Villa et al., 2018), and it is reinforced by the recent finding that basal mitophagy can occur independently of parkin in *Drosophila* (Lee et al., 2018).

This protocol was used to address the effect of *parkin* loss of function on mitophagy initiation in *Drosophila* dopaminergic neurons. However, it can be applied to a variety of mitophagy paradigms in flies. Mitophagy initiation can be quantified in a wide range of cell and tissue types by incorporating different GAL4 enhancers driving expression of fluorescent constructs. Such constructs should be chosen as follows: one construct must enable the visualization of the mitochondrial network (i.e., a mitochondrially-targeted GFP/mito-GFP), and a second construct should be an autophagy-related marker (i.e., mCherry-Atg8a). A microscope setup (spinning-disk or confocal) with compatible lasers – 488 nm and 532 nm – are needed to detect the fluorescent signal of the GFP and mCherry, respectively. CO<sub>2</sub> delivery to a blowgun and pad for anesthesia are required for fly sorting. An



image-processing software to analyze the degree of colocalization between GFP/mCherry is required. This protocol was developed for use with Image Pro Premier 3D.

### Generating *Drosophila* stocks

⌚ Timing: a minimum of 14 weeks

1. Obtain parent *Drosophila* stocks from Bloomington Drosophila Stock Center: <https://bdsc.indiana.edu/index.html>.
  - a. Fly stock containing the *GAL4* transcription activator construct and preferred promoter on the third chromosome (e.g., stock no. 8848, which has a tyrosine hydroxylase promoter for *GAL4*).
  - b. Fly stock containing the *UASmito-GFP* construct on the second chromosome (stock 8442).
  - c. Fly stock containing the *UASmCherry-Atg8a* construct on the second chromosome (stock 37750).
  - d. Fly stock 5439 containing a second chromosome balancer and a phenotypic marker gene on the non-balancer sister chromosome.

**Note:** Balancer chromosomes confer visible phenotypes, are homozygous lethal, and are engineered with multiple nested inversions to prevent recombination during mitosis. This stock contains a balancer that confers curly wing phenotype; the sister chromosome contains a gene that confers eye and body phenotypes to facilitate confirmation of chromosomal inheritance.

- e. Maintain stocks in vials on standard molasses food.
  - f. Transfer fly stocks to a new food vial at least once per month by rapidly removing the Flug® and inverting the fly vial over the new one. Tap flies into the new vial and close with a new Flug®. Use a computer mouse pad to tap flies to the bottom of the new vial. Vials should be  $\frac{1}{2}$  full of standard cornmeal and molasses food.
2. Generate genetic recombination stock harboring *UASmito-GFP* and *UASmCherry-Atg8a* on chromosome 2.
    - a. Place 5 to 10 CO<sub>2</sub>-anesthetized virgin females from one UAS stock in standard food vials with 3 to 5 anesthetized males from the other UAS stock. Vials should be  $\frac{1}{4}$  to  $\frac{1}{2}$  full of standard cornmeal and molasses food.

**Note:** Collecting females within 8 h of eclosion from pupa cases ensures that they have not been fertilized. For sexing and phenotyping images and information, we recommend the *Atlas of Drosophila Morphology: Wild-type and Classical Mutants* (Gompel, 2013).

- b. Transfer parental cross vials to new food vials two times per week. Keep original vials which have eggs, larvae and pupa from the F1 generation. Pupa are visible as soon as four days after parent flies are placed in a vial.

**Note:** Adult flies begin to eclose from pupa cases about ten days after parents are placed in a vial. Newly-eclosed adults are fertile, so parent cross vials must be discarded within 20 days after parents are placed in a food vial to avoid inter-generation contamination.

- c. After about 10 days, begin collecting F1 non-curly winged virgins.

**Note:** The chromosome carrying the gene conferring curly-wing phenotype is the sister of the chromosome carrying *UASmito-GFP*. *UASmCherry-Atg8a* stocks are homozygous for this transgene. Recombination of the two UAS transgenes will occur in a small portion of these females.

- d. Place the F1 generation virgins with new males from the original *UASmito-GFP* stock in a new food vial. Transfer flies to a new food vial two times per week. Keep original vials which have eggs, larvae and pupa from the F2 generation.
- e. Collect curly-winged F2 male progeny. If the UAS transgenes have recombined, they will be passed to the F2 generation.
- f. To create “clones,” place one F2-generation curly-winged male with five virgins from stock 5439. Transfer flies to a new food vial two times per week. Keep original vials which have eggs, larvae and pupa from the F3 generation.
  - i. Do this up to forty times until recombination of the *UASmito-GFP* and *UASmCherry-Atg8a* is successful.
- g. Collect and combine curly-winged, non-glazed eyed virgins and males from F3 progeny. Transfer flies to a new food vial two times per week. Keep progeny from one clone separate from that of another.
- h. To determine successful recombination, run standard PCR for GFP and mCherry on collected F3 progeny.
  - i. Crudely grind one F3 curly, non-glaze eyed fly per PCR sample in extraction buffer.
  - ii. Examples of primers for GFP are 5' AAGCTGACCCTGAAGTTCATCTGC (forward) and 5' CTTGTAGTTGCCGTCGTCCTTGAA (reverse) with a 201 bp product. Examples of primers for mCherry are 5' CCAAGCTGAAGGTGACCAA (forward) and 5' TCTTCTTCTGCAT TACGGGG (reverse) with a 288 bp product.

#### PCR cycling conditions GFP

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	30 s	30
Annealing	68°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4°C	indefinite	1

#### PCR cycling conditions mCherry

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	30 s	30
Annealing	55°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4°C	indefinite	1

- i. If GFP and mCherry are present in an F3 generation clone, flies can be transferred once per month. Constructs will not be lost with inter-generational fertilization because the balancer chromosome will maintain the mito-GFP and mCherry-Atg8a constructs.
3. Maintain stocks in vials on standard molasses food, transferring to new food vials at least once per month.
4. Generate flies expressing mito-GFP and mCherry-Atg8a in GAL4-producing cells.
  - a. Place about 5 males from mito-GFP and mCherry-Atg8a recombination step with about 10 virgins from the GAL4 stock.
  - b. Transfer flies to a new food vial two times per week and keep old food vials that contain eggs, larvae and pupa for experimental flies.
  - c. Begin collecting non-curly winged flies. These will harbor all three constructs.

**Optional:** Assessing mitophagy initiation in genetically modified flies will require additional crossing and possible recombination depending on which chromosome the additional gene is located. We used *park*-null *park*<sup>25/25</sup> (Greene et al., 2003) flies in our 2018 study (Cackovic et al., 2018). Experimental flies should always be backcrossed using balancer chromosomes, a variety of which are available at The Bloomington Drosophila Stock Center at Indiana University (<https://bdsc.indiana.edu/stocks/balancers/index.html>).

### Preparation of standard cornmeal molasses *Drosophila* food vials

⌚ Timing: 3.5 h

5. Prepare 10% methylparaben by adding 100 g of Tegosept®, an anti-fungal agent, to 1 L of molecular grade ethanol and mix well.
6. Add 3600 mL of reverse osmosis (RO) water to a large rice cooker and bring to a boil.
7. Add 490 mL of RO water to a 1 L graduated cylinder then slowly add 247.1 mL of molasses, 247.1 mL of light corn syrup and mix well.
8. Add 620 mL of RO water to a 1 L beaker. Add a stir bar followed by 41.2 g of NutriSoy® flour and mix well. Once the soy flour is thoroughly mixed, add 82.4 g of active dry yeast and mix until well combined.
9. Measure 411.8 g of yellow cornmeal and 41.2 g of NutriFly® *Drosophila* Agar Gelidium into a 2 L beaker, then add 1400 mL RO water and mix with a spoon.
10. Add in the following order to the boiling water in the rice cooker: cornmeal/agar mixture, soy flour/agar, molasses/corn syrup and continually stir with a spoon to avoid clumping and burning.
11. Bring to a total volume of 7 L with RO water.
12. Allow food to boil while stirring for 10 min.
13. Turn off the rice cooker and allow food to cool to 70°C.
14. Add 30.9 mL of propionic acid and 70 mL of 10% methylparaben and mix well.
15. Transfer food to a food pourer or pour by hand into standard *Drosophila* vials in racks.
16. Cover with cloth and allow to sit for at least 4 h to overnight.
17. Add Glad® Press and Seal® to the top of each rack; flip and store at 4°C.
18. Allow food to warm to 22°C to 25°C before transferring flies. Use Flugs® to cap each vial.

### Preparation of extraction buffer for PCR

⌚ Timing: 20 min

19. 10 mM Tris-HCl
20. 1 mM EDTA
21. 25 mM NaCl
22. supplement with 1 µL of Proteinase-K per 50 µL on the day of extraction

### Preparation of solutions for *Drosophila* brain dissection and fixation

⌚ Timing: 20 min

23. Phosphate buffer with Triton™ X-100 (PBT)
24. 1× phosphate buffered saline (PBS)

### Preparation of solutions for optional immunofluorescence

⌚ Timing: 20 min

25. 0.3% phosphate buffer with Triton™ X-100 (PBT)
26. 1× phosphate buffered saline (PBS)

**KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
(optional) Rabbit anti-tyrosine hydroxylase	EMD Millipore	AB152MI
(optional) Alexa Fluor 405-conjugated anti-rabbit	Invitrogen	A48254
<b>Chemicals, peptides, and recombinant proteins</b>		
Grandma's Molasses, 1 gal	<a href="https://www.amazon.com">amazon.com</a>	N/A
Agricore Corse Yellow Cornmeal	<a href="https://www.webrestaurantstore.com">webrestaurantstore.com</a>	N/A
Karo Light Corn Syrup, 1 gal	<a href="https://www.amazon.com">amazon.com</a>	N/A
Tegosept®	Genesee	20–258
Molecular grade EtOH	Sigma	E7148-1GA
NutriSoy® flour	Genesee	62–115
Red Star Active dry yeast		2751
NutriFly® Drosophila Agar Gelidium	Genesee	66–103
Aroma Housewares 60-Cup Rice Cooker	<a href="https://www.amazon.com">amazon.com</a>	ARC-1033E
Propionic acid	Fisher Scientific	AC149300025
10× Phosphate buffered saline (PBS)	Fisher Scientific	AM9625
Triton™ X-100	Sigma-Aldrich	T8787-100ML
37% formaldehyde	Sigma-Aldrich	252549-100ML
Normal goat serum	Vector Laboratories	S-1000
Molecular-grade glycerol	Fisher Scientific	AC158922500
Molecular Probes Probes™ ProLong™ Diamond Antifade Mountant	Fisher Scientific	P36961
<b>Experimental models: organisms/strains</b>		
Drosophila stock containing UAS-mito-GFP construct	Bloomington Drosophila Stock Center	8442
Drosophila stock containing UAS-mCherry-Atg8a construct	Bloomington Drosophila Stock Center	37750
Drosophila stock containing GAL4 with desired promoter	Bloomington Drosophila Stock Center	N/A
Drosophila stock containing second chromosome balancers	Bloomington Drosophila Stock Center	5439
<b>Oligonucleotides</b>		
GFP (forward) 5' AAGCTGACCCTGAAGTTCATCTGC	Integrated DNA Technologies	N/A
GFP (reverse) 5' CTTGTAGTTGCCGTCGTCCTTGAA	Integrated DNA Technologies	N/A
mCherry (forward) 5' CCAAGCTGAAGGTGACCAA	Integrated DNA Technologies	N/A
mCherry (reverse) 5' TCTTCTTGCAATTACGGGG	Integrated DNA Technologies	N/A
<b>Software and algorithms</b>		
Image-Pro Premier Plus 3D	Media Cybernetics	Version 9.3.3 or higher
Leica Application Suite X (LASX)	Leica Microsystems™	N/A
GraphPad Prism	GraphPad	Version 8.0 or higher
<b>Other</b>		
Glad® Press and Seal®		N/A
CO <sub>2</sub> Bubbler Kit	Genesee Scientific	59–180
T-fitting	Genesee Scientific	59–123
Flypad	Genesee Scientific	59–119
Flypad Frame	Genesee Scientific	59–120
Clear Drosophila polyurethane tubing 1/8 in (3 mm)	Genesee Scientific	59–124C
Inflating needles	Spaulding	58463S
Safety tip air blow gun with hook	Harbor Freight Tools	68263
Gas regulator	Fisher Scientific	10-575-140
Carbon dioxide UN1013 cylinder	N/A	N/A
Polyvinyl chloride tubing	VWR 3/16 × 5/16	89068-500
Polypropylene Erlenmeyer flask, 1 L	Fisher Scientific	10-041-10E
Dissecting microscope	Zeiss	SteREO Discovery V8

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dumont #5 forceps	Fine Science Tools	11295-10
Pyrex™ spot plates	Fisher Scientific	13-748B
Nunc™ 72-well MicroWell® plate	Fisher Scientific	12-565-154
Fine tip transfer pipette	Fisher Scientific	13-711-25
Orbital shaker	Benchmark Scientific	B3D1008-GROUP
Superfrost® microslides	VWR	48311-950
Coverglass 22 × 22 mm	VWR	48368 062
Clear nail polish	Sally Hansen	45077
Kim Wipes®	Fisher Scientific	06-666A
Leica Microsystems™ TCS PSE (or similar) confocal microscope with 405, 488, 532 nm excitation lasers	Leica Microsystems™	N/A
63× Confocal microscope objective	Leica Microsystems™	ACS APO, oil, NA = 1.3, WD = 0.16 mm
Optical table to reduce microscope vibration	Kinetic Systems	Series 9100
Zeiss™ Immersol™ 518F objective oil	Fisher Scientific	12-624-66A
Compressed air tank	Matheson	CD 50
CGA 346 air regulator and connecting hoses	Fisher Scientific	10-575-140
Lens paper	Fisher Scientific	11-996
Microscope slide box	VWR	82003-412

**MATERIALS AND EQUIPMENT**

**Drosophila food recipe**

Reagent	Final concentration	Amount
Molasses	3.53%	247.1 mL
Yellow cornmeal	5.88%	411.8 g
Light corn syrup	3.53%	247.1 mL
Tegosept® (10% in molecular grade EtOH)	0.010%	70 mL
RO H <sub>2</sub> O		6110 mL
NutriSoy® flour	0.059%	41.2 g
Active dry yeast	1.18%	82.4 g
1× PBS	0.071%	50 mL
NutriFly® Drosophila Agar Gelidium	0.059%	41.2 g
Propionic acid	0.044%	30.9 mL
RO H <sub>2</sub> O	85.64%	up to 7 L
<b>Total</b>		<b>7 L</b>

All reagents can be stored at 22°C to 25°C for at least one year. Acids and bases should be stored in separate cabinets.

**PCR extraction buffer recipe**

Tris-HCl pH 8.2 (1 M)	10 mM	500 μL
EDTA (500 mM)	1.0 mM	100 μL
NaCl (5 M)	25 mM	250 μL
Proteinase K		1 μL
RO H <sub>2</sub> O		Up to 50 mL
<b>Total</b>		<b>50 mL</b>

Proteinase K should be stored at –25°C to –15°C. All other reagents can be stored at 22°C to 25°C for at least one year. Acids and bases should be stored in separate cabinets.

**1× Phosphate buffer with Triton™ X-100 (PBT) recipe**

10× Phosphate buffered saline (PBS)	10%	10 mL
Triton™ X-100	0.1%	100 μL
RO H <sub>2</sub> O	90%	90 mL
<b>Total</b>		<b>100 mL</b>

All reagents can be stored at 22°C to 25°C for at least one year. Acids and bases should be stored in separate cabinets.

### Optional: 0.3% PBT recipe

10× PBS	1 ×	50 mL
Triton™ X-100	0.3%	1.5 mL
<b>Total</b>	<b>n/a</b>	<b>50 mL</b>

All reagents can be stored at 22°C to 25°C for at least one year. Acids and bases should be stored in separate cabinets.

### Optional: Blocking solution recipe

Normal goat serum	10%	500 µL
PBT	90%	4.5 mL
<b>Total</b>	<b>n/a</b>	<b>5.0 mL</b>

All reagents can be stored at 22°C to 25°C for at least one year. Acids and bases should be stored in separate cabinets.

PBT can be stored at 22°C to 25°C for at least one year. Normal goat serum should be stored at –20°C for up to two years. Solution should be freshly prepared and about 500 µL transferred to each glass well. Acids and bases should be stored in separate cabinets.

### Image processing minimum computer system requirements

Windows 7, 8.1, or 10 64-bit operating system	n/a	1 each
Multi high-speed SATA hard disks or SSDs	n/a	1 each
8 GB free on installation drive	n/a	1 each
20+ GB free space for image storage	n/a	1 each
2.8 GHz CPU Intel quad-core processor or better	n/a	1 each
16 GB+ RAM	n/a	1 each
NVIDIA GeForce GTX cards with 4 GB graphics memory	n/a	1 each
Open GL 4.2 or higher	n/a	1 each
USB port	n/a	1 each
Internet connection	n/a	1 each
Internet Explorer version 9 or higher	n/a	1 each

**△ CRITICAL:** Formaldehyde is flammable, corrosive to skin and can cause serious eye damage. It also causes respiratory sensitization and is acutely toxic by inhalation and oral or dermal contact. Use with nitrile gloves under a fume hood. Dispose properly with hazardous waste.

**Alternatives:** Any confocal microscope can be used with this protocol. Several models of orbital shakers are compatible with this protocol. Most graphing and statistical analysis programs can be used in place of GraphPad Prism.

## STEP-BY-STEP METHOD DETAILS

All incubations are carried out at 22°C to 25°C unless otherwise stated.

### Aging flies

⌚ **Timing:** 1 to 60 days

1. Collect female and/or male flies from fly cross in beginning step 2g (Generating *Drosophila* stocks), which express mito-GFP, mCherry-Atg8a and the desired GAL4 driver.
2. Maintain in standard food vials, transferring to new food vials two times per week until flies reach desired age. Discard old vials.

⏸ **Pause point:** 3 to 4 days



### **Drosophila brain dissection and fixation**

⌚ **Timing:** 20 min per brain

3. Anesthetize aged experimental flies on a CO<sub>2</sub> pad a few at a time to avoid excessive anesthesia. Using dissecting forceps, pick up an anesthetized fly and place it on a dissecting plate near a droplet of 0.3% PBT.
4. Quickly decapitate the fly with forceps and place the head into the droplet. Dissect the brain under a dissecting microscope by removing eye discs and cuticle.

**Note:** A useful instructional video can be found here: <https://www.youtube.com/watch?v=j4rVa7JCzdg&t=1s>.

5. Place brains in a glass spot plate well that contains 3.7% formaldehyde for 15 min.
6. Using forceps, transfer the brain from fixing solution to another well containing 0.3% PBT. Up to five brains of the same genotype can be transferred to a single well.
7. When all brains have been placed in 0.3% PBT, place the glass well plate on an orbital shaker on the lowest setting for 5 min. Repeat this washing step four times by using a fine tip transfer pipette to discard used solution and add fresh 0.3% PBT to wells.

**Note:** When adding new solution into a well, ensure the brains are in solution before the plate to the shaker. Brains that stick to the glass above liquid level will become dry and unusable.

**Optional:** Immunostaining *Drosophila* brain (This step can be eliminated if using a GAL4 enhancer that allows easier mito-GFP and mCherry-Atg8a visualization.)

⌚ **Timing:** 2 days

8. Under the dissecting microscope, use a fine tip transfer pipette to remove 0.3% PBT from each well and add 500 μL blocking solution to brain-containing wells. Keep the glass spot plate on the orbital shaker for at least 30 min.
9. Prepare a fresh primary antibody solution (We use 1:100 anti-tyrosine hydroxylase primary antibody to blocking solution). Place 10 μL of the primary antibody solution to each microtiter well.
10. Remove the glass spot plate from shaker and use forceps to transfer brains from the glass wells to antibody-containing microtiter wells. Each well can hold up to 5 brains.
11. Place the microtiter plate in an empty pipette box with a moist towel on the bottom to keep brains from drying and keep the box at 4°C overnight.

⏸ **Pause point:** 1 day

12. Using forceps, transfer the brains from the microtiter plate to a glass well containing 0.3% PBT. Using a fine tip transfer pipette to change solutions, wash the brains in 0.3% PBT four times for 5 min on the orbital shaker.
13. Remove 0.3% PBT and add 500 μL of blocking solution. Place on shaker for at least 30 min.
14. While the brains are in the blocking phase, prepare a 1:200 solution of Alexa Fluor® 405-conjugated secondary antibody to blocking solution.

**Note:** Limit light exposure to light-sensitive secondary antibody solution.

15. Remove blocking solution and add 400 μL of secondary antibody solution to each well. Protect from light and place on orbital shaker for 2 h.

⏸ **Pause point:** 1 day

- Replace secondary antibody solution with 0.3% PBT and incubate for five minutes on shaker. Repeat washing procedure four times.

### Mounting *Drosophila* brains on microscope slides

⌚ **Timing:** 30 min

- Label a microscope slide with genotype, antibody label, fly age, treatment, and date.
- Place a small drop of 70% glycerol onto the slide near the frosted left side. Drag the glycerol droplet to the right along the slide with the transfer pipette. The thin glycerol line will stabilize the brains.
- Use forceps to remove one brain at a time from the final wash and place it in the left region of the glycerol line.

**Note:** Dab the forceps on a Kim Wipe® to minimize the transfer of washing solution to the slide.

- Carefully drag the brain to the right, maintaining orientation for ease of imaging. Align brains in the glycerol line.
- Remove excess glycerol from the slide using a Kim Wipe®. This is easiest to do under the dissecting microscope. Re-orient displaced brains.
- Place a small horizontal line of Prolong Antifade® above the line of brains. Carefully place a coverslip over the top of the mounted brains. Allow the mounting media to spread for 1 to 2 min, avoiding light exposure.
- Seal the coverslip to the slide with clear nail polish.
- Using a marker, draw a line indicating the location of the brains so they can be easily located during imaging.
- Place slide in a slide box and store at  $-20^{\circ}\text{C}$ .

⏸ **Pause point:** up to 5 days

### Capturing z-stacks of mito-GFP and mCherry-Atg8a

⌚ **Timing:** 10 min per brain

- Turn on computer, confocal microscope and open LASX application. The following steps are executed in the "Acquisition" panel of the LASX application unless otherwise indicated.
- Turn on the 488 nm and 532 nm excitation lasers.

**Optional:** Turn on the \*405 nm excitation laser if capturing z-stacks for Alexa Fluor 405.

- Open 3 sequences and set the conditions as listed below.

Sequence	Excitation Laser (nm)	Laser Intensity (%)	Emission (nm)	Gain	Offset
1	488	20	500 to 590	800	-1
2	532	55	590 to 700	800	0
3*	405	12	410 to 500	550	-2

- Set pinhole to 1 AU.
- Set the image resolution to  $512 \times 512$  pixels and speed to 600 Hz for fast scanning while locating volume of interest (VOI) boundaries.

31. Using the 63× oil objective, locate the brain and the region of interest (ROI) on the confocal microscope. Use the setting for Sequence 3 if the brains have been immunostained, as excitation laser exposure can cause photobleaching. If not using immunostaining, locate the ROI as rapidly as possible using the fluorescence of GFP or of mCherry.

**Note:** ROI selection will vary depending on the brain region or cell type of interest. A large ROI may require acquisition of multiple z-stacks and/or development of a standardized selection sampling process.

**Note:** A small amount of photobleaching may occur while locating the ROI. Since the Alexa Fluor 405 is not being measured, it is best to expose this fluorophore to excitation while locating the ROI.

32. If using antibody labeling, use the antibody signal to define z-plane VOI boundaries. Use the mCherry signal in the absence of antibody staining, as the GFP signal fades rapidly with excitation.
33. Click the “Live” button in the LASX application and manually manipulate the z position around your VOI. At the top of the VOI, click the “Begin” button. Move the stage to the bottom of the VOI and click the “End” button.

**Note:** VOI boundary in the z-plane will vary depending on the brain region or cell type of interest. A large VOI will increase acquisition time and file size and may increase protocol time unnecessarily. Researchers may choose to capture multiple z-stacks or develop a standardized selection sampling process.

34. Change the pixels per image and image capture speed to 1024 × 1024 and 400 Hz, respectively, to capture a higher-resolution z-stack.
35. Set the z-stack step to 340 nm. The z-slice thickness should remain constant for all samples; the number of slices per sample will vary with the VOI.
36. Select “Between Lines” under the “Sequential Scan” tab so that each z slice is excited by all lasers (sequences) before the confocal moves to the next slice. This pattern continues until all z slices in the VOI have been captured.
37. Click “Start” to take image – this can take several minutes depending on the size of the VOI in the z plane.
38. Save the image in “lif” format with a descriptive title.

⏸ Pause point: indefinite

### Detecting 3D colocalization of mCherry-Atg8a and mito-GFP

⌚ Timing: 10 min per brain

39. Open Image-Pro Premier 3D Application and open lif files.
40. Under 3D View tab, click the “Reload” button to open the “Load Image” window. The voxel sizes should read: X = 0.114, Y = 0.114, Z = 0.340 (μm). These settings are determined by image capture settings and should be constant among all samples.
41. In the same window, uncheck “Auto” and click “Reset” to change all values to 1.
42. Under the “3D View” tab, click the “Show VOI” and “Show VOI handles” icons to allow adjustment of volume of interest (VOI). Using the arrow tool from the “3D View” panel, grab the green square handles on the corners of the image to adjust the VOI.
43. Under 3D Measure tab, click types.
44. Add “volume:volume,” and set minimum volume to 0.01 and maximum volume to 1.5 μm.
45. Click “Ranges” button to “On.”

46. Click the "Add Iso-Surface" icon in the right panel.
  - a. Select channel 2 (532 nm, sequence 2 during image capture) from the drop-down menu.
  - b. Uncheck "Auto" and click "Reset" to change all values to 1.
  - c. Select "Lo-Pass 3×3×3" from "Filter" the drop-down menu.
  - d. Select "Auto Bright" from the "Threshold type" drop-down menu.
47. Under "Volume Surface Channel 2," Click the "Count" button to select puncta that fit volume and threshold criteria. An "isosurface" will appear on selected voxels.
48. Adjust minimum "Volume Surface Channel 2" intensity range to collect visible puncta.
49. Click data table under 3D Measure tab.

**Optional:** If collecting data in immunolabeled cells, manually remove puncta outside of labeled regions: in the "3D View" tab, select the arrow tool and click on each unwanted object and press "delete."

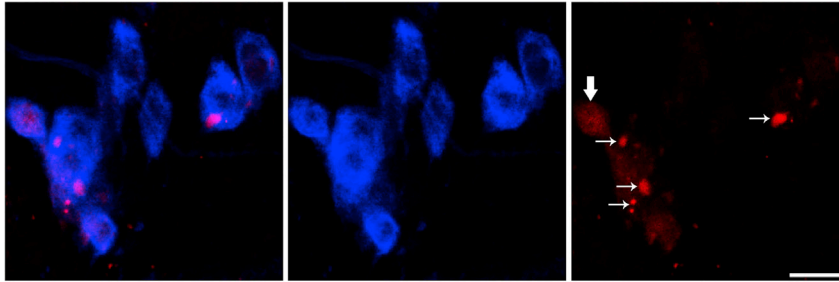
50. Under the "3D Measure" tab, click "Data Table," then click on Microsoft Excel icon to export the data to an Excel spreadsheet.
51. Click on the disk icon to save this isosurface file, which represents the autophagosomes. Delete channel 2 isosurface from the viewing window.

**Note:** If it is not deleted, these data will be exported on additional export attempts.

52. If visible puncta fall out of detection range, repeat steps 46 to 51, adjusting minimum "Volume Surface Channel" range in step 48 to collect visible puncta. Manually remove puncta that have already been exported to Excel.
53. Click "Types" on 3D Measure tab and add, "Coloc Pearson."
54. Click on "Add 3D Colocalization" isosurface. From the drop-down menu, select the "channel red, channel green" option to determine how much green mito-GFP signal is in red puncta.
55. Click "Reset," and enter "1" for each pixel dimension.
56. Select "Lo-Pass 3×3×3" filter, "Auto Bright", "Threshold Type" and check box for close edges. Uncheck execute count.
57. Set the threshold for the red channel using same minimum threshold for red isosurface in step 48 for each saved isosurface.
58. Click folder icon (next to disk icon used to save red puncta) under colocalization and open the saved isosurface file. (Note: on previous versions of Image Pro Premier 3D, data occasionally are all "0." If this occurs, re-open to apply saved isosurface). Data are automatically added to the data table.
59. Click on data table and manually remove identified objects that have Pearson's correlation coefficients that are less than or equal to zero.
60. Export to Excel.
61. Repeat steps 54 to 60 for each saved isosurface.
62. Count the number of colocalization measurements.

### EXPECTED OUTCOMES

The GAL4 driver chosen determines the structures that will express mito-GFP and mCherry-Atg8a. In this protocol, dopaminergic neurons are expected to be the only cells co-expressing the two fluorescent constructs. Among dopaminergic neurons, protocerebral posterior lateral region 1 (PPL1) clusters are easily identifiable using an anti-TH antibody. If other tissue- or cell-specific drivers are used, the first step is to verify that the expression of mito-GFP and mCherry-Atg8a is specific to the desired cells. The second aspect is to evaluate the appearance of the mitochondrial network using mito-GFP. Control flies should present interconnected, ribbon-like mitochondria, while damaged mitochondria like those observed in *park*<sup>-/-</sup> fly PPL1 neurons should be swollen and fragmented, not organized in a network (Figures 3A and 3C) (Cackovic et al., 2018; Greene et al., 2003).



**Figure 1. Identifying soluble and lipidated mCherry-Atg8a**

Blue represents TH-positive neurons and red represents mCherry-Atg8a. Bold arrows indicate diffusely distributed, soluble mCherry-Atg8a, while small arrows point to lipidated mCherry-Atg8a in autophagosomes. Scale bar represents 10  $\mu\text{m}$ .

Indeed, the physical separation of defective mitochondria through fragmentation events have been shown to precede mitophagy (Poole et al., 2008; Riparbelli and Callaini, 2007; Whitworth and Palanck, 2017). However, this dramatic change in mitochondrial morphology is a hallmark of parkin-dependent mitophagy. In parkin-independent mitophagy paradigms, it is important that organelle morphology is evaluated on a case-by-case basis. The third aspect to consider is the presence or absence of mCherry-Atg8a-positive autophagosomes. mCherry-Atg8a distribution can either be found as a diffused cytosolic labeling, which is associated with inactive Atg8a, while vesicular, punctate mCherry-Atg8a corresponds to the fraction of the protein that is associated with autophagosomes (Klionsky et al., 2016) (Figure 2). These distribution patterns are highlighted in Figure 1. The presence or absence of parkin does not affect the overall quantity of autophagosomes in PPL1 neurons (Cackovic et al., 2018). If parkin-independent mitophagy paradigms are involved, it is important to independently assess the total number of autophagosomes for each specific cell type.

In cells showing clusters positive for mito-GFP and mCherry-Atg8a, colocalization analyses between the two fluorescent markers can then be performed. The association of mCherry-Atg8a with mito-GFP was previously shown to decrease in PPL1 neurons from day 5 and 10 *park*<sup>-/-</sup> adult flies (Figure 3) (Cackovic et al., 2018). This substantiates the role of parkin as a key mediator of mitochondrial turnover events.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Positive Pearson colocalization values are entered into GraphPad Prism 9.0.0 (121), which generates graphs and compares means using paired *t*-tests when data are normally distributed. Mann-Whitney tests to compare ranks should be performed if data are not normally distributed.

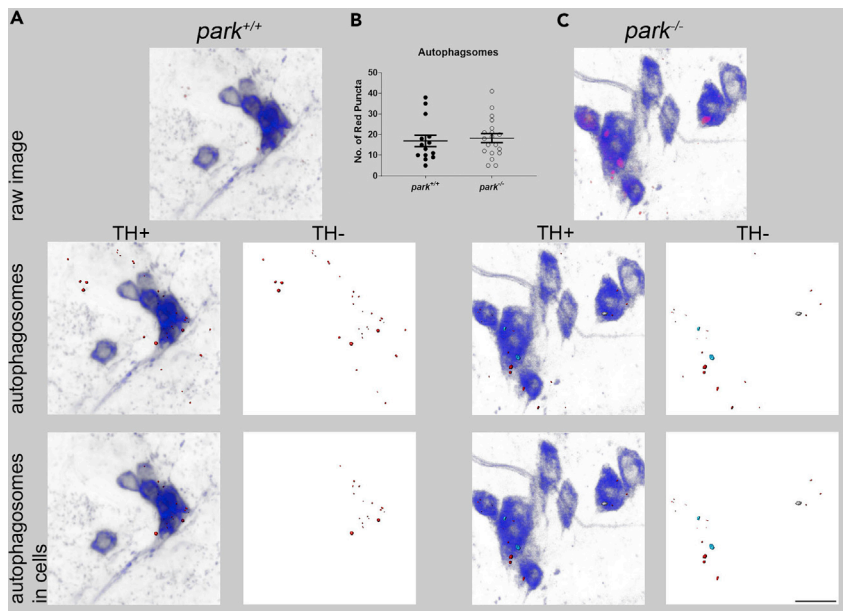
## LIMITATIONS

### Using a commercially-available software vs an open-source solution

This protocol was originally performed using the Image-Pro Premier 3D software, which consists of a comprehensive framework for the semi-automatic detection of autophagosomes positive for mCherry-Atg8a and their colocalization with mitochondria positive for mito-GFP. Using a predefined, commercially-available software for data analysis could potentially reduce the diffusion of the present protocol to study mitophagy. However, similar analyses can be performed using an open-source, community-driven software as Fiji (<https://imagej.net/Fiji>) with plugins that perform colocalization analyses on individual objects, such as JaCoP (Bolte and Cordelières, 2006; Cordelières and Bolte, 2008). When using this solution, the user must pre-determine the optimal parameters allowing the detection and segmentation of autophagosomes.

### Intensity-based vs object-based colocalization analysis

The choice of the method to perform colocalization analyses could depend on two factors: (i) the dimensions of autophagosomes and their distribution, which may vary by cell type, and (ii) the relative



**Figure 2. Quantification of autophagosome formation**

Top row images show raw data projections for a representative sample of (A) *park*<sup>+/+</sup> and (C) *park*<sup>-/-</sup> PPL1 neurons expressing mCherry-Atg8a and TH-antibody staining (blue). Red puncta indicate lipidated mCherry-Atg8a. In the second row, red, blue and grey isosurfaces highlight mCherry-Atg8a puncta selected by the imaging software. We counted and compared the number of puncta located within TH-labeled region (bottom row) and did not detect an effect of the *park*-null mutation on autophagosome formation (B). Scale bar represents 10  $\mu$ m. Data are represented as mean  $\pm$  SEM. The effect of parkin loss of function was determined using a Mann-Whitney test. This figure has been modified from (Cackovic et al., 2018) and falls under terms of the Creative Commons Attribution License (CC BY).

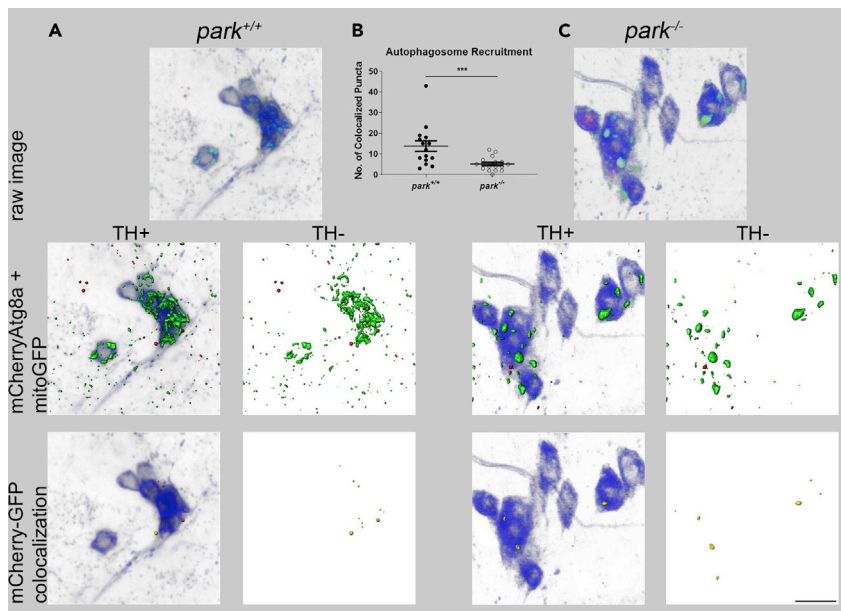
fluorescence intensity of mito-GFP and mCherry-Atg8a. As discussed above, the GAL driver chosen for the expression of fluorescent constructs might yield differential intensities for mito-GFP and mCherry-Atg8a.

A key-point for correct data interpretation relies on determining the average dimensions of autophagosomes in control tissues by the user. If the autophagosomes have constant dimensions, the minimal and maximal dimensions can be set as standards and applied to all the images analyzed (See step 44). If autophagosomes vary in their dimensions or if they are clustered together and individual vesicles cannot be detected, the user should apply more stringent criteria/image analysis algorithms to identify (segment) individual autophagosomes. A guide to object segmentation can be found on the Fiji/ImageJ website (<https://imagej.net/Category:Segmentation>). Once autophagosomes have been correctly segmented, the user must evaluate the relative fluorescence intensity of the mito-GFP and mCherry-Atg8a labeling. If the relative intensity of both constructs is similar as in the present protocol, a particularly suitable method is Pearson's colocalization analysis (Bolte and Cordelieres, 2006). On the contrary, the user could benefit from the vesicle-like structure of mitochondria and autophagosomes and run an object-based colocalization analysis. This method does not take into account the relative fluorescence intensity of objects, but it calculates colocalization using the object coordinates (Bolte and Cordelieres, 2006). This method outperforms when the vesicular-like objects are present, but it shows poor accuracy if one of the two stainings is diffused.

## TROUBLESHOOTING

### Problem 1

Generating *Drosophila* stock, steps 2a, c, d, e, f, g, 4a, c: the desired progeny do not eclose after 10 days.



**Figure 3. 3D colocalization of mCherry-Atg8a puncta with mito-GFP indicates level of autophagosome-to-mitochondria recruitment**

Raw data projections for representative samples of (A) *park*<sup>+/+</sup> and (C) *park*<sup>-/-</sup> PPL1 neurons expressing mCherry-Atg8a, mito-GFP and blue TH label (top row). Red and green isosurfaces depict mCherry-Atg8a puncta and mitochondria selected by the imaging software (second row). Images in the third row illustrate distribution of puncta colocalized with mito-GFP (yellow) in the cell bodies; that is, the colocalized objects had positive Pearson's coefficient. (B) We found that *park*-null flies had decreased autophagosome-to-mitochondria recruitment in dopaminergic neurons. Scale bar represents 10  $\mu$ m. Data are represented as mean  $\pm$  SEM. The effect of parkin loss of function was determined using a Mann-Whitney test.  $p < 0.001$  for \*\*\*. This figure has been modified from (Cackovic et al., 2018) and falls under terms of the Creative Commons Attribution License (CC BY).

### Potential solution

Continue to supplement parent vials with males and virgin females and transfer flies every 3 to 4 days. The number of female and male parent flies per vial can increase to up to 20 total flies. Although the fly lifecycle is about ten days, eclosion of flies with desired phenotypes/genotypes varies depending on viability of flies with desired combination of chromosomes. Researchers should allow up to 4 weeks to collect phenotypes of interest.

### Problem 2

Capturing z-stacks of mito-GFP and mCherry-Atg8a, step 28, 35: the fluorescence intensity of mito-GFP and mCherry-Atg8a is low.

### Potential solution

Intensity of mCherry-Atg8a and mito-GFP will depend on GAL4 promotor activity. Therefore, while z-stack capture parameters (e.g., laser intensity) must be constant for each fluorophore between samples, parameters can be altered in order to accommodate differences in fluorophore expression.

### Problem 3

Capturing z-stacks of mito-GFP and mCherry-Atg8a, step 35: The VOI has a large z dimension, making image capture time burdensome or unrealistic.

### Potential solution

The z-slice thickness should remain constant for all samples; the number of slices per sample will vary with the VOI. Z-slice thickness can be increased for larger VOIs; however, detection sensitivity decreases as z-slice thickness increases. User should optimize z-slice thickness based on sample size.



### Problem 4

Detecting 3D colocalization of mCherry-Atg8a and mito-GFP, step 44: autophagosomes do not show constant sizes and/or their sizes vary according to the tissue analyzed.

### Potential solution

Since autophagosome sizes may vary by cell type, user should adjust upper and lower limit for red puncta volume to optimize puncta selection according to the specific tissue analyzed.

### Problem 5

Some cell types may have low or no levels of autophagosome formation and/or mitophagy initiation, and therefore, they will have no or few detectable mCherry-positive puncta. If no or very few puncta are visible when viewing the z-stack projection in the imaging software, colocalization analysis cannot be conducted. Non-lipidated mCherry-Atg8a is soluble and can sometimes be observed in z-stack projections. Soluble mCherry-Atg8a is not found in autophagosomes and is usually excluded from analysis by volume size limitations (Figure 1).

### Potential solution

Depending on hypotheses being addressed, researchers can induce mitophagy via exposure to hypoxia or rotenone (Kim et al., 2019).

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Giulia Bertolin, [giulia.bertolin@univ-rennes1.fr](mailto:giulia.bertolin@univ-rennes1.fr).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

The datasets supporting the current study have not been deposited in a public repository because the deposit was not required. Data are available from the corresponding author on request.

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## AUTHOR CONTRIBUTIONS

S.J.G.L. developed the protocol, conducted experiments, and analyzed the data. A.N.J. contributed to protocol optimization and data collection; she also helped compile the Key Resources Table and the Material and Equipment section. G.B. consulted on the project, shared manuscript writing responsibilities, and communicated with the journal. L.M.B. directed the project and shared manuscript writing responsibilities.

## DECLARATION OF INTERESTS

The authors declare no competing interests.



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