New methods for monitoring mitochondrial biogenesis and mitophagy *in vitro* and *in vivo*

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Impact statement

Removal of damaged mitochondria through mitophagy is critical for maintaining cellular homeostasis and functions. However, reliable quantitative assays to monitor mitophagy, particularly *in vivo*, are just emerging. This review will summarize the current novel quantitative assays to monitor mitophagy *in vivo*.

Abstract

Removal of damaged mitochondria through mitophagy is critical for maintaining cellular homeostasis and functions. Increasing evidence implicates mitophagy in red blood cell differentiation, neurodegeneration, macrophage-mediated inflammation, ischemia, adipogenesis, drug-induced tissue injury, and cancer. Considerable progress has been made toward understanding the biochemical mechanisms involved in mitophagy regulation. However, few reliable assays to monitor and quantify mitophagy have been developed, particularly *in vivo*. In this review, we summarize the recent development of three assays, MitoTimer, mt-Keima and mito-QC, for monitoring and quantifying mitophagy in cells and in animal tissues. We also discuss the advantages and limitations of these three assays when using them to monitor and quantify mitophagy.

Keywords: Autophagy, mitophagy, MitoTimer, Mt-Keima, mito-QC

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Introduction

Macroautophagy (hereafter referred to as autophagy) is a catabolic process that degrades cellular proteins and damaged or excess organelles through the formation of a double-membrane autophagosome. Autophagosomes engulf these proteins and organelles in the cytoplasm and then fuse with lysosomes to degrade their components via lysosome proteases.[1] Autophagy can be both a non-selective and selective process. Non-selective autophagy is a protective process that provides the cell with nutrients in response to starvation to produce energy. Selective autophagy removes protein aggregates and damaged organelles using specific receptors, and it occurs in both nutrient-rich and poor conditions.[2]

Mitophagy is a form of selective autophagy that is specific for the degradation of damaged mitochondria.[3,4] The most studied pathway for mitophagy induction is regulated by PTEN-induced putative kinase 1 (PINK1) and Parkin, where PINK1 recruits Parkin to depolarized mitochondria. Once recruited to mitochondria, Parkin ubiquitinates outer mitochondrial membrane proteins for recognition and engulfment by autophagosomes and subsequent degradation in lysosomes. Several Parkin-independent pathways also induce mitophagy. These mitophagy pathways have been extensively reviewed.[5–8] Mitophagy is balanced by mitochondrial biogenesis for generation of new mitochondria.[9,10] Removal of damaged mitochondria via mitophagy is essential for prevention of cellular dysfunction and death. Impaired mitophagy has been implicated in many neurodegenerative diseases such as Parkinson's disease,[11] Alzheimer's disease,[12,13] amyotrophic lateral sclerosis (ALS),[14,15] and Huntington's disease.[16,17] In addition, mitophagy may also protect the liver during acetaminophen overdose and alcoholic liver disease by removing damaged mitochondria.[7,18–22] Mitophagy also has protective functions in the heart.[23–26]

There has been tremendous progress in the mitophagy field over the past several years leading to a better understanding of mechanisms involved in the pathway and its role in disease. However, most of this knowledge came from *in vitro* experiments involving treatment of cells with mitochondrial damaging agents. Even though these experiments led to many breakthroughs in the mitophagy field, methods for studying mitophagy *in vivo* would provide even more critical information regarding regulation of mitophagy and its role in various diseases. The lack of reliable quantitative assays to monitor mitophagy *in vivo* has been a big hurdle in the mitophagy research field. Excitingly, several novel methods have recently been developed for

monitoring mitophagy both in cells and *in vivo,* which include use of the fluorescent probes MitoTimer, mt-Keima, and mito-QC (quality control). These new methods for studying mitophagy are the focus of this review.

Methods for monitoring mitophagy

The most commonly used methods to study mitophagy in yeast and mammalian cells include electron microscopy (EM), fluorescence microscopy for co-localization of mitochondria with autophagosomes or lysosomes, Western blotting to measure mitochondrial protein degradation, quantification of mtDNA and citrate synthase activity.[5] EM is an excellent tool for visualization of individual mitochondria engulfed by autophagosomes and lysosomes during mitophagy. However, it is not very quantitative due to limited numbers of sections and cells. EM also requires a trained eye for accurate identification of mitochondria, autophagosomes, and lysosomes and may have a wide range of sample variability. Fluorescence microscopy for co-localization of mitochondria with autophagosomes and lysosomes is a useful tool and can provide results for a large number of cells for quantification. However, the formation of green fluorescence protein (GFP)-microtubule-associated protein light chain 3 (LC3) aggregates can be misleading. In addition, this assay is the best to indicate the association of LC3 positive autophagosomes with mitochondria but cannot be used to reflect the degradation of mitochondria. Measuring mitochondrial protein degradation by Western blot can determine if mitophagy is activated. However, high levels of mitophagy may be required to detect mitochondrial protein degradation. In addition, outer mitochondrial membrane proteins are degraded by both autophagy and the proteasome, which makes data interpretation very complicated. However, mitochondrial inner membrane or matrix proteins have been recommended for mitophagy assessment by Western blot analysis.[5,27] Several recently developed assays for measuring mitophagy including MitoTimer, mt-Keima, and mito-QC may help solve these issues. More importantly, these assays can be used to monitor and quantify mitophagy in *in vivo* in different tissues.

MitoTimer

MitoTimer is a novel tool for monitoring real-time mitochondrial aging, turnover and biogenesis, and it can be used to evaluate individual mitochondria or mitochondrial populations within a cell. MitoTimer is the Timer fluorescent protein targeted to the mitochondrial matrix.[28,29] Timer protein, also known as DsRed1-E5, is a mutant form of the red fluorescent protein drFP583 that irreversibly changes its emitted fluorescence from green to red over time with protein maturation, typically within 48 h after expression.[30] The Timer protein was fused to the mitochondrial targeting sequence of the COX8A subunit to create MitoTimer, which co-localizes with mitochondria as shown by co-localization with the outer mitochondrial membrane protein TOM20 in mouse embryonic fibroblast (MEF) cells.[28] Changes in green MitoTimer expression levels suggest differences in mitochondrial protein synthesis, import, or biogenesis. Changes in red expression levels without changes in green expression levels suggest alterations in mitochondrial degradation.[31] MitoTimer has been used in several cell culture studies to investigate mitochondrial aging, biogenesis, and damage during stress.[28,29] We found that MitoTimer can also be used to monitor mitochondrial biogenesis in primary cultured mouse hepatocytes, which display green-only mitochondria (newly synthesized mitochondria), red-only mitochondria (old/aging mitochondria) as well as yellow mitochondria (represent intermediate stage of mitochondria) (Figure 1). The majority of mitochondria are yellow, likely indicating the dynamic fusion process of matured/old mitochondria (red) with newly synthesized mitochondria (green) in hepatocytes.

To monitor mitochondrial aging with MitoTimer in cells, Ferree *et al.* used a doxycycline (DOX)-inducible vector to activate MitoTimer transcription in MEF cells, and MitoTimer green fluorescence was seen 8 h after addition of DOX by fluorescence microscopy. Green fluorescence in MEF cells was changed to a mixture of red and green fluorescence at 24 h, which further changed to a mostly red fluorescence at 48 to 72 h after transcription induction.[28] MitoTimer induced in HEK293 cells also

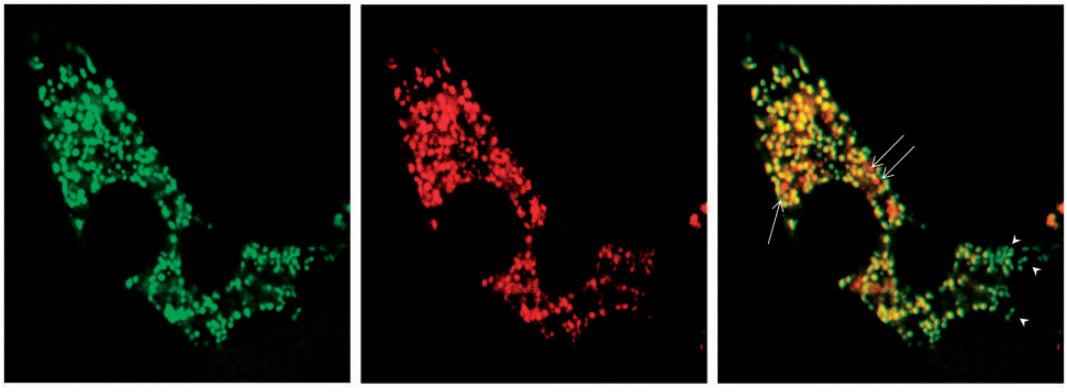

Figure 1 Monitoring mitochondria biogenesis in primary hepatocytes using AAV-MitoTimer. Primary mouse hepatocytes were infected with AAV-TBG-MitoTimer (1000 GC/cell) for 72 h followed by confocal microscopy. Arrows denote red-only old mitochondria and arrow heads denote green-only newly synthesized mitochondria.

showed a shift in green to red fluorescence within 48 h of MitoTimer transcription by fluorescence microscopy.[29]

In addition to monitoring mitochondrial aging, MitoTimer may be used to assess mitochondrial damage and degradation in cells or *in vivo* if mitochondria become stressed or depolarized because depolarized mitochondria cannot import newly synthesized and green-expressing MitoTimer protein. Laker *et al.* treated MitoTimer-transfected C2C12 myoblasts with the mitochondrial reactive oxygen species (ROS)-inducing drugs rotenone, antimycin, or paraquat for 6 h, which all caused transition from green to red florescence, suggesting mitochondrial damage. They confirmed drug-induced mitochondrial stress in these cells using a Seahorse analyzer, which showed reduced basal mitochondrial respiration, ATP-linked respiration, and mitochondrial reserve capacity after drug treatment.[32] Stotland and Gottlieb used the mitochondrial uncoupler carbonylcyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) to depolarize mitochondria in C2C12 myoblasts. Upon depolarization with FCCP, the mitochondria population displayed a shift to red fluorescence in myoblasts constitutively expressing MitoTimer. In addition, mitochondria with green-fluorescing MitoTimer did not have any evidence of previous red-fluorescing MitoTimer, suggesting that all green-fluorescing mitochondria were newly formed organelles or mitochondria that were still capable of importing the MitoTimer protein after FCCP treatment.[33] MitoTimer may also be used for monitoring effects of autophagy modulation on the mitochondria population in cells and *in vivo*. Inhibition of autophagy in stably expressing MitoTimer COS cells by bafilomycin or chloroquine resulted in an increased ratio of red-to-green fluorescence compared to control cells without affecting the number of green-fluorescing cells, suggesting an accumulation of aged mitochondria due to inhibition of their degradation.[28]

Laker *et al.* demonstrated that MitoTimer may also be used *in vivo* in *Drosophila* to monitor mitochondrial aging. MitoTimer flies were generated by subcloning the coding region of MitoTimer into a UAS vector, which was then used to generate UAS-MitoTimer transgenic flies. Flies that were 70-days old had increased red fluorescence in their heart tubes compared to seven-day-old flies, indicating that mitochondrial turnover decreases in flies as they age likely due to decreased mitophagy.[32] Stotland and Gottlieb produced conditional MitoTimer cardiac transgenic mice by pronuclear injection of MitoTimer driven by a cardiac alpha myosin heavy chain (α-MHC) promoter, and they found similar mitophagy results during aging in these mice as Laker *et al.* found in flies. MitoTimer mice that were three-weeks old had a greater population of green-fluorescing mitochondria compared to 16-week-old mice, suggesting that mitochondria turnover decreases with age in mouse hearts.[33] Furthermore, Stotland and Gottlieb demonstrated that many red-expressing cardiac mitochondria in α-MHC MitoTimer mice were targeted for degradation by autophagy because they co-localized with the autophagosome marker LC3.[33]

MitoTimer was also used to demonstrate benefits of exercise for mitochondria in *Drosophila* and mice. MitoTimer transgenic *Drosophila* were exposed to a mechanized platform, known as a ''Power Tower''[34] to induce exercise, which resulted in improved exercise capacity and a significant shift to green MitoTimer fluorescence in their heart tubes.[32] Mice with somatic gene transfer of MitoTimer in their flexor digitorum brevis (FDB) muscles underwent voluntary wheel running for six weeks, which led to a significant increase in their mitochondrial content and a shift toward MitoTimer green fluorescence.[32] However, there was no significant change in red-fluorescing mitochondria for either mice or *Drosophila* after exercise, suggesting that the number of aged or stressed mitochondria were not decreased by exercise.[32] Nevertheless, an increase in MitoTimer green-expressing mitochondria suggests an increase in newly synthesized mitochondria during exercise, which is a definite health benefit. Interestingly, a MitoTimer shift to red fluorescence caused by high fat diet (HFD) in mice was prevented by three weeks of voluntary wheel running.[32]

Fluorescence microscopy and flow cytometry may both be used for MitoTimer analysis. Plotting red to green fluorescence using either method gives an idea of the ratio of aged and newly generated mitochondria over time.[28,29] A benefit of using fluorescence microscopy for MitoTimer analysis is that fixation of MitoTimer stabilizes green and red fluorescence, allowing for convenient and simultaneous analysis of all samples in an experimental group by fluorescence microscopy.[29] However, one must use caution to avoid bleaching of MitoTimer during analysis. Imaging settings should also be consistent among time points and treatment groups.[28,31] For flow cytometry analysis, excitation at 488 nm and 543 nm and emission at 497–531 nm and 583–695 nm should be used for green and red-fluorescing mitochondria, respectively.[31] MitoTimer results should always be compared to a control group when analyzing by either fluorescence microscopy or flow cytometry.

Overall, MitoTimer is a very useful tool for monitoring both mitophagy and mitochondrial biogenesis with some caveats. For example, mitochondrial half-lives vary among different tissues. MitoTimer protein matures from green to red within 48 h and is retained in the mitochondria after maturation for several days. Mitochondrial half-life in hepatocytes is estimated to be two days, which is similar to MitoTimer maturation time. However, cardiac mitochondrial half-life is much longer. Therefore, in tissues with longer mitochondrial half-lives, it may be hard to distinguish new red-fluorescing mitochondria from mitochondria that imported MitoTimer and retained red fluorescence.[35]

Changes in MitoTimer fluorescence do not necessarily indicate mitochondrial damage or specific changes in mitochondrial degradation or biogenesis. Therefore, results using this assay must be combined with other methods to accurately determine causes of changes in red or green fluorescence levels. In addition, inhibitors of mitophagy or mitochondrial biogenesis should be incorporated into experiments when measuring mitophagy as a control.[28,35] It is also important to establish experimental controls and synchronization of expression when monitoring MitoTimer expression in cells, such as use of a DOX-inducible vector

to activate expression of MitoTimer. It was suggested that DOX be used every two days for continuous expression of MitoTimer.[31] In addition, MitoTimer should be stably expressed in cells for *in vitro* experiments, and its expression should be monitored for at least a week before beginning experiments to confirm stability of red and green expression levels. MitoTimer effects on mitochondrial structure and function in cells or *in vivo* should also be evaluated before performing experiments to ensure that MitoTimer itself does not cause mitochondrial damage or inhibit mitochondrial function.[31] It is also important to note that changes in transition from green to red fluorescence are affected by temperature, oxygen, and light exposure.[29,30,35] Finally, MitoTimer has not been ubiquitously expressed in mice like mt-Keima or mito-QC, which are discussed in later sections. Instead, MitoTimer studies so far have been limited to cells, flies or mouse skeletal muscle/heart. However, conditional MitoTimer expression in other mouse tissues is possible and would provide a useful tool for monitoring mitophagy. Development of whole-body transgenic MitoTimer mice would enhance use and benefits of MitoTimer significantly.

Mt-Keima

Mitophagy can be quantified by fluorescence imaging using a mitochondria-matrix targeted Keima (mt-Keima) fluorescent protein in cells and *in vivo*. Similar to MitoTimer, Keima is targeted to the mitochondrial matrix via fusion with COX8 to make mt-Keima.[36] The mt-Keima protein changes color depending on if it is in an acidic (red color) or neutral (green color) pH environment, like when being transferred from an autophagosome (green color) to a lysosome (red color) for example. Therefore, mitochondria labelled with mt-Keima that are undergoing mitophagy and degradation in the lysosome will produce a red signal. The mt-Keima protein is stable in lysosomes, and the excitation peak of mt-Keima shifts from 440 nm to 586 nm upon lysosome delivery, indicating mitophagy activation and delivery of mitochondria to lysosomes.[36] The specificity of mt-Keima for mitophagy activation in cells was confirmed in several experiments. Sun *et al.* induced mitophagy in HeLa cells stably expressing mt-Keima via hypoxia. Hypoxic conditions resulted in an increased red-to-green mt-Keima fluorescence ratio, suggesting increased mitophagy and degradation of mitochondria in lysosomes. They used 458 nm laser excitation and 561 nm laser excitation for the green-neutral and red-acidic quantification of mt-Keima, respectively. Sun *et al.* also treated Parkin and mt-Keima expressing HeLa cells with FCCP and oligomycin to induce mitochondrial depolarization and subsequent mitophagy, which resulted in loss of mt-Keima green fluorescence with a corresponding increase in red mt-Keima florescence in Parkin expressing cells.[27] Carbonyl-cyanide *m*-chlorophenyl hydrazine (CCCP) and oligomycin treatment in MEF cells co-transfected with mt-Keima and GFP-Parkin also resulted in increased puncta with red mt-Keima expression at 550 nm and a corresponding decrease in GFP-Parkin signal.[36] Therefore, mt-Keima is a useful and novel method for determining mitophagy activation in cells, and it can be combined with GFP-Parkin expression for assessing mitophagy activation.

A transgenic mouse expressing mt-Keima was recently developed for monitoring mitophagy *in vivo*. To make these mice, Sun *et al.* inserted a single copy of the mt-Keima transgene into the Hip11 locus on chromosome 11. Isolated MEFs from these mice had green tubular mitochondria when examined at 458 nm excitation and had red puncta that overlapped with labeled lysosomes at 561 nm excitation.[27] Sun *et al.* performed several experiments to ensure mt-Keima red signal was specific to degradation of mitochondria in lysosomes. When these mice were treated with chloroquine to block lysosome degradation, red mt-Keima fluorescence decreased, suggesting decreased degradation of mitochondria in lysosomes.[27] In addition, mt-Keima embryonic brains from Atg7 knockout mice and brains from Atg5 conditional knockout mice, two genes required for autophagosome formation, also had decreased red mt-Keima signal compared to control mice, indicative of decreased mitochondrial degradation.[1,27] Furthermore, mice placed in hypoxic conditions for 10 days had increased red mt-Keima signal in their livers, suggestive of increased mitophagy.[27] Interestingly, mt-Keima mice showed that levels of mitophagy varied among different mouse tissues with high levels of basal mitophagy in the heart and low levels in the thymus.[27]

Mt-Keima mice may also be used to study mitophagy during aging and in disease models. Sun *et al.* also observed a decline in mitochondrial turnover with aging, similar to results produced in MitoTimer mice and flies.[27,32,35] mt-Keima mice that were three months old had greater red signal than mice that were 21 months old, indicative of increased mitochondrial turnover in younger mice.[27] However, authors mentioned that decreased red signal seen in older mice may also be due to changes in cellular composition or decreased neural stem cell numbers, which also occur with aging. Cellular abundance was similar between young and old mice by DAPI staining. mt-Keima mice have also been used to monitor mitophagy in disease models. Interestingly, mt-Keima mice expressing the Huntingtin's transgene (HTT), a model of Huntingtin's disease, had decreased levels of red signal compared to control mice, suggesting decreased levels of mitophagy in Huntingtin's disease. mt-Keima mice fed a HFD for 18 to 20 weeks also had reduced mitophagy in their livers, which was shown by decreased red mt-Keima signal compared to control-fed mouse livers. Therefore, mt-Keima mice may be used to monitor mitophagy levels in various disease states or treatment conditions.

Mt-Keima has proven useful for monitoring mitophagy in cells and *in vivo*, and it can monitor mitophagy in a live cell setting using a large population of cells without having a direct effect on mitochondrial function.[27] It is also likely more specific for monitoring mitophagy than MitoTimer. However, there are some downfalls to assessing mitophagy levels using mt-Keima. Unlike MitoTimer, mt-Keima cannot be used to monitor mitochondrial biogenesis. Unlike MitoTimer and mito-QC analysis (discussed in the next section), mt-Keima samples cannot be fixed because fixation disrupts the lysosome pH gradient. In addition,

Table 1 Advantages and limitations of MitoTimer, mt-Keima, and mito-QC

Assays	Advantages	Limitations	References
MitoTimer	Assess mitochondria turnover/ mitophagy & mitochondrial biogenesis; fixed and live cells	Only cell culture and tissue expression via electroporation; not specific for autophagy; no transgenic mice yet	28,29, 31–33, 35
Mt-Keima	Assess mitochondria turnover/ mitophagy; live cells & tissues; transgenic mice available	Only live cells, no fixation; overlapping spectra; not for mitochondria biogenesis	27, 36
Mito-QC	Assess mitochondria turnover/ mitophagy; mitochondrial network architecture; live cells & tissues; transgenic mice available	Not for mitochondrial biogenesis; target mitochondrial outer membrane which could also be degraded by proteasome	37

the Keima excitation spectrums for red and green fluorescence partially overlap, which may sometimes result in an orange color when expressed in the lysosome instead of red and can cause confusion during data interpretation. Like MitoTimer, long exposure times of mt-Keima samples to fluorescent microscope lasers may influence the results. Another challenge for this assay is in determining the precise location of analysis in mouse tissue, which may be difficult unless a separate fluorescence reporter is used or there are easily identifiable morphological landmarks present in the tissue being analyzed. Inhibitors of mitophagy should be used as a control in this assay, and this assay should be combined with other methods of measuring mitophagy to confirm the results.

Mito-QC

The mito-QC mouse is another new mechanism for studying mitophagy as well as visualizing the overall mitochondrial network. Similar to mt-Keima, mito-QC is a pH-sensitive mitochondrial fluorescent probe. Mito-QC is made up of a tandem mCherry-GFP tag fused to the mitochondrial targeting sequence of the outer mitochondrial membrane protein FIS1. Mito-QC displays red and green fluorescence during steady-state conditions, but the mCherry signal becomes stable when mitophagy is induced because mitochondria are delivered to the lysosome where the GFP signal is quenched. Therefore, mCherry-only puncta are seen during mitophagy activation.[37] Mito-QC transgenic mice were developed by knockin of mCherry-GFP-FIS1101-152 in the Rosa26 locus of C57BL/6 mice, which allows expression of mito-QC in all mammalian tissues. However, expression levels of the transgene vary between heart, skeletal muscle, and liver with lowest expression in the liver. Mito-QC does not seem to affect mitochondrial structure or function.[37]

Mito-QC mice have been used for monitoring mitophagy levels during stress in addition to comparing mitochondrial networks and mitophagy levels among different tissues. McWilliams *et al*. determined that mitophagy occurs at similar rates in heart and skeletal muscle using mito-QC mice.[37] They also showed that lowest levels of mitophagy occurred in the spleen while highest levels of mitophagy occurred in the liver and kidney among the tissues analyzed. Interestingly, high expression levels of mitophagy were also seen in the muscle fibers of the tongue in mito-QC mice.[37] An advantage of mito-QC is that mitochondria populations within different areas of a particular tissue can be monitored. For example, young mice had higher mitophagy levels in their kidney glomeruli while adult mice had lower levels of mitophagy in their glomeruli and higher levels in their cortical tubules. Entire mitochondrial networks can also be compared using mito-QC mice.[37] In addition, mito-QC mice can be used to monitor changes in mitophagy during stress. Treatment of MEFs isolated from mito-QC mice with the iron chelator deferiprone significantly increased the levels of mCherry puncta compared to control cells, indicative of increased mitophagy levels.[37]

The mito-QC mouse is a promising approach for studying mitophagy that has advantages compared to the mt-Keima and MitoTimer mice. For example, mito-QC mice allow for visualization of entire mitochondrial networks among different tissues. In addition, like tissues from MitoTimer mice, tissues from mito-QC mice can be fixed before analysis if the fixative used is maintained at a pH of 7.0.[35,37] Mito-QC mice are also likely more specific for mitophagy activation than MitoTimer mice. Furthermore, there is no overlap in emission spectra using the mito-QC mouse, which is an advantage over the mt-Keima mouse.[27,37] However, there are also some drawbacks to using mito-QC mice for analysis of mitophagy. Like mt-Keima mice, analysis of specific cell populations affected within a tissue sample from mito-QC mice may be challenging unless an additional reporter is used. In addition, fluorophore selection for additional immunolabeling is limited because mito-QC mice already use red and green fluorescence.[37] Additional methods for measuring mitophagy should be used with mito-QC mice to confirm the results. In addition, inhibitors of mitophagy should be used as a control with this assay.

Concluding remarks

Mitophagy plays critical roles in maintaining cellular homeostasis and functions. Impaired mitophagy may lead to metabolic syndrome and pathogenesis of many human diseases. With the development of these reliable

quantitative assays to monitor mitophagy by using MitoTimer, mt-Keima and mito-QC transgenic animals, we will be able to reveal and better understand basic biochemical mechanisms regulating mitophagy *in vivo*. While these tools have great values for monitoring and quantifying mitophagy in particular in tissues *in vivo*, they also have their limitations (see Table 1) and more efforts should be put to continue to develop better and more reliable assays for assessing mitophagy. These efforts may ultimately lead to new avenues for treatment of diseases characterized by dysfunctional mitochondria.

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