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Facts and artefacts on the oxygen dependence of hydrogen peroxide production using Amplex UltraRed

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Reviewer 2

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Reviewer 2

The work of Komlódi and colleagues investigates the methodological issues associated with the quantification of cellular hydrogen peroxide (H_2O_2) production by using the fluorescent probe Amplex UltraRed through high resolution fluororespirometry. The results are quite clear in pointing out the artifacts generated by phosphate or potassium-based buffers on H_2O_2 production, but not on Mir05 media when yeast cells are challenged by a hypoxic stress. The authors also assessed the oxygen dependency of H_2O_2 production and, unexpectedly, they observed that in Mir05 yeast cells under hypoxia generate less H_2O_2 than under normoxia. This is a remarkable observation as it contrasts with the concept of reductive stress and hypoxia-driven superoxide production in other cellular models. The work was carefully conducted, and the manuscript is very well written, and I addressed specific points below to help authors strength their conclusions.

Major comments:

1) Given that Amplex UltraRed reacts with carboxyesterases, representing a source of HRP-independent signal of this probe (https://www.sciencedirect.com/science/article/pii/S0891584915011090), the authors should consider how these activities might contribute to the background fluorescence of yeast cells in their measures. Additional controls such as test the effect of carboxyesterases inhibitors like PMSF to check their contribution in yeast cells seems a valuable approach. Alternatively, the authors could add a cautionary note at the discussion section to consider this point.

2) While the authors state at the first paragraph of page 15 in the discussion section, "…These side-effects can be practically excluded in our experiments with living yeast" it is not clear why the potential role of carboxyesterases can be ruled out in their experiments.

3) While the effect of media on background Amplex UltraRed fluorescence is clearly demonstrated in figures 5b and 5f, one might think on how potential artifacts related to this signal may contribute to yeast Amplex UltraRed fluorescence under Mir05. Is it possible that under DPBS yeast carboxyesterase activity is higher than under Mir05 which would explain higher unspecific yeast fluorescence observed in figure 5b? Interestingly, carboxyesterase activity seems to be induced by hypoxia in mammalian cells (https://pubmed.ncbi.nlm.nih.gov/17397102/), which, in combination of a media-specific effect, might explain the non-specific fluorescence signal of Amplex UltraRed.

Authors

2.1.-2.3. We addressed these arguments with the following modification in the Discussion: "If AmR does not cross the cell wall and plasma membrane of yeast cells, these side-effects can be excluded in our experiments. If, however, AmR reacts with intracellular carboxylesterases, this would not explain the absence of the hypoxic peak in MiR05 (Figure 8) nor the induction of the hypoxic peak in DPBS at high fluorescence intensity (Figure 3)."

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4) While the presented results provide a detailed analyses and corrections of the potential artifacts when using the Amplex UltraRed method to quantify H_2O_2 production by yeast cells, the authors could add a general comment at the discussion section to explain whether similar artifacts might also affect the detection of H_2O_2 by this method in other systems (mammalian/plant/insect cells).

Authors

2.4. We extended the end of the Discussion: "This provides the basis for correction for background fluorescence slope and evaluation of the O_2 dependence of H_2O_2 flux not only in yeast but generally in applications of the AmR assay in living and permeabilized cells, and isolated mitochondria including mammalian cell models."

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5) At first paragraph of page 16, the authors should explain why the hypoxic-driven H_2O_2 production is not observed in yeast cells under Mir05. Is there literature evidence indicating that in this media yeast cells do not undergo reductive stress upon hypoxia as a mechanistic explanation for the absence of increased H_2O_2 production?

Authors

2.5. The controversy on reductive stress is not restricted to yeast but is a hot topic in the literature on mammalian mitochondria and cells. This we clarified now in the second sentence in the Discussion by adding "This observation is in line with studies on mammalian mitochondria…".

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Minor comments:

The graphs on figure 5 should clearly indicate which experiments were performed with and without yeast cells.

Authors

See our response to Reviewer 1 (Section 1.2).