Short Technical Communication

Cite
Davis MS, Barrett MR, Bayly WM, Bolinger A (2023) Effect of selected fluorophores on equine skeletal muscle mitochondrial respiration. MitoFit Preprints 2023.5. https://doi.org/10.26124/mitofit:2023-0005

Author contributions
Data collection and evaluation was performed by MSD, MRB, WMB, and AB. All authors wrote the manuscript.

Conflicts of interest
MSD, MRB, WMB and AB have declared no conflict of interest.

Online 2023-07-07

Data availability
Data available upon request to the corresponding author.

Keywords
horse;
Amplex UltraRed;
Magnesium Green;
tetramethylrhodamine;
LEAK respiration

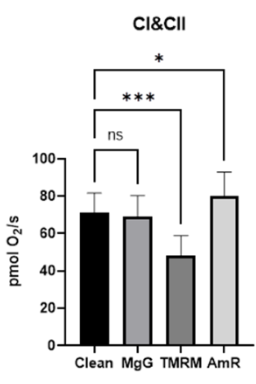
Effect of selected fluorophores on equine skeletal muscle mitochondrial respiration

Michael S. Davis[1], Montana R. Barrett[1], Warwick M. Bayly[2], Amanda Bolinger[1]

[1] Department of Physiological Sciences, College of Veterinary Medicine, Oklahoma State University, OK, USA
[2] Department of Veterinary Clinical Sciences, Washington State University, Pullman, WA, USA

* Corresponding author: michael.davis@okstate.edu

Summary



Equine skeletal muscle provides a rich source of mitochondria, suitable for both basic and applied studies of cellular energetics. Inclusion of fluorophores and the associated endpoints provides valuable information to complement measurements of mitochondrial oxygen flux, but investigators must be aware of the possible artifacts created by those fluorophores. Our studies demonstrate that Magnesium Green has no significant effect on respiration of isolated mitochondria, whereas tetramethylrhodamine inhibits phosphorylating respiration and increases leak respiration. Amplex UltraRed increases phosphorylating and non-phosphorylating respiration through Complex I. The effect of Amplex UltraRed is a novel finding, and further study is necessary to determine the mechanism underlying this artifact.

1. Introduction

Fluororespirometry is the analytical technique of using a combination of oxygen sensors and fluorescent dyes to quantify the metabolic activity of living cells or cellular organelles. Although the use of both techniques has been well-established in the scientific literature for many decades, recent advances in the precision of the analytical equipment has allowed for growing adoption of these tools in scientific laboratories around the

world. Concurrent with this expansion has been the recognition of subtle differences in cellular metabolic properties between species and tissues (Doerrier et al 2018), highlighting the need for species- and tissue-specific analytical protocols for fluororespirometry.

Extrapolation of data obtained *ex vivo* to the relevant *in vivo* condition is best achieved when there is minimal analytical artifact, and in instances in which analytical artifact is unavoidable, it is important to know the precise nature of the analytical artifact. The use of fluorescent dyes in fluororespirometry provides a clear example of this challenge – fluorescent dyes, while absolutely necessary for measuring the endpoints of interest, may alter the cellular metabolism responsible for those endpoints (Krumschnabel et al 2014; Makrecka-Kuka et al 2015). To address this aspect of fluororespirometry, we compared the effects of 3 different common dyes used in fluororespirometry – Amplex UltraRed (used to quantify production of hydrogen peroxide), Magnesium Green (used to quantify ATP synthesis) and tetramethylrhodamine methylester (TMRM, used to quantify mitochondrial membrane potential) on oxygen flux in freshly-isolated equine skeletal muscle mitochondria.

2. Experimental methods

Study #1 was approved by the Washington State University Institutional Animal Care and Use Committee, and Study #2 was approved by the Oklahoma State University Institutional Animal Care and Use Committee.

2.1. Study population and sample preparation

Study #1 used 7 healthy Thoroughbred horses (2 mares, 5 geldings, 8.3±3.5 yr, range 4-13 yr). Biopsies were obtained before and after a 9-week aerobic conditioning program intended to increase fitness. Study #2 used 6 healthy adult Thoroughbred geldings (15±5.8 yr, range 7-25 yr). Horses were at minimal aerobic fitness at the time of the study, having been housed together in pasture with no compulsory exercise for at least 24 months.

2.2. Sample preparation and high-resolution respirometry

Skeletal muscle biopsies were obtained using sterile technique from the center of the semitendinosus muscle using a 12 ga UCH biopsy needle while under light sedation and using local anesthesia. Biopsies were immediately transferred into vials with ice-cold BIOPS solution (2.77 mM CaK_2-EGTA, 7.23 mM K_2-EGTA, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP, and 15 mM phosphocreatine, adjusted to pH 7.1) and transported to the laboratory for analysis. Mitochondria were isolated using a commercial kit (MITOISO1, Sigma Aldrich) according to the manufacturer's instructions, with the exception of using substrate-free media (225 mM mannitol, 75 mM sucrose, 1 mM EGTA) for suspension of the isolated mitochondria at the end of the procedure. The final pellet of isolated mitochondria was resuspended using 80 µL of suspension media per 100 mg of muscle used for mitochondria isolation. Maximum interval from the time of the biopsy procedure to the isolation of mitochondria was less than 30 minutes. Samples were kept at 0-4 °C throughout the processing until added to the high-resolution respirometers, and all analysis was completed within 12 hr of the initial biopsy.

High-resolution respirometers (Oxygraph O2K, Oroboros Instruments, Innsbruck, Austria) were used to analyze the effects of different fluorophores on mitochondrial oxygen consumption as previously described (Davis et al 2023). Samples were analyzed in duplicate for each tested condition. Respirometer chambers (2 mL) were filled with low-Mg MiR05 (0.5 mM EGTA, 1 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA essentially fatty acid-free, adjusted to pH 7.1) and instruments were set at 38 °C to represent basal temperature of equine skeletal muscle. Instrument oxygen sensors were calibrated daily, and oxygen consumption was calculated as the negative slope of the oxygen measurement and reported as pmol $\times$ s^{-1} $\times$ mL^{-1}. In Study #1, samples were analyzed with either Magnesium Green (1.1 µM) or Amplex UltraRed (10 µM), along with associated assay reagents (superoxide dismutase (5 U/mL) and horseradish peroxidase (1 U/mL)) in separate high-resolution respirometers concurrently. In Study #2, the effects of the different fluorophores on mitochondrial respiration were measured using 4 high-resolution respirometers operated in parallel. The first machine did not contain any fluorophore and served as the control (Clean) for the remaining 3 machines containing Magnesium Green, TMRM (0.4 µM), or Amplex UltraRed. For both studies, 15 µL of isolated mitochondria suspension was added to each incubation chamber, and the sample was vortexed between each titration to maintain uniform suspension of the sample. Residual oxygen consumption (*Rox*) was measured prior to the addition of any substrates, and this value was subtracted from the oxygen consumption values of each step in the Substrate/Uncoupler/Inhibitor Titration (SUIT) protocol. The SUIT consisted of sequential titrations of pyruvate (5 mM), glutamate (10 mM), and malate (2 mM) into each chamber to produce NADH and stimulate non-phosphorylating (LEAK) respiration supported by NADH oxidized through Complex I (L_{N}). ADP (5 mM) was then added to stimulate phosphorylating respiration through Complex I (P_{N}). The addition of succinate (10 mM) resulted in phosphorylating respiration through the combination of Complex I and Complex II (P_{NS}). Finally, rotenone (0.5 µM) was added to block Complex I, with the resulting oxygen flux representing the capacity of Complex II to support mitochondrial oxygen consumption through the oxidation of succinate alone (P_{S}).

2.3. Data reduction and analysis

For all protocols, LEAK respiration was expressed as both *Rox*-corrected oxygen flux and as the percentage of the corresponding *Rox*-corrected phosphorylating respiration ($FCR_L = L_{\mathrm{N}}/P_{\mathrm{N}} \times 100$). The flux control ratio for NADH-supported and succinate-supported respiration (FCR_{N} and FCR_{S}, respectively) was calculated as the proportion of corrected maximal respiration supported by Complex I ($P_{\mathrm{N}}/P_{\mathrm{NS}} \times 100$) and Complex II ($P_{\mathrm{S}}/P_{\mathrm{NS}} \times 100$), respectively, and oxidative phosphorylation efficiency was calculated as $1\text{-}L_{\mathrm{N}}/P_{\mathrm{NS}}$. Results for each parameter of interest were calculated for individual respirometer chambers, then duplicates were averaged by subject for each experimental condition. In Study #1, data were analyzed using paired student's t-test. In Study #2, data were analyzed using repeated measures one-way ANOVA (Prism 9.5.1, GraphPad Software, San Diego, CA). If the overall ANOVA yielded $p<0.05$, then post-hoc pairwise comparisons were performed using Fisher's Least Squares Difference to compare the effects of the different fluorophores to the control. Finally, the AmR and MgG data from both studies were analyzed together for effects of study and fluorophore using 2 way repeated measures ANOVA, with subjects within a single study as the repeating factor.

3. Results

Table 1. Comparison of respirometry variables obtained with either Magnesium Green or Amplex UltraRed. Data from Study #1, N=7 subjects, 27 biopsies (one horse was biopsied 3 times, all other horses biopsied 4 times) *p<0.05, ***p<0.001 using paired student's t-test.

	MgG	AmR
L_N	2.78±0.96	3.25±1.13***
P_N	59.99±20.40	75.03±32.02***
P_{NS}	89.27±29.54	113.70±40.12***
P_S	49.82±15.70	52.47±17.24
FCR_L	4.71±0.66	4.69±1.64
FCR_N	66.88±3.86	64.87±9.70
FCR_S	64.87±9.70	46.62±4.53***
Efficiency	0.969±0.004	0.971±0.006*

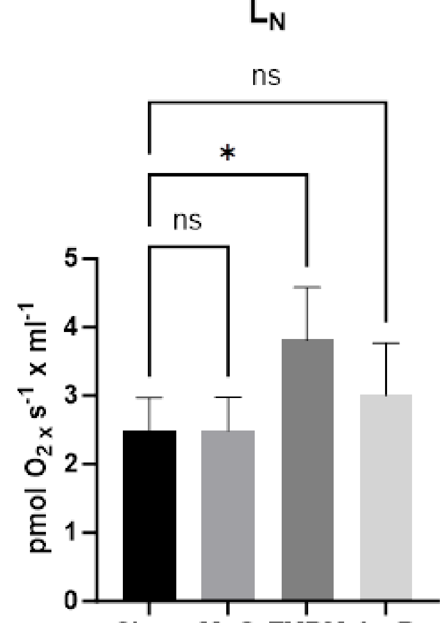


Figure 1. Effect of fluorophores on LEAK respiration. TMRM increased LEAK respiration by approximately 50 %. N=6 subjects, *=significantly different from control assay, p<0.05 on one-way repeated measures ANOVA and post-hoc Fisher's Least Squares Differences.

In Study #1, Amplex UltraRed was associated with higher L_N, P_N, and P_{NS} values, a lower FCR_S value, and a slightly higher value for calculated Efficiency (Table 1). In Study #2, there was no effect of MgG on any measured or calculated respirometry value. TMRM increased L_N by 52 % (Figure 1) and decreased $P_{(N+S)}$ by 32 % (Figure 2), the latter due to inhibition of both P_N and P_S (Figures 3 and 4). P_N was inhibited to a much greater degree than P_S (41 % and 11 %, respectively), resulting in a decrease in FCR_N but an increase in FCR_S (Table 2) despite decreased flux through CII. In addition, there was a modest decrease in OXPHOS efficiency. FCR_L tripled in the presence of TMRM due to the combination of increased L_N and decreased phosphorylating respiration through Complex I. AmR caused a 12 % increase in P_{NS} (Figure 2) despite causing a 13 % decrease in CII-based respiration (Figure 4) and corresponding decrease in FCR_S, and no significant change in CI-based respiration.

Table 2. Effect of fluorophores on calculated respirometry indices. Data from Study #2, N=6 subjects, *=significantly different from control assay, p<0.05 on one-way repeated measures ANOVA and post-hoc Fisher's Least Squares Differences. See Materials and Methods for term definitions.

	Clean	MgG	TMRM	AmR
FCR_L	5.312±0.439	5.365±0.618	15.630±2.522*	6.280±1.656
FCR_N	64.67±1.84	65.37±0.98	55.30±1.60*	61.78±1.69
FCR_S	61.31±1.87	60.33±1.37	83.66±4.65*	47.28±0.64*
Efficiency	0.9654±0.0035	0.9648±0.0043	0.9150±0.0141*	0.9611±0.0104

The combined analysis of the Study #1 and Study #2 data found an effect of fluorophore on L_N, P_N, P_{NS}, and FCR_S (p=0.0015, p=0.0415, p=0.0002, and p<0.0001, respectively), and a significant interaction between study and fluorophore on P_S (p=0.0432). Furthermore, there were trends (p<0.1) towards an independent effect of Study on P_{NS}, P_S, FCR_L, FCR_S, and Efficiency.

4. Discussion

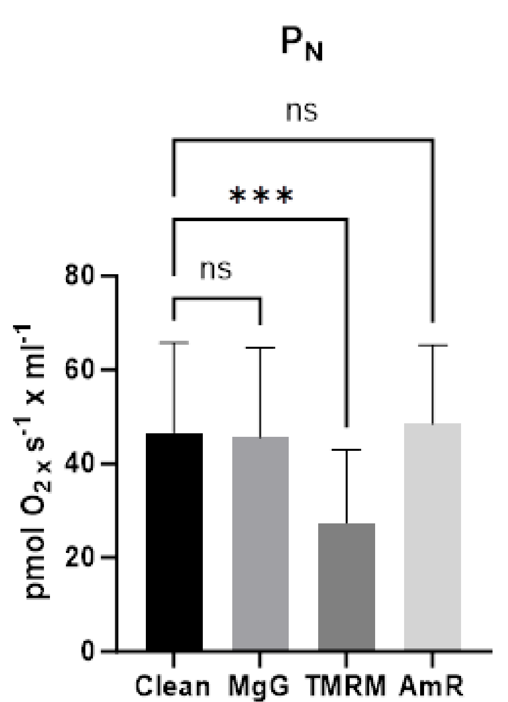


Figure 2. Effect of fluorophores on maximum phosphorylating respiration. *N*=6 subjects, ***=significantly different from control assay, *p*<0.001 on one-way repeated measures ANOVA and post-hoc Fisher's Least Squares Differences.

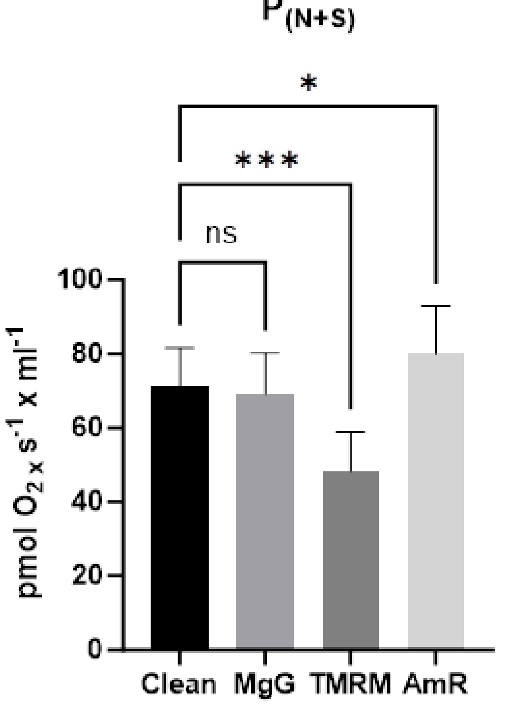


Figure 3. Effect of fluorophores on phosphorylating respiration through Complex I. *N*=6 subjects, *=significantly different from control assay, *p*<0.05, ***=significantly different from control assay, *p*<0.001 on one-way repeated measures ANOVA and post-hoc Fisher's Least Squares Differences.

An ideal *ex vivo* assay accurately replicates the corresponding in vivo phenomenon. In that regard, the use of Magnesium Green (MgG) is a useful fluorophore for studies of mitochondrial physiology in that its presence in the assay media did not have any detected effects on respiration of equine skeletal muscle mitochondria compared to the fluorophore-free control. This finding is consistent with what has been reported by Cardoso et al for mitochondria isolated from mouse cardiac muscle (Cardoso et al 2021). It is important to note that the assay using MgG to quantify the rate of ATP synthesis (Chinopoulos et al 2014) employs a lower concentration of magnesium in the incubation media than is typically recommended. Although this is 1/3rd the concentration typically used for high-resolution respirometry, previous studies have demonstrated that the addition of 1 M total Mg^{2+} results in concentrations of free Mg^{2+} that is comparable to that found in living cells.

TMRM inhibited phosphorylating respiration through both Complex I and Complex II, although not equally: respiration through Complex I was affected to a greater extent than respiration through Complex II. This is similar to the pattern reported for safranin, which like TMRM is used to measure mitochondrial membrane potential (Krumschnabel et al 2014). In the case of safranin, the inhibition of phosphorylating respiration is believed to be due primarily to inhibition of phosphorylation and, to a lesser extent, inhibition of respiration because the addition of uncoupler results in partial recovery of respiratory capacity. However, our previous work has demonstrated that when incubating at 38 °C, the samples are sufficiently uncoupled to preclude control of phosphorylating respiration by the components of the phosphorylation system (Davis et al 2020). Thus, TMRM appears to have a greater inhibition of respiration than does safranin. Also in contrast to safranin, TMRM caused a 3-fold increase in LEAK respiration, comparable in magnitude to that caused by physiological hyperthermia (Davis et al 2020). The mechanism for TMRM-induced proton leak is unknown but is mechanistically consistent with the progressive uncoupling of phosphorylating respiration that has been previously reported (Sumbalova et al 2015). The fluorescent signal from TMRM displayed the predicted changes in relative membrane potential, at least at a qualitative level, but due to the fluorophore-associated changes in mitochondrial respiration, TMRM may not be the best tool for measurement of membrane potential. Inhibition of respiration by fluorophores is typically dose-dependent; therefore, additional testing may be needed to minimize the concentrations used while maintaining sufficient fluorescent signal for quantification of mitochondrial membrane potential.

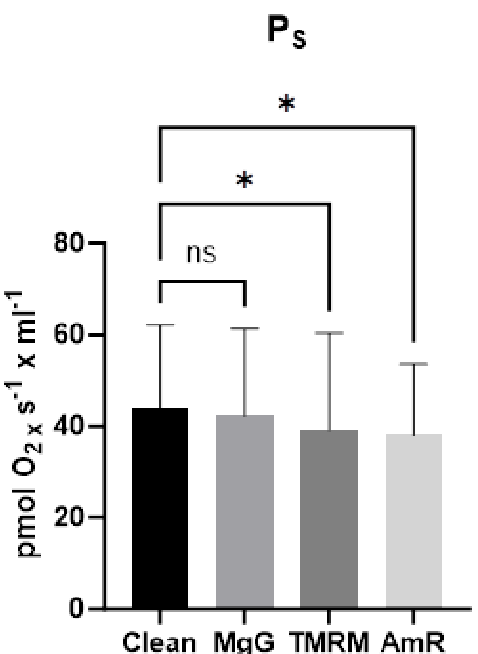


Figure 4. Effect of fluorophores on phosphorylating respiration through Complex II. *N*=6 subjects, *=significantly different from control assay, $p<0.05$ on one-way repeated measures ANOVA and post-hoc Fisher's Least Squares Differences.

The reagents that make up the Amplex UltraRed assay for mitochondrial ROS production were associated with an increase in respiration (both non-phosphorylating and phosphorylating) supported by NADH oxidation through Complex I in younger horses that were in an exercise conditioning program; however, the AmR assay was associated with inhibition of phosphorylating respiration through Complex II in older, unfit horses. The difference in fitness history is a logical explanation for the trends towards greater mitochondrial respiration in the subjects of Study #1, and may also be responsible for the difference in sensitivity to the AmR assay reagents. A 2015 study by Makrecka-Kuka et al reported that, at concentrations greater than 10 µM, Amplex Red had an inhibitory effect on respiration through a combination of Complex I and II in HEK 293T cells (Makrecka-Kuka et al 2015). The stimulatory effect reported here is a novel and unexpected finding. Differences in sample type and preparation, and the additional presence of superoxide

dismutase and horseradish peroxidase in this study compared to the previously published study, may be responsible for the different results. The specific mechanism for the Amplex UltraRed stimulation of respiration is unknown and requires further research, but the artifact created by this assay on mitochondrial respiration should be considered when assessing experimental results of similar or smaller magnitude.

Abbreviations

AmR	Amplex UltraRed	FCR_S	Complex II flux control ratio
BIOPS	Biopsy preservation media	MgG	Magnesium Green
FCR_L	LEAK flux control ratio	TMRM	Tetramethylrhodamine
FCR_N	Complex I flux control ratio	ROX	Residual oxygen consumption

Acknowledgements

Study was supported using funds from the Grayson Jockey Club Research Foundation (Grant) and the John and Debbie Oxley Endowed Chair in Equine Sports Medicine.

References

Cardoso LHD, Doerrier C, Gnaiger E (2021) Magnesium Green for fluorometric measurement of ATP production does not interfere with mitochondrial respiration. Bioenerg. https://doi.org/10.26124/bec:2021-0001

Chinopoulos C, Kiss G, Kawamata H, Starkov AA (2014) Measurement of ADP-ATP exchange in relation to mitochondrial transmembrane potential and oxygen consumption. https://doi.org/10.1016/B978-0-12-416618-9.00017-0

Davis MS, Barrett MR (2023) High-resolution fluoro-respirometry of equine skeletal muscle. https://doi.org/10.3791/65075

Davis MS, Fulton MR, Popken AA (2020) Effect of hyperthermia and acidosis on equine skeletal muscle mitochondrial oxygen consumption. https://doi.org/https://doi.org/10.3920/CEP200041

Doerrier C, Garcia-Souza LF, Krumschnabel G, Wohlfarter Y, Meszaros AT, Gnaiger E (2018) High-resolution fluorespirometry and OXPHOS protocols for human cells, permeabilized fibers from small biopsies of muscle, and isolated mitochondria. https://doi.org/10.1007/978-1-4939-7831-1_3

Krumschnabel G, Eigentler A, Fasching M, Gnaiger E (2014) Use of safranin for the assessment of mitochondrial membrane potential by high-resolution respirometry and fluorometry. https://doi.org/10.1016/B978-0-12-416618-9.00009-1

Makrecka-Kuka M, Krumschnabel G, Gnaiger E (2015) High-resolution respirometry for simultaneous measurement of oxygen and hydrogen peroxide fluxes in permeabilized cells, tissue homogenate and isolated mitochondria. https://doi.org/10.3390/biom5031319

Sumbalova Z, Gnaiger E (2015) High-resolution measurement of mitochondrial membrane potential and respiration – comparison of potentiometric and fluorometric methods. https://wiki.oroboros.at/index.php/Sumbalova_2015_Abstract_MiP2015

Copyright: © 2023 The authors. This is an Open Access preprint (not peer-reviewed) distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original authors and source are credited. © remains with the authors, who have granted MitoFit Preprints an Open Access publication license in perpetuity.