European Journal of Cell Biology 102 (2023) 151371

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

MitoTracker: A useful tool in need of better alternatives

Kit Neikirk [a,1], Andrea G. Marshall [a,1], Bartosz Kula [b], Nathan Smith [b], Sharonda LeBlanc [c,d], Antentor Hinton Jr. [a,*]

[a] Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232, USA
[b] Del Monte Institute for Neuroscience, Department of Neuroscience, University of Rochester, School of Medicine and Dentistry, Rochester 14642, USA
[c] Department of Physics, North Carolina State University, Raleigh 27695, NC, USA
[d] Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill 27599, NC, USA

ARTICLE INFO

Keywords:
MitoTracker
Mitochondria
Fluorescent probes
Membrane potential

ABSTRACT

The fluorescence viewing of mitochondria is commonly performed by MitoTracker, a lipophilic cationic dye that is taken up by the mitochondria. In this forum, we highlight several issues that may occur with MitoTracker, including staining of other organelles. Our aim is to offer alternative dyes and discuss their advantages and disadvantages. We also offer options for software with alternatives to MitoTracker to expedite future experimental design.

1. Introduction

Mitochondria are essential organelles that perform various metabolic functions and regulate cellular homeostasis. Their visualization is critical to understanding many physiological and pathological processes, such as energy production, oxidative stress, apoptosis, aging, and neurodegeneration (Doherty and Perl, 2017; Brand et al., 2013). First developed in the 1990 s, MitoTrackers are fluorescent probes that are commonly used in cell biology to visualize mitochondria (Chazotte, 2011). These probes are made up of a lipophilic cationic dye, which can pass the plasma membrane of the mitochondria in live cells, with accumulation due to the high membrane potential across the mitochondrial inner membrane (Xiao et al., 2016). MitoTracker offers an advantage through its permanency in the mitochondria as it is retained after membrane potential is lost during fixation, due to the covalent bond formed at the thiol group of a cysteine residue inside the mitochondria (Chazotte, 2011). The structure of MitoTracker depends on the specific dye, which includes red, green, deep-red, or orange (Table 1), but, generally, it consists of a large planar aromatic structure with two positively charged nitrogen atoms. These cationic species enable MitoTracker to accumulate in active mitochondria in a potential-dependent manner (Doherty and Perl, 2017). In tandem, the chloromethylphenyl functional group enables MitoTracker to react with thiols in the mitochondria and permanently bind to them (Zhitomirsky et al., 2018). In theory, once inside the mitochondria, the dye binds to mitochondrial proteins, which results in the fluorescence of the probe, with an intensity proportional to the concentration of the dye within the mitochondria, thus visualizing the mass and morphology of mitochondria (Chazotte, 2011). In addition, there is a range of MitoTracker dyes (Table 1) available such as Mitotracker Red Fei Mao (FM) and Mitotracker Green FM, which are dependent and independent of membrane potential, respectively (Nick, 2018). Given the high specificity, stability, brightness, and photostability of MitoTracker along with its flexibility to be used in combination with other probes, MitoTracker is considered a powerful technique (Chazotte, 2011; Zhitomirsky et al., 2018; Little et al., 2020).

2. Challenges

Past studies have shown that commonly used calcium probes, such as Fura-2 AM and Rhod-2 AM, impede cell metabolism and Na,K-ATPase activity (Smith et al., 2018). These studies highlight the importance of investigating and verifying the accuracy of the precision of current tools. Critically, research performed can only be as accurate as the techniques that are used. While reliable and versatile methods for mitochondrial imaging are of great interest to researchers, MitoTracker poses certain issues that researchers may not be aware of. MitoTracker depends on the mitochondrial membrane potential for its accumulation in the

* Correspondence to: Department of Molecular Physiology and Biophysics, Vanderbilt School of Medicine Basic Sciences, The Vanderbilt Diabetes Research and Training Center, 750 Robinson Research Building, 2200 Pierce Ave, Nashville, USA.
E-mail address: antentor.o.hinton.jr@vanderbilt.edu (A. Hinton).
[1] Indicates co-first authorship.

https://doi.org/10.1016/j.ejcb.2023.151371
Received 16 August 2023; Received in revised form 10 October 2023; Accepted 30 October 2023
Available online 31 October 2023

Table 1
Comparison of types of MitoTrackers as well as their use cases and drawbacks. Information utilized from https://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook/probes-for-organelles/probes-for-mitochondria.html and http://101.200.202.226/files/prod/manuals/029/1323157570_10003.pdf.

MitoTracker Type	Advantage (s)	Disadvantage (s)
Mitotracker Green FM	- Independent of membrane potential - Background fluorescence is negligible - Live cells	- Easily washed out, not applicable for immunofluorescence (Nick, 2018) - Altered by oxidative state (Doherty and Perl, 2017)
Mitotracker Red FM	- Carbocyanide base that depends on membrane potential - Possible for multicolor labeling experiments - Live cells	- Cannot label dysfunctional mitochondria or mitochondria with altered membrane potential (Nick, 2018)
MitoTracker DeepRed	- Retained following permeabilization - Shows activity in tissues (Dan et al., 2020)	- Potentially preferentially targets highly-active mitochondria (Sargiacomo et al., 2021)
MitoTracker Orange	- Retained following permeabilization	- Can inhibit Complex I
MitoTracker Red	- Retained following permeabilization	- Dependent on mitochondrial potential
Mitotracker Deep Red FM	- Possible for multicolor labeling experiments	- Can inhibit Complex I
MitoTracker Red CMXRos	- Possible for multicolor labeling experiments	- Binds to mitochondrial lipids and proteins, not purely dependent on mitochondrial potential (Buravkov et al., 2014)

mitochondria (Xiao et al., 2016). Thus, MitoTracker cannot label mitochondria that have lost their membrane potential due to damage or dysfunction. Therefore, MitoTracker may underestimate or miss some populations of mitochondria that are relevant for certain biological questions or conditions. For example, MitoTracker may not detect mitochondria undergoing fission or fusion events or those involved in intercellular transfer, which can have a temporary loss of membrane potential (Dong et al., 2022). Furthermore, oxidant burden can influence MitoTracker uptake (Buckman et al., 2001). It should be kept in mind by researchers using MitoTracker that while its initial uptake is dependent on membrane potential, its retention is not, so MitoTracker is an inadequate tool for measuring long-term membrane potential (Kholmukhamedov et al., 2013).

Once inside the mitochondria, MitoTracker should serve as an analog for mitochondrial mass, but this is not always the case (Xiao et al., 2016). Importantly, MitoTracker Green has been suggested to be dependent on both redox status as well as membrane potential, thus not serving as a good measurement of mitochondrial mass (Doherty and Perl, 2017; Xiao et al., 2016; Little et al., 2020) (Fig. 1). This behavior has important implications, as cristae, the inner folds of mitochondria that house ATP synthases, may display differential mitochondrial membrane potentials (Wolf et al., 2019). While MitoTracker is commonly understood to be initially dependent on membrane potential for entry into the mitochondria, further modification by independent cristae membrane potential may interfere with its usefulness. Additionally, MitoTracker can be affected by protein degradation caused by autophagic processes (Xiao et al., 2016). Finally, while MitoTracker should be specific to mitochondria, the tag is attracted to the high membrane potential of other organelles; for example, MitoTracker can "escape" into the endoplasmic reticulum or lysosomes, staining organelles with membrane potentials outside of the mitochondria (Klier et al., 2022). As the diversity of mitochondrial morphology and cristae integrity are becoming increasingly understood (Glancy et al., 2020), it remains unclear if morphology can affect MitoTracker uptake. For example, cristae membrane potentials may be dependent on mitochondrial morphology or cellular environment (Wolf et al., 2019). Pertinently, mitochondria are now understood to display diverse morphologies that are influenced by factors including aging (Vue et al., 2023a; Crabtree et al., 2023; Vue et al., 2023b), which may make MitoTracker an unreliable indicator of certain 3D phenotypes of mitochondria. Thus, future research is needed to broaden understanding of this potential relationship.

MitoTracker may be unsuitable for live cell imaging as it can interrupt mitochondrial processes including the electron transfer chain (Buckman et al., 2001). While similar effects have been noted in

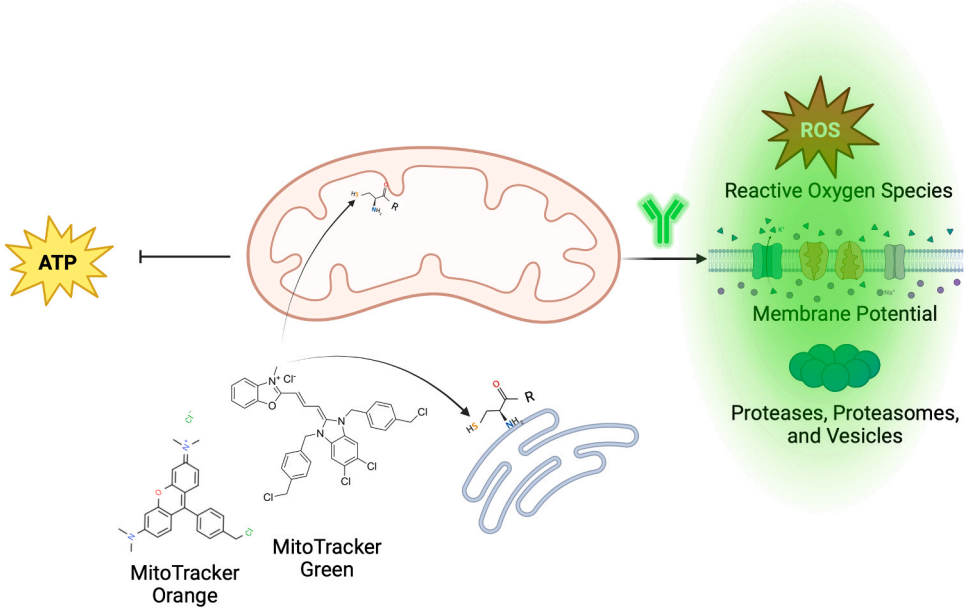

Fig. 1. Simple mechanism of action of MitoTracker and potential interferences or issues. Ideally, MitoTracker Green will enter the mitochondria and bind with a chloromethyl group to inner mitochondrial membrane thiols. Yet, off-target binding to the ER may occur, while retained MitoTracker may also be dependent on membrane potential, ROS, and other factors, while inhibiting mitochondrial OXPHOS.

Table 2
List of potential alternative dyes, tags, and probes, for common *in vitro* usage. Additionally, software that can be utilized for their analysis. Non-comprehensive and representative of the variety available in the field. Full use of methods of the dyes/tag/probes are available at the indicated reference.

Name of Dye / Tag / Probe:	Usage:	Reference:
10-N-nonyl-acridine orange	Fluorescence dye specific for Cardiolipin in the inner mitochondrial membrane. However, while some references suggest it is independent from membrane potential, it has been shown that retention is not completely independent from membrane potential.	Keij, J. F., Bell-Prince, C., & Steinkamp, J. A. (2000). Staining of mitochondrial membranes with 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green is affected by mitochondrial membrane potential altering drugs. Cytometry, 39 (3), 203–210. https://doi.org/10.1002/ (sici)1097–0320 (20000301)39:3 < 203::aid-cyto5 > 3.0.co;2-z
BrdU	Thymidine analog that can be incorporated into newly synthesized mitochondrial DNA (mtDNA) during cell division, which allows for mitochondria to be traced across their replication and synthesis, but better suited for metabolic purposes than structural purposes. However, its usage requires anti-BrdU antibody and its incorporation isn't specific to only mtDNA.	Calkins, M. J., & Reddy, P. H. (2011). Assessment of newly synthesized mitochondrial DNA using BrdU labeling in primary neurons from Alzheimer's disease mice: Implications for impaired mitochondrial biogenesis and synaptic damage. Biochimica et biophysica acta, 1812 (9), 1182–1189. https://doi.org/10.1016/j.bbadis.2011.04.006
CellLight Mitochondria-GFP	Ability to be used in combination with traditional organic dyes, combines with BacMam to localize fluorescence to mitochondria independent from mitochondrial membrane potential.	Bertolini, I., Keeney, F., & Altieri, D. C. (2021). Protocol for assessing real-time changes in mitochondrial morphology, fission and fusion events in live cells using confocal microscopy. STAR protocols, 2 (3), 100767. https://doi.org/10.1016/j.xpro.2021.100767
Chromabodies	Also known as fluorescent or labeled nanobodies. At a much smaller size than traditional antibodies, fluorescent nanobodies have a far greater ability to detect endogenous proteins without causing alteration in host cell function. While specific nanobodies for mitochondria are still limited and need developing, nanobodies have been developed for Miro1, an outer mitochondrial membrane protein. Anti-GFP nanobodies have also been utilized as a sensor for calcium in close proximity to the mitochondria.	Fagbadebo, F. O., Kaiser, P. D., Zittlau, K., Bartlick, N., Wagner, T. R., Froehlich, T., Jarjour, G., Nueske, S., Scholz, A., Traenkle, B., Macek, B., & Rothbauer, U. (2022). A Nanobody-Based Toolset to Monitor and Modify the Mitochondrial GTPase Miro1. Frontiers in molecular biosciences, 9, 835302. https://doi.org/10.3389/fmolb.2022.835302 de Beer, M. A., & Giepmans, B. N. G. (2020). Nanobody-Based Probes for Subcellular Protein Identification and Visualization. Frontiers in cellular neuroscience, 14, 573278. https://doi.org/10.3389/fncel.2020.573278
Cox8 Snap Tag	Specific SNAP tag for the cytochrome C oxidase inner mitochondrial membrane protein that can allow for distinct populations of mitochondria to be tagged independent of membrane potential and without affecting mitochondrial function. Especially relevant for labeling of cristae.	Pirooznia, S. K., Wang, H., Panicker, N., Kumar, M., Neifert, S., Dar, M. A., Lau, E., Kang, B. G., Redding-Ochoa, J., Troncoso, J. C., Dawson, V. L., & Dawson, T. M. (2022). Deubiquitinase CYLD acts as a negative regulator of dopamine neuron survival in Parkinson's disease. Science advances, 8 (13), eabh1824. https://doi.org/10.1126/sciadv.abh1824
$DiOC_6$ (3)	Commonly used method in flow cytometry to measure mitochondria. Carbocyanine dye to measure mitochondrial membrane potential. High mitochondrial toxicity. Also has been suggested it is not as reliable as other alternatives such as JC-1.	Salvioli, S., Ardizzoni, A., Franceschi, C., & Cossarizza, A. (1997). JC-1, but not DiOC6 (3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS letters, 411 (1), 77–82. https://doi.org/10.1016/s0014-5793 (97)00669–8
IraZolve-Mito	Luminescent iridium complex that selectively stains mitochondria with low cytotoxicity and deep tissue penetration. While it can also be combined with other probes, in contrast to MitoTracker, it has not been shown to affect metabolism or mitochondrial function, with the principal drawback being the necessity of a two-photon microscope for its use as a probe.	Sorvina, A., Bader, C. A., Darby, J. R. T., Lock, M. C., Soo, J. Y., Johnson, I. R. D., Caporale, C., Voelcker, N. H., Stagni, S., Massi, M., Morrison, J. L., Plush, S. E., & Brooks, D. A. (2018). Mitochondrial imaging in live or fixed tissues using a luminescent iridium complex. Scientific reports, 8 (1), 8191. https://doi.org/10.1038/s41598-018–24672-w
JC-1	Only dependent on mitochondrial membrane potential. Exists as simple monomer at low-membrane potential, with broad fluorescence at high-membrane showing sensitive measure of mitochondrial membrane potential. Not applicable in all experimental conditions and less complete mitochondrial analysis compared to MitoTrackers.	Chazotte B. (2011). Labeling mitochondria with JC-1. *Cold Spring Harbor protocols*, 2011 (9), pdb.prot065490. https://doi.org/10.1101/pdb.prot065490 Marcondes, N. A., Terra, S. R., Lasta, C. S., Hlavac, N. R. C., Dalmolin, M. L., Lacerda, L. A., Faulhaber, G. A. M., & González, F. H. D. (2019). Comparison of JC-1 and MitoTracker probes for mitochondrial viability assessment in stored canine platelet concentrates: A flow cytometry study. *Cytometry. Part A: the journal of the International Society for Analytical Cytology*, *95* (2), 214–218. https://doi.org/10.1002/cyto.a.23567
JC-9	Similar to JC-1 with potential-dependent actions. However, while red fluorescence intensity is dependent on membrane potential, green fluorescence intensity remains mostly independent from hyperpolarization.	Cottet-Rousselle, C., Ronot, X., Leverve, X., & Mayol, J. F. (2011). Cytometric assessment of mitochondria using fluorescent probes. Cytometry. Part A: the journal of the International Society for Analytical Cytology, 79 (6), 405–425. https://doi.org/10.1002/cyto.a.21061
mito-Dronpa	Photoswitchable fluorescent protein that may be used with mito-Orange.	Kremers, G. J., Hazelwood, K. L., Murphy, C. S., Davidson, M. W., & Piston, D. W. (2009). Photoconversion in orange and red fluorescent proteins. Nature methods, 6 (5), 355–358. https://doi.org/10.1038/nmeth.1319
mito-PA-mCherry1	Photoactivatable fluorescent marker applicable to a wide-range of organelles and uses including mitochondrial networks (through targeting of Cox8). Well adapted for 3D localization.	York, A. G., Ghitani, A., Vaziri, A., Davidson, M. W., & Shroff, H. (2011). Confined activation and subdiffractive localization enables whole-cell PALM with genetically expressed probes. Nature methods, 8 (4), 327–333. https://doi.org/10.1038/nmeth.1571
Mito-QC	pH-sensitive probe consisting of mCherry1 targeting to outer mitochondrial membrane of FIS1. Allows for both imaging of mitochondrial networks and monitoring mitophagy with high specificity.	Williams, J. A., Zhao, K., Jin, S., & Ding, W. X. (2017). New methods for monitoring mitochondrial biogenesis and mitophagy in vitro and in vivo. Experimental biology and medicine (Maywood, N.J.), 242 (8), 781–787. https://doi.org/10.1177/1535370216688802
MitoBlue	Fluorescent bisamidine commonly used to image mitochondria-lysosome communication. Stains functional mitochondria with low toxicity relatively independent of the membrane potential.	Sánchez, M. I., Vida, Y., Pérez-Inestrosa, E., Mascareñas, J. L., Vázquez, M. E., Sugiura, A., & Martínez-Costas, J. (2020). MitoBlue as a tool to analyze the mitochondria-lysosome communication.

(*continued on next page*)

Table 2 (*continued*)

Name of Dye / Tag / Probe:	Usage:	Reference:
	In comparison to MitoTracker, persists to monitor until it is transferred to lysosomes, allowing for tracing of autophagy.	Scientific reports, 10 (1), 3528. https://doi.org/10.1038/s41598-020–60573-7
Mitorotor-1	Fluorescence lifetime-based molecular motor mitochondrial probe that reports solvent viscosity and ordering of the inner mitochondrial membrane. Suitable for live cell imaging.	https://www.pnas.org/doi/10.1073/pnas.2213241120
MitoSOX	Binds to Mitochondrial Superoxide produced by oxidative phosphorylation, using hydroethidine, to showcase ROS generation. However, issues have been raised with unreliability.	Zielonka, J., & Kalyanaraman, B. (2010). Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. Free radical biology & medicine, 48 (8), 983–1001. https://doi.org/10.1016/j.freeradbiomed.2010.01.028
MitoTimer	Tool for measuring mitochondrial aging, biogenesis, and protein synthesis. Dependent on polarization, so serves as a probe to monitor mitochondrial damage and degradation.	Williams, J. A., Zhao, K., Jin, S., & Ding, W. X. (2017). New methods for monitoring mitochondrial biogenesis and mitophagy in vitro and in vivo. Experimental biology and medicine (Maywood, N.J.), 242 (8), 781–787. https://doi.org/10.1177/1535370216688802
Mt-Keima	Fusion with COX8, to have a pH dependent dye. Specificity of mitophagy activation, and retains in lysosomes. Cannot be fixed or measure mitochondrial biogenesis.	Williams, J. A., Zhao, K., Jin, S., & Ding, W. X. (2017). New methods for monitoring mitochondrial biogenesis and mitophagy in vitro and in vivo. Experimental biology and medicine (Maywood, N.J.), 242 (8), 781–787. https://doi.org/10.1177/1535370216688802
PK Mito Orange (PKMO)	Conjugation of cyclooctatetraene to a benzo-fused cyanine dye. This marker exhibits stimulated emission depletion at 775 nm, strong photostability, and has much lower phototoxicity than other options. Because of this, PKMO can be utilized to collect high-resolution live-cell imaging of mitochondrial inner membrane dynamics in immortalized cells, primary cells, and organoids.	Liu, T., Stephan, T., Chen, P., Keller-Findeisen, J., Chen, J., Riedel, D., Yang, Z., Jakobs, S., & Chen, Z. (2022). Multi-color live-cell STED nanoscopy of mitochondria with a gentle inner membrane stain. Proceedings of the National Academy of Sciences of the United States of America, 119 (52), e2215799119. https://doi.org/10.1073/pnas.2215799119
Rhodamine 123	Measures mitochondrial membrane potential, but is washed out upon loss of membrane potential. However, susceptible to intracellular/intramitochondrial modification and has a small amount of ETC inhibition.	Uribe, P., Villegas, J. V., Boguen, R., Treulen, F., Sánchez, R., Mallmann, P., Isachenko, V., Rahimi, G., & Isachenko, E. (2017). Use of the fluorescent dye tetramethylrhodamine methyl ester perchlorate for mitochondrial membrane potential assessment in human spermatozoa. Andrologia, 49 (9), 10.1111/and.12753. https://doi.org/10.1111/and.12753
SNARF-1 dye	Carboxy pH indicator that allows for changes in mitochondrial pH to be measured through a radiometric fluorescent probe.	Ramshesh, V. K., & Lemasters, J. J. (2018). Imaging of Mitochondrial pH Using SNARF-1. Methods in molecular biology (Clifton, N.J.), 1782, 351–356. https://doi.org/10.1007/978–1-4939–7831-1_21
SPLICS	Split-GFP-based contact site sensor that can measure mitochondria endoplasmic reticulum contact sites up to 50 nm distance. Unique in specifically fluoresces when organelles are in proximity.	Cieri, D., Vicario, M., Giacomello, M., Vallese, F., Filadi, R., Wagner, T., Pozzan, T., Pizzo, P., Scorrano, L., Brini, M., & Calì, T. (2018). SPLICS: a split green fluorescent protein-based contact site sensor for narrow and wide heterotypic organelle juxtaposition. Cell death and differentiation, 25 (6), 1131–1145. https://doi.org/10.1038/s41418-017–0033-z
styryl dyes DASPMI	Dynamic uptake and fluorescence intensity of fluorescence dye based on the membrane potential of mitochondria with high sensitivity that follows the Nernst relationship.	Ramadass, R., & Bereiter-Hahn, J. (2008). How DASPMI reveals mitochondrial membrane potential: fluorescence decay kinetics and steady-state anisotropy in living cells. Biophysical journal, 95 (8), 4068–4076. https://doi.org/10.1529/biophysj.108.135079
Terpyridyl cyclometalated Ir (III) complexes	High photostability, two-photon dye as a non-invasive mechanism. Does not stain other organelles or display a quenching effect like MitoTracker. Exact mechanism likely based on lipophilicity and positive charge, but not fully elucidated.	Huang, H., Zhang, P., Qiu, K., Huang, J., Chen, Y., Ji, L., & Chao, H. (2016). Mitochondrial Dynamics Tracking with Two-Photon Phosphorescent Terpyridyl Iridium (III) Complexes. Scientific reports, 6, 20887. https://doi.org/10.1038/srep20887
Tetra-methylrhodamine Methylester	Measures mitochondrial membrane potential, but is washed out upon loss of membrane potential. Accumulates exclusively in hyperpolarised mitochondria. Cytometry technology capabilities. For TMRM, low ETC inhibition.	Uribe, P., Villegas, J. V., Boguen, R., Treulen, F., Sánchez, R., Mallmann, P., Isachenko, V., Rahimi, G., & Isachenko, E. (2017). Use of the fluorescent dye tetramethylrhodamine methyl ester perchlorate for mitochondrial membrane potential assessment in human spermatozoa. Andrologia, 49 (9), 10.1111/and.12753. https://doi.org/10.1111/and.12753
Various Mito-targeted fluorescent proteins including red, yellow, orange, green, blue, and cyan.	Generally, depend on cytochrome c oxidase subunit 8a with various specific use cases. Full list available on Addgene.	Full List available at: Harwig, M. C., Viana, M. P., Egner, J. M., Harwig, J. J., Widlansky, M. E., Rafelski, S. M., & Hill, R. B. (2018). Methods for imaging mammalian mitochondrial morphology: A prospective on MitoGraph. Analytical biochemistry, 552, 81–99. https://doi.org/10.1016/j.ab.2018.02.022https://doi.org/10.1016/j.ab.2018.02.022
ZP1-HaloTag	More distinguishable and works longer than MitoTracker. Self-labeling protein tags that can label fusion proteins to allow for multiplexing. HaloTags have high efficiency and can use proteins such as rhodamine to label multiple targets (e.g., nucleus, mitochondria, and Golgi apparatus). High flexibility and developing technology of HaloTags allow for fluorescence imaging of discrete subcellular compartments.	Zastrow, M. L., Huang, Z., & Lippard, S. J. (2020). HaloTag-Based Hybrid Targetable and Ratiometric Sensors for Intracellular Zinc. ACS chemical biology, 15 (2), 396–406. https://doi.org/10.1021/acschembio.9b00872 Frei, M. S., Tarnawski, M., Roberti, M. J., Koch, B., Hiblot, J., & Johnsson, K. (2022). Engineered HaloTag variants for fluorescence lifetime multiplexing. Nature methods, 19 (1), 65–70. https://doi.org/10.1038/s41592-021–01341-x

Software:	Usage:	Reference:
CellProfiler	Open-source plugin for a variety of purposes including the analysis of fluorescent antibody staining. New plugins allow for machine learning to expedite localization studies.	Carpenter, A. E., Jones, T. R., Lamprecht, M. R., Clarke, C., Kang, I. H., Friman, O., Guertin, D. A., Chang, J. H., Lindquist, R. A., Moffat, J., Golland, P., & Sabatini, D. M. (2006). CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome biology, 7(10), R100. https://doi.org/10.1186/gb-2006–7–10-r100

(*continued on next page*)

Table 2 (*continued*)

Software:	Usage:	Reference:
ImageJ/FIJI (Fiji Is Just ImageJ)	Widely available open-source software with plugins for many applications, allows for cell fluorescence intensity and localization to be measured through a variety of workflow. Macros such as Mitochondrial Morphology can allow for specifically automated methods of fluorescence. Mitochondrial Network Analysis is another alternative that can measure 2D mitochondria networks and branch lists.	Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature methods, 9(7), 671–675. https://doi.org/10.1038/nmeth.2089
Imaris	Generally, allows for processing of live-cell imaging. Machine learning capabilities with ability to perform automatically generated tracks in 2D and 3D.	Bros, H., Hauser, A., Paul, F., Niesner, R., & Infante-Duarte, C. (2015). Assessing Mitochondrial Movement Within Neurons: Manual Versus Automated Tracking Methods. Traffic (Copenhagen, Denmark), 16(8), 906–917. https://doi.org/10.1111/tra.12291
MATLAB	Coding framework which allows for high-flexibility, while requiring more user expertise. Especially useful for niche- or custom applications of deep-learning techniques.	Sobie E. A. (2011). An introduction to MATLAB. Science signaling, 4(191), tr7. https://doi.org/10.1126/scisignal.2001984
MetaMorph	Widely available imaging software that has high flexibility. Increasingly has been utilized for purposes including photomanipulation and multiwavelength images of regions of interest.	Araki, N., Ikeda, Y., Kato, T., Kawai, K., Egami, Y., Miyake, K., Tsurumaki, N., & Yamaguchi, M. (2014). Development of an automated fluorescence microscopy system for photomanipulation of genetically encoded photoactivatable proteins (optogenetics) in live cells. Microscopy (Oxford, England), 63(3), 255–260. https://doi.org/10.1093/jmicro/dfu003
Mito Hacker	Set of three tools: Cell Catcher, and Mito Catcher, and MiA. These three applications isolate individual cells from regions of interests, utilize pixel intensity to remove background, and analyze mitochondrial network quantifications and distribution to enable high-throughput, automated analysis of mitochondria.	Rohani, A., Kashatus, J. A., Sessions, D. T., Sharmin, S., & Kashatus, D. F. (2020). Mito Hacker: a set of tools to enable high-throughput analysis of mitochondrial network morphology. Scientific reports, 10(1), 18941. https://doi.org/10.1038/s41598–020–75899–5
MitoGraph	Based on C+ +, open-source automated platform for analyzing and predicting 3D mitochondrial structures and networks based on fluorescent images. Specifically adapted to be able to image diverse mitochondrial phenotypes such as entirely fragmented or hyper-elongated. While originally made for yeast, has been shown to be effective in other models.	Harwig, M. C., Viana, M. P., Egner, J. M., Harwig, J. J., Widlansky, M. E., Rafelski, S. M., & Hill, R. B. (2018). Methods for imaging mammalian mitochondrial morphology: A prospective on MitoGraph. Analytical biochemistry, 552, 81–99. https://doi.org/10.1016/j.ab.2018.02.022
Napari	Based on Python, library to allow for viewing of multi-dimensional images. Especially useful for 2D, 3D, or n-dimensional analysis. In specific, the empanada-napari plugin is an accessible plugin for the machine-learning based segmentation of EM mitochondria.	Chiu, C. L., & Clack, N. (2022). napari: a Python Multi-Dimensional Image Viewer Platform for the Research Community. Microscopy and Microanalysis, 28(S1), 1576–1577. https://empanada.readthedocs.io/en/latest/empanada-napari.html

MitoTracker Red and Deep Red, MitoTracker Orange has been noted to inhibit Complex I and potentially cause cellular death by activation of the permeability transition pore (Doherty and Perl, 2017; Scorrano et al., 1999). In general, all different colors of MitoTrackers share similarities with the principal difference in the chromophore cores (e.g., xanthene, rosamine, silicon-rhodamine, and benzoxazole), which in the case of MitoTracker Orange, may affect some Complex I activity. For example, this property of ATP inhibition has been exploited, with high concentrations of MitoTracker showing promise with anti-tumor activity in cancer stem cells (Sargiacomo et al., 2021). As MitoTracker forms epoxy resin once inside the mitochondria (Chazotte, 2011), it may also affect other functions of the mitochondria aside from the electron transport chain. For example, co-linking with the endoplasmic reticulum and interfering with mitochondrial inner-membrane proteins, such as cristae proteins, may disrupt calcium homeostasis, especially at mitochondria-endoplasmic reticulum contact sites. While greater studies into off-target interactions are necessary, Mitotrackers may artificially alter mitochondria during imaging.

3. *In vitro* alternatives

While rhodamine 123 and tetramethylrhodamine methylester are common alternatives, neither dye is retained after the loss of membrane polarity (Huang et al., 2007). Therefore, new alternatives are necessary for MitoTracker. Possible developments include organic molecules, nanobodies, or luminescent complexes. Novel dyes with utility for staining specific sub-organelle features of the mitochondria have also recently been developed. In general, these alternatives may offer fewer drawbacks for more specific use cases (Table 2). Past reviews have demonstrated that many alternatives can be utilized for the specific study of membrane potential that may not leak into organelles or cause other problems common to MitoTracker (Perry et al., 2011). Beyond this, a recent review by Wang and colleagues has demonstrated an excellent list of potential therapeutic targeting agents (Wang et al., 2021). Notably, several transition metal complexes have been used, along with cutting-edge techniques such as carbon quantum dots and nanoparticles which offer remarkably low cytotoxicity. The mechanisms of actions of these materials, often targeting receptors that may not interfere with mitochondrial function, suggest that there is a possibility of repurposing them for mitochondrial imaging. However, studies on these techniques are still ongoing, and while their development is promising, they may cause potential complications that would make them unfit for imaging. Currently, emerging technologies for imaging mitochondria non-invasively such as nanobody-based probes are still limited (de Beer and Giepmans, 2020). In tandem, there is a multitude of potential alternatives to MitoTracker with more specific use cases, as well as potential software that may aid in the usage of alternatives (Table 2).

4. *In vivo* considerations

Increasingly, it is clear that certain behaviors of mitochondria, such as their bidirectional horizontal bidirectional transfer between cells, are best studied *in vivo* (Dong et al., 2023). While MitoTracker is commonly used for *in vitro* experimentation, the necessity of transmembrane potentials mitigates its usage for fixed tissue samples (Sorvina et al., 2018). Notably, IraZolve-Mito uniquely allows for *in vivo* staining in tissue fixed by paraformaldehyde or flash frozen due to its molecular target not relying on membrane potential (Sorvina et al., 2018). Similarly, HaloTags are a promising technique that is continuously improving; HaloTags have been used with fixed cells to label mitochondria (Kompa et al., 2023), while in other fields for proteins HaloTags have been used for labeling *in vivo* (Masch et al., 2018). Together, this makes HaloTags an important technique that may be valuable for the *in vivo* study of mitochondria.

Outside of fixation studies, murine models with insertion into the genome of a genetic construct targeted to mitochondria alongside a fluorescent signal are promising techniques. Specifically, one main construct is the CAG/su9-DsRed2 transgene, which is designed to be ubiquitously expressed, with su9 targeting mitochondria while DsRed2

provides a fluorescent signal (Dong et al., 2017). Notably, murine models with this construct have been used to study horizontal transfer (Dong et al., 2017). Yet, an overarching limitation of *in vivo* techniques is limited markers, especially when it comes to tissue-specific labeling. One recent study has overcome this by creating mitochondrially targeted cyan fluorescent protein and mitochondrially targeted yellow fluorescent protein controlled by regulatory elements derived from the Thy1 and nse (Eno2) genes to specifically target neurons (Misgeld et al., 2007). Similarly, another mouse model has been devised specifically for the study of mitophagy *in vivo*, which contains a mCherry-GFP-FIS1 with the relative pH differences in mCherry and GFP allowing for the detection of mitochondria undergoing mitophagy (McWilliams et al., 2016). Another mouse model devised is focused on the implementation of mito::mKate2, which uniquely allows for "Fucci" cell cycle reporters to visualize different stages of the cell cycle using distinct fluorescent markers and ROSA26R-mTmG heterozygotes enable for imaging of murine models that have undergone a genetic alteration (Barrasso et al., 2018). Alternatively, as *in vivo* techniques remain in development, models such as zebrafish, which are naturally translucent, alongside mitochondrially-localized TagRFP, can offer unique ways to visualize mitochondria without disrupting the organelle system (Mandal et al., 2018).

5. Conclusion

While MitoTracker is useful, limitations in usability with fixation techniques, as well as accuracy in certain conditions highlight the need for consideration and development of *in vitro* and *in vivo* alternatives. Equally important, beyond only looking at the dye being utilized, is considering the entire experimental design to critically ask whether MitoTracker is the tool necessary. While MitoTracker can cause issues, some of these issues may be mitigated by changes in software or analysis (Table 2). With that being acknowledged, MitoTracker is a useful but sometimes problematic tool for mitochondrial imaging that may affect mitochondrial function and miss some mitochondrial populations. It is important to evaluate the advantages and disadvantages of each type of MitoTracker (Table 1) and alternative methods (Table 2) to determine the best option for each specific case.

CRediT authorship contribution statement

Kit Neikirk: Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Andrea G. Marshall**: Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Bartosz Kula**: Writing – review & editing. **Nathan Smith**: Writing – review & editing. **Sharonda LeBlanc**: Visualization, Writing – original draft, Writing – review & editing. **Antentor Hinton, Jr.**: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The UNCF/Bristol-Myers Squibb E.E. Just Faculty Fund, Career Award at the Scientific Interface (CASI Award) from Burroughs Welcome Fund (BWF) ID # 1021868.01, BWF Ad-hoc Award, NIH Small Research Pilot Subaward to 5R25HL106365–12 from the National Institutes of Health PRIDE Program, DK020593, Vanderbilt Diabetes and Research Training Center for DRTC Alzheimer's Disease Pilot & Feasibility Program and CZI Science Diversity Leadership grant number 2022- 253529 from the Chan Zuckerberg Initiative DAF, an advised fund of the Silicon Valley Community Foundation (AHJ). This work was supported by the National Institutes of Health Grant K01NS110981 to N. A.S. and the National Science Foundation NSF1926781 to N.A.S. The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Barrasso, A.P., et al., 2018. The mito::mKate2 mouse: a far-red fluorescent reporter mouse line for tracking mitochondrial dynamics in vivo. Genesis 56.
de Beer, M.A., Giepmans, B.N.G., 2020. Nanobody-based probes for subcellular protein identification and visualization. Front. Cell. Neurosci. 14.
Brand, M.D., et al., 2013. The role of mitochondrial function and cellular bioenergetics in ageing and disease. Br. J. Dermatol. 169, 1–8.
Buckman, J.F., et al., 2001. MitoTracker labeling in primary neuronal and astrocytic cultures: influence of mitochondrial membrane potential and oxidants. J. Neurosci. Methods 104, 165–176.
Buravkov, S.V., et al., 2014. Comparison of mitochondrial fluorescent dyes in stromal cells. Bull. Exp. Biol. Med 157, 654–658.
Chazotte, B., 2011. Labeling mitochondria with MitoTracker dyes. Cold Spring Harb. Protoc. 648.
Crabtree, A., et al., 2023. Defining mitochondrial cristae morphology changes induced by aging in brown adipose tissue. Adv. Biol. https://doi.org/10.1002/adbi.202300186.
Dan, L., et al., 2020. Comparison between different mitochondrial staining methods in cell and tissue samples. scdxxbyxb 51, 388–392.
Doherty, E., Perl, A., 2017. Measurement of mitochondrial mass by flow cytometry during oxidative stress. React. Oxyg. Species 4, 275–283.
Dong, F., et al., 2022. Mitochondrial fusion and fission are required for proper mitochondrial function and cell proliferation in fission yeast. FEBS J. 289, 262–278.
Dong, L.-F., et al., 2017. Horizontal transfer of whole mitochondria restores tumorigenic potential in mitochondrial DNA-deficient cancer cells. eLife 6, e22187.
Dong, L.-F., et al., 2023. Mitochondria on the move: horizontal mitochondrial transfer in disease and health. J. Cell Biol. 222, e202211044.
Glancy, B., et al., 2020. The functional impact of mitochondrial structure across subcellular scales. Front. Physiol. 11.
Huang, M., et al., 2007. Mitochondrial inner membrane electrophysiology assessed by Rhodamine-123 transport and fluorescence. Ann. Biomed. Eng. 35, 1276–1285.
Kholmukhamedov, A., et al., 2013. Mitotracker probes and mitochondrial membrane potential. Shock 39, 543.
Klier, P.E.Z., et al., 2022. Fluorescent indicators for imaging membrane potential of organelles. Curr. Opin. Chem. Biol. 71, 102203.
Kompa, J., et al., 2023. Exchangeable halotag ligands for super-resolution fluorescence microscopy. J. Am. Chem. Soc. 145, 3075–3083.
Little, A.C., et al., 2020. High-content fluorescence imaging with the metabolic flux assay reveals insights into mitochondrial properties and functions. Commun. Biol. 3, 1–10.
Mandal, A., et al., 2018. Analyzing neuronal mitochondria in vivo using fluorescent reporters in Zebrafish. Front. Cell Dev. Biol. 6.
Masch, J.-M., et al., 2018. Robust nanoscopy of a synaptic protein in living mice by organic-fluorophore labeling. Proc. Natl. Acad. Sci. 115, E8047–E8056.
McWilliams, T.G., et al., 2016. mito-QC illuminates mitophagy and mitochondrial architecture in vivo. J. Cell Biol. 214, 333–345.
Misgeld, T., et al., 2007. Imaging axonal transport of mitochondria in vivo. Nat. Methods 4, 559–561.
Nick, P.B. 2018 KIT - Botanisches Institut - Nick-LaborMitotracker [Online]. Available: https://www.jkip.kit.edu/botzell/2408.php. [Accessed: 17-Jul-2023].
Perry, S.W., et al., 2011. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. BioTechniques 50, 98–115.
Sargiacomo, C., et al., 2021. MitoTracker Deep Red (MTDR) is a metabolic inhibitor for targeting mitochondria and eradicating cancer stem cells (CSCs), with anti-tumor and anti-metastatic activity in vivo. Front. Oncol. 11.
Scorrano, L., et al., 1999. Chloromethyltetramethylrosamine (Mitotracker Orange) induces the mitochondrial permeability transition and inhibits respiratory complex I. Implications for the mechanism of cytochrome c release. J. Biol. Chem. 274, 24657–24663.
Smith, N.A., et al., 2018. Fluorescent Ca2+ indicators directly inhibit the Na,K-ATPase and disrupt cellular functions. Sci. Signal 039.
Sorvina, A., et al., 2018. Mitochondrial imaging in live or fixed tissues using a luminescent iridium complex. Sci. Rep. 8, 8191.
Vue, Z., et al., 2023a. 3D Reconstruction of murine mitochondria exhibits changes in structure across aging linked to the MICOS Complex. Aging Cell. https://doi.org/10.1101/2022.03.22.485341.
Vue, Z., et al., 2023b. Three-dimensional mitochondria reconstructions of murine cardiac muscle changes in size across aging. Am. J. Physiol. Heart Circ. Physiol. https://doi.org/10.1152/ajpheart.00202.2023.
Wang, H., et al., 2021. Recent advances in chemical biology of mitochondria targeting. Front. Chem. 9.
Wolf, D.M., et al., 2019. Individual cristae within the same mitochondrion display different membrane potentials and are functionally independent. EMBO J. 38, e101056.
Xiao, B., et al., 2016. Flow cytometry-based assessment of mitophagy Using Mitotracker. Front. Cell. Neurosci. 10.
Zhitomirsky, B., et al., 2018. LysoTracker and MitoTracker Red are transport substrates of P-glycoprotein: implications for anticancer drug design evading multidrug resistance. J. Cell. Mol. Med. 22, 2131–2141.