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TITLE:

Measuring Trans-Plasma Membrane Electron Transport by C2C12 Myotubes

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SHORT ABSTRACT:

The goal of this protocol is to spectrophotometrically monitor trans-plasma membrane electron transport utilizing extracellular electron acceptors and to analyze enzymatic interactions that may occur with these extracellular electron acceptors.

LONG ABSTRACT:

Trans-plasma membrane electron transport (tPMET) plays a role in protection of cells from intracellular reductive stress as well as protection from damage by extracellular oxidants. This process of transporting electrons from intracellular reductants to extracellular oxidants is not well defined. Here we present spectrophotometric assays by C2C12 myotubes to monitor tPMET utilizing the extracellular electron acceptors: water-soluble tetrazolium salt-1 (WST-1) and 2,6-dichlorophenolindophenol (DPIP or DCIP). Through reduction of these electron acceptors, we are able to monitor this process in a real-time analysis. With the addition of enzymes such as ascorbate oxidase (AO) and superoxide dismutase (SOD) to the assays, we can determine which portion of tPMET is due to ascorbate export or superoxide production, respectively. While WST-1 was shown to produce stable results with low background, DPIP was able to be re-oxidized after the addition of AO and SOD, which was demonstrated with spectrophotometric analysis. This method demonstrates a real-time, multi-well, quick spectrophotometric assay with advantages over other methods used to monitor tPMET, such as ferricyanide (FeCN) and ferricytochrome c reduction.

INTRODUCTION:

The ability of purified plasma membranes to reduce electron acceptors has led to the view that the plasma membrane has an inherent redox capacity¹. Previously seen in fungi, plants, and

animals, tPMET is a process common to multiple organisms²⁻⁵. Specifically, this process has been demonstrated in *Saccharomyces cerevisiae*, carrot cells, erythrocytes, lymphocytes, osteosarcoma, melanoma, macrophages, skeletal muscle, and neutrophils²⁻⁷. In a process that transports electrons across the plasma membrane to reduce extracellular oxidants, tPMET is involved in many cellular functions including: cell growth^{5,8}, cell viability⁹, iron metabolism¹⁰, cell signaling¹¹⁻¹³, and protection from reactive oxygen species^{12,14,15}. Due to tPMET's involvement in many cellular functions, an imbalance of tPMET has been hypothesized to contribute to the development of some serious health conditions, including cancer¹⁶, cardiovascular disease¹⁷, and metabolic syndrome¹⁸.

There are multiple ways to monitor the transfer of electrons across the plasma membrane, but the most widely used technique is to assess the reduction of extracellular electron acceptors through colorimetric assays. Commonly used extracellular electron acceptors are tetrazolium salts, DPIP, FeCN, and ferricytochrome c^{19,20}. The most commonly used tetrazolium salt is a second-generation salt known as WST-1¹⁹. This compound is easier to utilize in colorimetric assays compared to first generation tetrazolium salts due to two sulfonate groups, which increase its water solubility²¹. WST-1, in conjunction with the intermediate electron acceptor 1-methoxyphenazine methosulfate (mPMS), is reduced in two single-electron transfer events. This reduction changes the weakly-colored oxidized form of WST-1 to a more intense, yellow formazan^{20,22}. WST-1 has a high molar extinction coefficient of $37 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$, leading to a high assay sensitivity^{21,22}. DPIP is also utilized as an extracellular electron acceptor to monitor tPMET. It has been shown that DPIP can be reduced extracellularly by tPMET without the aid of intermediate electron acceptors^{23,24}. Due to the lack of intermediate electron acceptors, DPIP can directly pick-up electrons from the plasma membrane, unlike WST-1²⁴. Similar to DPIP, FeCN has been shown to be reduced extracellularly to ferrocyanide by tPMET without the aid of intermediate electron acceptors^{19,24}. Unlike WST-1 and DPIP, FeCN has a low molar extinction coefficient leading to a lower assay sensitivity⁹. Another commonly used extracellular electron acceptor to monitor tPMET is ferricytochrome c. Similar to WST-1, ferricytochrome c reduction increases with the use of intermediate electron acceptor, mPMS²². Unlike WST-1 though, the ferricytochrome c method is less sensitive due to a high background and a low molar extinction coefficient²².

Here we present a method for real-time analysis of tPMET through spectrophotometric assays. The method utilized the extracellular electron acceptors WST-1 and DPIP, as they both have a high molar extinction coefficient while being less expensive compared to the other commonly used extracellular electron acceptors such as ferricytochrome c. We utilized phenazine methosulfate (PMS) instead of mPMS they have a similar chemical makeup and PMS is far less expensive. mPMS is photochemically stable which is an important characteristic for a commercial kit that needs a long shelf life. However, we make PMS fresh for each assay, so stability should not be an issue. We also present a method to evaluate possible enzymatic interactions (see **Figure 1**) between the extracellular electron acceptor and enzymes that may be utilized to further characterize the process of tPMET. Specifically, the enzymes AO and SOD can be used determine which portion of tPMET is due to ascorbate transport or extracellular superoxide release, two common methods for electrons to be transported across the plasma membrane.

PROTOCOL:

Note: See **Figure 1**.

1. WST-1 Reduction Assay

1.1. Grow and differentiate C2C12 adherent cells using standard cell culture procedures⁷ in a 96-well plate utilizing rows A–F.

1.1.1. Use a differentiation medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% horse serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Incubate the cells at 37 °C with 5% CO₂.

1.1.2. When monitoring ascorbate involvement in tPMET, supplement differentiation media with 100 µM ascorbic acid. Allow cells to incubate in differentiation media for ~24–48 h.

1.2. Prepare stock WST-1 and PMS solutions.

1.2.1. To make a 10 mM stock of WST-1, dissolve 0.033 g of WST-1 (Formula Weight (FW): 651.34 g/mol) in 5 mL of phosphate buffered saline (PBS). Store at 4 °C.

1.2.2. To make a 5 mM stock of PMS, dissolve 0.0023 g of PMS (FW: 306.34 g/mol) in 1.5 mL of deionized H₂O (diH₂O). Store at -20 °C and protect from light.

1.3. Add 0.0108 g of glucose for a final concentration of 5 mM to 11.4 mL of PBS or HEPES buffered saline (HBS; 20 mM HEPES sodium salt, 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO₄, 1 mM CaCl₂). Add 480 µL of 10 mM WST-1 for a final concentration of 400 µM and 48 µL of 5 mM PMS for a final concentration of 20 µM.

1.4. When monitoring ascorbate involvement in tPMET, divide the solution into two 6 mL aliquots. To one aliquot, add 6 µL of diH₂O and to the other aliquot add 6 µL of 2 kU/mL AO for a final concentration of 2 U/mL.

1.5. When monitoring superoxide involvement in tPMET, divide the solution into two 6 mL aliquots. To one aliquot, add 55 µL of 0.1 M KPO₄ buffer and to the other aliquot add 55 µL of 6.5 kU/mL SOD for a final concentration of 60 U/mL.

1.6. Wash the cells with PBS. Aspirate the media and add 150 µL PBS. Then aspirate the PBS.

Note: The assay is done with plated, attached cells. Thus, there is no centrifugation or use of trypsin in the washing process.

1.7. Add 100 μ L of WST-1 solution (-) SOD or (-) AO to columns 1–6 and add 100 μ L of WST-1 solution (+) SOD or (+) AO to columns 7–12 in the 96-well plate. Use rows G and H as background controls (*i.e.*, to monitor change in absorbance in the reagent alone in wells without cells).

1.8. Measure the absorbance values using the spectrophotometer every 10 min for 1 h at 438 nm.

1.9. After reading, aspirate the media and wash each well with 150 μ L of PBS. Then aspirate the PBS.

1.10. Calculate the change in absorbance for each well by subtracting the initial absorbance for a well from the absorbance at any time point for that well. Correct this change for the change in absorbance (if any) observed in the background wells (*i.e.*, wells with assay solution but no cells).

1.11. For analysis, normalize the absorbance data to the 60 min control measurement or wash wells with PBS or HBS and then perform a bicinchoninic acid (BCA) protein assay.

1.12. Add 2 mg/mL bovine serum albumin (BSA) standard (range from 0.5–2 μ L for the standard curve, depending on the degree of confluence of the cells) to the empty wells (rows G and H), then add the BCA reagent to all wells.

1.13. Quantify the data via nmol of WST-1 reduction per μ g of protein. Use $37 \text{ mM}^{-1}\text{cm}^{-1}$ ²² as the extinction coefficient for reduced WST-1 at 438 nm.

2. DPIP Reduction Assay

2.1. Grow and differentiate C2C12 adherent cells with the same procedure as step 1.1.

2.2. Prepare stock 10 mM DPIP solution as follows. Dissolve 0.029 g of DPIP (FW: 290.08 g/mol) in 10 mL of diH₂O. Confirm the concentration of DPIP by measuring absorbance at 600 nm with a spectrophotometer. Use $1 \text{ mM}^{-1}\text{cm}^{-1}$ ²³ as the extinction coefficient for reduced DPIP at 600 nm. Store at 4 °C.

2.3. Add 0.0108 g of glucose to 11.880 mL of PBS for a final concentration of 5 mM. Add 120 μ L of 10 mM DPIP for a final concentration of 100 μ M.

2.4. When monitoring ascorbate involvement in tPMET, divide the solution into two 6 mL aliquots. To one aliquot, add 6 μ L of diH₂O and to the other aliquot add 6 μ L of 2 kU/mL AO for a final concentration of 2 U/mL.

2.5. When monitoring superoxide involvement in tPMET, divide the solution into two 6 mL aliquots. To one aliquot, add 55 μ L of 0.1 M KPO₄ buffer and to the other aliquot add 55 μ L of 6.5 kU/mL SOD for a final concentration of 60 U/mL.

2.6. Aspirate the media and wash cells in 150 μ L of PBS. Aspirate the PBS and add 100 μ L of DPIP solution (-) SOD or (-) AO to columns 1–6 and add 100 μ L of DPIP solution (+) SOD or (+) AO to columns 7–12 in the 96-well plate. Use rows G and H will as background controls (*i.e.*, to monitor change in absorbance in the reagent alone in wells without cells).

2.7. Measure the absorbance at 600 nm using spectrophotometer every 10 min for 1 h. Quantify the change in absorbance relative to the control at 60 min similar to steps 1.9–1.13.

3. Determination of Whether Reduced Electron Acceptors Are Substrates for AO or SOD

3.1. To 5.436 mL of PBS or HBS, add 240 μ L of 10 mM WST-1 for a final concentration of 400 μ M and 24 μ L of 5 mM PMS for a final concentration of 20 μ M.

3.1.1. For DPIP, add 60 μ L of 10 mM DPIP, for a final concentration of 100 μ M, to 5.940 mL of PBS or HBS.

3.2. Add 100 μ L of solution to each well in a flat-bottom 96-well plate in the absence of cells and measure the absorbance in a spectrophotometer at 438 nm, for WST-1, or 600 nm, for DPIP.

3.3. Add 1 μ L of 10 mM ascorbate to half of the wells for a final concentration of 100 μ M. Monitor the absorbance until it stabilizes.

3.4. Upon stabilization, add 1 μ L of 200 U/mL AO for a final concentration of 2 U/mL to each well or add 1 μ L of 6 kU/mL SOD for a final concentration of 60 U/mL to each well and monitor the absorbance for 1 h.

REPRESENTATIVE RESULTS:

Statistics were performed with ANOVA with repeated measures using RStudio statistical software²⁵. Sample sizes are indicated in the figure legends.

To monitor tPMET, C2C12 myotubes were utilized along with extracellular electron acceptors, WST-1 and DPIP. AO was used to determine which portion of WST-1 and DPIP reduction was due to ascorbate efflux and SOD was used to determine which portion of WST-1 reduction was due to extracellular superoxide release. As shown in **Figure 2**, C2C12 myotubes are capable of tPMET as seen by the reduction of WST-1. With the addition of AO, WST-1 reduction was suppressed by ~30% indicating that ~30% of tPMET was due to the export of ascorbate (**Figure 2A**). With the addition of SOD, WST-1 reduction was suppressed by ~70%, indicating that ~70% of tPMET was due extracellular superoxide release (**Figure 2B**). When DPIP was utilized as an extracellular electron acceptor with the addition of AO, DPIP reduction was suppressed over 100% (**Figure 3**). This raised questions regarding the validity of using DPIP with AO to assess contribution of ascorbate.

To look into whether reduced WST-1 is a substrate for AO or SOD, WST-1 was reduced with ascorbic acid. AO or SOD was added, and the absorbance was monitored. After 1 h, there was no

change in absorbance between the reduced form of WST-1 without AO or SOD and the reduced form of WST-1 with AO or SOD (**Figure 4A, B**). Therefore, the reduced WST-1 is not a substrate for these enzymes, and the use of AO or SOD in conjunction with WST-1 is appropriate for assessment of roles of ascorbate or superoxide, respectively.

To determine if reduced DPIP is a substrate for AO or SOD, DPIP was reduced with ascorbic acid. After 20 min, AO was added, and the absorbance was monitored. Reduced DPIP was shown to be a substrate for AO, as seen in **Figure 5A**, where DPIP returns to its oxidized form within 40 min. When SOD was added, DPIP was also re-oxidized (**Figure 5B**). Therefore, reduced DPIP is a substrate for these enzymes and not an appropriate extracellular electron acceptor to be utilized with these enzymes.

FIGURE AND TABLE LEGENDS:

Figure 1: A schematic representation of the key steps of the electron transport assays and determination of enzymatic interactions with the extracellular electron acceptors.

Figure 2: The addition of AO and SOD suppresses WST-1 reduction by C2C12 myotubes. (A) tPMET was analyzed for 60 min using 400 μ M WST-1 and 20 μ M PMS with the addition of 2 U/mL AO or diH₂O (N = 18/group). (B) tPMET was analyzed for 60 min with the addition of 60 U/mL SOD or 0.1 M KPO₄ buffer (N = 36/group). * $p \geq 0.05$ at the indicated time point. Error bars represent standard error of the mean and may be too small to visualize.

Figure 3: The addition of AO suppresses DPIP reduction by C2C12 myotubes. tPMET was analyzed for 60 min using 100 μ M DPIP with or without the addition of 2 U/mL AO (N = 36/group). * $p \geq 0.05$ at the indicated time point. Error bars represent standard error of the mean and may be too small to visualize.

Figure 4: Reduced WST-1 is not a substrate for AO or SOD. (A) WST-1 was reduced with ascorbate. After 45 min, AO was added, and the absorbance was monitored for 1 h. No change in absorbance was observed. (B) The procedure is the same as above, except SOD was added after 45 min. No change in absorbance was observed (N = 24/group). Error bars represent standard error of the mean and may be too small to visualize.

Figure 5: Reduced DPIP is a substrate for AO and SOD. (A) DPIP was reduced with ascorbate. After 20 min, AO was added, and the absorbance was monitored. Within 40 min, the samples returned to their original absorbance, indicating that reduced DPIP is a substrate for AO. (B) DPIP was reduced with ascorbate. After 20 min, SOD was added, and the absorbance was monitored. Over time, the reduced DPIP was re-oxidized, indicating that reduced DPIP is a substrate for SOD (N = 24/group). Error bars represent standard error of the mean and may be too small to visualize.

Figure 6: Schematic depicting tPMET. In this model of tPMET, ascorbate (or another electron carrier) exported by the cell can reduce extracellular PMS. In addition, NADPH oxidases generate extracellular superoxide, which can reduce WST-1 directly or reduce it indirectly via reduction of

PMS. Electrons from other plasma membrane donors can also reduce PMS, which can donate electrons to either WST-1 or O₂ with subsequent WST-1 reduction.

DISCUSSION:

We have presented two methods for utilizing extracellular electron acceptors, WST-1 and DPIP, in spectrophotometric assays to monitor tPMET in C2C12 myotubes. With the growth of cell lines in standard culture procedures and a spectrophotometer plate reader, it is possible to monitor tPMET with these electron acceptors in a simple microplate assay. WST-1 reduction is reproducible from well-to-well within an assay, but there is day-to-day variability. The day-to-day coefficient of variation (CV) utilizing PBS as the buffer is 0.18 and the CV utilizing HBS as the buffer is 0.23.

From previous literature utilizing mPMS and WST-1, we have modified this protocol, specifically, for the use of PMS. Thus, troubleshooting included determining the appropriate concentrations of PMS and WST-1 for use with the myotubes described. We also determined that temperature (in a range of 23–37 °C) is not a critical component for the assay, while shaking the plate prior to each absorbance reading is important. However, these issues should be investigated for other cell lines to which the assay is applied. The findings regarding the ability of SOD and AO to directly oxidize reduced WST-1 suggest that screening of new assay components for this property is a key aspect of troubleshooting.

Our data suggests that PMS should be utilized in conjunction with WST-1 in order to elicit optimal WST-1 reduction. We found that in a 1 mL volume, 100 µM ascorbate reduces 322 nmol of WST-1 per minute in the presence of PMS, which is required for direct reduction of WST-1 by ascorbate. In a 1 mL volume, 2 U/mL of AO oxidizes ascorbate at a rate of 4,400 nmol/s, or 800 times faster than the reduction of WST-1 by ascorbate. Clearly, enzymatic activity of AO is much faster than direct reduction of PMS/WST-1 by ascorbate. We expect that the same would follow for superoxide and SOD, though we do not have a system for measuring the absolute rate of WST-1 reduction by superoxide.

To determine whether inclusion of PMS affects the reduction of WST-1 by superoxide, we used a xanthine oxidase (4.5 mU/mL)/hypoxanthine (0.1 mM) superoxide generating system as previously described^{26,27}. Inclusion of PMS approximately doubles the rate of WST-1 reduction by this system. Thus, it appears that PMS mediates transfer of electrons from superoxide to WST-1.

To further evaluate the requirement of PMS for cellular WST-1 reduction, we assayed reduction in the presence or absence of PMS and WST-1, and found that PMS is required for cellular WST-1 reduction. When cells are incubated in assay reagent containing PMS alone (in the absence of WST-1), there is an increase in absorbance at 438 nm. This likely reflects an accumulation of the semiquinone form of PMS (PMS-SQ) that has a peak absorbance at 440 nm²⁸. Accumulation of PMS-SQ suggests that PMS is being reduced faster than it can pass electrons to O₂ to produce superoxide. Thus, the accumulation of PMS-SQ is consistent with the reported slow reduction of O₂ by PMS-SQ compared to the rate for fully-reduced PMS²⁸.

We have also presented a protocol to determine if the electron acceptors are substrates for the enzymes that may be utilized to monitor tPMET. By monitoring the reduction of the extracellular electron acceptors in a spectrophotometer followed by the addition of the enzyme of interest, it is possible to determine if the electron acceptor is a substrate for these enzymes. Through this method, we have shown that reduced DPIP is a substrate for AO and SOD indicating that it is not a suitable electron acceptor for monitoring ascorbate efflux and superoxide export.

The procedure for testing whether or not assay components such as SOD or AO create artifacts is a significant improvement over existing methods. For example, reduced DPIP is a substrate for SOD, suggesting that published data obtained with use of DPIP and SOD should be interpreted accordingly. Our findings support previous research conducted by Dayan and Dawson in which the absorbance of leuco 2,6-dichloroindophenol was monitored after the addition of AO: DPIP was oxidized indicating that it is a substrate for AO²⁹. However, our findings add context to research conducted by Tan and Berridge²⁴, as well as Bellavite *et al.*³⁰, that utilized SOD to inhibit DPIP reduction. In a study conducted by Tan and Berridge comparing the electron acceptors of WST-1, DPIP, and FeCN in HeLa cells, they saw that the reduction of some of the electron acceptors was suppressed in the presence of SOD. The reduction of WST-1, in conjunction with mPMS, was inhibited ~80% in the presence of SOD while the reduction of DPIP was inhibited by 40%. The reduction of FeCN, in conjunction with an alternative intermediate electron acceptor, was not inhibited in the presence of SOD²⁴. When evaluating the mode of electron exchange between NADPH oxidases and DPIP or cytochrome c, Bellavite *et al.* found that DPIP and cytochrome c are reduced by NADPH oxidases and that this reduction is significantly inhibited by SOD³⁰. Berridge and Tan have also demonstrated that SOD can inhibit WST-1 reduction by 80% in mouse cell lines of 32Dcl23, WEHI3B, and J774 as well as human cell lines of Jurkat, MALME3M, and 143B^{6,31}. With human primary neutrophil cells, WST-1 reduction was inhibited 95% in the presence of SOD^{6,32}.

The data here suggest that it is important to verify that enzymes used to assess specific contributions of exported molecules to tPMET cannot re-oxidize the reduced form of the extracellular electron acceptor. We found that (data not shown) ferrous chloride and ferrous sulfate were not soluble in PBS. In HBS and phenol red-free DMEM, they were rapidly oxidized, demonstrating incompatibility with HBS or DMEM as assay media. In contrast, AO and SOD do not use potassium ferrocyanide as a substrate, suggesting that it is a suitable extracellular electron acceptor if its low extinction coefficient is not an issue.

One of the main limitations of this assay is that PMS/WST-1 and DPIP can be reduced by multiple electron donors, such as NAD(P)H, ascorbate, and flavonoids such as quercetin and myricetin^{19,33,34}. However, this can be overcome by the addition of enzymes (*e.g.*, SOD, AO) or inhibitors to determine specific contributors to PMS/WST-1 or DPIP reduction. Another limitation is that DPIP can act as a substrate for AO and SOD. Thus, these enzymes should not be utilized in conjunction with DPIP to monitor tPMET. An additional important limitation of this study is the ability of PMS and other redox cyclers to produce superoxide^{26,35,36}. This is illustrated in **Figure 6**. On the other hand, PMS itself reportedly has no direct effect on tPMET³². Additionally, Tan and Berridge³² have shown that the NADPH oxidase (NOX) inhibitor, diphenyleneiodinium chloride

(DPI), can suppress the majority of WST-1 reduction, suggesting that DPI-sensitive tPMET can be a specific measure of NOX contribution to tPMET. Another limitation is that we see small changes in absorbance when utilizing WST-1. We have found that when WST-1 is made fresh and cells are confluent, the greatest change in absorbance is seen. Previous studies conducted in P388 cell lines have shown that the amount of formazan produced positively correlates with cell number²¹. Therefore, the more confluent the cells are then the greater the reduction in WST-1. This can be corrected for by normalizing to microgram of protein for each well.

One caveat regarding this assay is that it is primarily designed to assess global tPMET. Although it can be manipulated to monitor multiple forms of tPMET, such as the example of ascorbate efflux, more specific assays might be more appropriate when the goal does not require measurements in the context of tPMET. For example, if superoxide is the main interest, there are a number of other avenues to monitor superoxide, such as use of probes including fluorescent impermeable probes, aconitase, and nitroblue tetrazolium³⁷.

Existing probes to monitor tPMET such as DPIP, FeCN and ferricytochrome *c*, present disadvantages. For example, reduced DPIP is a substrate for AO and SOD, FeCN has a low molar extinction coefficient that limits its utility in real-time assays, and cytochrome *c* is costly. Additionally, some of these probes can produce high background leading to low sensitivity when measuring a change in absorbance³². WST-1 has been shown to not be a substrate for enzymes such as AO and SOD. Additionally, WST-1 has a high molar extinction coefficient and a low background reaction, creating a more sensitive tPMET assay. Moving forward, this assay can be utilized with a wide range of inhibitors to better understand the process of tPMET, map the path of electron transport in a given cell line, and lead to better understanding of how a cell's redox environment is maintained.

Critical steps of this protocol include determining that the cells are ready for experimentation (~70% confluent) as well as making the assay reagents, specifically WST-1 and PMS, fresh for each assay. It is also critical to wash the cells before the addition of assay reagents. The DMEM utilized in this protocol does not contain ascorbate, although we supplement differentiation medium with ascorbate. A number of available media do contain ascorbate. However, the cells are washed before the addition of the assay reagents, which are ascorbate-free, to remove any residual ascorbate from the medium. As a portion of tPMET is attributable to molecular efflux (*e.g.*, ascorbate export), it is important to start spectrophotometric readings immediately after the addition of assay reagent so as not to miss the first moments of efflux. Also, it is critical to include background wells for each individual reagent in an experiment. The assay constituents described in this paper caused negligible background change in absorbance. However, some additives (*e.g.*, phloretin) can promote a substantial background reaction. Thus, it is important that the background is subtracted out when the data are analyzed to eliminate any confounding effects that the reagents themselves may produce.

In summary, the protocol presented here provides a means of assessing tPMET and suggests a number of caveats and aspects of the protocol that should be taken into account when applying

the assay to different cell lines and/or assessing specific contributors (ascorbate and superoxide used here) to tPMET.

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DISCLOSURES:

The authors have nothing to disclose.

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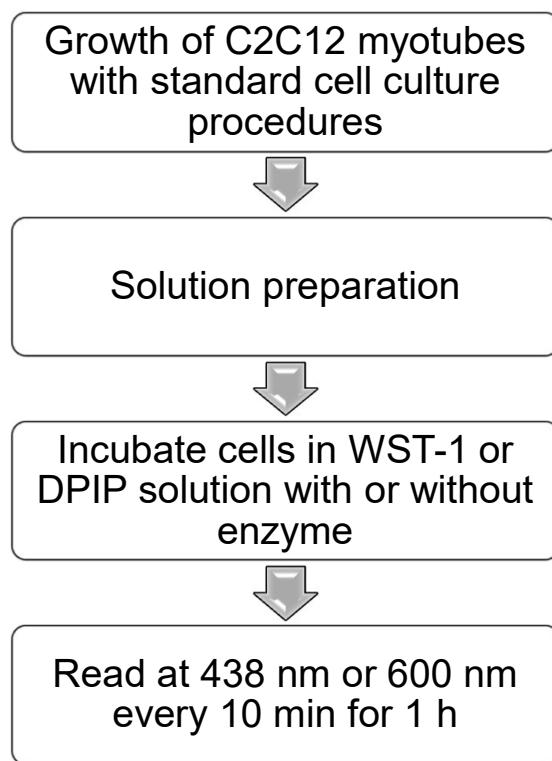
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509

Electron Transport Assay



Determine Enzymatic Interactions

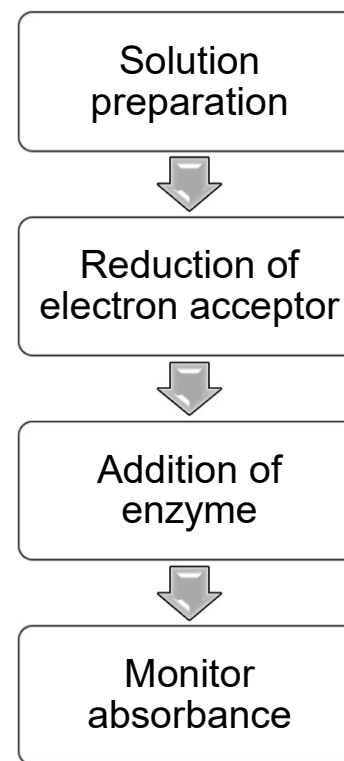


Figure 2

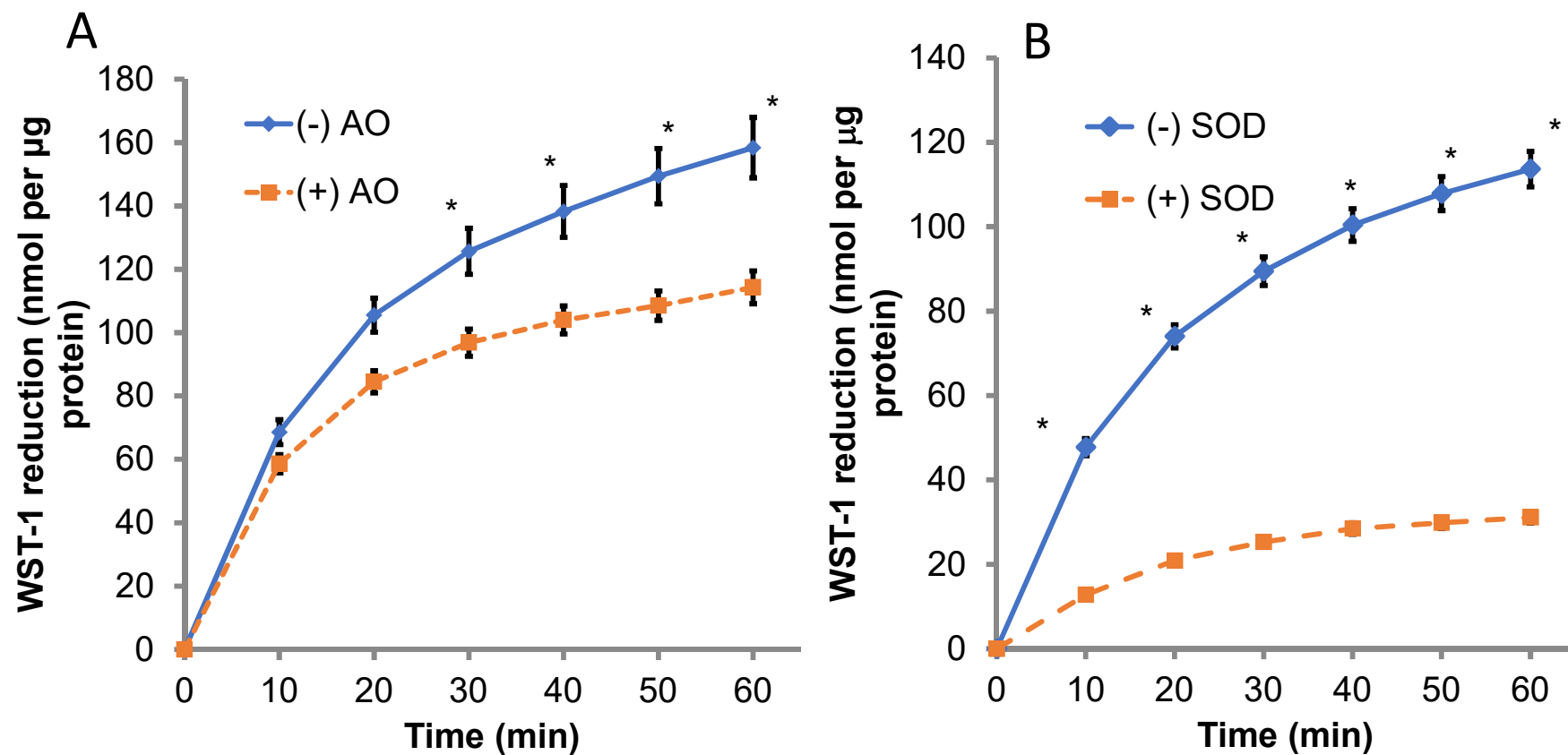


Figure 3

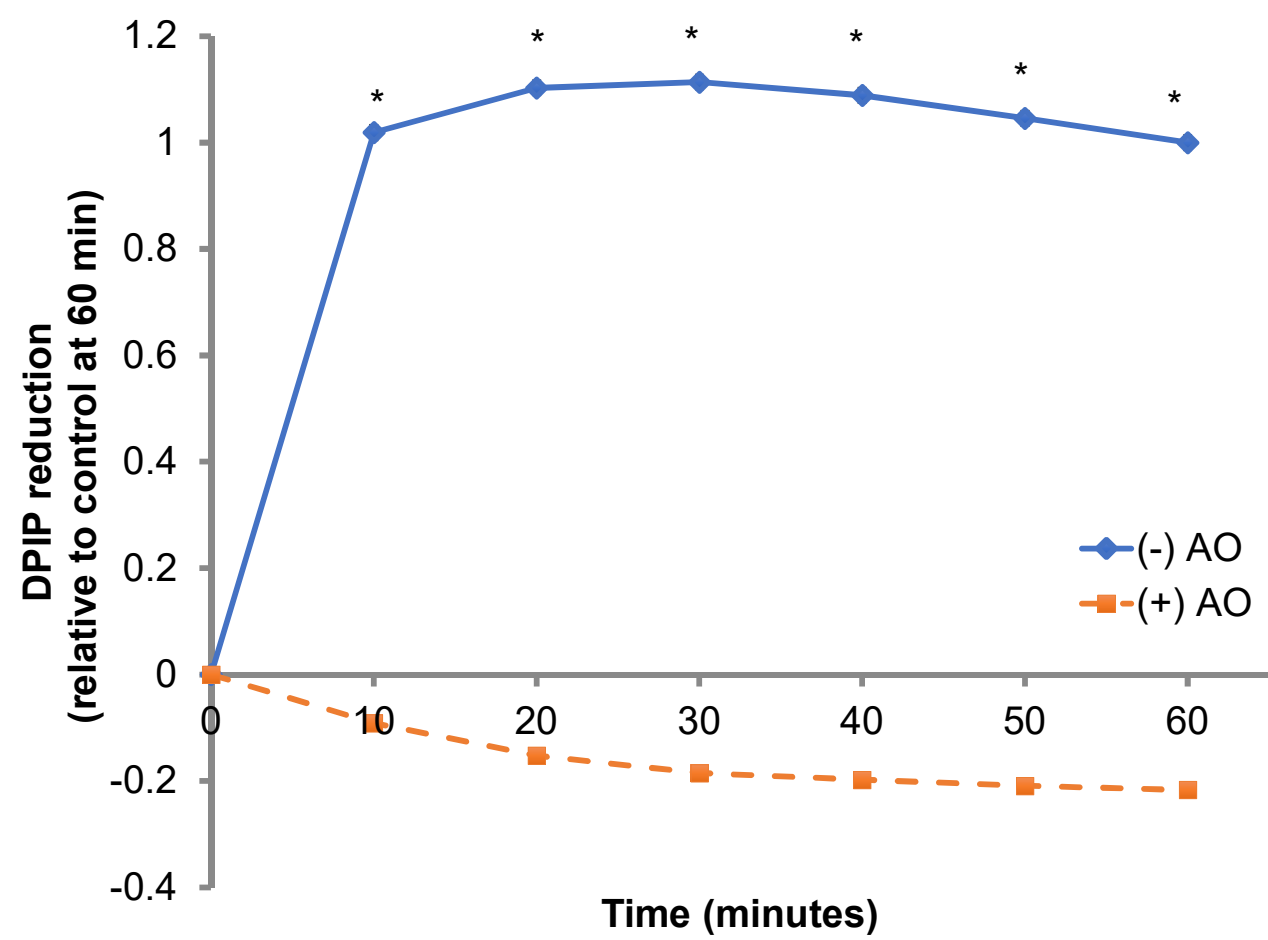


Figure 4

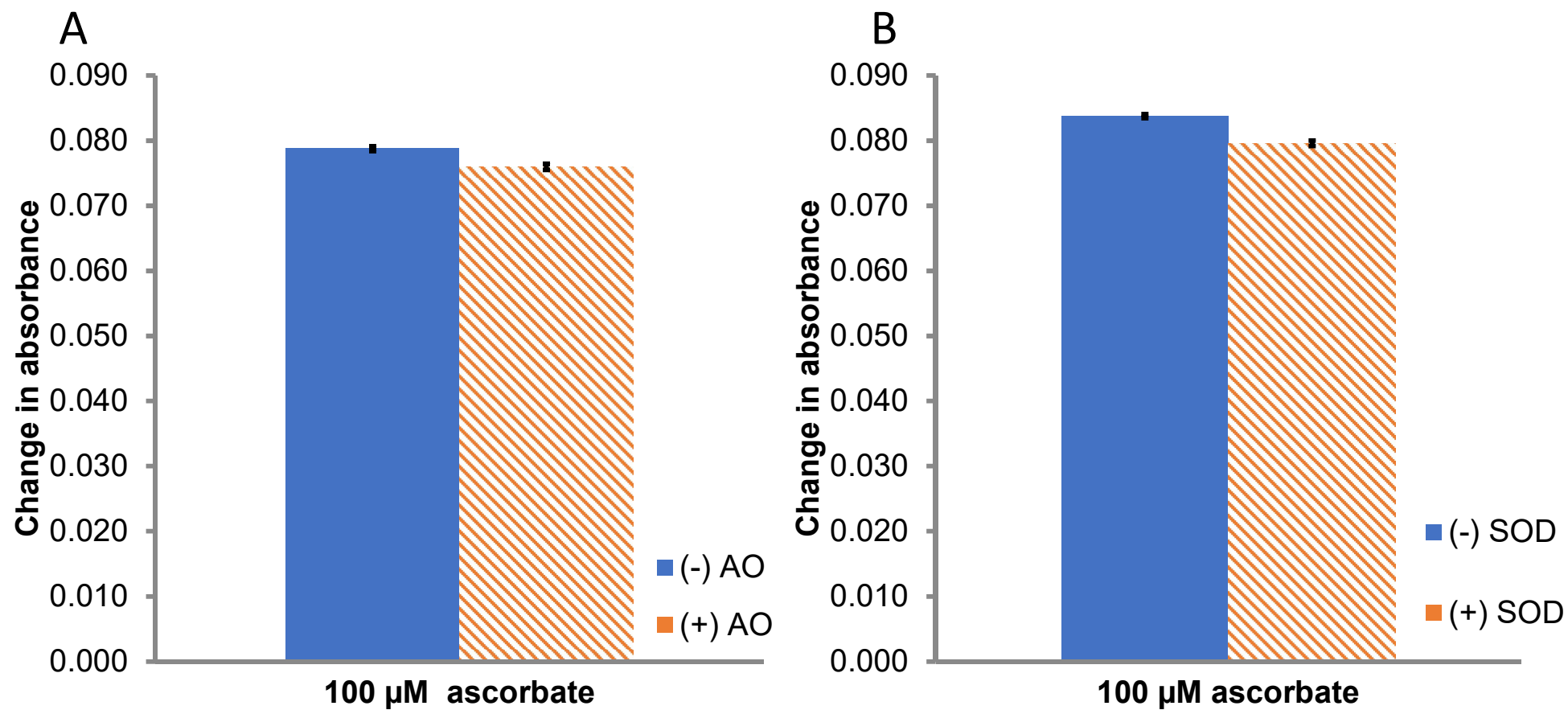
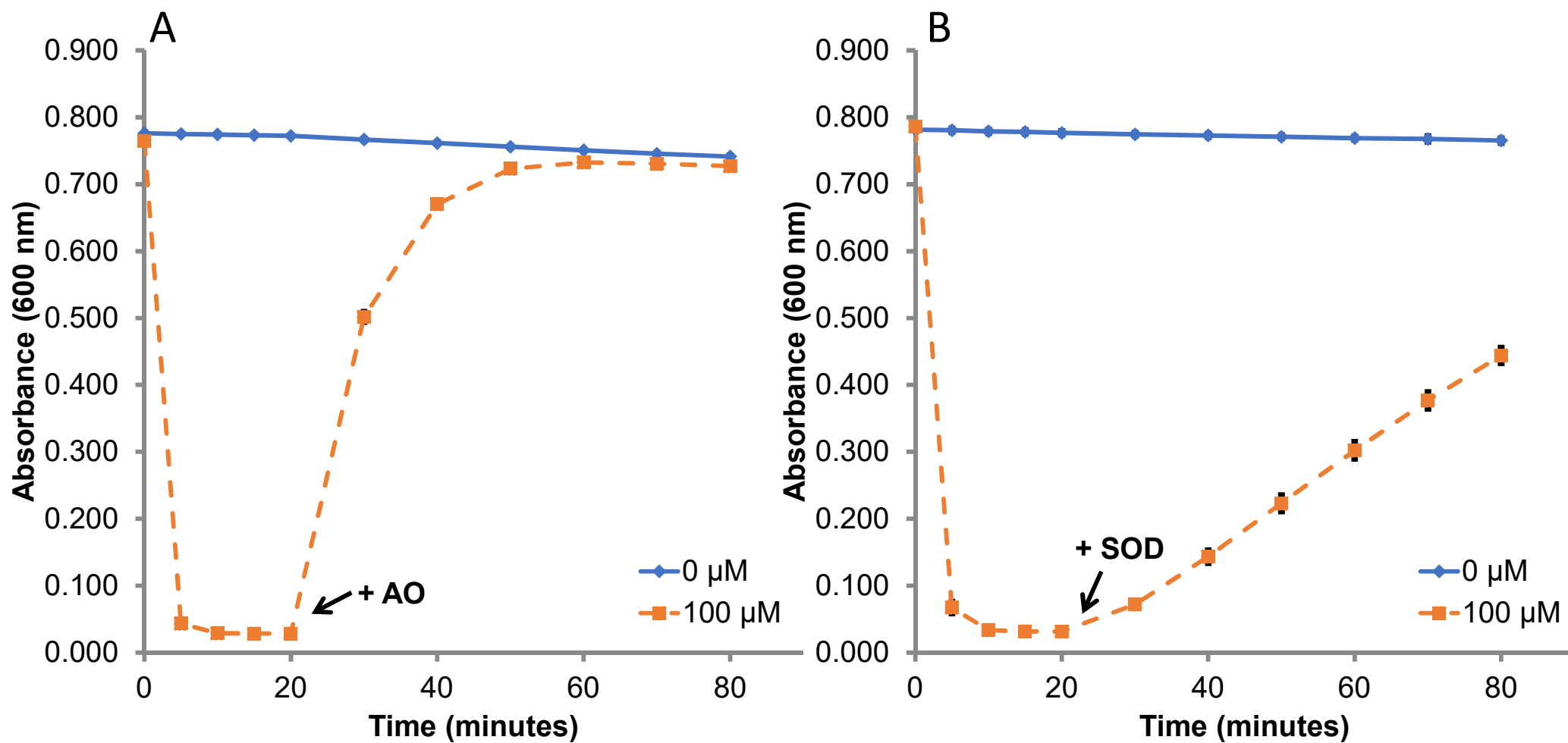
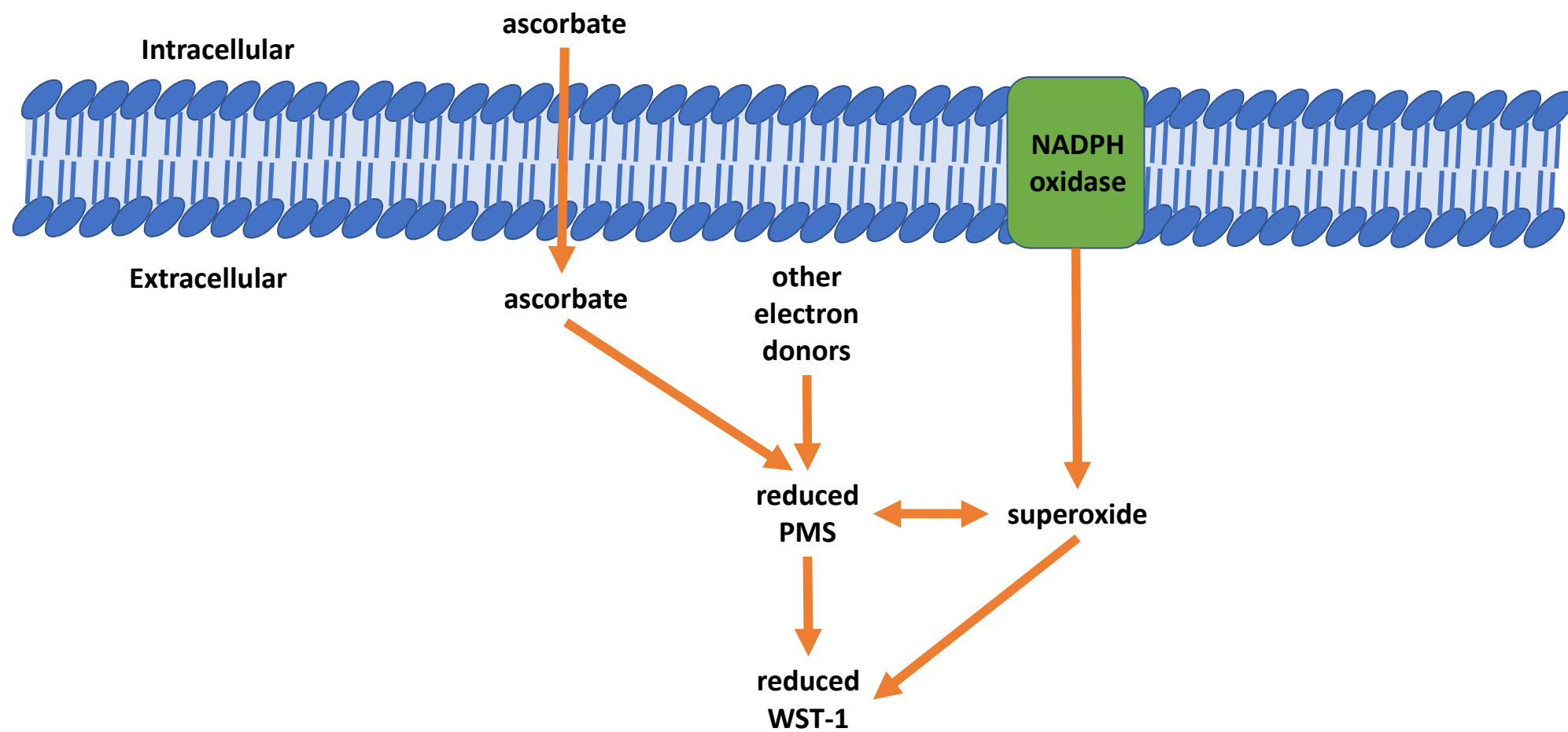


Figure 5





Name of Material/ Equipment	Company	Catalog Number
C2C12 myoblasts	American Type Culture Collection	CRL-1772
Dulbecco's modified eagle's medium - low glucose	Sigma	D6046
Fetal Plex animal serum complex	Gemini Bio-Products	100-602
penicillin-streptomycin	Sigma	516106
horse serum	Gibco Technologies	16050-130
Dulbecco's phosphate buffered saline	Sigma	D8537
trypsin-EDTA	Sigma	T4049
15 cm culture dishes	TPP	93150
96 well culture plates	TPP	92096
2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium Sodium Salt (WST-1)	Accela ChemBio Inc	SY016315
phenazine methosulfate	Sigma	P9625
L-ascorbic acid	Sigma	A5960
ascorbate oxidase	Sigma	A0157
superoxide dismutase	Sigma	S5395
2,6-dichloroindophenol sodium salt	ICN Biomedicals	215011825
D-(+)-glucose	Sigma	G7528
HEPES sodium salt	Sigma	H3784
sodium chloride	Sigma	S7653
potassium chloride	Fisher Scientific	BP366
magnesium sulfate heptahydrate	Sigma	M5921
calcium chloride dihydrate	Sigma	C7902
potassium phosphate	Fisher Scientific	BP363
Pierce BCA Protein Assay Kit	Thermo Scientific	23225
Powerwave X-I spectrophotometer	Biotek Instruments	discontinued
Spectronic Genesys 5 Spectrophotometer	Thermo Scientific	336001
PureGrade 96-well microplate, F-bottom, clear, untreated, non-sterile	MidSci	781602

Iron (II) chloride tetrahydrate	Sigma	220299
Iron (II) sulfate heptahydrate	Sigma	215422
hypoxanthine	Sigma	H9636
xanthine oxidase	Sigma	X4500
Excel	Microsoft	
R Studio	Rstudio	
KC4	Biotek Instruments	discontinued

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Author(s):

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Dear Dr. DSouza and Reviewers,

Thank you for constructive comments and insights. We greatly appreciate the feedback, and we believe that your suggestions have substantially improved the manuscript. Below is our line-by-line response to the Scientific Review Editor's and Reviewers' comments.

Thanks for your consideration,
and, Best regards,

Jonathan Fisher, Saint Louis University

Changes recommended by the JoVE Scientific Review Editor:

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have thoroughly proofread the manuscript for spelling and grammatical errors.

- **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more details to the following protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

1) 1.1: What is the composition of the differentiation medium? Mention incubation temperature and environmental conditions.

The differentiation medium consists of Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 100 units/ml penicillin with 0.1 mg/ml streptomycin. The cells are incubated at 37°C with 5% CO₂. These details have been added to the revised document.

2) 1.6: How are the cells washed? Do you centrifuge them? Do you need to trypsinize the cells? If so, please describe the amount of trypsin used and also mention how trypsin is neutralized.

The cells are washed with PBS. The media is aspirated, and PBS is added. The PBS is then aspirated. The assay is done with plated, attached cells. Thus, there is no trypsinization or centrifugation of cells before the assay. These issues have been clarified in the revised document.

3) 1.7: Measure absorbance signals?

"Read plate" has been replaced with "measure absorbance values."

4) 1.8: Again, how is washing done?

The procedure for washing cells by aspirating media, washing cells with PBS, then aspirating PBS has been clarified in the revised document.

5) 1.8: what is calculated? Unclear what the “data” represents.

"Data" represents the change in absorbance. The change is calculated by subtracting the initial absorbance for a well from the absorbance at any time point for that well. This change is then corrected for the change in absorbance (if any) observed in the background wells (i.e. wells with assay solution but no cells). This has been clarified in the text.

6) 2.1: “All procedures are as follows for the WST-1 assay above except” this statement is contradicting itself. Unclear which steps from section 1 are to be performed, please check and clarify.

We have clarified the DPIP reduction assay as follows:

2. DPIP Reduction Assay

2.1.-Grow and differentiate C2C12 adherent cells with the same procedure as 1.1

2.2. Prepare stock DPIP solution. To make a 10 mM stock of DPIP: dissolve 0.029 g of DPIP (F.W. 290.08 g/mol) in 10 mL of diH₂O. Confirm the concentration of DPIP with a spectrophotometer at 600 nm. The extinction coefficient for reduced DPIP at 600 nm is 21 mM⁻¹cm⁻¹²³. Store at 4°C.

2.3. Add 0.0108 g of glucose to 11.880 mL of PBS for a final concentration of 5 mM. Add 120 µL of 10 mM DPIP for a final concentration of 100 µM.

2.4. When monitoring ascorbate involvement in tPMET, divide solution into two 6 mL aliquots. To one aliquot, add 6 µL of diH₂O and to the other aliquot add 6 µL 2 kU/mL ascorbate oxidase (AO) for a final concentration of 2 U/mL.

2.5. When monitoring superoxide involvement in tPMET, divide solution into two 6 mL aliquots. To one aliquot, add 55 µL of 0.1 M KPO₄ buffer and to the other aliquot add 55 µL of 6.5 kU/mL superoxide dismutase (SOD) for a final concentration of 60 U/mL.

2.6. Aspirate media and wash cells in 150 µL of PBS. Aspirate PBS and add 100 µL of DPIP solution (-) SOD OR (-) AO to columns 1-6 and add 100 µL of DPIP solution (+) SOD OR (+) AO to columns 7-12 in 96 well plate. Rows G and H will be utilized as background controls (i.e. to monitor change in absorbance in the reagent alone in wells without cells).

2.7. Measure absorbance in spectrophotometer every 10 minutes for 1 hour at 600 nm. Quantify the change in absorbance relative to the control at 60 minutes similar to step 1.8.

7) 2.1.2: Unclear what the “data” represents. Is it “absorbance”?

"Data" has been clarified as the change in absorbance (as described for 1.8 above).

8) 3.2 : Is this the culture dish containing cells? Please reference the appropriate steps.
This assay plate does not contain cells. This is specified in the new document.

- **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

The protocol section is within the 10-page limit. The protocol steps have been highlighted.

- **Results:** Please mention the statistical tests performed and report sample sizes.
We used the statistical test of an ANOVA with repeated measures. Sample sizes are indicated in figure legends. This information is now provided in the Representative Results section.

- **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The following is now included in the discussion in the revised document.

1. Modifications and troubleshooting: From previous literature utilizing mPMS and WST-1, we have modified our protocol, specifically, for the use of PMS. Thus, troubleshooting included determining appropriate concentrations of PMS and WST-1 for use with the myotubes described. We also determined that temperature (in a range of 23-37° C) is not a critical component for the assay, while shaking the plate prior to each absorbance reading is important. However, these issues should be investigated for other cell lines to which the assay is applied. The findings regarding the ability of SOD and AO to directly oxidize reduced WST-1 suggests that screening of new assay components for this property is a key aspect of troubleshooting.

2. Limitations of the technique: One of the main limitations of this assay is that PMS/WST-1 and DPIIP can be reduced by multiple electron donors, such as NAD(P)H, ascorbate and flavinoids such as quercetin and myricetin (del Principe et al 2011, Phillips et al 2011, Altundag et al 2016). However, this can be overcome by the addition of enzymes (e.g. SOD, AO) or inhibitors to determine specific contributors to PMS/WST-1 or DPIIP reduction. Another limitation of the

assay is that DPIP can act as a substrate for AO and SOD. Thus, these enzymes should not be utilized in conjunction with DPIP to monitor tPMET. An additional limitation of the assay is the ability of PMS to produce superoxide (Halaka 1982). On the other hand, PMS itself reportedly has no direct effect on tPMET (Tan and Berridge 2000).

3. Significance with respect to existing methods: The procedure for testing whether or not assay components such as SOD or AO create artifacts is a significant improvement over existing methods. For example, reduced DPIP is a substrate for SOD, suggesting that published data obtained with use of DPIP and SOD should be interpreted accordingly. These previous studies are addressed in the discussion.

4. Future applications: Future applications of this assay include utilizing a wide range of inhibitors to better understand the process of tPMET, map the path of electron transport in a given cell line, and lead to better understanding of how a cell's redox environment is maintained.

5. Critical steps within the protocol: Critical steps of this protocol include determining that the cell are ready for experimentation (~70% confluent) as well as making the assay reagents, specifically WST-1 and PMS, fresh for each assay. As a portion of tPMET is attributable to molecular efflux (e.g. ascorbate export), it is important to start spectrophotometric readings immediately after addition of assay reagent in order not to miss the first moments of efflux. Also, it is critical to include background wells for each individual reagent in an experiment, as some additives (e.g. phloretin) can promote a substantial background reaction.

- **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as media, spectrophotometer etc.

The table now includes name, company, catalog number, and software of all relevant materials in separate columns in an xls/xlsx file. This is excluding the catalog number for the Biotek PowerwaveX-1 spectrophotometer which has been discontinued.

- Please define all abbreviations at first use.

All abbreviations have now been defined at first use.

- Please use standard abbreviations and symbols for SI Units such as μL , mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

We have checked abbreviations, symbols, and units.

- If your figures and tables are original and not published previously or you have already

obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All figures and tables are original and not published previously.

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

Here, Kelly and colleagues outline a method for the spectrophotometric measurement of trans-plasma membrane electron flux from the cytoplasm to the extracellular milieu in cultured C2C12 myotubes. Overall, I found the paper was well written but there are certain areas that need to be clarified and improved. My concerns with this submission are listed below:

Major Concerns:

1) The authors cite that the WST-1 method for detecting electron flow changes does not suffer from the same limitations as the use of ferricytochrome c or ferricyanide. This may very well be true but I am wondering why the authors do not use fluorescent membrane impermeable probes that are specific for superoxide? Indeed fluorescent probes overcome a lot of limitations associated with spectrophotometric assays including sensitivity and specificity issues. Another point is the use of PMS and WST-1. Other spectrophotometric methods for detecting superoxide include the indirect method, aconitase, which is known to be far more sensitive than any other method since its Fe-S cluster is disassembled in $\sim 10^7$ M $^{-1}$ s $^{-1}$. There is also nitro-tetrazolium blue and spin traps. The authors need to justify why this method for detecting extracellular superoxide is superior to all other known methods for superoxide detection.

While there are other means to detect extracellular superoxide, the current method is easily manipulated to monitor multiple forms of tPMET, such as ascorbate efflux. This assay method allows global electron transport to be monitored. If superoxide is the main interest, there are as the reviewer suggests a number of other avenues to monitor superoxide such as fluorescent impermeable probes, aconitase, and nitroblue tetrazolium (Maghzal et al 2012). We have added this information to the limitations section of the discussion.

2) It would be important for the authors to comment on the kinetics for the reaction of superoxide or ascorbate with WST-1. This, to me, is important since control reactions include 60 U/mL of SOD. SOD dismutates superoxide at a rate of 1.8×10^9 M $^{-1}$ s $^{-1}$.

In a 1 ml volume, 100 μ M ascorbate reduces 322 nmol WST-1 per minute in the presence of PMS, which is required for direct reduction of WST-1 by ascorbate. In a 1 ml volume, 2 U/mL of ascorbate oxidase oxidizes ascorbate at a rate of 4,400 nmol per second, or 800 times faster

than reduction of WST-1 by ascorbate. Clearly, enzymatic activity of ascorbate oxidase is much faster than direct reduction of PMS/WST-1 by ascorbate. We expect that the same would follow for superoxide and SOD, though we do not have a system for measuring the absolute rate of WST-1 reduction by superoxide.

3) It has been documented that reduced PMS and other redox cyclers can produce superoxide through the formation of semi-radical intermediates. This is a great point. We have now pointed out the ability of PMS and other redox cyclers to produce superoxide (Ukeda et al 1999, Peskin and Winterbourn 1999, Halaka et al 1982). This is an important limitation of this method. However, Tan and Berridge have shown that the NADPH oxidase inhibitor, DPI, can suppress WST-1 reduction indicating that extracellular superoxide production may not solely be attributable to PMS.

Can the authors comment, specifically, on the background absorbance one is expected to observe in this assay? Do any of the components of the assay system interfere with detection of tPMET through their autooxidation?

The average change in background seen in the assay is negligible. The background is subtracted out when the data is analyzed as to eliminate any confounding effects the reagents themselves may produce.

Also, it would be prudent to comment on the specificity of WST-1 and PMS since both can be reduced and oxidized by a number of redox pairs (NAD(P)H for example).

The reviewer correctly identifies that a limitation of this study is that WST-1 and PMS can be reduced by a number of redox pairs, and we have now acknowledged this in the Discussion.

4) A number of cell growth and differentiation mediums already contain ascorbate. Is this an issue in these assays? Can the authors comment on the exact contents of their medium?

DMEM does not contain ascorbate, although we supplement differentiation medium with ascorbate. A number of media types do contain ascorbate, but the cells are washed before the addition of the assay reagents, which are ascorbate-free.

5) N=36 does this mean 36 wells were monitored, also can the authors demonstrate the assay is reproducible between plates? And for a lot of figures the error bars are so small they cannot be visualized. Is this to mean that this spectrophotometric cell based assay, that uses tetrazolium dyes, is highly reproducible?

The sample size of 36 is indicative of 36 wells per treatment. WST-1 reduction is reproducible from well to well within an assay, but there is day-to-day variability. The day-to-day coefficient of variation (CV) utilizing PBS as the buffer is 0.18 and the CV utilizing HBS as the buffer is 0.23.

And what kind of stats were performed? The statistical analyses performed were ANOVAs with repeated measures.

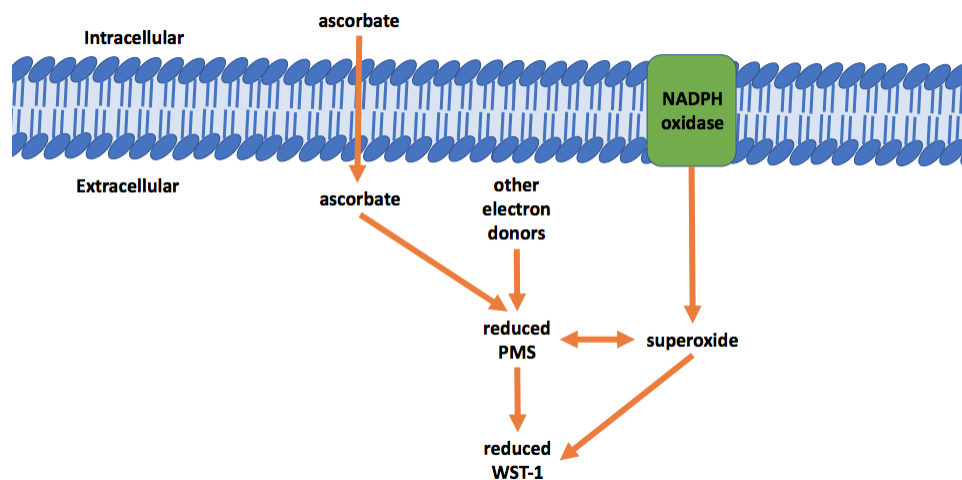
The CV information and a description of the statistical methods have been added to the manuscript.

6) The limitations sections is sparse and there is no discussion on troubleshooting, what the critical steps are in the protocol, and its significance with respect to existing methods.

As described in our response to the JoVE Scientific Review Editor above, we have fleshed out these sections of the Discussion.

7) The paper would benefit greatly from a scheme depicting tPMET and how the assay works. Below is a scheme depicting tPMET and how this process contributes to WST-1 reduction.

In this model of tPMET, ascorbate (or other electron carrier) exported by the cell can reduce extracellular PMS. In addition, NADPH oxidases generate extracellular superoxide, which can reduce WST-1 directly or reduce it indirectly via reduction of PMS. Electrons from other plasma membrane donors can also reduce PMS, which can donate electrons to either WST-1 or O_2 with subsequent WST-1 reduction.



Reviewer #2:

Manuscript Summary:

This manuscript compares two cell-impermeable dyes for measuring tPMET. Using C2C12

myotubes it is shown that WST-1 reduction in the presence of the intermediate electron acceptor, PMS, is the preferred method with 70% of dye reduction accounted for by extracellular superoxide and 30% by ascorbate efflux.

Major Concerns:

1. The WST-1 procedure described for measuring tPMET has used PMS (5-methyl-phenazine methosulfate, Sigma P9625) as an intermediate electron acceptor (IEA) throughout, whereas the original Boehringer Mannheim WST-1 reagent and subsequent publications have used mPMS (1-methoxy, 5-methyl-phenazine methosulfate) as a "photochemically stable electron mediator with greater efficacy and lower background than PMS" (see ref 22). Publications referred to that used mPMS should be altered to reflect this (e.g. lines 63, 74 and 236). The authors should discuss their choice of IEA and whether background with PMS alone + cells, was an issue relative to WST-1 alone + cells.

In lines 63, 74, and 236, it has been clarified that previous studies have utilized mPMS. To the introduction in the revised document we have clarified that we have utilized PMS instead of mPMS based on cost. mPMS is photochemically stable, which is an important characteristic for a commercial kit that needs a long shelf life. However, we make PMS fresh for each assay, so stability shouldn't be an issue.

In our hands, PMS is required for cellular WST-1 reduction. When cells are incubated in assay reagent containing PMS alone (in the absence of WST-1), there is an increase in absorbance at 438 nm. This likely reflects an accumulation of the semiquinone form of PMS (PMS-SQ) that has a peak absorbance at 440 nm (Zaugg 1964). Accumulation of PMS-SQ suggests that PMS is being reduced faster than it can pass electrons to O_2 to produce superoxide. Thus, the accumulation of PMS-SQ is consistent with the reported slow reduction of O_2 by PMS-SQ compared to the rate for fully-reduced PMS.

As described above, PMS is required for WST-1 reduction by ascorbate. In addition, PMS doubles the rate of WST-1 reduction by a xanthine oxidase/hypoxanthine superoxide generating system. Thus, it appears that PMS mediates transfer of electrons from superoxide to WST-1.

2. It is important to distinguish between respiratory burst oxidase activity that reduces WST-1 directly at the plasma membrane via superoxide (i.e. in the absence of mPMS/PMS) and the tPMET activity that requires mPMS/PMS and is variably and indirectly SOD-inhibitable. In the absence of mPMS/PMS, was there any reduction of WST-1 over 60 min? (see also point 1).

As stated above, in the absence of PMS, there is minimal cellular reduction of WST-1. However, our data suggest that PMS mediates transfer of electrons from superoxide to WST-1.

3. In the first sentence of the abstract, protection from intracellular reductive stress may be as important as protection from damage by extracellular oxidants.

This is an excellent point. We have now mentioned that tPMET can protect cells from intracellular reductive stress (Del Principe et al 2011) as well as protect the cells from damage by extracellular oxidants.

Minor Concerns:

1. line 38; replace "previous" with "other"

We have made this replacement.

2. line 44; replace "belief" with "view"

We have made this replacement.

3. line 91; briefly mention the method used to differentiate mouse C2C12 cells. Ref 25 relates to rat L6 cells and uses 2% horse serum.

We have now described the method of differentiation and cited a study that uses identical cell culture procedures with C2C12 cells (Eccardt et al 2017).

4. abbreviations should be defined and include DPIP=DCIP

The abbreviation for DPIP has now been updated to include DCIP.

5. line 230; ref 27 should follow Bellavite et al and ref 24 Tan and Berridge.

Thanks for noticing this. The text in line 230 now includes the citation numbers.

6. line 242; ref 29 should be included as it presents more comprehensive information on reduction of WST-1 by activated neutrophils.

We have now cited the Tan&Berridge 2000 paper here.