

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.

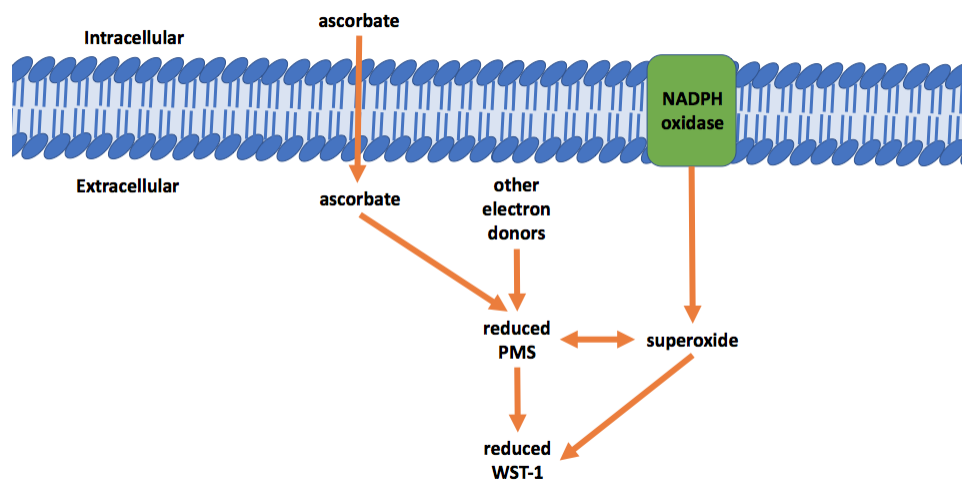
The CV information and a description of the statistical methods have been added to the manuscript.

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.