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ARTICLE

Methodology for measuring oxidative capacity of isolated peroxisomes in the Seahorse assay

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Abbreviations used: CPF, crude peroxisome fraction; Cpt1B, carnitine palmitoyl transferase 1B; ER, endoplasmic reticulum; MACS, magnetic-activated cell sorting; PPF, purified peroxisomal fraction; ROS, reactive oxygen species

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ABSTRACT

The regulation of cellular energetics is a complex process that requires the coordinated function of multiple organelles. Historically, studies focused on understanding cellular energy utilization and production have been overwhelmingly concentrated on the mitochondria. While mitochondria account for the majority of intracellular energy production, they alone are incapable of maintaining the variable energetic demands of the cell. The peroxisome has recently emerged as a secondary metabolic organelle that complements and improves mitochondrial performance. Although mitochondria and peroxisomes are structurally distinct organelles, they share key functional similarities that allows for the potential to repurpose readily available tools initially developed for mitochondrial assessment to interrogate peroxisomal metabolic function in a novel manner. To this end, we report here on procedures for the isolation, purification and real-time metabolic assessment of peroxisomal β-oxidation using the Agilent Seahorse® system. When used together, these protocols provide a straightforward, reproducible and highly quantifiable method for measuring the contributions of peroxisomes to cellular and organismal metabolism.

Keywords: peroxisome, seahorse assay, fatty acid metabolism

INTRODUCTION

Akin to mitochondria, peroxisomes are an essential organelle common to nearly all eukaryotic cells [1,2]. Unlike mitochondria, however, peroxisomes lack their own DNA encoded genes and instead depend on nuclear expressed gene products to generate the functional peroxisomal population [2,3]. Both organelles rely on the endoplasmic reticulum (ER) for generation of their lipid bilayer, with peroxisomes directly budding off the ER structure, while mitochondria receive phospholipids manufactured within the ER matrix [4,5]. Organelle specific proteins decorate the membranes of each organelle that in turn support their function, regulation, transport, and even maturation [2,3]. Interestingly, both the mitochondria and peroxisome routinely interact with other cellular components that ideally position them within proximity to cellular lipid depots. The functional connections between peroxisomes and mitochondria to the ER have been well documented, while the association between peroxisomes and lipid droplets has only recently been revealed [5-8]. This commonality between organelles highlights a shared functionality between peroxisomes and mitochondria that centers on lipid metabolism.

Traditionally, peroxisomes perform "house-keeping" duties by leveraging specialized enzymes that scavenge reactive oxygen species (ROS) byproducts derived from mitochondrial oxidative phosphorylation [9,10]. However, it is now appreciated that peroxisomes also facilitate the conversion of very long-chain fatty acids into shorter chained fatty acids that further supply the mitochondria with substrates for ATP production [11]. In addition to these lipid preprocessing duties, peroxisomes can also compensate for defects in mitochondrial function that are often associated with conditions such as carnitine deficiency, obesity, and inborn errors of metabolism [12-14]. Such metabolic compensation can be accomplished through direct upregulation of β-oxidative processes to metabolize lipid species beyond long-chain fatty acids, such as short-chain fatty acids or TCA cycle intermediates, like succinate, for utilization by the mitochondria [12,15]. A similar metabolic fate can be achieved indirectly by engaging ω-oxidation in the ER, which functions as a backup for β-oxidation by producing dicarboxylic acids from medium- and long-chain fatty acids that are uniquely metabolized by the peroxisome [16]. Although no ATP is produced from

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peroxisomal β-oxidation, when defects in mitochondrial β-oxidation occur, this process provides an alternative means for supplying a pool of preprocessed substrates that can more readily be utilized for ATP synthesis *via* alternate metabolic entry points. Based on this knowledge, we reasoned that technologies that enable the accurate measurement of functional peroxisomes would likely provide key insights into the metabolic health of the cell and the overall organism.

The current standard for assessing peroxisomal β-oxidation is accomplished by either mass spectrometry-based quantification of peroxisomal-specific lipid species or measurement of NAD^+ levels, a coenzyme that serves as a proton acceptor during fatty acid metabolism [13,17,18]. Because NAD^+ functions as a transient cofactor for cellular signaling and metabolism, it is often an unreliable proxy for the accurate assessment of peroxisomal contributions to lipid oxidation unless peroxisomes are first purified. Therefore, to establish a more definitive method for measuring peroxisomal β-oxidation, we developed a methodology for the real-time assessment of peroxisomal oxygen consumption rate (OCR) using the Agilent Seahorse® assay, the recognized gold-standard for interrogating mitochondrial respiration [19,20]. Peroxisomes can account for up to 20% of cellular total oxygen consumption in rat hepatocytes, indicating these organelles are amenable for use in the Seahorse assay similar to mitochondria [9]. To this end, we devised an optimized protocol for the purification of in-tact peroxisomes and the ideal Seahorse assay conditions to directly measure peroxisomal capacity for lipid metabolism.

METHODS

Isolation of functional peroxisomes

To isolate functionally pure peroxisomes, modifications were made to an existing published protocol from Sigma (Perox1) [18,21]. An overview of our purification scheme, including these modifications, are outlined in **Figure 1A**. Buffers and solutions used in the purification of peroxisomes are summarized in **Table 1**. Briefly, following euthanasia with isofluorane, liver tissue was isolated from C57BL6/J male mice ($N = 4$). Approximately 4 g of total liver tissue (1 g/mouse) was utilized for isolation of peroxisomes. All reagents were prechilled on ice prior to start of the assay and all steps were performed on ice to preserve organelle viability and integrity. To obtain the crude peroxisome fraction (CPF), liver tissue was cut into small pieces using a razor blade in a petri dish. Processed livers were then transferred to a prechilled glass Dounce® homogenizer and homogenized with 15 strokes of the glass pestle. The remainder of the Sigma protocol was followed verbatim until the final centrifugation step, which was carried out at 14000 × *g* for 35 min. Optimal centrifugation times and speed determinations were made using the following formula (**Eqn. 1**):

$$T_n = T_p\left(\frac{S_p}{S_n}\right) \quad \text{(Eqn. 1)}$$

where *Tn* is the new spin time, *Tp* is the protocol spin time, *Sp* is the protocol spin speed, and *Sn* is the new spin speed. Following isolation of the CPF, further purification was accomplished by density gradient centrifugation as outlined in the Sigma protocol, using the provided volumes and methods (**Fig. 1B**). Centrifugation was carried out in a fixed-angle rotor. The upper layers were carefully removed by aspiration and the remaining bottom layer containing peroxisomes, termed the purified peroxisomal fraction (PPF), was transferred, along with the floating pellet, to a new tube in an approximate volume of 1 ml. Purity of this fraction was assessed by immunoblotting with antibodies raised against organelle-specific proteins: LCA3A/B (lysosome), Serca2 (endoplasmic reticulum), cytochrome C (mitochondria) and catalase (peroxisome). Immunoblot analysis of the PPF revealed the presence of mitochondria (**Fig. 2A**), which is problematic for the accurate assessment of peroxisomal metabolic activity as mitochondria are highly active in the Seahorse assay.

Table 1. Buffers and solutions used in the purification of peroxisomes. Buffers were freshly prepared prior to harvest and kept on ice. The 60% OptiPrep density gradient medium was maintained at 4°C until preparation of the gradient solutions.

Buffer	1× Peroxisome extraction buffer	1× OptiPrep dilution buffer	27.5% OptiPrep density gradient solution	22.5% OptiPrep density gradient solution with CPF	20% OptiPrep density gradient solution
Components	5 ml 5× peroxisome extraction buffer	1.25 ml 20× OptiPrep dilution buffer	4.58 ml 60% OptiPrep density gradient medium	1.69 ml 60% OptiPrep density gradient medium	3.33 ml 60% OptiPrep density gradient medium
	20 ml ultrapure water	23.75 ml ultrapure water	5.42 ml 1× OptiPrep dilution buffer	1.61 ml 1× OptiPrep dilution buffer	6.67 ml 1× OptiPrep dilution buffer
				1.2 ml CPF	

To facilitate the removal of mitochondrial contamination from the peroxisomal fraction, a magnetic-activated cell sorting (MACS) column purification was used. Specifically, the purified PPF was incubated with 2 µl of biotinylated carnitine palmitoyl transferase 1B (Cpt1B, BiossUSA.com, bs-5045R-biotin) antibody for 10 min at a 1:500 dilution. In the liver, Cpt1 controls approximately 80% of the metabolic flux resulting from fat oxidation under normal homeostatic conditions [22]. Moreover, Cpt1 is localized to the outer mitochondrial membrane and was therefore chosen to selectively retain mitochondria on the MACS column while peroxisomes remain in the flowthrough. This strategy also limited mechanical stress on the peroxisomal fraction to preserve organelle integrity and function. Next, the PPF fraction was applied to a MACS column containing anti-biotin microbeads (MiltenyiBiotec 130-090-485) (**Fig. 1C**). The resulting suspension was applied to prepped LD columns (MiltenyiBiotec 130-042-901) and the flowthrough was collected and saved. Immunoblotting of the resulting purified fraction was repeated as described above and no mitochondrial contamination was detected (**Fig. 2B**).

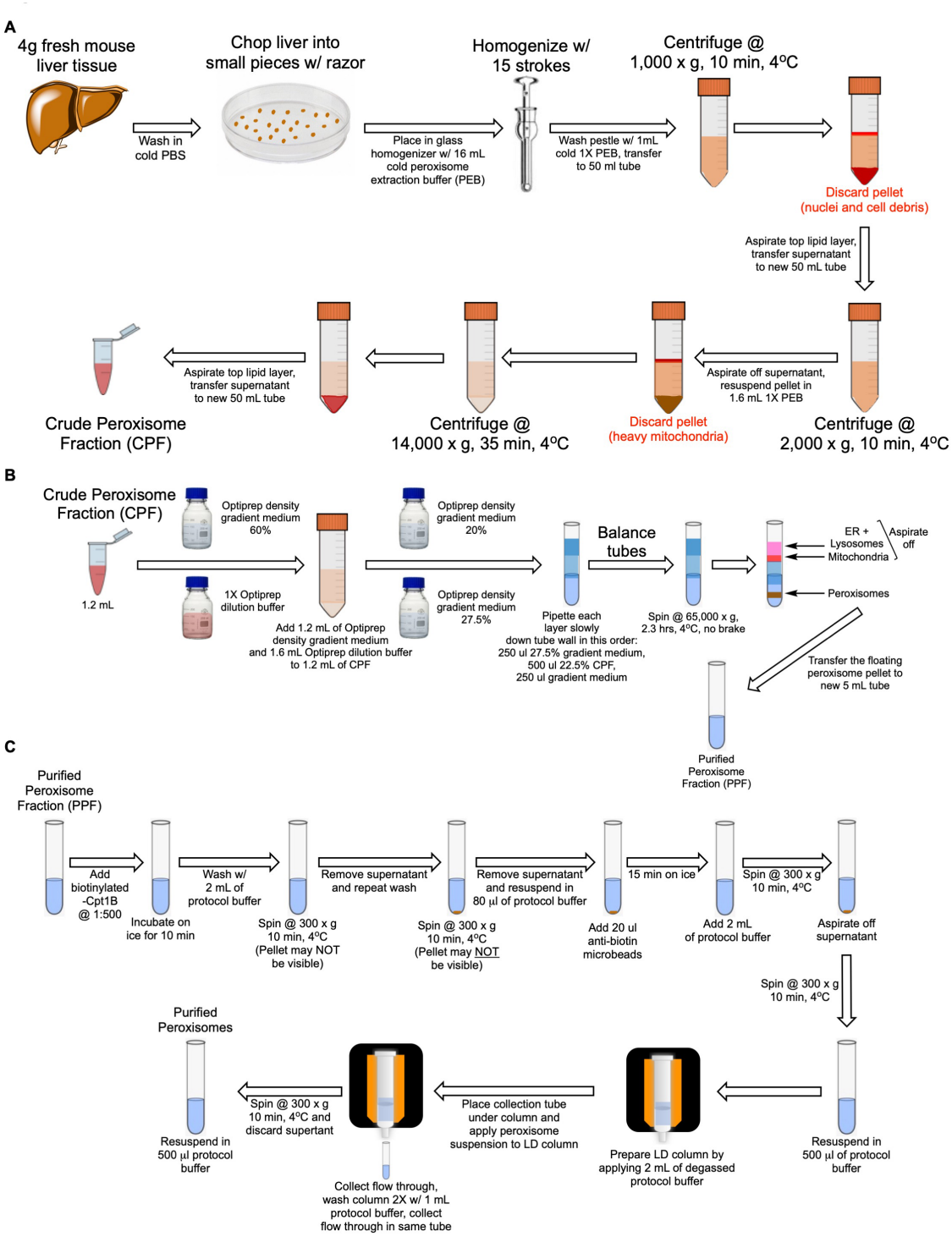

Figure 1. Purification scheme for isolation of peroxisomes. Purification was accomplished in a three step process using a combination of the Sigma peroxisome purification protocol (Perox1) and MACs system. **A.** Initial purification of the liver lysate to obtain the crude peroxisomal fraction (CPF), which contains peroxisomes, lysosomes, mitochondria, and the endoplasmic reticulum; **B.** Purification of the CPF to the purified peroxisome fraction (PPF), which contains peroxisomes with minimal mitochondrial contamination; **C.** Purification of the PPF using MACs LD columns to deplete mitochondria to obtain purified peroxisomes.

Immunoblot analysis

Immunoblot analyses were performed on purified peroxisome fractions separated by SDS-PAGE. Briefly, 20 and 40 µg of peroxisomal protein was loaded onto a 4% to 20% gradient SDS-PAGE page gel, separated at 200 V for 45 min, transferred using the iBlot system for 4:30 min to nitrocellulose membranes, and blocked in 5% milk in Tris-buffered saline with 0.1% Tween-20 (TSBT). Primary antibodies (LCA3A/B (lysosome), rabbit, Cell Signaling # 12741; Serca2 (endoplasmic reticulim), rabbit, Cell Signaling # 9580; Catalase (peroxisome), rabbit, Cell Signaling # 12980, Cytochrome C (mitochondria), rabbit, Cell Signaling # 11940; and Pex14 (peroxisome), rabbit, Sigma # SAB4502176) were diluted in 1% milk in TBST at 1:1000. Nitrocellulose membranes were incubated with primary antibodies overnight with rotation at 4°C. Immunoblots were incubated with appropriate secondary antibodies coupled to horseradish peroxidase (Digital anti-Rabbit-HRP, R1006, 1:1000, Kindle Biosciences), reacted with ECL reagents per the manufacturer's instructions (Thermo), and detected by autoradiography or KwikQuant® Imager (Kindle Biosciences).

Seahorse assay parameters

To formulate media for the measurement of peroxisomal metabolism that could be compatible with the Seahorse instrumentation, we began with the Seahorse XF Base Medium as it contains a proprietary assortment of salts and amino acids. To account for the fact that peroxisomes are unable to synthesize ATP, Seahorse XF Basal Media was supplemented with 100 µM ATP (Promega, E601B). Similar to Seahorse conditions using purified mitochondria, no glucose was added to the peroxisomal assay media, as these organelles lack the required machinery to directly metabolize glucose. Next, we supplemented basal Seahorse media with a mixture of palmitic, oleic, and linoleic fatty acids, each at a concentration of 1%, for a total fatty acid concentration of 3%. Consistent with the homeostatic concentration of acetyl-CoA in the liver, which has been reported at approximately 50 nmol/gram of liver tissue [23], we also supplemented the basal Seahorse media with 50 nM of acetyl-CoA (Sigma, A2056). As a control, we employed the use of enoximone (Tocris, Cat. # 1693, CAS # 77671-31-9), an inhibitor of peroxisomal β-oxidation [17]. Theoretically, the addition of enoximone would be expected to modulate oxygen consumption rate (OCR) similar to how oligomycin, FCCP, and antimycin A are used to interrogate functional aspects of the mitochondrial electron transport chain in the traditional Seahorse assay. Previous reports using enoximone suggest that a concentration of 250 µM is sufficient to inhibit nearly all oxidative output [17]. We selected a concentration of 300 µM to achieve maximal inhibition of peroxisomal β-oxidation. The complete media conditions are summarized in detail in **Table 2**.

Metabolic measurement of purified peroxisomes in the Seahorse assay

The Seahorse assay was carried out as outlined in **Figure 3**. The calibration plate was set up the night before the assay per the manufacturers' recommendation. The day of the assay, the Seahorse XL96 instrument was programmed to include 6 measurement cycles with no mixing or injections. During the calibration step, 50 µg of purified peroxisomes were plated on ice in a Seahorse 96-well plate and centrifuged at 12000 × *g* for 15 min at 4°C. The appropriate media, as indicated in the figure legend for **Table 2**, was added to each well in a final volume of 125 µl. Purified peroxisomes were incubated in media cocktail containing 3% fatty acids alone, or in combination with increasing concentrations of acetyl-CoA and/or enoxomine, an inhibitor of peroxisomal β-oxidation. The prepared plate was maintained on ice until the calibration step was complete.

H_2O_2 measurements

Purified peroxisomes treated with either fatty acids alone, or in combination with increasing concentrations of acetyl-CoA and/or enoxomine in the Seahorse assay were used to measure H_2O_2 concentration. More specifically, 30 µg of the purified peroxisome fraction was treated exactly as described in the Seahorse assay and 50 µl of the resulting reaction was used to measure the concentration of H_2O_2 in a colorimetric assay per the manufacturer's recommendations (AbCaM, Cat #: AB102500) at 570 nm on UV plate reader (Tecan Infinite M200 Pro).

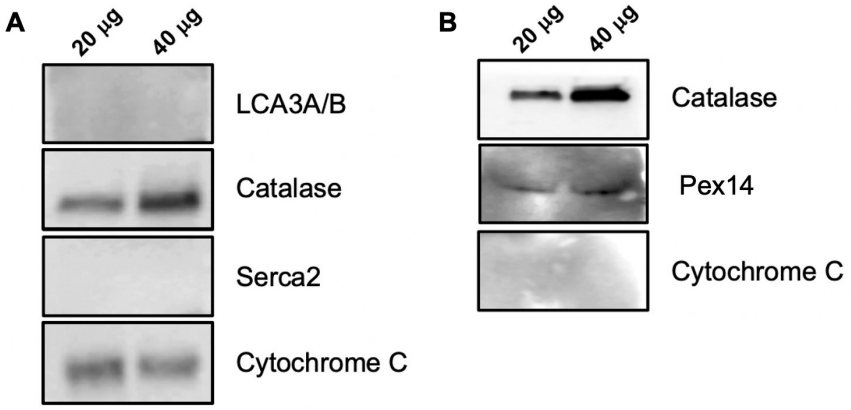

Figure 2. Immunoblot validation of peroxisomal fraction purity. A. Approximately 20 and 40 µg of protein from the purified peroxisomal fraction (PPF) was separated on a 4% to 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The resulting membrane was immunoblotted with organelle-specific antibodies: LCA3A/B (lysosome), catalase (peroxisome), Serca2 (ER), and cytochrome C (mitochondria). **B.** The PPF was subjected to a MACS column purification step using a biotinylated Cpt1B antibody. Approximately 20 and 40 µg of protein from the resulting PPF was separated on a 4% to 12% SDS-PAGE gel and transferred to a nitrocellulose membrane and immunoblotted as described in (A). The resulting membrane was immunoblotted with organelle-specific antibodies: catalase (peroxisome), Pex14 (peroxisome) and cytochrome C (mitochondria).

Table 2. Media and supplements used to assess the metabolic activity of purified peroxisomes in the Seahorse assay. All reagents were fresh the day of the assay. Seahorse Basal XF Media was used for the preparation of all supplemental treatment groups.

Media	BSA + DMSO	Fat + DMSO	Fat + enoxomine	Fat + acetyl-CoA + DMSO	Fat + acetyl-CoA + enoximone
Components	3% Fatty acid- free BSA	3% long-chain fats (1% each palmitate, oleate, and linolate)	3% long chain fats (1% each palmitate, oleate, and linolate)	3% long chain fats (1% each palmitate, oleate, and linolate)	3% long chain fats (1% each palmitate, oleate, and linolate)
	100 µM ATP	100 µM ATP	100 µM ATP	100 µM ATP	100 µM ATP
	DMSO	DMSO	300 µM enoximone in DMSO	DMSO	300 µM enoximone in DMSO
				50 nM Acetyl-CoA	50 nM Acetyl-CoA

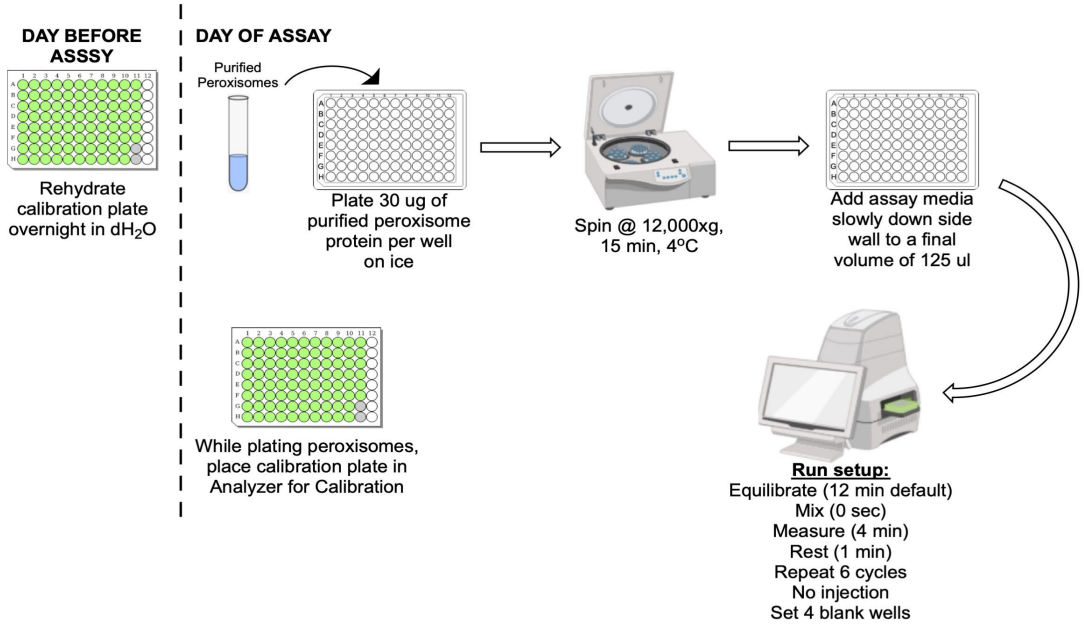

Figure 3. Schematic for the peroxisome Seahorse assay. The calibration plate was rehydrated overnight in sterilized dH_2O per the standard Seahorse protocol in the absence of CO_2. The day of the assay, the calibration plate was switched to calibration fluid 1 h prior to start of the assay and then placed in the Seahorse instrument for calibration. Peroxisomes were plated on ice during calibration and centrifuged to position them at the bottom of the well. The appropriate media was added to each well for a final volume of 125 µl. A total of 6 measurement cycles of 4 min each were conducted without mixing or injections.

RESULTS

As traditional methods of measuring peroxisomal β-oxidation either require complex analytical equipment and expertise or rely on indirect assessments of proxy metabolites, we developed an improved approach for peroxisomal purification that leverages the real-time assay capabilities of the Seahorse platform. Using the peroxisomal purification scheme outlined in **Figure 1**, we measured β-oxidation in purified liver peroxisomes using optimized conditions for the Seahorse assay (**Fig. 3**). Since peroxisomes are capable of metabolizing long chain fatty acids, we compared the oxidative potential of purified peroxisomes treated with either a BSA cocktail containing 3% fatty acids or BSA alone (**Fig. 4A**). We found that addition of fatty acid increased peroxisomal OCR approximately 2.5-fold when compared to BSA alone (**Fig. 4A**). Similarly, addition of acetyl-CoA in combination with fatty acids significantly bolstered peroxisomal OCR by an additional 2-fold when compared to fatty acid alone (**Fig. 4A-4D**). Importantly, peroxisomal OCR resulting from either fatty acids alone or in combination with acetyl-CoA was suppressed upon addition of enoximone, an inhibitor of peroxisomal β-oxidation (**Fig. 4E** and **4F**). Consistent with these findings, measurement of peroxisomal peroxide (H_2O_2) production as a proxy of β-oxidation identified that fatty acids and acetyl-CoA significantly increase H_2O_2 production that is suppressed by increasing concentrations of enoximone (**Fig. 4G**). Taken together, these findings highlight the amendability of the Seahorse platform for the accurate and reproducible measurement of the oxidative capacity in purified peroxisomes.

DISCUSSION

Though most notable as scavengers of reactive oxygen species (ROS), peroxisomes have gained recent attention for their contributions to fatty acid metabolism. For example, mutations in peroxisomal (PEX) proteins have been shown to produce neurological diseases that stem from lipid buildup rather than ROS accumulation [24]. As the majority of peroxisomal research has been carried out in plants and yeast, perhaps it is not surprising that there remains a deficit in our understanding of the metabolic contributions of peroxisomes in mammalian cell biology. However, the recent discovery that Zellweger syndrome is caused by underlying defects in peroxisomal function has sparked new interest into the roles this critical organelle may play in metabolic health and disease [25].

In normal healthy cells, peroxisomes complement mitochondrial function by preprocessing very long chain fatty acids into species that can be more readily used by the mitochondria for energy production *via* β-oxidation [26]. Conversely, cells housing damaged or dysfunctional mitochondria typically express peroxisomes that further metabolize lipid substrates into shorter chain fatty acids or TCA cycle intermediates that can then be shunted into alternate metabolic entry points for ATP synthesis in the mitochondria [1,13]. Thus, peroxisomes carry out essential metabolic roles that are vital to the proper maintenance of cell functionality.

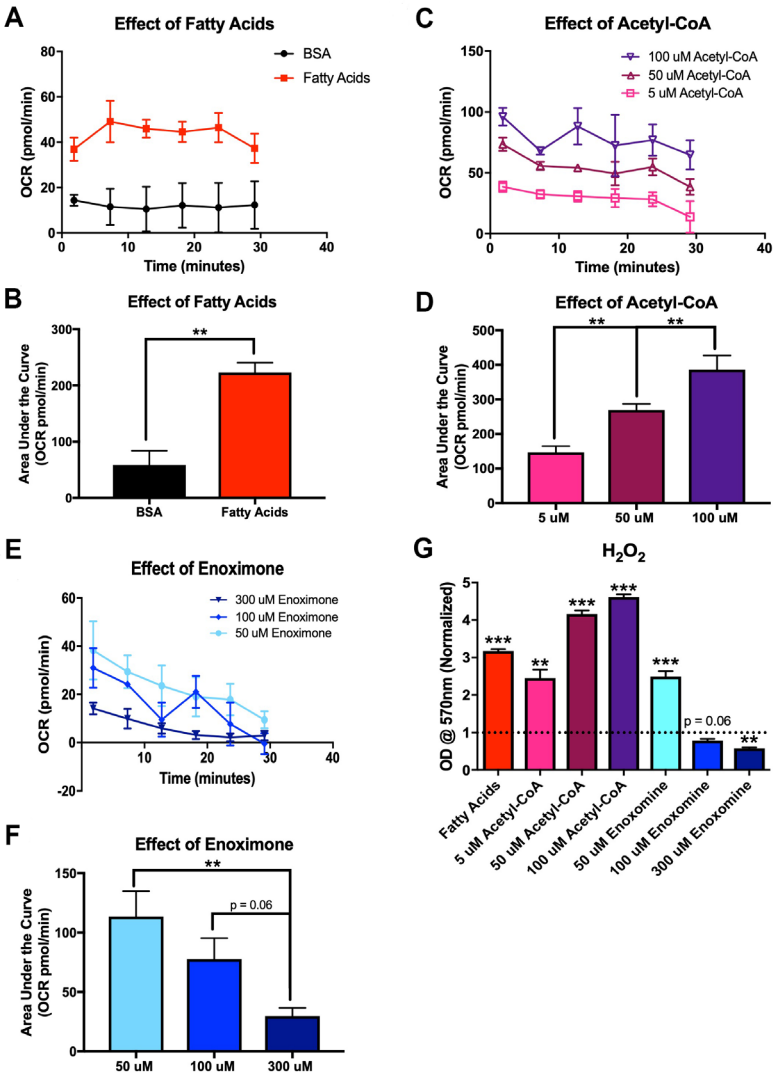

Figure 4. Peroxisomes respond to metabolic treatments in the Seahorse assay. Approximately 30 µg of purified peroxisomes were plated per well and centrifuged at 12000 x *g* for 15 min at 4°C, and brought up to a final volume of 125 µl using the appropriate media. Media treatments were performed in triplicate. The Seahorse assay was run for 6 measurement cycles with no injections or mixing. **A.** The addition of 3% fatty acids (1% each palmitic, oleaic, and linoleic acids) to the base media significantly increased the oxygen consumption rate (OCR). **B.** Area under the curve for (A). **C.** The addition of acetyl-Co-A to the base media with fatty acids significantly increased the OCR in a dose-dependent manner. **D.** Area under the curve of (B). **E.** The addition of enoximone to the base media with fatty acids and 50 nM Acetyl-CoA significantly decreased the OCR in a dose-dependent manner. **F.** Area under the curve of (C). **G.** H_2O_2 production of purified peroxisomes from the Seahorse assay normalized to H_2O_2 production of peroxisomes in base media only, which was set equal to 1 as indicated by the dotted line. Data are represented as the mean ± SEM unless otherwise specified. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Currently available methodologies for assessing peroxisomal metabolic function were first developed half a century ago and rely on measuring NAD^+ reduction [18]. In isolated primary hepatocytes, reduction of NAD^+ as a proxy for peroxisomal metabolic capacity fails to distinguish between other oxidative processes that also culminate in the reduction of NAD^+[17]. More recently, mass spectrometry-based methods have been successfully commissioned to quantitatively measure the abundance of peroxisomal-specific lipid species [13,25]. However, this approach is expensive and requires specialized sample processing, equipment and expertise, thus limiting its broad utility. We sought to overcome these limitations in measurement of peroxisomal metabolism by developing an assay amendable to Seahorse® technology that detects changes in oxygen consumption as an index of oxidative capacity. The Seahorse assay has quickly emerged as the current gold standard for the automated and accurate assessment of metabolic capacity in cells and isolated mitochondria [19,20]. Our findings here are the first to report on a protocol that expands the capability of the Seahorse technology to include purified peroxisomes and their metabolic contributions.

Historically, protocols for the separation and isolation of cellular organelles have relied heavily on ultracentrifugation to improve purity of these fractions while preserving their functionality. The primary challenge with current protocols for the isolation of either mitochondria or peroxisomes is cross-contamination, as these two organelles often resolve into adjacent layers during centrufigation. Since mitochondrial oxidation is significantly higher than all other organelles, any contamination with non-mitochondrial structures has minimal impact on the true measurement of mitochondrial oxidative potential. Conversely, any mitochondrial contamination of a purified peroxisomal preparation would likely complicate the accurate interpretation of peroxisome contributions to oxidative metabolism, reinforcing the importance of accruing pure peroxisome fractions for study and the need for improved methodologies to do so. To first isolate functional peroxisomes, we began with an established protocol from Sigma (PEROX1). Unfortunately, our assessment of the purity of the resulting peroxisomal fraction identified mitochondrial contamination. Although published protocols suggest mitochondria are fragile and structurally compromised by high centrifugation speeds, we sought alternative methods to resolve our contamination issue that would allow for the further purification of peroxisomes that preserve their functionality [27]. To selectively eliminate mitochondria from our isolated fractions, we included an additional purification step that leveraged the MACS column purification system. The MACS purification system has been successfully used by our laboratory to isolate individual populations of cells, and presented a viable option that could be similarly applied to organelles [28]. We carefully selected carnitine palmitoyl transferase 1B (Cpt1B) as an integral outer mitochondrial membrane protein that could be exploited to specifically capture mitochondria while allowing peroxisomes to remain unaltered in the purification scheme. This approach proved incredibly successful as immunoblot analysis following this supplemental purification step revealed no detectable mitochondrial contamination in our peroxisomal isolate (**Fig. 2B**), which permitted their use of the Seahorse assay to directly determine the oxidative capacity of this organelle alone.

Adapting the Seahorse system to measure the oxidative metabolism of purified peroxisomes required a reformulation of the standard assay buffer to supply peroxisomes with usable fuel substrates. We focused our attention on formulating the assay media with two primary goals: (1) to preserve peroxisomal viability and (2) to provide substrates that support peroxisomal function *in vitro*. To this end, we began by supplementing the Seahorse XF base media with ATP, as it is required for peroxisomal β-oxidation but cannot be synthesized de novo by the peroxisome. Next, we sought to provide a suitable fuel substrate to support peroxisomal oxidative metabolism. One primary motivation for the development of this methodology was to assess the effect of different lipid species on peroxisomal activity. Previous studies have reported that addition of fatty acids increases the oxidative activity of peroxisomes, while the use of the imidazolone derivative, enoximone, serves to attenuate peroxisomal metabolism [17,18]. Consistent with these reports, we found that addition of fatty acids (*i.e.*, palmitic, oleic, and linoleic acids and acetyl-CoA) was sufficient to significantly bolster the oxidative metabolism of purified peroxisomes, while treatment with enoximone blunted their OCR in a dose-dependent manner (**Fig. 4E**). Taken together, these findings demonstrate the successful utility of the Seahorse platform to interrogate the metabolic activity of purified peroxisomes.

The high degree of consistency between biological replicates and treatment groups in this study highlights the utility of measuring OCR to determine the oxidative activity of peroxisomes (**Fig. 4**). Mitochondria consume oxygen by producing carbon dioxide (CO_2) and peroxide (H_2O_2) as byproducts of the β-oxidation of lipids and synthesis of ATP through the electron transport chain *via* the release of water. By comparison, peroxisomes consume oxygen solely in the production of the H_2O_2 byproduct [1,2], which is enzymatically metabolized *via* catalase to produce O_2 and H_2O, a function that mitochondria lack. As such, we contend that the peroxisomal production of H_2O_2 should closely mirror β-oxidation rates. Supporting this notion, we found that relative to no treatment controls, addition of fatty acids and/or acetyl-CoA to purified peroxisomes significantly increased H_2O_2 production while treatment with enoxomine significantly suppressed this effect (**Fig. 4G**). Future investigations using isotopically labeled fatty acids where the donor oxygen is radiolabeled would provide a more selective methodology to directly correlate our Seahorse and peroxide data. However, the advent of platforms such as the Seahorse and Oroboros® now offers highly sensitive and reproducible instrumentation for accurately measuring subtle changes in oxygen consumption. Given also the popularity and accessibility of these systems for studying mechanisms of metabolism, the protocols and refinement of reagents defined in this study will help usher in a new era of studies on peroxisomal function.

We have demonstrated the successful implementation of an improved protocol that permits the real-time measurement of the oxidative capacity of purified peroxisomes. Additionally, the procedures reported here expand upon previously accepted and published methods for isolating peroxisomes that ensure minimal contamination from other cellular organelles. Moreover, we developed a straightforward and simple protocol for assessing the oxidative potential of isolated peroxisomes under a variety of metabolic conditions using the readily available Seahorse instrumentation. We strongly believe this application can be further utilized to compare peroxisomal function across different tissues and genotypes, opening up new avenues of study for understanding the organelle-specific contributions to metabolic disease. Furthermore, we anticipate that in the future, this protocol will be leveraged to investigate the metabolic connections and interplay between mitochondria and peroxisomes. This endeavor will likely produce key insights that would aid in further clarifying how these two organelles synergistically function and how disruptions to either organelle impact disease

progression and outcomes.

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