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Chapter 41

Methods in Molecular Biology

Investigating mitophagy and mitochondrial morphology *in vivo* using *mito-QC*

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Abstract

Autophagy evolved as a mechanism to sustain cellular homeostasis in times of nutrient deprivation. Mounting evidence has also clarified that under basal and stress conditions, selective autophagy pathways can target the destruction of specific organelles. Mitochondrial autophagy, or mitophagy, has emerged as a key quality control (QC) mechanism to sustain the integrity of eukaryotic mitochondrial networks. We recently reported the development of *mito-QC*, a novel reporter mouse model that enables visualisation of mitophagy with precision, in fixed and live preparations. This model holds significant potential to transform our understanding of mammalian mitophagy pathways *in vivo*, in a variety of physiological contexts. We outline a detailed protocol for use of our recently described *mito-QC* mouse model, including tips and troubleshooting advice for those interested in monitoring mitophagy *in vitro* and *in vivo*.

Key Words

Mitophagy, Autophagy, Mitochondria, Mouse Models, Neurodegeneration, Metabolism, Cancer, Immunology, Cardiology, Vascular Biology, Nephrology, Developmental Biology, Histology, Microscopy

Introduction

Mitochondria lie at the heart of eukaryotic metabolism. Mitochondrial damage and dysfunction has been implicated in the pathogenesis of many human diseases, ranging from inherited rare metabolic disorders to widespread conditions such as cancer and neurodegeneration **(1)**. The seminal discovery that the Parkinson's disease related proteins PINK1 (*PARK6*) and Parkin (*PARK2*) modulate a distinct type of mitochondrial quality control known as mitophagy placed this cellular pathway at the centre of efforts to understand mitochondrial neurodegeneration **(2)**. We now know that many other types of mitophagy exist, for example in response to hypoxia, metabolic remodelling, and iron chelation **(3)**. Although *in vitro* biochemistry and cell biology have formed the basis for much of these insights, little was known about the physiological basis of mitophagy until the recent advent of three mitophagy mouse models: *mito-QC*, *mt-Keima* and *mito-Timer* **(4-6)**. This is because monitoring mitophagy is difficult without a suitable reporter probe *in vivo*. Antibodies to mitochondrial proteins *in vivo* are not reliable, dye-based approaches are incompatible with fixed tissue and electron microscopy remains the gold standard. These limitations are summarised in Table 1. We recently described a reporter mouse model engineered to monitor this type of mitochondrial quality control (QC), called "*mito-QC*" **(4)**. *mito-QC* is based on

a tandem-fusion protein (mCherry-GFP) that is targeted to the outer mitochondrial membrane (OMM) *via* the mitochondrial targeting sequence (MTS) of the OMM-protein, FIS1. Under steady state conditions, all mitochondria fluoresce in both green and red, which when merged gives a yellow colour. However, under conditions where mitochondria are targeted to the lysosome in mitophagy, GFP fluorescence is quenched due to its acid-labile properties – yet mCherry fluorescence remains unaffected. This has the advantage of being able to visualise a mitophagic “end-point”, as red puncta can be scored or counted to provide an index of mitophagy in cells and tissues. Additionally, researchers can also benefit from being able to study mitochondrial morphology – which is a key determinant of mitochondrial and cellular homeostasis (see Figure 1). There are several major advantages of *mito*-QC over other mitophagy models. (1) It is compatible with fixation and a variety of histochemical approaches. This enables researchers to study mitophagy in labelled subsets of cells *in vivo* with ease. At first glance, this advantage may seem irrelevant, however this distinguishing feature of *mito*-QC from other available models is crucially important. This is because not all cells are equally susceptible to dysfunction *in vivo*. This is particularly true in the case of neurodegenerative diseases, where neural subpopulations exhibit multifactorial pathology accompanied by a high degree of selective vulnerability. Common examples of this include A9 dopaminergic neurons that degenerate in Parkinson’s disease (PD), GABA-ergic medium spiny neurons in Huntington’s disease (HD), entorhinal and hippocampal CA1 neurons in Alzheimer’s disease (AD), ventral motoneurons in amyotrophic lateral sclerosis (ALS) and cerebellar Purkinje neurons that degenerate in the rare

lysosomal storage disorder, Niemann-Pick Type C1 (NPC1). In the case of mitochondrial DNA (mtDNA)-associated diseases, patients present with a vast degree of clinical and tissue-specific heterogeneity. The cause of selective vulnerability across these aforementioned conditions remains unknown and thus, understanding the regulation of mitochondrial homeostasis between different cell subtypes *in vivo* remains a vital area of biomedical research. (2) This compatibility with fixation also enables a high-throughput approach to assessing mitophagy and mitochondrial biology in experiments where large sample sizes are required, without the uncertainty, low-throughput limitations and labour involved in live-tissue imaging methods. (3) *mito-QC* affords researchers the ability to visualise end-point turnover in the context of the entire mitochondrial network *in vitro* and *in vivo*. Thus in addition to mitophagy, mitochondrial dynamics and organelle crosstalk can also be investigated. (4) As *mito-QC* is a tandem-tag protein, it enables the measurement of mCherry-only signal to GFP. This ratio is useful for a variety of analyses, ranging from microscopy to FACS. (5) As excellent antibodies are available to detect GFP, researchers can obtain gold-standard verification in their specimens by immunogold electron microscopy. (6) GFP and mCherry work as an excellent tandem pair, due to their fast maturation times and photostability (7). Given that the delivery of mitochondria to lysosomes may occur over several hours, maturation times should not be a concern as mitolysosomes are identified as distinct mCherry-only structures. Furthermore, even in the case of suspected dequenching of the reporter signal - the distinct morphology of mitolysosomes enables a bona-fide readout of mitophagy in concert with LAMP1 (or equivalent) immunolabelling of

lysosomes. Recently, we used mito-QC to demonstrate that basal mitophagy is unaffected in mammalian tissues lacking a functional PINK1-Parkin signalling pathway (8). Demonstrating the power of this approach to monitor mitophagy *in vivo*, mito-QC was also used to demonstrate the evolutionarily-conserved and PINK1/Parkin-independent nature of basal mitophagy (9). We hope this protocol will serve as a useful reference for both experienced and newcomers to *in vivo* cell biology.

Materials

IMPORTANT: Obtain ethical and institutional approval for all procedures involving the use of animal subjects. In this study, all experiments were subjected to ethical approval from the University of Dundee, in addition to being performed by licensed and trained individuals in accordance with the UK Animals Act (ASPA) 1986, on a Home Office Project License.

Basic isolation of tissues

1. *mito*-QC mice at desired age
2. 1X PBS (Gibco) Life Technologies
3. Fixative: 3.7% Formaldehyde in 200 mM HEPES, at pH 7.0 –
 - **Prepare fresh on day or 24 h before; store at 4°C on ice**
4. 70% EtOH (for sterilisation)
5. Autoclaved, sterile surgical instruments – Fine Science Tools

Isolation of adult tissues following trans-cardial perfusion (or perfusion-fixation)

1. As above, but with additional appropriate surgical implements for procedure
2. Automated Perfusion: Suitable perfusion system (Perfusion One – Leica or equivalent) or Peristaltic Pump. For manual delivery of perfusate, use a 20 ml syringe.
3. 1X PBS (Gibco) Life Technologies
4. For *trans-cardial perfusion with PBS*:
 - a. Perfuse with 1X PBS (Gibco) Life Technologies until blood runs clear, rapidly excise organs of interest and place into labelled tubes containing 3.7% Formaldehyde in 200 mM HEPES, at pH 7.0 – make fresh on day or 24 h before; store at 4°C.
5. For *trans-cardial perfusion-fixation*:
 - a. Perfuse with 1X PBS (Gibco) Life Technologies and switch to 3.7% Formaldehyde in 200 mM HEPES, at pH 7.0 – make fresh on day or 24 h before; store at 4°C. Rapidly excise organs of interest and post-fix in labelled tubes containing 3.7% Formaldehyde in 200 mM HEPES, at pH 7.0.
6. Fixation times will vary depending on the age of the animal, type and size of specimen. For adult organs, we typically post-fix for 24 hours at 4°C. For embryos, fixation times will vary according to developmental stage.

Tissue sectioning

1. Sucrose (D-saccharose)
2. Peel-A-Way histology moulds (Ted Pella) or equivalent

- a. **NOTE: A range of different moulds are available. Select the one best for the tissue of interest, depending on size and sectioning setup.**
3. Cold room or fridge at 4°C
4. OCT – Sakura (Cryoprotectant – for cryosectioning)
5. SuperGlue (For vibrasectioning)
6. For sectioning:
 - a. Thaw-mounted sections - Cryostat: e.g. Leica CM3080
 - b. Free-floating sections – Vibratome: e.g. Leica VT2000
7. Leica SurgiPath slides
8. Ice-cold 1X PBS (if sectioning using vibratome)
9. 12 or 24-well-plastic dishes containing 1X PBS, Sable Paintbrush (To collect free-floating vibrasections)
10. Cryoboxes with desiccant, suitable for slide storage (cryosections)

NOTES:

- a. **We typically section adult brains on a vibratome, thickness depends on orientation and desired downstream application. For horizontal (transverse) vibrasections, we use 100-200 micron sections. Free-floating cryo-sections may be obtained using a sledge microtome with a freezing-stage attachment.**
- b. **On the cryostat, sections ranging from 5-50 microns can be acquired. It can be difficult to obtain consistent sections below 8 microns, although such thin tissue sections may be required for specialist microscopy applications. As a general rule of**

thumb, we typically acquire good cryosections between 12-20 microns.

- c. For cryosections: optimisation of tissue section acquisition/collection may vary between instruments. Consult an experienced histologist for practical guidance, as many factors (tissue type, chamber and specimen core temperature, anti-roll plate, adequate fixation and freezing of specimen, blades and technical expertise) can greatly influence the quality of tissue sections obtained. Air dry sections and store at -20°C to -80°C, or proceed to IHC.
- d. For vibrasections: free-floating sections are collected in appropriate 12-24 well dishes at 4°C. The addition of sodium azide to PBS will prevent putrefaction and microbial growth over time, without interfering with fluorescent signal.

Nuclear counterstaining

1. We typically always use DAPI or Hoescht (Blue); A far-red stain such as ToPro-3 may also suffice, although we have not tested this extensively.

Mounting of tissue sections

1. No.1.5 coverslips MenzerGlaser
2. Vectashield H-1000
 - **NOTE:** For all experiments with *mito-QC* tissues, Vectashield H-1000 provides excellent and consistent results, without any detectable loss of signal for up to 1

year. We have encountered unusual imaging artefacts with Vectashield “hard-set” formulations that contain a hard setting agent. For this reason, we advise against the use of mounting media containing a hard-setting agent.

3. Suitable quick-drying sealing agent: Rimmel 60-Second Shine (Transparent) or TopCoat.
4. Kimwipes/Absorbent Lint-Free Tissues
5. Slide Folder, storage space at 4°C.

Imaging of mito-QC tissues and cells

1. Zeiss LSM 710 META or LSM880 with Airyscan; Laser Scanning Confocal Microscopes or Equivalent, with 4x, 40x, 63x and 100x objectives
2. Multiphoton laser for Deep Tissue Imaging
3. For live cell imaging: Spinning-disk confocal microscopes may also offer a faster and milder platform for live-cell imaging experiments.

Verification of mitolysosomes by LAMP1 immunohistochemistry and immunocytochemistry

1. Antibody to LAMP1: Rat anti-LAMP1, clone 1D4B, Developmental Studies Hybridoma Bank.

Establishment of primary MEF cultures from mito-QC embryos

1. *mito*-QC embryos at desired/appropriate stage of gestation (E13.5-E17.5)
2. 10 cm Tissue Culture-Treated Petri Dishes
3. Autoclaved, sterile surgical instruments – Fine Science Tools

4. Sterile, disposable scalpels
5. DMEM containing 10% FBS, 2 mM L-Glutamine, 50 U/ml Penicillin, 50 ug/ml Streptomycin, 1X Sodium Pyruvate, 1X Non-Essential Amino Acids.

Immunohistochemical labelling of tissue sections

1. *mito*-QC tissue sections
2. Slide staining tray/system
3. 1X PBS
4. Disposable Pasteur pipettes/squeegee bottle
5. Primary and Secondary Antibodies
6. Detergent: Triton-X100
7. Optional: 1% SDS for antigen retrieval

Crucial: preparation of fixative and solutions & *mito*-QC stability

mito-QC exploits the acid-labile properties of GFP and the stability of mCherry to provide an end-point readout of mitophagy. The most critical aspect to the success and interpretation of the assay is the correct preparation of fixative at the appropriate pH. For all experiments, we use 3.7% formaldehyde in 200 mM HEPES buffer, pH 7.0. Experiments using *mito*-QC in cultured cells have demonstrated de-quenching of GFP in specimens processed with fixative that is not at pH 7.0. Heat-mediated de-quenching of GFP can also occur, and as such – heat-mediated antigen retrieval is currently not possible with *mito*-QC. For those wishing to optimise antibody staining, we recommend SDS-mediated antigen retrieval, as described in **(10)**. Researchers should also be mindful when using reagents that require heating e.g. use low-melting point

agarose that has cooled sufficiently. Nonetheless, even in a de-quenched sample, the morphology of mitolysosomes is distinct from that of mitochondria, and users can verify mitophagy by simply using IHC to a lysosomal marker such as LAMP1, which provides an excellent way to validate the lysosomal nature of mCherry-only puncta.

Preparation of fixative

3.7% formaldehyde is prepared by adding paraformaldehyde to water and heating to 60°C with stirring. All steps must be performed in a designated chemical fume hood as formaldehyde gas will be produced (CAUTION: carcinogenic). When up to temperature add drops of potassium or sodium hydroxide to clarify solution, and remove from heat to cool. The addition of 1M HEPES at pH 7.0 gives a final concentration of 200mM and 3.7% formaldehyde. The preparation of a 1M HEPES stock solution is therefore essential to ensure appropriate pH. Filter sterilise solution and store at 4°C in dark until use. For experiments involving freshly excised tissues or perfusion, formaldehyde must be prepared fresh and used within 24 hours. For cell-based experiments, aliquots from a freshly prepared batch of formaldehyde stored at -20°C for single use. **NOTE: for best results avoid the use of commercial fixatives, which often contain up to 10% (v/v) methanol (CH₃OH) as a stabilisation agent. Fixation using organic solvents such as methanol simultaneously precipitate proteins and extract lipids from specimens, and thus may affect organelle morphology and GFP expression.**

Considerations for experimental designs using *mito-QC*

mito-QC is expressed from the *Rosa26* locus, and is found ubiquitously in all tissues. Heterozygote animals with one copy of the transgene demonstrate fluorescence in all tissues, however homozygous animals (two copies) exhibit noticeably pronounced fluorescence. We recommend that for analyses of neural tissue (particularly brain), researchers utilise homozygous *mito-QC* mice. It is also worth noting that although *Rosa26*-mediated expression affords ubiquitous inter-tissue expression, we observe a mosaic-type pattern of expression in the liver, and a prominent expression of the reporter in blood vessels. This does not affect the ability of the reporter to provide an accurate readout of mitophagy, but users should optimise laser settings in order to obtain consistent results from these particular tissues. Endogenous signal from *mito-QC* usually provides excellent results without the need for amplification. However, as with all transgenic models that use fluorescent proteins such as GFP and mCherry, researchers can avail of commercial reagents (anti-GFP or anti-mCherry/RFP antibodies) to enhance signal should this be required. For immunofluorescence, we favour chicken anti-GFP (ab13909) from Abcam, Rabbit anti-GFP (A11122) from Life Technologies/Thermo Fisher Scientific and chicken anti-GFP (GFP-1020) from Aves. For immunoblotting experiments, mouse anti-GFP (1181460001) from Roche yields excellent results.

Establishment of MEF cultures to study mitophagy from the *mito-QC* mouse model

Despite being one of the most heterogeneous cell populations described in mammals **(11)**, MEFs have been used to as a tractable source of primary cells to interrogate cell biology and signalling mechanisms in myriad transgenic mouse lines. Indeed, their ease of culture and propagation makes them attractive for a variety of assays. We have established MEFs from *mito-QC* embryos at a range of gestational ages from E12-E17.5. This is a general protocol that should suffice for the routine generation and maintenance of *mito-QC* MEFs and the study of mitophagy and mitochondrial dynamics *in vitro*. Positive controls to assess stimulus-induced mitophagy are available. To study PINK1-dependent mitophagy, researchers can overexpress the RBR E3 ubiquitin ligase Parkin and use mitochondrial depolarisation agents to trigger mitophagy (data not shown). To study PNK1/Parkin-independent mitophagy, MEFs can be stimulated with the iron chelation agent deferiprone as described in **(4,10)**.

NOTE:

- **Heterozygote matings will yield wild-type (reporter negative), heterozygote and homozygote (reporter positive) genotypes. Mixed cultures can be established to perform comparative analyses in a variety of cell biology paradigms as described in McWilliams *et al.*, (2016). For general analyses, tend to favour the use of het and hom embryos. Wild-type embryos can be distinguished from *mito-QC* embryos using a microscope equipped with epifluorescence. However, diagnostic end-point PCR is required to distinguish between HET and HOM embryos.**

- **The isolation and dispatch of embryos is a regulated procedure, and thus we will not outline this protocol here. Perform in accordance with your institutional and national guidelines governing the ethics and treatment of animal subjects.**
- **Embryo staging practices may vary between institutes, however all of our experiments are performed with embryos staged according to the criteria of Theiler (1976).**
- **MEFs may be immortalised using T-cell antigen (SV40) however, be mindful that this transition may affect metabolic and mitochondrial status which could impact on the induction of mitophagy.**
- **In the event that mouse maintenance is not possible, Lentiviral and retroviral *mito-QC* constructs are available for the generation of stable cell lines as described in (12).**

To establish primary MEF cultures from mito-QC embryo litters:

1. With the aid of a dissection microscope in a laminar flow cabinet, remove all extra-embryonic tissues and decapitate embryos using a scalpel in cold sterile PBS or L-15 medium.
 - a. A separate scalpel may be required for each individual embryo if different genotypes are a concern.
2. Eviscerate embryos and remove a sample biopsy for genotyping by diagnostic PCR.
3. In a 10 cm petri dish, homogenise eviscerated tissue with scalpels into fine cubes. Change scalpels between embryos.
4. Incubate tissue with 4 ml 0.025% trypsin (Gibco) for 5-10 min at 37C.

5. Neutralise reaction with 7.5 ml complete MEF medium and transfer to a 15 ml falcon.
6. Centrifuge at 500 x g for 5 min at room temperature.
7. Carefully aspirate supernatant and re-suspend the tissue pellet in 10 ml of media.
8. Triturate briefly and transfer to a 10 cm tissue culture dish.
9. Gently distribute cells by mixing in a figure-of-eight motion, incubate at 37C.
10. Change media every two to three days as required.
11. When cells are confluent they are further propagated or frozen down.
12. Seed cells for analyses in appropriate dishes.

Isolating tissues from the *mito-QC* mouse to perform histological analyses

Tissues may be isolated fresh (i.e. after cervical dislocation or CO₂), however perfusion remains the gold standard to remove blood-containing immunogenic components and provides superior results for downstream analyses. This protocol will detail both methods, however users should be advised that the latter method is recommended to achieve consistent results – especially in the case of immunohistochemistry and to avoid haematopoietic breakdown products (e.g. lipofuscin) which can confound analyses due to autofluorescence.

1. For Direct Isolation of Tissues e.g. visceral tissues

1. Perform euthanasia by an approved/licensed method and confirm death.
2. Sanitise cadaver using 70% EtOH and prepare for laparotomy
3. Using surgical scissors and forceps, make a longitudinal incision along the abdomen
4. Identify organ(s) of interest and excise rapidly, taking care not to damage any other tissues
5. Wash excess blood from isolated tissue(s) using chilled tissue culture grade PBS and perform further subdissection if required.
6. Fix isolated tissue by immersion fixation.
 - a. NOTE: In keeping with good histological practice, the volume of fixative should be 20X that of the organ to ensure appropriate penetration of fixative. Fixative should be prepared fresh on the day and used within 24 hours.
 - b. Cutting tissues into smaller pieces will aid the fixation process, however this is at the expense of anatomical accuracy and can compromise spatial evaluation of mitophagy in a given region.
 - c. Depending on your cellular population of interest, fixation times may vary. It is important not to overfix tissues.
7. Following fixation: wash tissues in 3X in 1X PBS at 4°C.
8. Prior to cryosectioning, cryoprotect tissues in 30% w/v sucrose/1X PBS at 4°C. Cryoprotected tissues will sink to the bottom of the tube over time. At this point, fixed tissues can be stored at 4°C. Long-term storage may require the addition of a low concentration of sodium azide (NaN_3) to sucrose to prevent microbial growth and putrefaction.

- a. **NOTE:** The w/v percentage of sucrose may be varied depending on type, size and age of tissue specimen. Many histological protocols stipulate concentrations ranging from 10-30% (w/v). For consistency, we have used 30% throughout – however, increasing gradients of sucrose may also be used if preferred.
9. For Cryosectioning: remove tissue from sucrose and quickly remove excess sucrose by blotting with a lint-free tissue (e.g. kimwipe). Place tissue in a labelled cryomould with OCT, let equilibrate for 5-20 min at room temperature. Use a permanent marker to label cryomould. Ensure sure no bubbles are present near the tissue – these can be removed using a hypodermic needle. Orient specimen as desired. The bottom of the cryomould will be the primary sectioning surface.
- a. **NOTE (1):** Histological practices and styles often vary between laboratories and there is no “single correct method.” In our experience, equilibration steps at this stage may be shortened or omitted without compromising *mito-QC* signal.
 - b. **NOTE (2):** We advise researchers to be mindful at the embedding step. As the bottom of the cryomould will be the sectioning start point, the correct orientation of fixed tissue at this point is crucial to obtain satisfactory results downstream. Be mindful of tissue orientation and anatomical plane e.g. sectioning *mito-QC* tissues in different planes will yield different mitochondrial network

morphology. This will not affect the quantitation of mitophagy, but care is advised to achieve consistent results. Embedding multiple organs or organ biopsies within the same block also provides a high-throughput way of sectioning, in addition to valuable comparative analysis.

- c. NOTE (3): Many researchers often remove excess tissue at this point e.g. visceral fat overlying organs etc. Generally speaking, we do not do this as comparative analyses are extremely valuable.**

10. Freeze the mould containing OCT and tissue into a cryo-ready sectioning block. This is best achieved by partially lowering the mould into a suitable crucible containing chilled isopentane. The OCT will turn white, indicating a liquid to solid transition. This first begins at the periphery and continues around the tissue. Keep temperature constant at this step. When the entire block turns white (including the surface of OCT), the mould may be fully submerged into chilled isopentane for >1-2 min.

- a. NOTE: isopentane may be chilled using dry ice – some researchers favour the use of crushed dry ice for packing and more controlled freezing. Liquid nitrogen may also be used, although freezing may occur in a more rapid and unpredictable pattern. Tissue and OCT have been reported to crack using this method.**

11. Remove frozen block from isopentane, blot off excess liquid and place in cryostat chamber, ready for sectioning.

a. NOTE:

- i. If sectioning immediately, tissue block will need at least 20 min to equilibrate to cryochamber.**
- ii. If sectioning at a later date, store blocks on dry ice until finished. For long-term storage, wrap blocks individually in tinfoil, and label with tape. Place in sealed bag in a cryobox and store at -20°C for short term storage, or -80°C for long term storage.**

12. For cryosectioning advice, consult an experienced histotechnologist.

Each cryostat is variable and a range of different parameters can greatly influence the integrity and quality of sections obtained. We use a thaw-melting technique to mount cryosections on slides, with minimal manipulation of sections. Slides are air-dried at room temperature for several hours before proceeding to staining/mounting or long term storage in a cryobox.

- a. NOTE: Each tissue behaves differently when cut, and will exhibit different characteristics when mounted to a slide e.g. some will adhere more easily, others prone to imperfections such as wrinkles/bubbles etc. Smaller biopsies may prove easier to handle for beginners, however this sacrifices a wealth of spatial information that can be obtained from an anatomically intact organ.**

1. Isolating Tissues and Trans-cardial Perfusion e.g. Adult Brain

Trans-cardial perfusion-fixation remains the gold-standard for immunohistochemical analyses. However, in the absence of a suitable setup (appropriately vented chemical fume cupboard, equipped for surgery), trans-cardial perfusion with PBS combined with immersion fixation provides an equally effective way to assess mitophagy combined with immunohistochemical labelling.

NOTE: this procedure should only be conducted by experienced professionals licensed to perform anaesthesia and surgery, and thus we will not detail the specifics of perfusion surgery. Record any adverse side effects or events during anaesthesia that may inform results obtained from downstream analyses. Obtain ethical approval and consult your institutional named veterinary surgeon and compliance officer before proceeding.

1. Commence terminal anaesthesia using I.P. administration of pentobarbital (Euthetal) we have not tested the effect of different agents on *mito-QC*, and so users who wish to utilise different compounds should be mindful of any potential effects on downstream analyses).
2. Proceed with trans-cardial perfusion once animal has reached a surgical plane of anaesthesia.
3. Use temperature-equilibrated buffer (1X PBS or equivalent) as the initial perfusate
 - a. If you are not performing perfusion-fixation, and are only using PBS – perfuse animal with PBS until blood is removed.

- b. Note, other physiological solutions may be used e.g. Krebs, Ringers/Tyrodes etc. – however we do not know the effect of these with the reporter.
4. When perfusate becomes clear, switch to fixative.
5. Observe for standard signs of fixation throughout surgery e.g. tremor, clearing of the liver.
6. For brain, decapitate animal and perform craniotomy – taking care not to damage tissue. Tissues should appear white, with vasculature minimally (if at all) apparent. If the perfusion has worked correctly – tissues should appear devoid of blood and characteristic vascular morphology.
7. Remove brain and proceed to processing for sectioning.
 - a. **NOTE: For vibrasectioning, brain should be post-fixed as usual and then washed in PBS. Vibrasectioning will take place in PBS, although vibrasectioning can also be performed on cryo-protected specimens.**
 - b. **For brain or other organs, biopsies can be removed and snap frozen in liquid N₂ from freshly excised organs. This facilitates parallel biochemical/other measurements from a single animal.**
 - c. **Organs may be bisected or cut to enable penetration of fixative if desired, however we have found this is not necessary.**

Notes on sectioning with *mito-QC*

Typically, cryosections should suffice for the majority of investigations. Excellent resolution can be obtained in the majority of tissues using cryosections from 12-20 μm . Very thin cryosections (5-6 μm) can be useful with tissues that can be difficult to immunolabel. However, in the case of brain tissue where thicker sections combined with free-floating immunohistochemistry can facilitate the resolution of entire axon tracts and neural pathways – we have found vibratome sectioning to provide excellent results. We routinely use Leica SurgiPath slides for all experiments.

Standard immunostaining protocol using mito-QC sections on slides

1. Defrost slides with tissue sections in a slide tray at room temperature (30 min-1 hour).
2. Rehydrate and wash off residual OCT using 3 x 5 min washes with 1X PBS.
 - a. **NOTE: at this point, detergent can be included in the PBS (e.g. 0.3% Triton-X100) to permeabilise sections.**
3. Incubate using block containing detergent for defined amount of time
 - a. **NOTE: each antibody will have different blocking requirements. Some antibodies require specific sera, some do not require blocking. Consult the relevant literature for your epitope of interest. Over-blocking can compromise the fluorescence of your target epitope.**
 - b. **Detergent is a key factor in the success of immunostaining. We find that 0.3% Triton-X100 works well in the majority of cases. However, researchers should optimise according to**

their needs. Different detergents, combinations and concentrations may yield better results e.g. NP-40, Saponin, Digitonin, Tween-20 etc.

4. Incubate with primary antibody for defined amount of time, in block solution containing detergent

a. Practices vary according to laboratory and antibody. For most antigens, room temperature incubation overnight will yield satisfactory results. More controlled staining can be conducted at 4°C, or for shorter incubation times at RT. Incubation of slides at 37°C can also be used in the case of certain antibodies.

b. Block concentration may be reduced e.g. 5% to 1% BSA. Consult the relevant literature to determine the optimal concentration in your field, bearing in mind that a high concentration of primary antibody over a long incubation period may result in sub-optimal bleed-through. Take time to titrate and optimise conditions according to your needs.

c. If incubating overnight, use a humified chamber. Ensure slides do not dry out as this will be detrimental to the success of the staining protocol. Use strips of parafilm to prevent drying out.

d. This is one staining method, however staining systems exist that exploit capillary action to use less antibody. Furthermore, pap-pens can be used to reduce amounts of

primary antibody and perform multiplex labelling using different primary antibodies on the same slide.

e. Many factors can influence staining success, including section thickness. Detergent concentration may also play a factor in mediating antibody penetration. Consider free-floating sections for troublesome antigens.

f. In addition to detergent, some investigators favour adding a low concentration of inert serum (e.g. BSA) to their wash buffers to help reduce background.

5. Wash primary antibody off with PBS containing detergent at room temperature – 3 x 5 min washes.

6. Incubate with secondary antibody in appropriate sera and detergent, following manufacturers concentration of 1-1.5 hours max. We find 30-45 min using 1:500 secondary antibody can yield satisfactory results in most cases.

7. Wash secondary antibody off with PBS containing detergent at room temperature – 3 x 5 min washes.

8. If possible, perform nuclear counterstaining to aid anatomical identification. We typically use DAPI/Hoescht at 1:5,000 for 5 min at RT, followed by brief washing.

a. NOTE: We advise against the use of mounting media containing nuclear counterstains.

9. Blot off excess PBS and mount slides using Vectashield H-1000 and Deckgläser - Menzel-Gläser 1.5 coverslips.

10. Remove excess vectashield with a kimwipe and seal neatly using transparent TopCoat or Nail Polish.

- a. **NOTE: Drying can be accelerated by placing slides on a protected surface inside a chemical fume cupboard with appropriate airflow.**
- b. **To minimise refraction index artefacts, wait at least 3 hours before imaging. We typically mount slides and leave overnight to equilibrate.**
- c. **Place slides in a folder at 4°C for storage.**

Considerations for multiplex immunolabelling using *mito-QC*

As *mito-QC* works by fluorescence in the green and red spectra, users are restricted to using blue and far-red spectra to selectively label other cellular components. We have had success with Alexa Fluor-conjugated antibodies, particularly with Pacific Blue, 633 nm and 647 nm fluorophores that we routinely use at manufacturers recommended concentration (1:500). Users are advised to optimise the concentration of their primary antibody to avoid spectral overlap between blue/green and far-red/red channels. Failure to do this can pose a problem during image acquisition and prevent the reliable identification of subcellular structures. Single labelling pilot experiments in wild-type (non *mito-QC*) tissues can be useful to understand the pattern of labelling before proceeding with multiplex IHC.

Mouse-on-mouse immunolabelling using *mito-QC*

In certain circumstances, it may be necessary to use a primary antibody raised in mouse to detect an antigen of interest in tissue. Mouse-on-mouse staining is typically associated with higher background fluorescence, due to cross-reactivity of mouse primary antibodies with endogenous Ig in tissues. Although perfusion aims to deplete tissues of blood and thus reduce immunogenicity, the complete elimination of all erythrocytes from an adult mammal can never be guaranteed. In this instance, we have found that mouse-on-mouse blocking serum (Vector) yields satisfactory results.

Imaging *in vivo* mitophagy with *mito-QC*

No special imaging setups are required to obtain visually striking images with *mito-QC*. In general, confocal microscopy provides the gold standard with additional advances such as Airyscan (Zeiss) and Hyvolution (Leica) aiding ultraclear resolution of mitochondria and mitolysosomes *in vivo*. When imaging GFP and mCherry using identical laser settings, mitochondrial networks of cultured cells and tissue specimens will appear yellow. Equal intensities will usually result in saturation of the red-only signal in mitolysosomes, which then appear larger than normal and can obscure mitochondrial network morphology. For this reason, we recommend users take time to optimise laser settings using a range indicator with their particular microscope.

Quantitation of mitophagy using *mito-QC*

A variety of quantitative approaches are compatible using *mito-QC*, and these range in sophistication, speed and complexity. As there is no spectral overlap

between GFP and mCherry, no corrections have to be applied using our model. The most basic readout using our model is to monitor mitophagy by simply counting mitolysosomes per cell or per field. This can be performed manually or using an object counter application, although this method is laborious and not amenable to high-throughput adaption.

We routinely analyse mitophagy using Volocity software (Perkin Elmer). Defining a protocol in this software enables consistent, semi-automated batch processing and a multiparametric measurement of mitophagy in tissue sections. Large numbers of tissues and conditions can be assessed in this way. For example, numbers, shape factors and size of mitolysosomes can be quantitated in tissue. This is particularly valuable when quantifying mitophagy in labelled cells or structures e.g. in a particular population of neurons, or quantitating overlap of mCherry-only puncta with LAMP1-positive lysosomes to obtain numbers of *bona fide* mitolysosomes *in vivo*. Tissue expression levels can sometimes vary between animals, and as such – normalising mCherry only signal to GFP area provides a way to obtain a consistent measurement of mitophagy between tissues and subjects. We advise users to experiment with the approach best suited and directly available to them. Quantitation is also possible using freeware such as NIH ImageJ/FIJI. Obtaining the size of mitolysosomes may provide valuable information about lysosomal biology *in vivo*.

Investigating mitochondrial network morphology with *mito-QC*

Mitophagy is not a singular event and thus it is important to investigate its regulation in the wider context of mitochondrial network homeostasis. Mitochondria are dynamic and functionally pleiotropic organelles, and the modulation of mitochondrial morphology is believed to be important in understanding its function. This is particularly important given our recent discovery of mitophagic heterogeneity *in vivo*. Abnormal mitochondrial dynamics are associated with defective metabolism and a range of pathophysiological conditions. When laser signals have been optimised accordingly, the OMM signal of *mito-QC* enables the facile quantitation of mitochondrial network architecture *in vitro* and *in vivo*. We recently exploited this feature of our model to reveal the mitochondrial reticulum in skeletal muscle using light microscopy, previously only resolved by FIS-SIM. Tracing using appropriate software can highlight the diversity of mitochondrial morphologies within different tissues. Further analysis and quantitation of mitochondrial length and shape factor is also possible using Volocity. A representative figure demonstrating the range of mitochondrial networks is shown in Figure 2. This aspect of our model is an important one, as such ultrastructural characterisations were previously only possible by EM.

Modelling selective autophagy in space: Volume imaging and 3D rendering of mitolysosomes and mitochondrial network patterns *in vivo*

We previously employed *mito-QC* with iDISCO to visualise selective autophagy in the adult kidney (4). iDISCO and their variants (iDISCO⁺) are tissue clearing techniques that enable volume imaging of optically-cleared specimens (13). Users should be aware that the use of solvents can result in

dequenching in *mito*-QC tissue specimens, although for regions that exhibit high levels of mitophagy this should not be problematic (e.g. proximal tubules of kidney contain an abundance of mitolysosomes). The acquisition of z-stacks from immunolabelled tissue sections enables users to obtain a major amount of information in space, which is vital to obtain a comprehensive understanding of how mitochondrial homeostasis is orchestrated *in vivo*.

3D Volume Image Analysis Software (Imaris, Bitplane) can be used to reveal cell-specific mitochondrial networks *in vivo* in ever increasing detail. Using the isosurface rendering function, it is possible to emphasise the 3D differences between mitochondrial networks of different cell types *in vivo* in a variety of contexts e.g. during development, between genotypes, in different treatments etc. This approach should prove particularly valuable in understanding how mitochondrial homeostasis is regulated during health and disease (Figure 3).

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References

1. Nunnari J and Suomalainen A (2012) Mitochondria: in sickness and in health. *Cell* 16;148(6):1145-59
2. McWilliams TG and Muqit MMK (2017) PINK1 and Parkin: Emerging themes in mitochondrial homeostasis. *Current Opinion in Cell Biology*. 45: 83-91.
3. McWilliams TG and Ganley IG (2016) Life in lights: illuminating mitochondrial delivery to the lysosome in vivo. *Autophagy*. Vol. 12. No. 12, 2506-2507
4. McWilliams TG, Prescott AR, Allen GFG, Tamjar J, Munson MJ, Muqit MMK and Ganley IG (2016) mito-QC illuminates mitophagy and mitochondrial architecture in vivo. *The Journal of Cell Biology*. Aug 1;214(3):333-45.
5. Sun, N., J. Yun, J. Liu, D. Malide, C. Liu, Rovira, II, K.M. Holmstrom, M.M. Fergusson, Y.H. Yoo, C.A. Combs, and T. Finkel. 2015. Measuring In Vivo Mitophagy. *Molecular Cell*. 60:685-696.
6. Stotland A and Gottlieb RA (2016) α -MHC MitoTimer mouse: *In vivo* mitochondrial turnover model reveals remarkable mitochondrial heterogeneity in the heart. *J Mol Cell Cardiol*. 90: 53-58.
7. Nowotschin S et al., (2009) Live-imaging fluorescent proteins in mouse embryos: multi-dimensional, multi-spectral perspectives. *Trends in Biotechnology*. 27(5): 266–276.
8. McWilliams TG, Prescott AR, Montava Garriga L, Ball G, Singh F, Barini E, Muqit MMK, Brooks SP, Ganley IG (2018) Basal mitophagy occurs independently of PINK1 in mouse tissues of high metabolic demand. *Cell Metab* 27(2):439–449.e5.

9. Lee JJ, Sanchez-Martinez A, Zarate AM, Beninca´ C, Mayor U, Clague MJ, Whitworth AJ (2018) Basal mitophagy is widespread in *Drosophila* but minimally affected by loss of Pink1 or parkin. *J Cell Biol* 217 (5):1613–1622.
10. Brown D, Lydon J, McLaughlin M, Stuart-Tilley A, Tyszkowski R, Alper S (1996) Antigen retrieval in cryostat tissue sections and cultured cells by treatment with sodium dodecyl sulfate (SDS). *Histochem Cell Biol*. 105(4):261-7
11. Singhal PK, Sassi S, Lan L, Au P, Halvorsen SC, Fukumura D, Jain R, Seed B (2016) Mouse embryonic fibroblasts exhibit extensive developmental and phenotypic diversity. *Proc Natl Acad Sci* 113: 122–127.
12. Allen GFG, Toth R, James J and Ganley IG. (2013) Loss of iron triggers PINK1/Parkin-independent mitophagy. *EMBO Rep*, 14(12): p. 1127-35.
13. Renier, N., Z. Wu, D.J. Simon, J. Yang, P. Ariel, and M. Tessier-Lavigne. (2014) iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. *Cell*. 159:896-910.

Figure Legends

Figure 1: Basic principle of the *mito-QC* mouse model

The *mito-QC* assay is based on a tandem tag construct of mCherry-GFP. This tag is targeted to mitochondria through the addition of a 51 amino acid mitochondrial targeting sequence derived from the outer mitochondrial

membrane protein, FIS1 (FIS1^{mt101-152}). The resultant mCherry-GFP-FIS1^{mt101-152} (*mito-QC*) construct is observable as a red and green fluorescent signal, which when merged appears yellow. The engulfment of mitochondria by the phagophore and concomitant fusion of the mitophagosome with the lysosome results in the appearance of mCherry-only puncta, due to the acid-labile properties of GFP. This provides quantitative and facile end-point assessments of mitophagy and mitochondrial morphology both *in vitro* and *in vivo*. The bottom panel shows a micrograph of pancreatic acinar cells from *mito-QC*, highlighting actual mitochondria and mitolysosomes. Scale bar, 10 μm .

Figure 2: *mito-QC* enables studies of mitochondrial network morphology *in vivo*

The outer mitochondrial membrane localisation and ubiquitous expression of *mito-QC* enables the study of mitochondria and mitochondrial network morphology *in vivo*. Shown are camera lucida-style traces of mitochondrial networks from skeletal muscle, heart and kidney generated in Adobe photoshop, using the GFP channel as a mask. Pictures are modified from McWilliams *et al.*, (2016).

Figure 3: Simplifying the visualisation of *in vivo* mitophagy in space within complex tissues

In many instances, multiplex immunolabelling in complex tissues such as brain can prove challenging to interpret for even the most experienced investigators. The compatibility of *mito-QC* with fixation enables

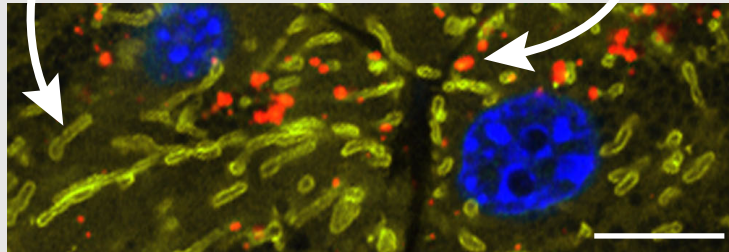
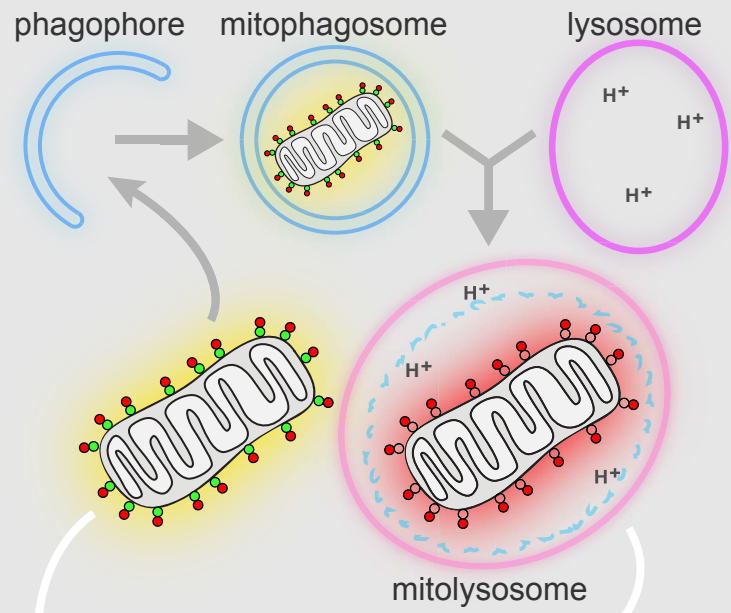
immunolabelling of cellular subsets *in vivo* as shown in a section of mouse cerebellum (left). We used 3D volume rendering of this z-stack to create an isosurface or volume render (right). This enables investigators to create an impactful and informative graphic that can simplify the interpretation of mitophagy within complex structures *in vivo*. Shown is the concentration of mitolysosomes present in Purkinje cell somata *in vivo*, as described in McWilliams *et al.*, (2016). With sufficient optimisation and z-resolution, this technique can be successfully applied to profile the spatial nature of mitophagy in both cleared and non-cleared preparations. Scale bar, 10 μm .

Method/Technique	Advantages	Disadvantages
Transition Electron Microscopy (TEM)	<ul style="list-style-type: none"> • Gold-standard method of detection • Enables direct visualisation of autophagosome membranes, autophagic bodies and other cellular compartments • Immunolabelling can localise proteins of interest to mitophagosomes 	<ul style="list-style-type: none"> • Laborious – many steps where error can be introduced • Requires high degree of technical expertise to implement & execute • Requires depth of experience to interpret • Not adaptable for high-throughput screening • Difficult to optimise for tissues • Requires perfusion-fixation for tissues • Immunolabelling can be challenging due to loss of epitopes during sample processing
Co-localisation of mitotracker and lysotracker	<ul style="list-style-type: none"> • Rapid, easy to use 	<ul style="list-style-type: none"> • Incompatible to detect mitophagy <i>in vivo</i> • Documented problems with specificity and variability in labelling • Off target effects in other membrane bound organelles
Anti-DNA antibodies	<ul style="list-style-type: none"> • Enables visualisation of loss of mt-DNA nucleoids following stimulus-induced mitophagy <i>in vitro</i> 	<ul style="list-style-type: none"> • Expensive • Antibody used in field (Progen) is highly inefficient: manufacturer recommends 1:10 dilution • Yields variable results <i>in vivo</i>
Biochemical analysis of mitophagic flux: <ul style="list-style-type: none"> • Immunoblotting • Citrate Synthase Assay 	<ul style="list-style-type: none"> • Rapid, easy to perform 	<ul style="list-style-type: none"> • Low Sensitivity i.e. high levels of mitophagy required • Lysosomal inhibitors required to interpret flux (e.g. Bafilomycin A1, hydroxychloroquine) and can have detrimental effects <i>in vivo</i>
Immunofluorescence-based colocalisation of mitochondria with LC3-positive autophagosomes	<ul style="list-style-type: none"> • Rapid, easy to perform 	<ul style="list-style-type: none"> • Difficult to implement for <i>in vivo</i> mitophagy • Non-specific autophagosomes thought to form at mitochondria • Not all mitophagy may be LC3-dependent
mito-Keima (mt-Keima)	<ul style="list-style-type: none"> • Provides end-point readout of mitophagy <i>in vitro</i> and <i>in vivo</i> • Compatible with intravital imaging 	<ul style="list-style-type: none"> • mt-Keima protein is incompatible with fixation and thus direct immunolabelling, only enables a regional analysis of mitophagy in complex tissues • Analysis must be conducted in live tissue slices – not amenable to high-throughput <i>in vivo</i> analysis and may necessitate further verification • mt-Keima signal is lost upon freezing – cryosectioning not possible • Long-term storage of tissue specimens and slices for reference is not possible • No antibodies yet available to Keima protein, thus electron

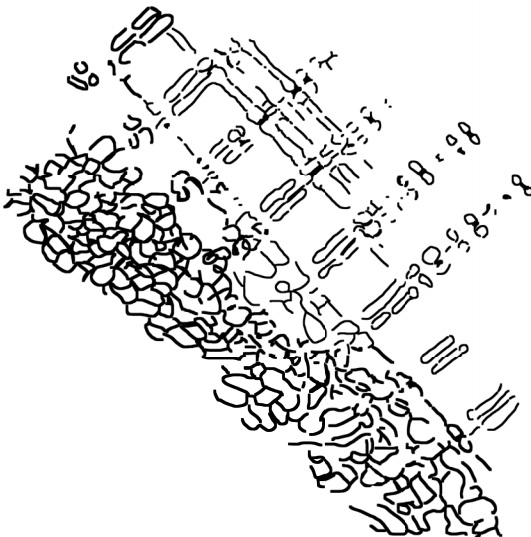
		<p>microscopy-based verification not possible</p> <ul style="list-style-type: none"> • Spectral overlap can complicate interpretation and quantitation • Level of reporter expression must be controlled to prevent mislocalisation
mito-Timer (DsRed1-E5)	<ul style="list-style-type: none"> • Enables a pulse-chase monitoring of mitochondrial half-life and mitophagy <i>in vitro</i> and <i>in vivo</i> • Useful model to understand biogenesis • Compatible with fixation and immunolabelling 	<ul style="list-style-type: none"> • Conditional model; requires the use of doxycycline to activate <i>in vitro</i> • Constitutive Mito-Timer expression in mice reported to be heterogeneous in tissue (heart: expression in ventricles, reduced in aorta) • Maturation of MitoTimer protein may be different across cells and tissues <i>in vivo</i>
mito-QC (mCherry-GFP-mtFIS¹⁰¹⁻¹⁵²)	<ul style="list-style-type: none"> • Provides end-point readout of mitophagy <i>in vitro</i> and <i>in vivo</i> • OMM labelling enables monitoring/characterisation of mitochondrial network and study of dynamics • Constitutive expression facilitates constant monitoring • Compatible with tissue fixation • Immunolabelling enables resolution of mitophagy in a vast array of cell subtypes <i>in vivo</i> • Compatible with freezing and samples stored long-term • No specialist microscopy setup required – compatible with simple and sophisticated systems • Antibodies to GFP and mCherry enable immunogold TEM • Compatible with iDISCO for volume imaging (although some dequenching may occur due to use of solvents) • Ease of measurement: enables multiparametric quantitation – mitolysosome number, size, mitochondrial shape, etc. 	<ul style="list-style-type: none"> • Fixation must be conducted using formaldehyde at pH 7.0: any deviation in pH of fixative will result in dequenching of GFP (yellow mitolysosomes instead of red-only) <ul style="list-style-type: none"> ◦ In this instance, LAMP1 immunostaining can be used to verify the lysosomal nature of presumptive mitophagic structures. • For fluorescent immunolabelling, secondary fluorophores limited to those outside of GFP/mCherry spectra • Incompatible with heat-mediated antigen retrieval <ul style="list-style-type: none"> ◦ SDS-mediated antigen retrieval possible • Level of reporter expression must be controlled to prevent mislocalisation

Table 1: Comparison of available methods to monitor mitophagy *in vitro* and *in vivo*

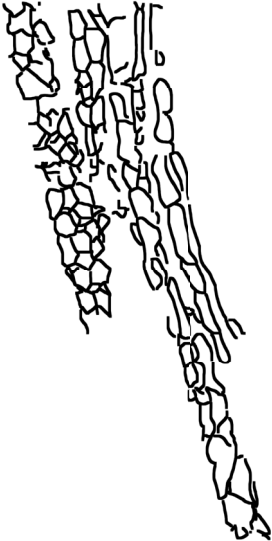
mito-QC



Skeletal Muscle



Cardiomyocyte

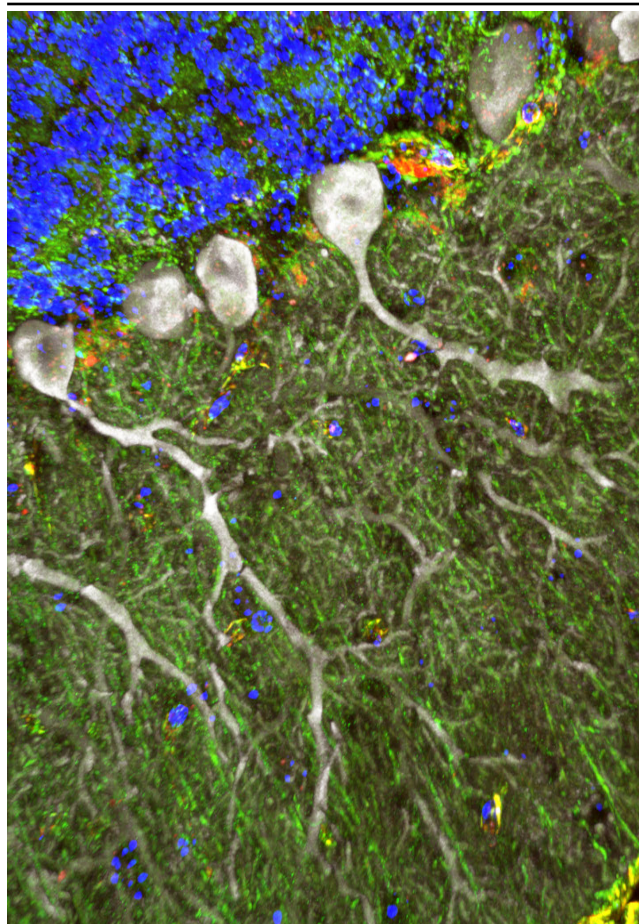


Renal Tubule

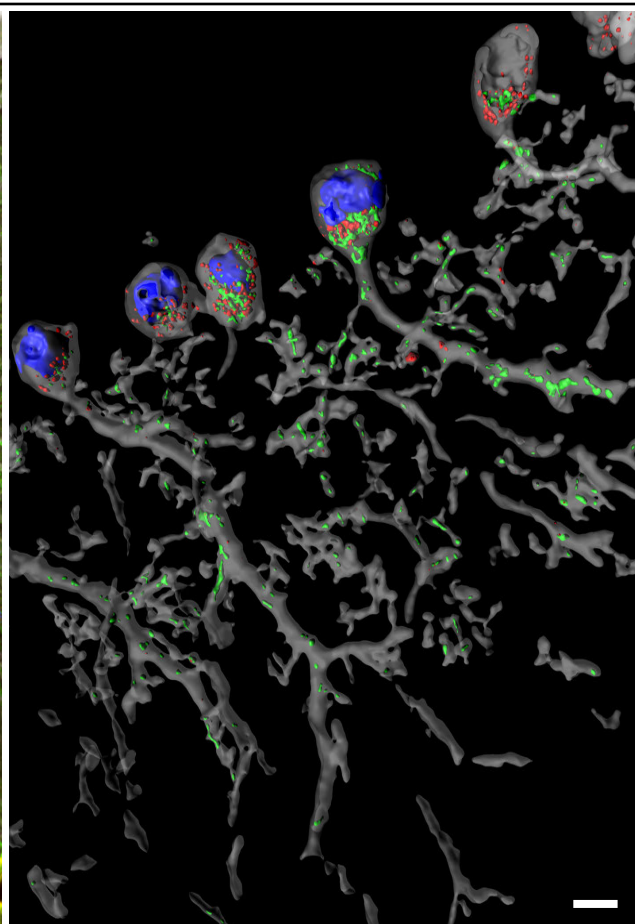


Adult Cerebellum

Purkinje Neurons Nuclei Mitochondria Mitolysosomes



Maximum Projection



Isosurface Render