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Research article

A novel *ex vivo* method for measuring whole brain metabolism in model systems

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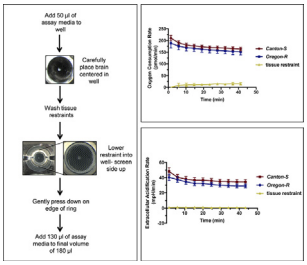
HIGHLIGHTS

- A novel method for measuring metabolism in *Drosophila* brains is presented.
- Method utilizes newly designed tissue restraints with XFe96 metabolic analyzer.
- Oxygen consumption and acidification rates are detected from single brains.
- Different metabolic responses are observed after drug treatment in transgenic brains.
- This method can be applied to other small model systems, as well as other tissues.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Many neuronal and glial diseases have been associated with changes in metabolism. Therefore, metabolic reprogramming has become an important area of research to better understand disease at the cellular level, as well as to identify targets for treatment. Model systems are ideal for interrogating metabolic questions in a tissue dependent context. However, while new tools have been developed to study metabolism in cultured cells there has been less progress towards studies *in vivo* and *ex vivo.*

New method: We have developed a method using newly designed tissue restraints to adapt the Agilent XFe96 metabolic analyzer for whole brain analysis. These restraints create a chamber for *Drosophila* brains and other small model system tissues to reside undisrupted, while still remaining in the zone for measurements by sensor probes.

Results: This method generates reproducible oxygen consumption and extracellular acidification rate data for *Drosophila* larval and adult brains. Single brains are effectively treated with inhibitors and expected metabolic readings are observed. Measuring metabolic changes, such as glycolytic rate, in transgenic larval brains demonstrates the potential for studying how genotype affects metabolism.

Comparison with existing methods and conclusions: Current methodology either utilizes whole animal chambers to measure respiration, not allowing for targeted tissue analysis, or uses technically challenging MRI technology for *in vivo* analysis that is not suitable for smaller model systems. This new method allows for novel metabolic investigation of intact brains and other tissues *ex vivo* in a quick, and simplistic way with the potential for large-scale studies.

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1. Introduction

An increasing number of neurological diseases and disorders with observed changes in metabolism have been identified in the last ten years. Mutations in metabolic enzymes were identified in Glioblastoma (GB) in 2008 (Parsons et al., 2008; Yan et al., 2009), and since more effective treatments have been developed by targeting metabolic vulnerabilities (Cairns et al., 2011; Hanahan and Weinberg, 2011; Ward and Thompson, 2012; Wolf et al., 2011; Zhao et al., 2013). Metabolic dysregulation in neurodegenerative disorders, such as Alzheimer's disease, Huntington's disease, and Parkinson's disease have also been identified (Atamna and Frey, 2007; Cai et al., 2012). Mitochondrial dysfunction and altered metabolism have been associated with mental health disorders, including major depressive disorder (MDD) (Brody et al., 1999), and schizophrenia (Prabakaran et al., 2004). This is not a new medical phenomenon, but instead reflects the progress made in the field of metabolism, including new tools for studying metabolic change.

Investigating metabolism in tissues following disease or genetic alteration has become a promising direction for new, targeted treatment options (Vander Heiden, 2011). Many new tools and instruments have emerged for studying metabolism in cells, but there has been less advancement in methods developed to analyze metabolic changes at the whole organ/tissue level. We are interested in the metabolic changes that occur in the brain, both glia and neurons, following genetic alterations. Using a small model system, such as *Drosophila*, we can investigate brains of transgenic animals expressing disease genes, specifically in glial cells and/or neurons. It is important to isolate the brain for this type of analysis since transgenes will only be expressed in this tissue, and slight changes in metabolism will likely not be detectable when measuring the whole animal. While methods exist to study metabolic reprogramming in cell culture, this technology does not enable the whole organ metabolism to be captured; missing interactions between different cell types that may contribute to the overall energy utilization of the tissue.

Current methodology either utilizes whole animal chambers to measure respiration, not allowing for targeted tissue analysis (Frappell et al., 1989; Lighton, 2000; Withers, 2001), or uses technically challenging MRI technology for *in vivo* analysis that is not suitable for smaller model systems (Liu et al., 2011). A user-friendly technology was developed in the early 2000′s by Seahorse Biosciences that non-invasively measures metabolic activity in cell culture (Ferrick et al., 2008). The XF Extracellular Flux Analyzer measures oxygen consumption and extracellular acidification simultaneously and is capable of delivering four separate injections of drugs and/or inhibitors to challenge cells metabolic response. These two parameters report on mitochondrial respiration and glycolytic respiration, respectively. Drug treatments provide further insight into a cells energy utilization, including fatty acid oxidation, nutrient preference, and the ability to shift metabolic program. This metabolic analyzer has greatly impacted the field of metabolism allowing for fast, reproducible results studying primary and established cell lines, including cancer, cardiac, and neuronal cell lines (Hardie et al., 2017; Kwang Kim et al., 2015; Xu et al., 2017). This instrument, however, cannot measure whole tissues (other than spheroids) in the 96-well format- a format that is crucial for the accurate measurement of small tissues. The XFe24 platform has larger wells that measure an increased volume of media above the cell sample. This does not allow for the sensitivity required to detect small metabolic changes in single brain samples. A requirement of this technology is a sample's ability to adhere to the bottom of the well during analysis. While non-adherent cells are easily plated using poly-lysine or other coating agents, the geometry of the brain and other whole tissues have difficulty adhering. This challenge has made this technology inaccessible for studies on whole brains in *Drosophila*. The ability to measure whole brains *ex vivo*, while still not an *in vivo* analysis, would provide close insight into how the organ is functioning at the metabolic level. Studies have shown that dissected *Drosophila* brains can be kept alive for hours in the proper media and under certain conditions (Williamson and Hiesinger, 2010). This suggests that short metabolic assays will yield biologically relevant data from live tissue.

In this study, we have designed and produced micro-tissue restraints (Tipping and Waters, 2017) that, when placed in the well of an XFe96 cell plate, hold the tissue in place for proper measurement. We have used this new technology to develop a method for measuring the energy utilization of whole *Drosophila* larvae and adult brains. We are able to chemically challenge these brains using the drug delivery ports of the XFe96 cartridge and observe changes in the metabolism of these organs. We also interrogated larval brains expressing genetic mutations that resulted in significantly different metabolic effects from wild type larval brains.

This new methodology can be used more broadly, extending to other insects and model systems, such as *C. elegans* (Fig. 5B). Small model systems have used the XFe24 platform for metabolic analysis (Gibert et al., 2013), but utilizing this new method will decrease the sample size required for assays and increase the sensitivity. This method can be applied beyond the brain to investigate other tissues in small model systems (Fig. 5A).

2. Materials and methods

2.1. Drosophila melanogaster stocks

The following *D. melanogaster* stocks were obtained from the Bloomington Drosophila Stock Center (Indiana University): *w[1118]; P{w[+mC]=UAS-Idh.R*$_{195}$*H·FLAG}3* [RRID:BDSC_56203], *IMP-L3 (LDH) RNAi* transgenic fly*: y[1]v[1];P{TRiP.HMS00039}attP2* [RRID:BDSC_33640], *w[1118]; P{w[+m*]=GAL4}repo/TM3, Sb[1]* [RRID:BDSC_7415], *Oregon-R-C* [RRID:BDSC_5], *Canton-S* [RRID:BDSC_64349]. All stocks were reared at 60% humidity and 25 °C in a cornmeal, molasses and yeast based food. Late third instar wandering larva were used for larval brain studies. Two to five day old adult male and female flies were used for adult brain studies.

2.2. C. elegans cultivation

N2 Bristol (strain VC2010) was used as wild-type and grown on NGM agar plates (For 1 l in water: 3 g NaCl, 17 g agar, 2.5 g Peptone, and 1 ml 1 M CaCl2, 1 ml 5 mg/ml cholesterol in ethanol, 1 ml 1 M MgSO4, and 25 ml 1 M KPO4 added after autoclaving) seeded with *E. coli* (strain OP50).

2.3. C. elegans synchronization for metabolic analysis

Gravid wild-type worms (egg bound) adults were treated with hypochlorite solution (for 5 ml in water: 0.5 ml 5N NaOH, 1 ml household bleach containing 5% sodium hypochlorite) for no longer than 3 min and washed 3 times with M9 media (for 1 l in water: 3 g Kh2PO4, 6 g NaHPO4, 5 g NaCl, 1 ml 1 M MgSO4, filter sterilized) to collect embryos. Embryos were placed in M9 media at 20° C overnight with agitation and allowed to hatch as starved L1s. L1 worms were then counted and plated on seeded NGM agar plates (as above) and grown to L4 stage. L4 worms were visually inspected and picked from the plate into M9 and washed 3 times in M9 to remove *E. coli* carried over from the plate. These L4 worms were then counted and added to the cell plates for analysis. Basal OCR rates were measured in M9 for 10 cycles according to the protocol described in Koopman et al., with each cycle consisting