α-MHC MitoTimer mouse: *In vivo* mitochondrial turnover model reveals remarkable mitochondrial heterogeneity in the heart

Aleksandr Stotland and **Roberta A. Gottlieb**[*]

The Cedars-Sinai Heart Institute, Barbra Streisand Women's Heart Center, Cedars-Sinai Medical Center, Los Angeles, CA, United States

Abstract

In order to maintain an efficient, energy-producing network in the heart, dysfunctional mitochondria are cleared through the mechanism of autophagy, which is closely linked with mitochondrial biogenesis; these, together with fusion and fission comprise a crucial process known as mitochondrial turnover. Until recently, the lack of molecular tools and methods available to researchers has impeded *in vivo* investigations of turnover. To investigate the process at the level of a single mitochondrion, our laboratory has developed the MitoTimer protein. Timer is a mutant of DsRed fluorescent protein characterized by transition from green fluorescence to a more stable red conformation over 48 h, and its rate of maturation is stable under physiological conditions. We fused the Timer cDNA with the inner mitochondrial membrane signal sequence and placed it under the control of a cardiac-restricted promoter. This construct was used to create the alpha-MHC-MitoTimer mice. Surprisingly, initial analysis of the hearts from these mice demonstrated a high degree of heterogeneity in the ratio of red-to-green fluorescence of MitoTimer in cardiac tissue. Further, scattered solitary mitochondria within cardiomyocytes display a much higher red-to-green fluorescence (red-shifted) relative to other mitochondria in the cell, implying a block in import of newly synthesized MitoTimer likely due to lower membrane potential. These red-shifted mitochondria may represent older, senescent mitochondria. Concurrently, the cardiomyocytes also contain a subpopulation of mitochondria that display a lower red-to-green fluorescence (green-shifted) relative to other mitochondria, indicative of germinal mitochondria that are actively engaged in import of newly-synthesized mito-targeted proteins. These mitochondria can be isolated and sorted from the heart by flow cytometry for further analysis. Initial studies suggest that these mice represent an elegant tool for the investigation of mitochondrial turnover in the heart.

Keywords

[*]Corresponding author at: Barbra Streisand Women's Heart Center, Cedars-Sinai Heart Institute, 127 S. San Vicente Blvd. AHSP9105, Los Angeles, CA 90048, United States., Roberta.Gottlieb@cshs.org (R.A. Gottlieb).

1. Materials and methods

1.1. Construction of plasmids and generation of a mouse line

The MitoTimer cDNA was cloned into pBMN.i.Blast (Courtesy of Dr. Roland Wolkowicz) retroviral transfer vector utilizing BamHI and XhoI sites of the vector and forward primer 5′-TATAGAATTCAGATCTCCACCATGTCCGTCCTGACGCCGCTG-3′ with reverse primer 5′-TATACTCGAGTCTAGACTACAGGAACAGGTGGTGGCG-3′. pC26 αMHC-MitoTimer plasmid was created by excising the mCherry-LC3 cDNA from the mCherry-LC3-mHC plasmid using the SalI and NheI restriction sites. MitoTimer cDNA was introduced by blunt 5'end of the insert and the NheI-compatible site XbaI, ligated into a Mung Bean nuclease-blunted SalI site and the NheI site of the vector.

MitoTimer transgenic mice were created in the C57/BL6 strain (Jackson Laboratories, Sacramento, CA) by pronuclear injection of murine alpha myosin heavy chain promoter-driven MitoTimer transgene (pC26 αMHC-MitoTimer).

1.2. Cell culture and flow cytometry

C2C12 skeletal myoblasts (ATCC, #CRL-1772) were maintained in growth media consisting of DMEM (Gibco, #11995-073) containing 10% fetal bovine serum (Life Technologies #16010-159) and antibiotic/antimycotic (Life Technologies, #15240-062). The C2C12-mitoTimer cells were established utilizing self-inactivating retroviral particles [1]. Transduced cells were sorted by flow cytometry on a BD FACSAria II.

C2C12 MitoTimer cells (2.5×10^5) were treated with either 10 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma-Aldrich, #C2920) or dequalinium chloride (DECA) (Sigma-Aldrich, #PHR1300) for 24 h. Cell media was replaced and cells were collected at indicated times by trypsinization with 0.05% Trypsin-EDTA (ThermoFisher Scientific, #25300-054). The cells were pelleted at 300 ×g for 5 min, washed once with phosphate-buffered saline (PBS) and resuspended in 4% paraformaldehyde in PBS for flow cytometry.

Mitochondria for FAOS were prepared as described [2] and then resuspended in 4% paraformaldehyde in PBS for flow cytometry. Anti-Mfn2 antibody (Abcam, #ab56889) and anti-mouse IgG Alexa Fluor 350 (Life Technologies, #A-11045) used in staining.

C2C12 MitoTimer cells (2.0×10^7) were incubated with 10 μM of EdU overnight; mitochondria were isolated by nitrogen cavitation followed by differential centrifugation. EdU labeling of mitochondria was performed using the Click-iT® EdU Flow Cytometry Assay Kit (Invitrogen, #C-10418).

BD LSR Fortessa (BD Biosciences) was used to collect the data from MitoTimer cells and isolated mitochondria. Data were collected on FACSDiva 8.0.1 and analyzed on FlowJo X 10.0.7r2 at the Cedars-Sinai Medical Center FACS core facility.

1.3. Immunocytochemistry and microscopy

Cells were seeded for microscopy on MatTek glass-bottom 35 mm dishes (MatTek, #P35G-1.5-14) and fixed with 4% formaldehyde in PBS for 10 min and then washed in PBS prior to imaging.

Freshly isolated whole hearts from α-MHC MitoTimer mice were fixed in 4% paraformaldehyde in PBS overnight, followed by an overnight incubation in 30% sucrose in PBS. The tissue was snap-frozen in OCT medium and 10 μm sections were stained with anti-LC3B/MAP1LC3B (1:100, Novus Biologicals #NB100-2220) primary and DyLight 350 conjugated anti-rabbit secondary antibody (Thermo Fisher–Pierce, #62270).

Fluorescence microscopy imaging was performed using a Keyence BZ-9000 microscope and analyzed with Keyence BZ-II Analyzer ver. 2.2. Confocal microscopy was performed using Leica TCS SP5 X White Light confocal microscope (Cedars Sinai Medical Center Microscopy Core). Images were collected and analyzed on Leica Application Suite X (LAS X) ver. 1.9.

2. Introduction

Maintenance of healthy mitochondria is critical for cardiac function. The organelles comprise one third of the volume of the heart, and under normal conditions, are responsible for nearly all ATP production required to meet the high energy demands of the heart [3]. Interestingly, mitochondria are not synchronized in constant production of ATP at maximal rates, but comprise a heterogeneous population with some producing ATP at high rates while others are relatively inefficient, quiescent, or producing significant amounts of reactive oxygen species (ROS). In order to maintain a healthy mitochondrial network, two processes—mitophagy and mitochondrial biogenesis—are employed by cells. Oxidative damage sustained as a by-product of oxidative phosphorylation (OXPHOS) is ordinarily handled by targeted protein quality control and by exchange of components during fusion followed by fission; in contrast, selective mitochondrial autophagy (mitophagy) eliminates entire damaged and dysfunctional mitochondria. This is accomplished by asymmetric fission to segregate damaged components into one daughter mitochondrion that is then isolated from the network and removed by mitophagy. To replenish the pool of healthy mitochondria, mitophagy is closely linked to mitochondrial biogenesis, a process of replacement of the organelles or alternately, synthesis of components and their insertion into the remaining mitochondria in the network [4,5].

Recent advances in assessing mitochondrial turnover include mass spectrometry analysis of the half-lives of mitochondrial proteins after deuterium pulse-chase, and mito-Keima, a fluorescent protein targeted to mitochondria that changes fluorescence in the acidic lysosome. Both techniques are invaluable in studying mitochondrial turnover on a tissue-wide level (isotopic labeling) or quantitative assessment of single mitophagy events and monitoring the rate of mitophagy in cells (Mito-Keima), but neither allow for the study of mitochondrial turnover (both mitophagy and biogenesis) at the level of a single mitochondrion [6].

To address this question, we modified the fluorescent protein Timer developed by Terskikh et al., that matures from green to red fluorescence over 48 h, which we targeted to the mitochondrial inner membrane [7]. Here, we demonstrate the utility of constitutively-expressed MitoTimer as a tool for monitoring mitochondrial turnover *in vitro* and *in vivo*.

3. Results and discussion

3.1. Constitutive expression of MitoTimer in vitro

High membrane potential is required for mitochondrial protein import; thus hypo-polarized mitochondria cannot import newly synthesized (green) MitoTimer protein and consequently exhibit a high red-to-green fluorescence ratio [8]. This is demonstrated in C2C12 myoblasts constitutively expressing MitoTimer, where isolated red fluorescent mitochondria can be observed following treatment with uncoupling agent FCCP. In addition, we observe a subpopulation of green fluorescent mitochondria which contain very little preexisting red fluorescent protein. These may represent newly-formed organelles or sites enriched for new protein import and mitochondrial protein synthesis, which are subsequently redistributed across the network through fusion (Fig. 1A, B). Flow cytometry of MitoTimer C2C12 cells treated with FCCP or an inhibitor of mitochondrial protein import DECA reveals a strong increase in red fluorescence due to the inability to import new (green) MitoTimer protein, while pre-existing MitoTimer protein continues to mature to red fluorescence (Fig. 1C, D) [9].

MitoTimer reveals mitochondrial turnover at the level of individual mitochondria: fluorescence-activated organelle sorting (FAOS) of mitochondria from FCCP-treated MitoTimer-C2C12 myoblasts demonstrates an increase in mostly-red mitochondria 24 h after FCCP treatment, and the advent of a subpopulation of mostly-green mitochondria (enriched for new protein import). By 48 h after the pulse, the red-to-green fluorescence ratios of the mitochondrial population begin to shift back to baseline as the recently-imported MitoTimer is distributed across the fusion-competent network and begins to mature to red (Fig. 1E).

Further analysis of isolated MitoTimer-positive mitochondria reveals that green-enriched organelles contain more mtDNA (DAPI) (Fig. 1F) which has recently replicated (as indicated by incorporation of the artificial nucleotide EdU) (Fig. 1G) during the preceding 24 h before harvest. If a subpopulation of mitochondria is specialized for new protein import and mtDNA replication, these distinctive mitochondria should also be able to share proteins and mtDNA with the rest of the mitochondrial network and therefore should be competent for fusion. We used immunostaining to assess expression of Mitofusin-2 (Mfn2), one of the key elements of mitochondrial fusion machinery [10], on red *vs* green MitoTimer populations, and found that indeed, mitochondria with the lowest red-to-green fluorescence had the highest expression of Mfn2 (Fig. 1H). These *germinal mitochondria*, identified by the lowest red-to-green fluorescence ratio, are engaged in new protein import, mtDNA replication, and are competent for fusion with the rest of the mitochondrial network.

3.2. Constitutive expression of MitoTimer in vivo

To monitor mitochondrial turnover *in vivo*, we expressed MitoTimer protein in mice under control of the cardiac α-MHC promoter [11]. MitoTimer was expressed in right and left ventricles, but to a much lower extent in the atria, despite the fact that αMHC is expressed in atrial cardiomyocytes [12] (Fig. 2A). This is consistent with the lower mitochondrial content in atrial myocytes [13]. Interestingly, we observed a high degree of heterogeneity in red-to-green fluorescence in ventricles, with longitudinally contiguous myocytes exhibiting synchronized MitoTimer ratios (Fig. 2B, C). The distribution of MitoTimer in ventricular cardiomyocytes resembled the typical pattern seen for cardiomyocyte mitochondria, consistent with the biochemically demonstrated subcellular distribution (Fig. 2C). Further, cardiomyocyte mitochondria displayed heterogeneity in MitoTimer fluorescence ratios within a cell, with solitary spherical mitochondria that were more red-fluorescent than the rest of the population (Fig. 2C). FAOS performed on isolated mitochondria from hearts of α-MHC MitoTimer mice confirmed the presence of discrete subsets of mostly-red and mostly-green mitochondria (Fig. 2D). As these solitary red mitochondria were isolated from the network and unable to import new MitoTimer protein, we hypothesized they were senescent and were likely to be targeted for autophagic elimination. Indeed, immunostaining with anti-LC3 revealed that solitary red mitochondria were often surrounded by autophagy protein LC3 (Fig. 2E, S2).

To monitor mitochondrial turnover in the context of cardiac development, we examined the hearts of two α-MHC MitoTimer littermates at different ages by microscopy (Fig. 2F) and FAOS (Fig. 2G). A slower rate of mitochondrial turnover with age is well characterized [14]; consistent with this, the mouse at 3 weeks of age (woa) has more green-predominant mitochondria than its littermate analyzed at 16 woa, as seen by the FAOS analysis of isolated cardiac mitochondria, indicating a faster rate of turnover in the younger animal. Future studies to examine later ages (out to 24 months) will yield additional insights.

Constitutively expressed MitoTimer allows us to assess mitochondrial turnover in cells and *in vivo*, based on the ratio of red:green fluorescence. Green-predominant mitochondria are actively engaged in import of newly-synthesized mito-targeted proteins, and are actively engaged in mtDNA replication; as mtDNA replication and transcription are concurrent, this is additional evidence that the green subpopulation is engaged in mitochondrial biogenesis. These so-called germinal mitochondria are also competent for fusion, allowing them to share their contents with the rest of the mitochondrial network. It is unknown whether germinal mitochondria are transient phenomena or whether there is a stable and distinct subpopulation of mitochondria specialized for biogenesis. We also observed solitary spherical red-predominant mitochondria, some of which were also decorated with LC3, supporting the notion that these senescent mitochondria were targeted for autophagic removal.

The remarkable heterogeneity of MitoTimer maturation ratios in the heart was unexpected. Interestingly, we noted that longitudinally contiguous cardiomyocytes have similar MitoTimer ratios whereas there is considerable variation across lateral neighboring cells. Moreover, with the exception of the occasional solitary red mitochondrion, the MitoTimer ratio was remarkably similar within a given cell, giving rise to the surprising interpretation

that mitochondrial biogenesis is synchronized within a cell, and perhaps also synchronized across cells connected by gap junctions.

While lower oxygen tension can delay MitoTimer maturation (prolonging the persistence of the green conformation), it seems unlikely that tissue oxygen tension varies appreciably from one cell to the next in the heart. However, given the high rate of oxygen consumption by cardiac mitochondria, tissue oxygen levels may be lower in the heart, which could delay MitoTimer maturation. If true, then the published Timer protein maturation kinetics may not be accurate; however, the ratio would still be highly informative as a relative indicator of mitochondrial turnover. Another caveat is a possible deleterious effect of MitoTimer expression on cardiac function with age. While founder mice have reached 18 months of age with no apparent health effects, detailed physiological characterization is ongoing.

The value of MitoTimer ratios as an index of mitochondrial turnover is supported by the images of heart sections and a comparison of the red:green ratios of cardiac mitochondria isolated from mice 3 weeks of age (woa) *vs* 16 woa. Mitochondrial biogenesis is quite brisk during the first month of postnatal life [14,15]. Consistent with this, the heart at 3 woa has a very low red:green ratio as compared to the older animal.

Mitochondrial turnover is essential to homeostatic quality control; it is the most important mechanism to compensate for oxygen radical-mediated mtDNA damage and to enable metabolic remodeling in response to alterations in cardiac workload arising from pressure overload hypertrophy or post-infarct remodeling. The MitoTimer mouse will provide new insights into cardiovascular physiology and pathophysiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2015.11.032.
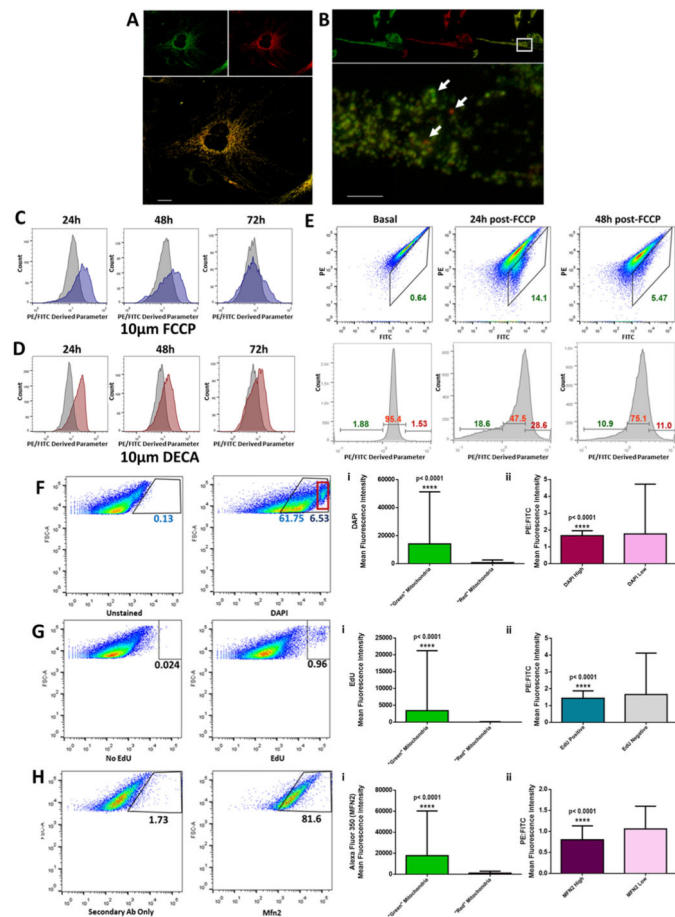
Fig. 1.
Constitutive expression of MitoTimer. A) Merged fluorescence microscopy (CFI Plan Apoλ 60xH) images of C2C12 myoblasts expressing MitoTimer. Smaller insets show the green and red channels. Scale bar: 20 μm. B) Fluorescence microscopy (CFI Plan Apoλ 60xH) merged image of MitoTimer C2C12 myoblasts following a 24 h pulse with 10 μM FCCP. Mitochondria engaged in new protein import (green) and mitochondria incapable of importing new protein (red) can be readily observed (white arrows). Scale bar: 5 μm. C) Flow cytometry histogram of derived red-to-green fluorescence of C2C12 MitoTimer cells treated with 10 μM FCCP. D) Flow cytometry histogram of derived red-to-green fluorescence ratio of C2C12 MitoTimer cells treated with 10 μM DECA. Control cells are in gray. Note the red-to-green ratio returning to baseline as mitochondria in cells begin to import newly-synthesized MitoTimer as they regain membrane potential (FCCP) or resume protein import (DECA). E) FAOS dot plots of isolated mitochondria from C2C12 MitoTimer cells 24 h after FCCP addition. The gate denotes newly synthesized green-fluorescent mitochondria. Derived histograms of the data from demonstrate the shift in the fluorescence ratio as the mitochondrial population is depolarized and allowed to recover. Orange number represents the % of mitochondria falling within the normal distribution (95%), while the green number represents the % of mitochondria that are green outliers and the dark red number the % of mitochondria that are red-shifted outliers. At 24 h, the red-shift is apparent, but a substantial increase in the green population is also present. At 48 h

the mitochondrial population is returning to equilibrium. F) FAOS dot plot shows mitochondria before (left) or after DAPI staining (right). Fi) Mitochondria that display a lower red:green fluorescence ratio have more DAPI binding (=more mtDNA, right panel, n = 3209, 6.61% of population, MFI PE/FITC = 0.88) than the red mitochondrial population (n = 2589, 4.43% of population, MFI PE/FITC = 5.33). Fii) The black ROI indicates DAPI-stained mitochondria (DAPI Low, n = 44,697, 61.75% of total mitochondria), and the red ROI indicates the brightest population (DAPI High, n = 3170, 6.53% of total mitochondria). (Indo-1 Violet channel used for DAPI) G) Similarly, mitochondria from cells incubated overnight with EdU (right panel) and without EdU (left panel) were subjected to Click Chemistry with Pacific Blue. The black ROI shows the subpopulation of mitochondria that incorporated EdU. Gi) Mitochondria that display a low red:green fluorescence ratio have more EdU incorporation (=more newly synthesized mtDNA, right panel, n = 1099, 2.24% of population, MFI PE/FITC = 0.88) than the mitochondrial population with a high red:green fluorescence (n = 3466, 7.06% of population, MFI PE/FITC = 2.73). Gii) Far-right graph demonstrates the red:green ratio for the Edu + (n = 669, 0.96% of total mitochondria) and EdU-gated subpopulations (n = 44,696), showing that EdU incorporation is higher in mitochondria that are more green-fluorescent. (Pacific Blue channel used for EdU detection) H) Dot plot shows MFN2 immunostaining of mitochondria (no Mfn2 antibody, secondary only, left; MFN2 immunostaining, right). The black ROI indicates Mfn2-positive mitochondria. Hi) MFN2 staining is highest in mitochondria that are mostly green fluorescent (n = 810, 7.52% of population, MFI PE/FITC = 0.60) compared to the mostly-red fluorescent mitochondria ((n = 711, 4.18% of population, MFI PE/FITC = 0.4.87). (Indo-1 Violet channel used to detect Alexa Fluor 350). Hii) MFN2-High (n = 430, 5.83% of the MFN2+ mitochondria) population displays a lower red:green fluorescence compared to MFN2-Low population (n = 6682, 90.8% of MFN2+ mitochondria). All FACS/FAOS experiments presented are plots and statistics from a representative experiment from multiple repeats. Statistical analysis performed from n = 50,000 events (cells or mitochondria). Unpaired t-test used for calculation of significance.
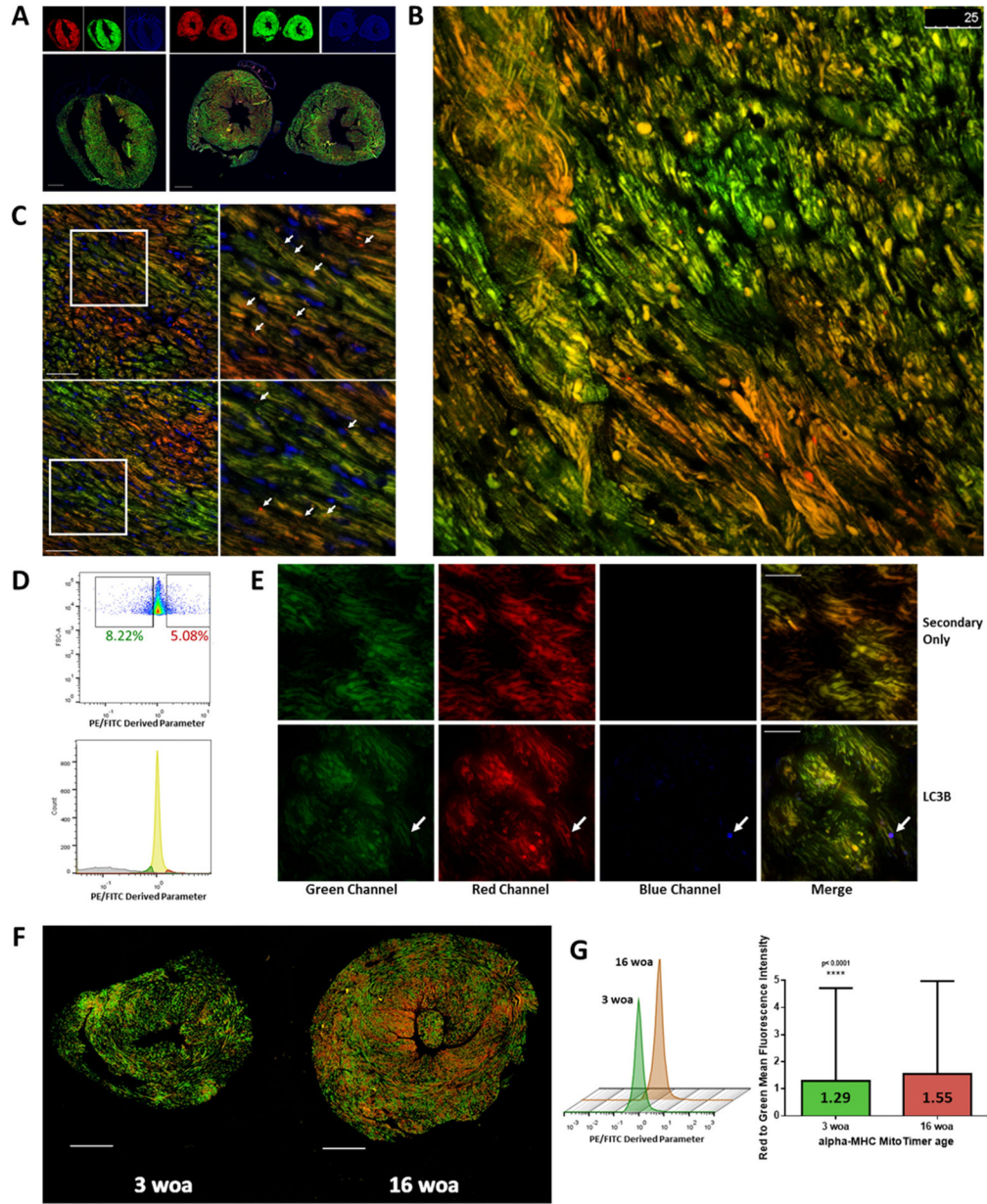
Fig. 2.
α-MHC MitoTimer Mouse. A) Fluorescence microscopy images (CFI Plan Apoλ 4X, image merge) of α-MHC MitoTimer mouse heart transverse and coronal sections. Merge of red, green, and blue (DAPI) channels; smaller insets show the individual channels. Scale bar: 1 mm. B) Cardiac tissue, confocal microscopy merged images of green and red channels is shown. Scale bar: 25 μm. C) Merged fluorescence microscopy images (CFI Plan Apoλ 40X) of green, red and blue (DAPI) channels. Scale bar: 25 μm. Arrows point to solitary red mitochondria in cardiomyocytes. D) Dot plot of FSC (X-axis) *vs* derived parameter (MitoTimer red:green ratio, PE/FITC) and derived parameter histogram of mitochondria isolated from α-MHC-MitoTimer mouse cardiomyocytes. Subpopulations of mostly-green and mostly-red mitochondria are readily detected. E) Fluorescence microscopy images α-

MHC-MitoTimer mouse cardiac tissue immunostained for LC3 (Blue channel, Alexa-Fluor 350 secondary antibody). Arrow points to a solitary red mitochondrion surrounded by LC3. Scale bar: 20 μm. F) Fluorescence microscopy merged images of MitoTimer mouse hearts of littermates (n = 1) at 3 woa (left) and 16 woa (right). G) Derived parameter histogram overlay of the isolated mitochondria from the 3 woa and 16 woa mouse hearts. Statistical analysis performed from n = 50,000 events (mitochondria from each heart). Unpaired t-test used for calculation of significance. Scale bar: 1 mm.