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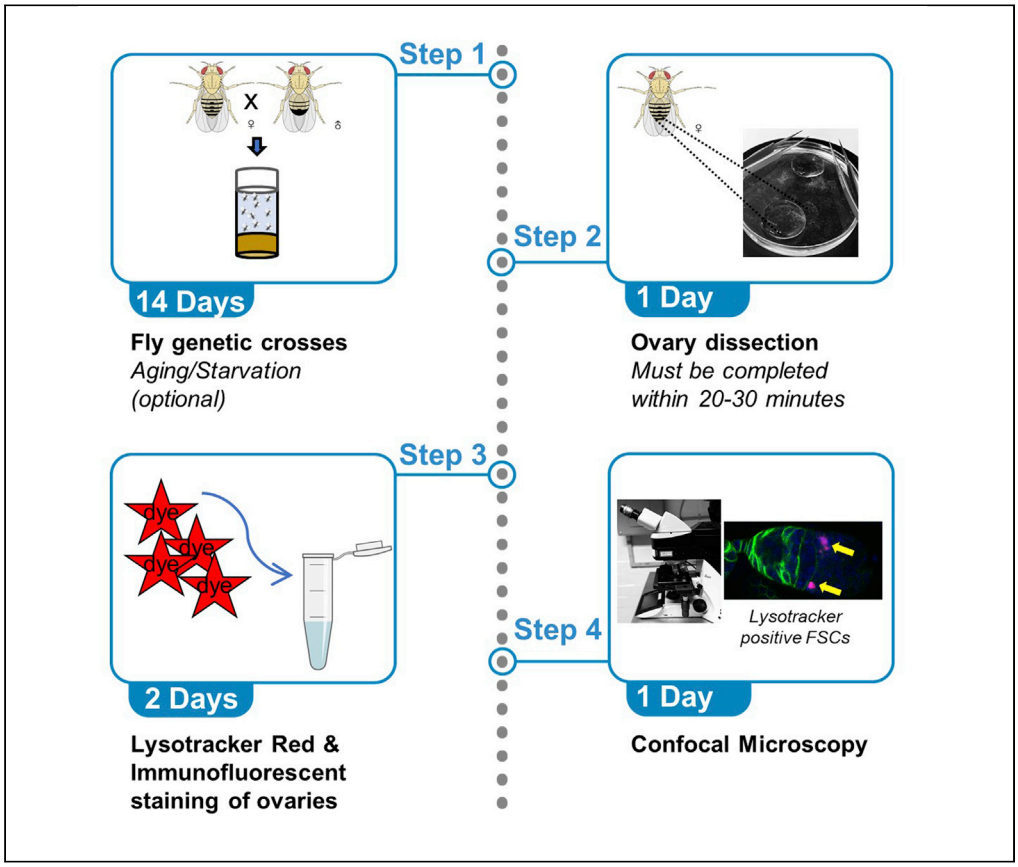
Protocol for evaluating autophagy using LysoTracker staining in the epithelial follicle stem cells of the *Drosophila* ovary



We have outlined the approach of visualizing autophagy specifically in the epithelial follicle stem cells of the *Drosophila* ovary using the LysoTracker dye. The advantage of using this protocol is that it details several techniques, including ovary dissection, immunofluorescence, and western blotting, which positively identify autophagy changes in a very small population of cells. One of the limitations of this protocol is that it needs to be combined with other genetic manipulations and positive markers of the autophagy pathway.

Iliana Correa, Melissa Wang, Eric H. Lee, Dara M. Ruiz-Whalen, Alana M. O'Reilly, Tanu Singh

Alana.OReilly@fccc.edu (A.M.O.)
Tanu.Singh@fccc.edu (T.S.)

Highlights

Protocol to monitor autophagy in the FSCs of the fly ovary using the LysoTracker dye

Protocol provides details on imaging the fly germarium using a confocal microscope

Features western blotting for the fly ovary and reiterates LysoTracker assay results

Protocol is applicable to diverse cells, tissues, and model systems

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Protocol for evaluating autophagy using LysoTracker staining in the epithelial follicle stem cells of the *Drosophila* ovary

Iliana Correa,[1,2] Melissa Wang,[1,2] Eric H. Lee,[1,3] Dara M. Ruiz-Whalen,[1] Alana M. O'Reilly,[1,4,*] and Tanu Singh[1,4,*]

[1]Fox Chase Cancer Center, Philadelphia, PA 19111, USA

[2]These authors contributed equally

[3]Technical contact

[4]Lead contact

*Correspondence: Alana.OReilly@fccc.edu (A.M.O.), Tanu.Singh@fccc.edu (T.S.)
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SUMMARY

We have outlined the approach of visualizing autophagy specifically in the epithelial follicle stem cells of the *Drosophila* ovary using the LysoTracker dye. The advantage of using this protocol is that it details several techniques, including ovary dissection, immunofluorescence, and western blotting, that positively identify autophagy changes in a very small population of cells. One of the limitations of this protocol is that it needs to be combined with other genetic manipulations and positive markers of the autophagy pathway.

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

BEFORE YOU BEGIN

Setup flies, crosses, media, and dissection equipment

Timing: 7–14 days

1. Collect virgin female flies, setup crosses, age, and starve the flies (if required, see below for details on how to raise flies under starvation conditions).
   a. For the purpose of this protocol, we will be using w^{1118} (wild-type) flies as controls, *w*; *109-30-Gal4*; *UAS-Atg5*^{RNAi} and *w*; *109-30-Gal4*; *Akt*^{RNAi} transgenic flies, the *109-30 Gal4* driver, which we previously demonstrated, enables strong expression (of RNAi) in FSCs and early follicle cell progenitors. Setup at least 10–15 female virgin flies for each genotype with 5 male flies in a yeasted fly food vial at 25°C.
   b. Check for progeny of the correct genotype once the flies have eclosed. Flip the progeny into a new yeasted vial with fly food to avoid sticking of adult flies to the food.
   c. Depending on the research question, flies from step 1b can also be raised under starvation or nutrient deprivation conditions (described in detail below). We nutrient restrict our control flies for a minimum of 3 days (Hartman et al., 2015), for example.
      i. Prepare agar grape juice plates (see recipe below under materials and equipment).

Note: Agar grape juice plates can be stored in a sealed storage bag with damp paper towels at 4°C for a month.

⚠ CRITICAL: Check the fruit juice plates for contamination or bacterial growth before usage.

ii. Take clean, sterile, 50 mL conical tubes, and perforate with a 22 1-1/2-inch gauge needle on the base side (refer to Figure 2).

iii. Anesthetize and collect at least 15–20 adult female flies and 10 adult male flies per starvation chamber, take the lid off of the perforated conical tube, transfer the flies to the tube and place the grape juice plates on the opening of the tube. Secure and seal the plate to the perforated tube with parafilm (also see Figure 2).

⚠ CRITICAL: Make sure the 50 mL conical tube starvation chambers have adequate air flow by ensuring the poked holes go all the way through the tube. Low oxygen has severe consequences for viability, fertility, and FSC function.

Note: FSCs become quiescent after 3 days on grape juice plates (Hartman et al., 2015). This resting state is associated with high levels of autophagy (Singh et al., 2018), which is well-known as a starvation response. Other types of nutrient-restriction protocols, such as culture on molasses plates, did not result in quiescence of FSCs even after 3 days of nutrient restriction (Hartman et al., 2015). For an optimal FSC proliferation arrest, grape juice plates are an effective tool (Hartman et al., 2015).

Note: A minimum of 15–20 adult female flies along with 5–10 adult male flies are required in order to achieve a good yield (number of ovarioles) because under nutrient deprivation conditions a fly's oogenesis process decreases. Flies exhibit nearly normal survival on grape juice plates relative to continuously accessible rich food (Hartman et al., 2010), so death of adult flies is not common. If death is observed during the time course, it is possible to a) either use more flies to start, b) combine the 109-30-Gal4 driver with a temperature-sensitive Gal80 to reduce RNAi expression levels (Singh et al., 2018), or c) take measurements at a shorter timepoint.

2. Wash and dry all material that will be used during the dissection of the fly ovaries, including buffers, media, Sylgard (DOW Chemical) coated petridish, surgical instruments (#5 watchmakers' fine forceps), and paintbrushes.

a. Prepare PBS and Graces' Medium (detailed step by step recipe under materials and equipment section below), use the Graces' medium at room temperature during the dissection step.

Note: Grace's medium is a composition that mimics the hemolymph inside the fly. We found that use of Grace's medium (Batista et al., 2005) maintains ovarian cells in their natural environment, whereas PBS alone can lead to disruption of cellular processes and cytoskeleton organization. Graces' media can be prepared in advance and stored for a week at 4°C. Check for bacterial growth before usage.

b. Wipe and clean the Drosophila CO_2 fly pad, used to anesthetize flies.

Note: Check CO_2 tank, making sure it's full or half full before you begin dissections. It's critical to not run out of CO_2 during dissections, as flies can wake up and escape. Connecting a porous "fly pad" to a source of CO_2 using rubber tubing provides a flattened surface that maintains flies in an anesthetized state on the stage of a stereomicroscope to enable sorting and other manipulations.

Prepare fixative, LysoTracker working stock, PBST, PBST + 0.5% BSA

Timing: less than 1 h

3. Prepare 4% paraformaldehyde in 1× PBS. Place 40 μg of paraformaldehyde powder in 1 mL of 1× PBS and heat at 98°C in a heat block until dissolved. Cool to room temperature before using to fix

tissue. PBST should be prepared with 0.3% Triton and for antibody dilution buffer; use 0.5% BSA in PBST.

4. Lysotracker dye comes packaged as a 1 mM stock solution in anhydrous DMSO. Thaw Lysotracker (1 vial contains 50 μL of lysotracker solution) at room temperature prior to dissection. We prepare a 1:100 working stock in PBS and wrap the tube with foil. Also see problem 2 for instructions on testing a range of lysotracker dilutions (1:50–1:2000) to optimize the working stock concentration for your model system.

Note: Protect Lysotracker from light, store the stock solutions at −20°C (not in a frost-free freezer), desiccate, avoid freeze-thaw cycles, and if possible, store in single-use aliquots.

⚠ CRITICAL: Formaldehyde is a toxic chemical, a carcinogen, eye, skin, nose irritant and must be handled with appropriate care, including correct PPE and should be handled in the fume hood. Any waste generated must be properly discarded as per your institute's policies.

⚠ CRITICAL: Prepare paraformaldehyde solution fresh on the day of the dissection.

Buffers for ovary lysis for western blotting

Timing: ~1–2 days

5. Prepare 10× RIPA lysis buffer and make 1× working stock (detailed recipe in table under materials and equipment section below).
6. Prepare 1× RIPA buffer containing Protease cocktail inhibitor, Sodium orthvanadate, Sodium Fluoride, and PMSF (see table below).
7. Prepare standards of BSA for the DC Protein Reagent Assay (BioRad) in 1× RIPA lysis buffer (see table below).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-rabbit Vasa	Santa Cruz Biotechnology (discontinued 2016)	Cat# sc-30210, RRID:AB_793874
Anti-mouse Fasciclin III	Developmental Studies Hybridoma Bank; Patel et al.,1987	Cat# 7G10 RRID:AB_528238
Anti-chicken GFP tag	Thermo Fisher Scientific	Cat # A10262, RRID: AB_2534023
Anti-rabbit Ref2P	Abcam	Cat# 178440
Anti-rabbit ß-actin	Abcam	Cat# ab8227, RRID:AB_2305186
Cy3-conjugated AffiniPure Donkey Anti-Rabbit IgG	Jackson ImmunoResearch Laboratories	Cat# 711-165-152, RRID:AB_2307443
Cy3-conjugated AffiniPure Donkey Anti-Mouse IgG	Jackson ImmunoResearch Laboratories	Cat# 715-165-151, RRID:AB_2315777
Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Mouse IgG	Jackson ImmunoResearch Laboratories	Cat# 715-095-151, RRID:AB_2335588
Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Chicken IgY	Jackson ImmunoResearch Laboratories	Cat# 103-095-155, RRID:AB_2337384
Cy5-conjugated AffiniPure Donkey Anti-Rabbit IgG	Jackson ImmunoResearch Laboratories	Cat# 711-175-152, RRID:AB_2340607
Alexa Fluor 647 goat anti-rat	Thermo Fisher Scientific	Cat# A21248, RRID:AB_11180200

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: organisms/strains		
D. melanogaster: w^{1118}	Bloomington Drosophila Stock Center	RRID:BDSC_3605
D. melanogaster: y[1] w*;P(GawB) 109-30/CyO	Bloomington Drosophila Stock Center	FlyBase: FBtp0000352
D. melanogaster: RNAi of Atg1: y[1] sc[*] v[1]; P{ *TRiP.JF02273*}attP2	Bloomington Drosophila Stock Center	RRID:BDSC_26731
D. melanogaster: RNAi of Atg5: y[1] sc[*] v[1]; P{ TRiP.HMS01244}attP2	Bloomington Drosophila Stock Center	RRID:BDSC_34899
D. melanogaster: RNAi of Akt1: y[1] sc[*] v[1]; P{ TRiP. HM04007}attP40	Bloomington Drosophila Stock Center	RRID:BDSC_31701
Chemicals, peptides, and recombinant proteins		
Grace's Insect Cell Culture Medium	Gibco	Cat# 11300-027
VECTASHIELD Mounting Medium	Vector Laboratories	Cat# H-1000, RRID:AB_2336789
LysoTracker™ Red DND-99	Thermo Fisher Scientific	Cat# L7528
DRAQ5™	Thermo Fisher Scientific	Cat# 62251
Sodium fluoride	Fisher Scientific	S299-100
COmplete Mini EDTA-free Protease Inhibitor Cocktail	Roche	Cat# 11836170001
Protein Assay Dye Reagent Concentrate	Bio-Rad	Cat# 5000006
PMSF	Sigma	P-7626
BSA	Sigma	A9647-100g
Bio-Rad DC Protein Assay Kit 1	Bio-Rad	5000111
Precision Plus Protein Dual Color Standards	Bio-Rad	Cat# 1610374
Methyl Paraben	Sigma	H3647-100g
Acetic Acid	Fisher Scientific	A38SI-212
Triton X-100	Fisher Scientific	BP151-500
Bacteriological Agar	Difco Laboratories: IBI Scientific	IB49170
Other		
Sylgard Petridish Pyrex 100 × 10mm	Fisher Scientific	08-747B
Juice Plate 33 × 10 mm petri dishes	Genesee Scientific	32-103
#5 watchmakers' fine forceps Dumont #5	Fine Science Tools	11254-20
#1.5 Coverslips (22 × 22)	Fisher Scientific	12-542-B
Slides, Frosted, pre-cleaned	Fisher Scientific	12-550-343
X-Ray Cassette 8 × 10 inch	Dupont Cronex Lightning-Plus EK	Cat# 200034
Classic Autoradiography Film	MIDSCI	Cat# BX1417
Hoefer™ Mighty Small™ II Mini Vertical Electrophoresis Systems	Hoefer	Cat# 03-500-483
Amerhsam Protran Premium Nitrocellulose Blotting Membrane	GE Healthcare Life Science	Cat# 10600003
Clear nail polish	Sally Hansen	N.A.
DM5000 confocal microscope	Leica	https://www.leica-microsystems.com/products/light-microscopes/upright-microscopes/details/product/leica-dm5000-b/
TCS SP5 Scanner, with HyD detector, dual emission filters (488/594), pinhole size at 1 Airy, 20× and 40×/1.3 N.A oil immersion objective	Leica	https://www.leica-microsystems.com/products/confocal-microscopes/details/product/leica-tcs-sp5/
Leica EZ4, stereomicroscope, with standard 10× eyepieces and objectives ranging from 0.8× to 3.5×.	Leica	https://www.leica-microsystems.com/products/stereo-microscopes-macroscopes/p/leica-ez4/
Hoefer TE 70, semi-dry transfer unit	Amersham Biosciences	Cat# 80-6210-34

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Electrophoresis power supply unit	Thermo Scientific	Model# EC-1000-90
Software and algorithms		
ImageJ/Fiji	Fiji	http://fiji.sc/
Photoshop	Adobe	https://www.adobe.com/products/
Prism	GraphPad	https://www.graphpad.com
LAS AF SP5	Leica	https://www.leica-microsystems.com/products/microscope-software/

MATERIALS AND EQUIPMENT

Equipment

A Leica confocal system SP5 with HyD detector is used in this study. We used the 488 nm and 594 nm excitation laser, dual emission filters (488/594), pinhole size at 1 Airy, 20× and 40×/1.3 N.A oil immersion objective.

A dissecting stereomicroscope Leica EZ4 is used in this study with standard 10× eyepieces and objectives ranging from 0.8× to 3.5×.

Agar fruit juice buffer recipe		
Reagent	Final concentration	Amount
Grape Juice (Warm)	50%	50 mL
Methyl Paraben (10%)	0.1%	1 mL
Acetic Acid	1%	1 mL
Bacto Agar (Difco)	3%	3 g
ddH_2O	50%	42 mL
Total	**n/a**	**50 mL**

Note: Plates can be stored in a sealed bag with damp paper towels at 4°C for a month.

Grace's medium recipe

- Dissolve 1 bottle of Grace's Insect Medium, 45.7 g of unsupplemented powder in 850 mL ddH_2O.
- Add 0.35 g of Sodium bicarbonate ($NaHCO_3$) to the above.
- Adjust pH to 6.1 and bring the volume to 1000 mL with ddH_2O.
- Filter the medium using a 0.2 μm filter.
- Make 50 mL aliquots in conical tubes and store at 4°C for a month.

Note: Check for contamination before using, if contaminated or precipitated do not use. Get a fresh aliquot or make a new batch.

RIPA lysis buffer recipe	
Reagent	Amount for 500 mL (final concentration)
1M Tris HCl pH 8.0	25 mL (50 mM)
20% SDS	2.5 mL (0.1%)
1M NaCl	75 mL (150 mM)
20% deoxycholic acid	25 mL (1%)
100% Triton X-100	5 mL (1%)
ddH_2O	12 mL
Total	**150 mL**
Store at −20°C for long term storage.	

Protease inhibitors (prepare fresh in RIPA buffer and keep on ice)	
Reagent	Final concentration, final amount depends on how much final RIPA is prepared
Protease Inhibitor Cocktail	1:100
Sodium orthovanadate	10 mM
Sodium fluoride	50 mM
PMSF (in ethanol)	2 mM
Store at −20°C in small aliquots for long term storage.	

BSA standards prepared in RIPA lysis buffer (prepare fresh and keep on ice)

Measure 15 mg of BSA and dissolve in 1 mL 1× RIPA lysis buffer. Dilute 15 mg/mL 1:100 to obtain 1.5 mg/mL (100 µL of 15 mg/mL BSA standard dissolved in 900 µL of 1× RIPA buffer. Perform serial dilutions in the same manner to make all the below listed BSA standards.

BSA standards in RIPA buffer
15 mg/mL
1.5 mg/mL
0.8 mg/mL
0.4 mg/mL
0.2 mg/mL
Make fresh before every use.

Buffers for western blotting	
6× sample buffer	Amount for 10 mL for a 6× final concentration
1M Tris pH 6.8	3.75 mL
Sodium dodecyl sulfate (SDS)	1.20 g
Glycerol	6 mL
DTT powder	0.9252 g
Bromophenol blue powder	6 mg
Bring volume to 10 mL with ddH_2O, store at −20°C for long term usage.	

⚠ CRITICAL: SDS is a flammable solid, causes skin, eye, and respiratory irritation. Harmful if swallowed or if inhaled. Wear protective PPE.

10× running buffer	Amount for 1000 mL for a 10× final concentration
Trizma Base	30.28 g
Glycine	144.13 g
ddH_2O	up to 950 mL
Sodium dodecyl sulfate (SDS)	10 g
Bring volume to 1000 mL with ddH_2O	
Use at 1×	Dilute in ddH_2O
Store at 30°C.	

⚠ CRITICAL: SDS is a flammable solid, causes skin, eye, and respiratory irritation. Harmful if swallowed or if inhaled. Wear protective PPE.

10× transfer buffer	Amount for 1000 mL for a 10× final concentration
Trizma Base	30.28 g
Glycine	144.13 g
ddH_2O	up to 950mL
Bring volume to 1000 mL with ddH2O, store at 4°C for a long-term storage (use at 1×, see below).	

1× transfer buffer	Amount for 1000 mL for a 1× final concentration
10× Transfer Buffer	100 mL
ddH_2O	600 mL
Methanol	200 mL
Bring volume to 1000 mL with ddH_2O, store at 4°C for a long-term storage.	

⚠ CRITICAL: Methanol is a toxic, flammable liquid, wear protective PPE, avoid inhalation and direct contact with skin or eyes. Dispose of any waste methanol and 1 × transfer buffer containing methanol safely according to your institution's policies.

10× TBS buffer	Amount for 1000 mL for a 10× final concentration
NaCl	87 g
KCl	2 g
Trizma base	30 g
ddH_2O	up to 800 mL
Adjust pH to 7.4	
Store at 4°C, bring volume to 1000 mL with ddH_2O, use at 1× (see below).	

1× TBST buffer	Amount for 1000 mL for a 1× final concentration
0.1% Tween-20	1 mL of 100%
	100 mL of 10× with 900 mL ddH_2O
Store at 4°C for a long-term storage.	

4× resolving gel buffer	Amount for 200 mL for a 4× final concentration
Trizma Base	36.3 g
ddH_2O	up to 150 mL
Adjust pH to 8.8	
Bring volume **to** 200 ml with ddH_2O, store at 4°C for a long-term storage.	

4× stacking gel buffer (.5M Tris, ph 6.8)	Amount for 50 mL for a 4× final concentration
Trizma Base	3.0 g
ddH_2O	up to 40 mL
Adjust pH to 6.8	
Bring volume to 200 mL with **ddH_2O**, store at 4°C for a long-term storage.	

Resolving (running) gel (for 1 gel), 10%	Amount for 5.5 mL for a 1× final concentration
ddH_2O	2 mL
4× Resolving Gel Buffer	1.25 mL
30% Acrylamide (37.5:1)	1.7 mL
10% SDS	50 μL
TEMED	5 μL
Make fresh before every use.	

Stacking gel mix	Amount for 500 mL for a 4× final concentration
4× Stacking Gel Buffer (.5M Tris, ph 6.8)	125 mL
30% Acrylamide (37.5:1)	67 mL
10% SDS	5 mL
ddH_2O	up to 300 mL
Bring volume to 500 mL with ddH_2O, store at 4°C.	

⚠ CRITICAL: Acrylamide is a toxic chemical, may cause cancer, fertility defects, is a skin and eye irritant. Wear protective PPE and dispose of safely according to your institution's policies.

Stacking gel (for 1 gel)	Amount for 2.1 mL for a 1× final concentration
Stacking Gel Mix	2 mL
10% APS	50 μL
Ponceau stain	20 μL
TEMED	20 μL
Make fresh before every use.	

⚠ CRITICAL: TEMED is a highly toxic flammable (vapor) chemical, harmful if swallowed or inhaled. Wear protective PPE and dispose of safely according to your institution's policies.

Blocking buffer (5% milk in TBST)	Amount for 50 mL for a 5% final concentration
Dry Milk Powder	2.5 g
TBST	up to 45 mL
Stir with stir bar until completely dissolved	
Bring volume to 50 mL with ddH_2O, make fresh before every use.	

STEP-BY-STEP METHOD DETAILS

Fly ovary dissection

Timing: ~1–2 h

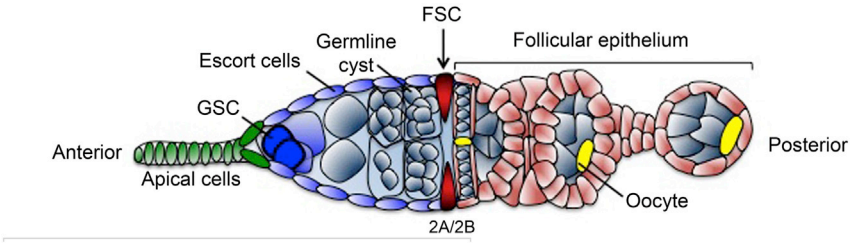

Figure 1. Fly Germarium Schematic: FSCs (red) reside in region 2A/2B and produce Follicle Stem Cells (FSCs, pink)
Germline stem cells (GSCs) (dark blue) reside anterior, adjacent to apical cells (green) and produce 16-cell germline cysts, including the oocyte (yellow). Germ cell-follicle cell units form egg chambers. Figure reprinted with permission from (Singh et al., 2018).

This major step outlines dissection of *Drosophila* ovaries, yielding intact ovarioles that are teased apart and ready to be fixed and fluorescently stained.

1. Pour 2 mL of Grace's Insect Medium into the center of your Sylgard coated petridish (DOW chemical).
2. Pour a small 1 mL "trash" pool of 1× PBS off center. Be aware of the location of the trash pool and make sure the two pools don't combine even if the dish gets bumped.
3. Anesthetize the females on the fly pad using CO_2.

⚠ CRITICAL: It is critical to finish the dissection within 30 min of anesthetizing the flies to avoid tissue damage. We recommend that beginners build up to dissection of 15–20 female flies within 30 min.

4. Using a pair of forceps, select a female fly (use your dominant hand).
5. Place the fly on the dish, outside of the Grace's pool. Gently pin the female to the dish with forceps in your non-dominant hand and quickly and carefully detach the head from the thorax to euthanize the fly. (Place forceps in dominant hand at the "neck" and slide away from body.)
6. Now move the fly to the Grace's and submerge. Gently hold the thorax (non-dominant hand) and grasp the posterior abdomen with the forceps of the dominant hand. Pull gently until the ovaries slide or pop out, along with intestines.

Note: In step 6, sometimes ovaries can be very plump (full of eggs) and easy to identify and dissect. Sometimes, due to genetic manipulations or nutrient restriction, ovaries can be underdeveloped and must be dissected carefully with precision.

7. Discard all parts of the fly that are not ovaries including as many large eggs as possible, in the "trash" pool. See problem 1.
8. Using your forceps, remove the sheath (black hair-like fiber bundling the ovary) and gently tease apart the ovaries in a brushing manner with the forceps to release individual ovarioles. To maintain the integrity of the stem cell compartment (germarium, Figure 1), run the forceps from posterior (egg side) toward the anterior (germarium side) in between adjacent ovarioles to separate the ovarioles, also see Figure 3.

Note: In step 8, be very gentle with the forceps. Don't pull out the individual ovarioles. Instead, gently tease the ovaries apart such that they are still bound at the top like a bunch of grapes (Figures 3 and 4).

9. Repeat until you have plenty of ovarioles sitting in the Grace's Insect Medium.

Figure 2. Starvation chamber setup depicting perforations (at the conical base of the 50 mL conical tube) for air flow for flies and mounted on a fruit juice plate using parafilm

10. Once finished, cut the end off a 200 μL tip, collect the ovarioles very carefully. To do this, fill up the cut-off tip with ~50 μL Graces before pipetting the ovarioles and pipette a small amount at a time to avoid sticking of sample to the walls of the tube and loss of material to the tip. Collect all the ovarioles in this manner into a labeled Microcentrifuge tube.
11. Tap the tube gently to nudge the ovarioles to settle down and let the tube rest on a rack for 2–3 min. Once the ovarioles settle to the bottom (hold the tube up to the light to see if there are any still floating in the liquid), remove as much liquid as possible.

Immunofluorescence of fly ovarioles

Timing: ~2 days

This major step immunostains ovarioles (*ex vivo*) with Lysotracker and antibodies of interest.

12. Add 200 μL of the Lysotracker working stock (1:100) per tube of ovarioles for 5 min and wrap the tube in foil. Wash with 1× PBS once and proceed to the next step.

Note: Lysotracker is a cell-permeable dye (Chazotte, 2011), it must be added to the tissue or cells prior to the fixation step.

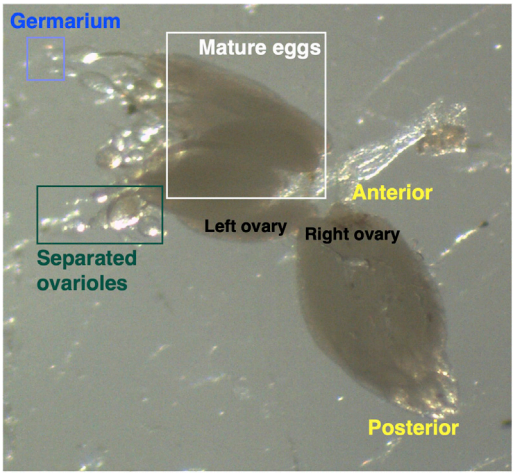

Figure 3. A pair of ovaries dissected and captured using the 10× objective of a dissecting stereoscope showing one ovary (right) with intact ovarioles and mature eggs versus the second ovary (left) with ovarioles teased apart from each other

13. Fix the ovarioles by adding 200 μL of 4% paraformaldehyde. Rock for 10 min at room temperature.
14. Wash thrice in 400 μL of PBS + 0.3% Triton (PBST) to permeabilize the tissue and rock for 10 min in between washes at room temperature. Be sure to let the tissue settle to the bottom of the tube after each wash to give the germaria time to settle to the bottom of the tube.
15. Make the working stock of your primary antibody of interest in PBST+0.5% BSA. For example: Fas III (at a concentration of 1:100) (Developmental Studies Hybridoma Bank, Cat.#7G10), is useful for staining the follicular epithelium (Zhang and Kalderon, 2000)). Let the ovaries settle, remove all the liquid and add 200–400 μL of primary antibody working stock per tube and rock overnight at 4°C.

Pause point: Fixed ovaries can be incubated with primary antibody either overnight or up to 2 days protected from light, on the shaker at 4°C.

16. Next day, wash thrice with PBST for 10 min each.
17. Let the ovaries settle, remove the PBST, then add working stock of secondary antibody (diluted in PBST+0.5% BSA) and rock the samples for 2 h at room temperature. Protect your samples from light from this point onward by covering the tubes with foil.
18. Incubate for 10 min in RNAse A to digest the RNA.

Note: It is not necessary to use RNAse, but this step will greatly enhance nuclear staining by reducing the background from mRNA in the cytoplasm which also binds to Draq5.

19. Add Draq5 or DAPI (1:1000) in PBST 100 μL, incubate and rock for 10 min.
20. Add 400 μL of PBS and incubate 5 min, rock and wash twice with PBS.
21. Take your samples off the rocker, let them settle, and carefully remove as much liquid as possible.
22. Add 30 μL Vectashield/sample, pipette up-down once to mix the Vectashield and gently pipette the contents of the tube on a slide and mount with 22 × 22 #1.5 coverslip.
23. Place folded Kimwipe on cover slip and put a weight on the top.

⚠ CRITICAL: It is critical in step 23 to avoid bubble formation. A gentle press can be applied to release the bubbles. In our hands, placing a Kimwipe on top of the slide, and then layering an upside-down cover slip box over the slide sets a weight-distributed platform for partially flattening the tissue. Place a half-filled 100 ml glass bottle on top of the coverslip

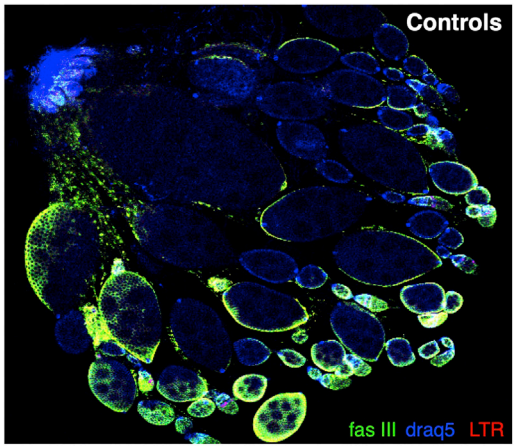

Figure 4. Immunofluorescence-stained ovary from a fly from the w^{1118} (wild-type) genotype with Fas III, Draq5 and Lysotracker (LTR)

to help spread out the individual ovarioles and improve imaging of the germaria as shown in Figures 3 and 4, with individual ovarioles stained with Fas III, Draq5 and Lysotracker (LTR). Too much pressure and weight can break the ovarioles.

24. Seal the edges of the cover slip with a thin layer of clear nail polish and dry for at least 5 min. Slides can be imaged immediately or at a later time, see pause point below.

Pause point: Slides can be stored in an airtight slide storage box at 4°C for short term and at −20°C for long term storage.

Confocal imaging of LysoTracker-stained fly ovarioles

Timing: ~1–2 days

This major step is important for quantifying Lysotracker stained FSCs and obtaining fluorescent images of the *Drosophila* germarium using a confocal microscope. For additional details also see Methods video S1, how to setup and use the Confocal Microscope to identify and score FSCs, positively stained for lysotracker.

25. Setup and turn on the lasers: for Fas III (stained with FITC secondary antibody) use 488 channel (green), and for Lysotracker dye use the 594 channel (orange).
26. Using the 20× objective and the FITC channel find germaria (see Methods video S1, how to setup and use the Confocal Microscope to identify and score FSCs, positively stained for lysotracker) and focus.
27. Switch to the 40× oil objective, focus and move to one end of the slide and start quantifying.
28. To count the germaria on the slide, go across the slide before slowly moving down and then going back across. Use a sweeping motion to make sure not to miss any germaria and avoid counting a germarium twice.
29. Switch back and forth between the green channel and red channel, to correctly quantify Lysotracker within the FSCs (Region 2A/2B), Fas III staining will help in identifying this location within each germarium. Also refer to Figure 1 of the fly germarium and Methods video S1(how to setup and use the Confocal Microscope to identify and score FSCs, positively stained for lysotracker) which describes in detail the identification and quantification of lysotracker positive FSCs in the region 2A/2B of the germarium.

30. For every germarium counted, quantify Lysotracker staining by designating '0' if no FSCs are stained with Lysotracker (negative) and '1' if at least one FSC is stained with Lysotracker (positive).
31. Periodically take representative images of germaria that have positively or negatively Lysotracker stained FSCs.

⚠ CRITICAL: Turn on the Confocal Microscope and warm up the lasers at least 30 min prior to quantification.

⚠ CRITICAL: Take out your slides from 4°C (short-term storage) or −20°C (long-term storage) before you turn on the confocal microscope and bring them to room temperature.

⚠ CRITICAL: If not sure that a FSC is positively or negatively stained with Lysotracker, use the live camera to take an image and or a z-stack to ensure you are quantifying correctly.

Prepping whole ovaries for western blotting

Timing: ~1–2 days

This major step prepares a protein sample from intact ovaries that can be analyzed using the Western blotting technique to observe changes in Ref (2)P associated with active autophagy. Western blotting for Ref (2)P serves as another positive measure of autophagy induction in fly ovaries (Lorincz et al., 2017; Singh et al., 2018).

32. Harvest the ovaries in Grace's media (as described in steps 1–11). Collect a minimum of 10 fed female flies (20 ovaries) or 20 female flies for nutrient restricted conditions and detach as many mature or large eggs from the ovaries as you possibly can by gently brushing/teasing them out using the 27Gx1/2 needle for each genotype/fly sample. Place the ovaries in a microcentrifuge tube.
33. Let the ovaries settle to the bottom for 5 min and remove as much liquid as possible.

Pause point: At step 33, you can snap freeze the ovaries in liquid nitrogen or by using ethanol and dry ice. In a cryobox with dry ice, pour some ethanol over dry ice to create an ultra-cool liquid pool. Using forceps, place your tubes (with ovaries) through an open rack to ensure the tissue is immersed in the cold ethanol to freeze the ovaries. The liquid pool created by ethanol addition freezes the tubes uniformly and quickly.

Note: Once frozen, samples can be stored at −80°C for a long term until ready to use for lysis and Western blotting. This temperature will preserve the ovary tissue for subsequent protein analysis.

34. Add 100 μL of 1× RIPA lysis buffer to each ovary sample in microcentrifuge tubes.
35. Use a fresh pestle to homogenize ovaries by hand. Grind the ovaries for a minute. Keep on ice. Assemble a 27Gx1/2 needle attached to a 1 mL syringe and suck up lysate to shear the sample at least five times.

Note: Avoid making bubbles and gently release the sheared ovaries into the tube.

36. Once your ovary samples are homogenized in 1× RIPA lysis Buffer, incubate samples on ice for 15 min.
37. Spin down the lysate samples at 14,000 RPM (high speed) 4°C for 10 min.
38. Collect and transfer the supernatant to a labeled microcentrifuge tube on ice.

39. Determine the protein concentration of your sample using the Bradford Assay (Biorad) and BSA standard curve.
40. Based on your protein sample concentration, calculate the volume of protein needed to load 10 ug of protein per sample (50 ug for nutrient restricted flies) on a 10% SDS-PAGE gel.
41. Calculate and add the volume of 6× sample buffer needed to prep each sample. Mix, vortex and place the samples on a 95°C heat block to denature proteins for 5 min.

Pause point: You can freeze your protein samples at −20°C for long term storage, or you can immediately load your samples into the SDS-PAGE gel (as described in the steps below).

42. Setup the vertical gel caster electrophoresis apparatus and clamp the glass plates and notched alumina plates with spacer in between on the lower buffer chamber. Prepare 10% resolving gel and pour into the two stacked plates, only fill 3/4th of the plate with the resolving gel to leave room for the stacking gel. Once the gel has polymerized (45–60 min) place a comb and prepare your stacking gel.
43. Pour the stacking gel and let it polymerize (30–45 min).
44. Dilute 10× Running Buffer to 1× concentration.
45. Using 1× Running Buffer, fill the cavity in the center of the tank (in between gels).
46. Carefully and smoothly remove the comb and use a 27Gx1/2 needle attached to a 1 mL syringe to wash out each well with the 1× running buffer without putting too much pressure or else the wells created by the comb can break.
47. Use a p20 pipet to load 5 μL of the protein ladder (Precision Plus Protein Dual Color Standards) in the first well.
48. Once all the samples are loaded, place the interlocking safety lid with leads on top of the gel caster chamber, black to black and red to red. Hook up electrodes to the electrophoresis power supply unit.
49. Turn on and run the gel at 120 Volts (constant milliAmps) for 1.5–2 h (the observed molecular weight of Ref(2)P is 92 kDa). This should be enough time to resolve Ref(2)P protein in your sample.
50. While the gel is running, dilute the 10× Transfer Buffer to make fresh, 1× Transfer Buffer with Methanol (see recipe under the materials and equipment section).

⚠ CRITICAL: Don't forget to add Methanol to your 1× transfer buffer, it significantly increases the absorption of proteins onto the membrane and enhances transfer efficiency.

⚠ CRITICAL: Methanol is a toxic, flammable liquid, wear protective PPE, avoid inhalation and direct contact with skin or eyes. Dispose of any waste methanol and 1× transfer buffer containing methanol safely according to your institution's policies.

51. While the gel is running, cut 4 pieces of Whatman chromatography paper and 1 piece of nitrocellulose membrane. Cut these to the gel size without any overhangs. Place your nitrocellulose membrane in a plastic dish containing 1× transfer buffer to pre-activate the membrane and soaked in methanol. Get your semi-dry transfer apparatus, forceps, flat spatula, a razor blade and pour 1× transfer buffer in plastic dishes.
52. When dye front is about 1 mm from the bottom of the gel, stop the run and turn off the machine. Take the gel out of the caster by using a flat spatula to pry open.
53. Remove the perimeter of the gel (not required part) with a razor blade. Place the gel in a plastic dish containing 1× transfer buffer to thoroughly equilibrate the gel. Wet two stacked pieces of Whatman paper in transfer buffer and let excess buffer drip out.
54. Make a transfer sandwich between on the base (anode) plate of the two plate electrodes of your semi-dry transfer apparatus. To make the sandwich, sequentially place the stack of two wet (not dripping) Whatman Chromatography papers, followed by wet (pre-activated) nitrocellulose membrane, then place the gel on top followed by two wet Whatman Chromatography papers

on the top, gently squeeze out bubbles using a 15 mL conical tube or wooden pencil between each layer.

55. Place and secure the cathode plate of the transfer apparatus on top of the anode plate with the sandwich in between the two plates. Perform electro-transfer using 100 mAmps, constant volts for 1.5 h.
56. During this time, prepare your blocking buffer (5% Milk in TBST).
57. After the transfer is finished, gently open the lid (cathode plate). Cut the membrane into two; in between the observed molecular weight of Ref (2)P, which is 92 kDa, and for ß-actin (used as a loading control) at 43 kDa. To denote the orientation of each membrane transferred, make a cut in the upper right corner for each membrane.
58. Gently transfer each membrane from the transfer apparatus to a tray containing 3 mL of blocking buffer using forceps. Block the membranes for 1 h at room temperature on a rocker.

Note: Make sure the membranes are completely submerged in the blocking solution and are able to freely oscillate back and forth in the plastic box.

59. After blocking, dilute your antibodies in blocking buffer. We use 1:100 concentration for Ref (2)P and 1:5000 for ß-actin primary antibodies for at least 2 h or overnight on a rocker at 4°C.

Pause point: In step 59, you can incubate your membranes in primary antibodies overnight on a rocker at 4°C.

60. Next day, wash your membranes in 1× TBST thrice for 5 min each.
61. Incubate in secondary antibody, anti-Rabbit (1:3000) for Ref (2)P and b-actin for at least 1 h.
62. Wash your membranes in 1× TBST thrice for 5 min each.
63. Prepare and mix chemiluminescence- ECL reagents A and B (1:1).
64. Place a piece of plastic wrap on the bench top and secure it with tape. Remove excess liquid from your membranes by gently dabbing the corners on a Kim wipe and place the membranes on the saran wrap.
65. Cover the membranes with ECL and incubate for 1 min at room temperature.
66. Gently dab the excess ECL liquid off your membrane on another Kim wipe and place in the X-ray film cassette.
67. Expose your membrane to X-ray film for appropriate length of time starting at 30 min and gradually decrease the exposure time and develop your films.

EXPECTED OUTCOMES

Ovarioles dissected with precision yield 16–18 ovarioles per ovary as shown in Figure 4, stained with Fas III and Draq 5. If the starting number of flies dissected is a minimum of 10, then approximately 400–500 ovarioles will be collected.

Lysotracker dye strongly binds to acidic organelles (Chazotte, 2011). We found Lysotracker to be a reliable method to assess active autophagy in single cells, resident within a tissue (Singh et al., 2018) for the following reasons: A) It was easy to identify and gave a bright signal for positive controls (such as starved ovaries in which autophagy is active in FSCs (Figure 5B) We observed and quantified Lysotracker signal with predictions from the literature, with reduced lysotracker staining observed upon loss-of-function of core autophagy genes including *Atg5* (Singh et al., 2018), (Figure 5C) and increased lysotracker staining upon reducing Insulin/TOR signaling or *Akt* in FSCs (Figure 5D)(Singh et al., 2018), suggesting that increases in lysotracker-positive vesicles are a good indication of active autophagy in ovarioles/FSCs.

A successful ovariole stained with Lysotracker yields a crisp confocal image of individual ovarioles with a stained epithelial layer, cell nuclei and a Lysotracker foci(s) in the FSC niche. Fas III is a

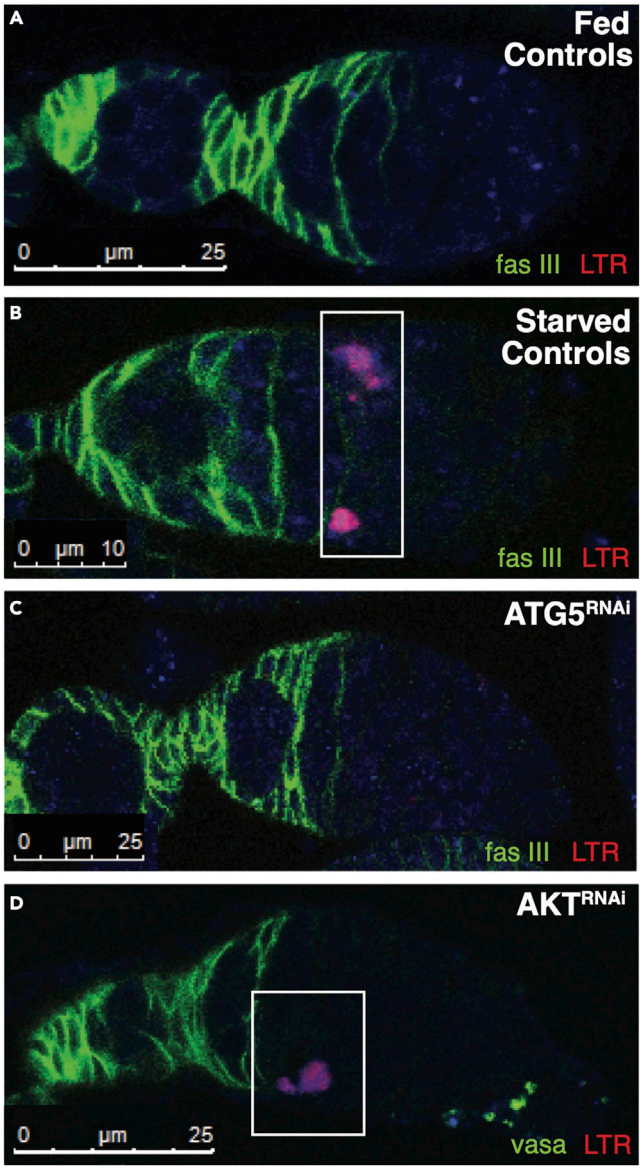

Figure 5. Nutrient restriction and genetic manipulations to confirm Lyostracker staining as a tool to study autophagy in FSCs

(A) Germaria from the ovary of fed control flies stained with Fas III and LTR. Scale bar, 25 µm. (B) Germaria from the ovary of 3d starved control flies stained with Fas III and LTR. Scale bar, 10 µm. (C) Germaria from a fly ovary expressing *Atg5 RNAi* (*w*; *109-30-Gal4*, $Atg5^{RNAi}$) in the FSCs, stained with Fas III and LTR. Scale bar, 25 µm. (D) Germaria from a fly ovary expressing *Akt RNAi* (*w*; *109-30-Gal4*, Akt^{RNAi}) in the FSCs, stained with Fas III and LTR. Scale bar, 25 µm. Boxes depict Lysotracker staining in the region 2A/2B of the germaria.

good antibody to stain the ovarian follicular epithelium. Alternative antibodies can also be used which work well for the 488-laser channel. FSCs are located just anterior to the strong Fas III staining the germarium (see Figure 1 and Methods video S1, how to setup and use the Confocal Microscope to identify and score FSCs, positively stained for lysotracker). Classically defined FSCs are 2 cell diameters anterior to the Fas III stain, at the border of Region 2A and 2B in the germarium (also called "Layer 2")(Margolis and Spradling, 1995; Nystul and Spradling, 2007; Reilein et al., 2017). Cells one cell diameter (Layer 1) or three cell diameters (Layer 3) anterior to the strong Fas III staining also have been shown to exhibit stem cell characteristics, albeit at a lower frequency than cells in Layer 2

(Melamed and Kalderon, 2020; Reilein et al., 2017; Reilein et al., 2018; Rust et al., 2020). Germ cell labeling with anti-Vasa antibodies (Aruna et al., 2009; Hay et al., 1990) also can help pinpoint the location of FSCs, at the anterior side of the fully flattened 16-cell germline cyst (Margolis and Spradling, 1995).

LIMITATIONS

Lysotracker is routinely used in many systems to stain lysosomes and autolysosomes (Mizushima et al., 2010). Lysotracker staining can also give artifacts and autofluorescence. This method alone cannot be used to assess autophagic changes, and other assays must be employed to validate the findings.

During staining with Lysotracker, some cell components and neighboring tissues such as mature eggs can autofluoresce and give false positive results (see Methods video S1, how to setup and use the Confocal Microscope to identify and score FSCs, positively stained for lysotracker). (See problems 1, 2 and 3).

TROUBLESHOOTING

Problem 1

Mature and large eggs within the ovariole can generate autofluorescence with Lysotracker stain and can obscure the quantification of Lysotracker positive germarium. Relevant to step 7.

Potential solution

To avoid autofluorescence and artifactual Lysotracker staining, attention must be paid to the duration of incubation and amount of stain used. Remove as many mature and large eggs from the ovaries during the ovary dissection step. For our studies we performed a dilution series of lysotracker and determined the ideal concentration (1:100 in PBS) and incubation time (5 min). Relevant to step 7, (also see Methods video S1, how to setup and use the Confocal Microscope to identify and score FSCs, positively stained for lysotracker).

Problem 2

False positive staining with high concentration of Lysotracker can lead to autofluorescence and very high background (relevant to step 4).

Potential solution

An optimization step to establish the correct dilution, ranging from 1:50 to 1:2000 for the Lysotracker stain must be employed every time a new system is used to study autophagy using the Lysotracker dye (relevant to step 4). In order to use this protocol in tissues other than the ovary, a dilution series must be performed to determine the appropriate concentration and incubation time.

Validate your results with other assays such as Western blotting or co-staining with known autophagy markers to confirm that the results obtained are autophagy pathway related. This is not always feasible when studying a single cell or a small population in a tissue. Genetic manipulation such as expressing RNAi targeting components of the core autophagy pathway (e.g., Atg1/ULK1, Atg5, Atg13, etc.) or its upstream regulators (Insulin Receptor, Akt, TOR, AMPK, LKB1)(Singh et al., 2018) or use of CRISPR-cas9 or mutant clones to reduce expression of autophagy pathway components should also be used to confirm specificity and corroborate the results obtained by Lysotracker staining. Loss of core autophagy components should abrogate formation of autophagic vesicles but have no impact on lysosomes. Relevant to step 4.

A positive control for Lysotracker staining/autophagy must be used along with the above-mentioned assays, to confirm the results. In our tissue of interest, nutrient restriction is a positive inducer of autophagy and was used to optimize lysotracker usage conditions (see Figure 5B). It is possible that starvation or nutrient restriction might not work in other tissues or model systems, thus genetic

manipulations such as decreasing AKT/TOR or increasing Atg1 expression should be used as positive controls for autophagy activation and validating Lysotracker dye in other systems.

Other assays for validation of autophagy must be performed to substantiate results obtained by Lysotracker staining. In our study, we used P62/Ref(2)P expression changes via Western blotting to confirm autophagic changes (DeVorkin and Gorski, 2014). Ref(2)P is degraded in cells undergoing autophagy. Loss of Ref(2)P protein indicates active autophagy, and the degree of activity can be measured based on the relative amount of Ref(2)P protein as assessed by Western blotting (DeVorkin and Gorski, 2014). The caveat of using Western blots for confirmation of autophagy in FSCs is that we are no longer measuring a single cell but a collection of cells in the ovariole. We carefully dissected the tissue to enrich for germaria in our preparation, resulting in mainly cells from the germarium in the resulting lysate. We were able to correlate in situ Lysotracker staining with observed changes in Ref(2)P levels using this approach, but this a heterogenous pool containing other cells types (germ cells, inner germarial sheath/escort cells, terminal filament, and cap cells, and differentiating follicle cells) (see Figure 1) in the lysate. Expression of Ref(2)P-GFP in FSCs by crossing to 109-30-Gal4 or other drivers expressed in FSCs may be a more cell-specific alternative way to confirm the Lysotracker staining via Ref(2)P expression levels (Nezis, 2012; Pircs et al., 2012)

Problem 3

There are a number of fly constructs available in the fly database that are frequently used to genetically label cells undergoing autophagy. One of the most widely used autophagy markers is Atg8a-GFP, which fluoresces in cells undergoing autophagy (Mauvezin et al., 2014). We found that expression of Atg8a-GFP in FSCs using the 109-30 Gal4 driver disrupted autophagic flux, confounding interpretation of the assay for measurement of autophagy induction. Initially, we found that Atg8a-GFP expression gave us false negative results on co-staining with Lysotracker. To evaluate this further, we tested Ref(2)P staining via Western blotting in the Atg8a-GFP background flies and noted that Ref(2)P degradation was opposite to our expected results in the positive controls, as well as in various genetic backgrounds where autophagy activation using Lysotracker positivity and Ref(2)P degradation had been observed. In FSCs, strong overexpression of Atg8a-GFP may either accelerate autophagy, reducing the number of autophagic vesicles available for staining by Lysotracker, or disrupt autophagic flux, preventing continuous autophagic activity even under conditions where autophagy would be constitutive.

Potential solution

Genetic constructs must be used with caution and thoroughly tested with positive controls and genetic confirmation in order to avoid false negative results. We do not recommend using Atg8a-GFP as a method for autophagy analysis in FSCs. Some systems can use Electron Microscopy (EM) to detect double-membrane vesicles associated with active autophagy. In our system, it was very challenging to locate a small portion of the ovary (germarium) and difficult to identify FSCs under EM specific conditions. Ref(2)P-GFP has been used successfully to measure autophagy in fly brains (DeVorkin and Gorski, 2014). Many scientists use dually tagged Atg8a to measure autophagic flux (Lorincz et al., 2017). We did not test the dually tagged Atg8a in FSCs.

Genetic crosses and manipulations using the model system must be employed to confirm autophagy pathway activation in the cells under study. For our study, we evaluated Lysotracker and Ref(2)P staining under conditions where core autophagy pathway components were reduced (Lorincz et al., 2017): We expressed RNAi targeting *Atg5* under UAS control in FSCs using the *109-30 Gal4* driver, which we previously demonstrated enables strong expression in FSCs and early follicle cell progenitors (Hartman et al., 2015; Hartman et al., 2010). Reduction of core autophagy genes abrogated the Lysotracker signal during aging; for example, decreased in old compared to young wild-type flies, as well as in genetic backgrounds that promote ectopic autophagy induction (e.g., reduced AKT or increased Hedgehog signaling) (see Figure 5). Combination of genetic confirmation with Ref(2)P analysis ensures correct interpretation of lysotracker results.

Problem 4

Hard to identify Ref (2)P protein doublet in a western blot (relevant to steps 32 and 40).

Potential solution

Dissect ten ovaries per genotype and twenty ovaries for nutrient restricted flies. Don't overload the gel and use a 10% SDS-PAGE gel to run the western blot.

Problem 5

Ovarian tissue disintegration while dissecting (relevant to steps 3–11).

Potential solution

Flies should be dissected in room temperature Graces' medium to keep the microtubules intact. Anesthetized flies must be dissected within 30 min and stained with lysotracker for five minutes followed immediately by fixation. Longer times from dissection to fixation can result in initial evidence of tissue disintegration.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alana M. O'Reilly (Alana.OReilly@fccc.edu).

Materials availability

Materials generated in this study are available from the lead contact or can be purchased [details listed in the key resources table].

Data and code availability

This study does not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100592.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.S. and A.M.O.'R.; investigation, T.S., E.H.L., D.M.R.-W., I.C., and M.W.; writing - original draft, T.S. and D.M.R.-W.; writing - review and editing, T.S. and A.M.O.'R.; visualization, T.S., E.H.L., D.M.R.-W., M.W., and I.C.; supervision, T.S., E.H.L., and A.M.O.'R.; project administration, T.S.; funding acquisition, A.M.O.'R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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