Video Article

Metabolic Analysis of *Drosophila melanogaster* Larval and Adult Brains

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Abstract

This protocol describes a method for measuring the metabolism in *Drosophila melanogaster* larval and adult brains. Quantifying metabolism in whole organs provides a tissue-level understanding of energy utilization that cannot be captured when analyzing primary cells and cell lines. While this analysis is *ex vivo*, it allows for the measurement from a number of specialized cells working together to perform a function in one tissue and more closely models the *in vivo* organ. Metabolic reprogramming has been observed in many neurological diseases, including neoplasia, and neurodegenerative diseases. This protocol was developed to assist the *D. melanogaster* community's investigation of metabolism in neurological disease models using a commercially available metabolic analyzer. Measuring metabolism of whole brains in the metabolic analyzer is challenging due to the geometry of the brain. This analyzer requires samples to remain at the bottom of a 96-well plate. Cell samples and tissue punches can adhere to the surface of the cell plate or utilize spheroid plates, respectively. However, the spherical, three-dimensional shape of *D. melanogaster* brains prevents the tissue from adhering to the plate. This protocol requires a specially designed and manufactured micro-tissue restraint that circumvents this problem by preventing any movement of the brain while still allowing metabolic measurements from the analyzer's two solid-state sensor probes. Oxygen consumption and extracellular acidification rates are reproducible and sensitive to a treatment with metabolic inhibitors. With a minor optimization, this protocol can be adapted for use with any whole tissue and/or model system, provided that the sample size does not exceed the chamber generated by the restraint. While basal metabolic measurements and an analysis after a treatment with mitochondrial inhibitors are described within this protocol, countless experimental conditions, such as energy source preference and rearing environment, could be interrogated.

Video Link

The video component of this article can be found at https://www.jove.com/video/58007/

Introduction

Metabolic reprogramming has been identified in many neurological diseases, including Glioblastoma Multiforme (GBM), Huntington's disease, and Major Depressive Disorder (MDD)[1,2,3]. As metabolism becomes the focus of therapeutic strategies, tools for basic metabolic research have advanced. However, most of these methods were designed to study cell lines and primary cells or to analyze larger tissues post-fixation or -freezing. Some approaches have relied on kits to simplistically measure specific metabolites, while others have utilized more costly and complex analyses utilizing chromatography in combination with mass spectrometry[4] for the same goal. To understand the larger metabolic landscape, metabolic profiling[5,6] and metabolic flux analysis (MFA)[7] emerged to complement the large-scale proteomic and genomic studies. Profiling provides a quantitative representation of metabolites in a cell or tissue at one point in time, while MFA expands upon this by allowing the tracking of labeled metabolites over time. The latter has been useful in revealing how energy sources are utilized differently in a cell or tissue when in a disease state[8]. However, these methods do not include the measurement of the overall metabolic rate.

To interrogate the overall metabolic state of the small model systems, traditional methods, such as the Clark electrode[9] and indirect calorimetry[10], can be used to measure oxygen consumption or configured for stop flow respirometry, to measure oxygen and carbon dioxide concentration, respectively. These techniques, while accurately providing insight into the readout of metabolic reprogramming at the organism level, have limitations. The use of the Clark electrode can be technically challenging and is not designed for high-throughput studies. The stop flow respirometer cannot function with the sensitivity required to assay cells or tissues. Several years ago, new technology was developed specifically for these smaller applications[11]. These instruments were initially designed to measure the oxygen consumption and extracellular acidification of cell lines and primary cells in a 24- or 96-well format. The simplicity of the set-up and data output established this method as an alternative to the traditional approaches. This methodology is a powerful tool for cells, and recent advances have allowed for the measurement of tissue punches[12,13,14]. However, the methods utilized in these assays do not allow for the measurement of whole organs from small model systems.

The metabolic analysis of disease models often involves the interaction between cells of specialized function residing within the same tissue. For example, glial cells produce metabolites utilized by neurons. These metabolic interactions are required for the neuronal survival[15]. Small model systems are advantageous for investigating questions such as these. Studies to measure the metabolism of a whole organ, containing a variety of cell types, will add to the understanding of the energy utilization *in vivo*. Recently, Becker *et al.* reported a method of measuring the oxygen

consumption in whole fly heads[16]. By pooling a number of heads together in one well of a 24-well cell plate, oxygen consumption readings were obtained using a metabolic analyzer. While this works well for heads, which are easily separated from the body, it is much more difficult to use for organs such as larval brains, because a large quantity needs to be dissected for this method. Therefore, a method utilizing a 96-well cell plate, which increases the sensitivity of the oxygen consumption and extracellular acidification readings, was developed to assay a single whole larval brain.

The metabolic analyzer used in this study requires the sample being measured to remain at the bottom of the well of a 96-well cell plate. For cells and relatively flat tissues, this is not a challenge; however, for *D. melanogaster* larval and adult brains, it is not possible using traditional plate coating protocols. The spherical three-dimensional shape of the brains will not reliably adhere to the plate surface, and those that do are often damaged while attempting to place them properly in the well. A critical part of this new protocol is the design and development of micro-tissue restraints[17] that provide a small chamber for the brain to reside in without interfering with the metabolic measurements. The chamber generated is approximately 0.016 in in height and provides enough space to easily hold many *D. melanogaster* brains. The restraints consist of nylon mesh attached to an inert polymer ring and will be discussed further in the **Representative Results**. Using these restraints minimizes the time required to prepare dissected brains for the metabolic measurement. Optimizing the measuring procedure generates steady, reproducible oxygen consumption and extracellular acidification rates after six measurement cycles (of 25 min). The brains remain metabolically active under these conditions for a minimum of 2 h, which enables injections of therapeutics, inhibitors, or other treatments *via* the metabolic analyzer cartridge drug delivery ports. This protocol can also be easily adapted for other tissues and small model systems[18].

The protocol detailed below describes how to assay the oxygen consumption in a whole *Drosophila* larval brain when challenged with mitochondrial stress. To stress the brain, oligomycin is added to inhibit the ATP synthase[19]. The decrease in oxygen consumption from basal readings shows a measurement of the amount of ATP-depending respiration in the brain. Further decreases in the oxygen consumption after treatments with rotenone[20] and antimycin A[21], inhibitors of electron transport chain complexes I and III, respectively, indicate the amount of non-mitochondrial oxygen consumption in the brain. This assay is one example of how mitochondrial respiration in a fly brain might be compared and how this protocol can be used to compare the metabolism in varying genotypes. However, this protocol can be adapted to measure simply basal oxygen consumption and extracellular acidification rates, as well as to design more elaborate studies investigating specific energy utilization or substrate utilization hypotheses.

Protocol

1. Assay Preparation (Day 1)

1. Place the metabolic analyzer (**Table of Materials**) in an incubator or a temperature-controlled room set to 11 °C.
   NOTE: This temperature is ideal for measurements taken at 25 °C, as it allows for the ~14 °C temperature fluctuation that occurs while running the assay. This is caused by the heat generated by the instrument during the run.
2. Open the appropriate software on the computer attached to the metabolic analyzer. Click on the numerical temperature in the bottom left side of the screen. In the new window that opens, click on the box next to the "Heater on" command and ensure a check mark appears. Adjust the temperature to 25 °C and adjust the tolerance range to 0.2 °C using the arrows.
   NOTE: Several hours are required to achieve stable temperatures.
3. Open the cartridge container (**Table of Materials**) and remove the cartridge from the utility plate without touching the probes.
   NOTE: The utility plate is used during the calibration of the cartridge and is not used while the metabolic assay is run.
4. Add 200 µL of a calibrant solution (**Table of Materials**) to the utility plate and place the cartridge back on top of the utility plate to rehydrate the sensor probes on the cartridge. Do not touch the probes and protect them from light.
5. Seal the cartridge with the paraffin film.
6. Incubate the cartridge overnight at 25 °C.

2. Assay Media Preparation (Day 2)

1. Add 10 mM glucose and 10 mM sodium pyruvate to the assay medium.
2. Incubate the medium at 25 °C until the liquid has equilibrated to this temperature.
3. Adjust the pH to 7.4 using a pH meter.

3. Setup of the Software for the Metabolic Analysis of the *Drosophila melanogaster* Brains (Day 2)

1. Set up the metabolic software on the metabolic analyzer used in step 1.2.
2. Select "Template" on the software home screen.
3. Double-click on "Blank" to start a new assay design.
4. Click on the "Plate Map" button in the top toolbar to view the design of the cell assay plate.
   NOTE: The cell assay plate will contain the brain samples and will be paired with the cartridge before beginning the metabolic assay run.
5. **Click the Add groups button 3x.**
   1. Double-click on the "Group 1" label and rename it to "Experimental".
      NOTE: This group will contain a fly brain and a micro-tissue restraint treated with drugs.
   2. Double-click on the "Group 2" label and rename it to "Control".
      NOTE: This group will contain a fly brain and a micro-tissue restraint with no drug treatment.
   3. Double-click on the "**Group 3**" label and rename it to "**Restraint only**".
      Note: This group will contain a micro-tissue restraint without any brains or drug treatment.

6. **Assign the wells to each group based on where samples in the cell plate are intended to be placed.**
   1. Click on the "Experimental" label and then highlight wells B2 - B8 on the plate map.
   2. Click on the "Control" label and then highlight wells C2-C8 on the plate map.
   3. Click on the "Restraint only" label and then highlight wells D2-D8 on the plate map.
   4. Check that all four corners (A1, A12, H1, and H12) are designated as background.
      Note: Wells along the perimeter of the plate are not used to reduce any edge effects. This is a default setting in the software.

7. **Click on the "Protocol" button in the top toolbar to set up the protocol.**
   1. Click on the "Edit measurement details" in the baseline measurement box.
   2. Adjust the time that the metabolic analyzer will measure the brain by changing the basal measure cycle time to "3:00" by clicking the up and down arrows.
   3. Adjust the time that the metabolic analyzer will wait between brain measurements by changing the basal wait cycle time to "0:00" by clicking the up and down arrows.
   4. Adjust the time that the metabolic analyzer will mix the media prior to measuring the brain by changing the basal mix cycle time to "1:00" by clicking the up and down arrows.
   5. Adjust the total number of basal cycles, which includes the measure, wait, and mix cycles, to "7" by clicking the up and down arrows.
      NOTE: Stable Oxygen Consumption Rate measurements are obtained after ~25 min from the start of the assay.
   6. Click on the "Add injection" button, located in the upper left toolbar.
   7. Click on the "Injection label" and change it to "Oligomycin".
   8. Adjust the injection measure, wait, and mix times to match those for the basal.
   9. Adjust the total number of injection cycles to "6" by clicking the up and down arrows.
   10. Repeat steps 3.7.6 - 3.7.8 but change the label to "Rot/AA" and adjust the total number of injection cycles to 7 by clicking the up and down arrows.
   11. Click on the "Run Assay" button in the top toolbar.
   12. "Save" the assay and begin setting up the assay cartridge (**Table of Materials**).

4. Preparation of the Cartridge for the Calibration (Day 2)

1. **Remove the cartridge from the 25 °C incubator and take off the cover.**
   1. Add 20 µL of 100 µM oligomycin to the small upper left circular injection port, port A, in the cartridge for all corresponding experimental wells on the cell plate and add 20 µL of assay media to port A in all other wells, including the control and background wells.
      NOTE: The cartridge contains 4 ports (A-D) for each of the 96 wells corresponding to the cell plate what will contain the samples. This step is done prior to adding the samples. Adding 20 µL of 100 µM oligomycin results in a final oligomycin concentration of 10 µM in the cell plate well once the drug is injected by the metabolic analyzer. Oligomycin is an inhibitor of ATP synthase. The resulting oxygen consumption value will reflect the amount of ATP-linked respiration in the brain. In order to properly inject the drugs into the specified wells, all ports of the same letter must be filled with the same volume of liquid, even if not in use.
   2. Add 22 µL of 50 µM rotenone/antimycin A to the small upper right circular port, port B, in the cartridge for all corresponding experimental wells on the cell plate, and add 22 µL of assay media to port B of all other wells, including the control and background wells.
      NOTE: This results in a final rotenone/Antimycin A concentration of 5 µM in the cell plate well once the drug is injected by the metabolic analyzer. Rotenone and antimycin A are inhibitors of electron transport chain complexes I and III, respectively. The resulting oxygen consumption value will reflect non-mitochondrial respiration in the brain.
   3. Click "Start run" to place the loaded cartridge with the utility plate into the metabolic analyzer with well A1 positioned in the upper left corner when facing the instrument, with the plate loader extending from the right side of the machine.

2. Select "I'm Ready" in the software to begin the equilibration and calibration of the cartridge prior to beginning the metabolic assay with the cell plate containing the samples.
   Note: The program will ask to save the file and then begin equilibrating and calibrating. These steps can take up to 1 h. Steps 5–8 are conducted during the equilibration and calibration time.

5. Preparation of the Micro-tissue Restraints (Day 2)

1. Select 1 micro-tissue restraint per brain that is being measured, plus at least 3 for use as restraint-only controls, from the storage container (contains 70% ethanol).
2. Rinse the restraints with fresh 70% ethanol and wash them with deionized water 3x for 2 min each, using a mesh basket and 6-well plate (**Table of Materials**). Add additional water washes if the restraints still give off an alcohol odor.
3. Wash the micro-tissue restraints in assay media and leave them in this solution until they are ready for use.

6. Dissection of the *Drosophila melanogaster* Larval Brains (Day 2)

1. Select late (wandering) third instar larvae from a vial of cultured *Oregon-R* flies using high precision, style 5 (0.10 x 0.06 mm^2) tweezers.
2. Place the larva in the well of a clean dissecting spot plate (**Table of Materials**) containing 500 µL of 1x PBS.
   NOTE: A spot plate is a glass plate with depressions, used to dissect small tissues and organisms.
3. Wash the larva by gently shaking it in the well, using tweezers.
4. Move the larva to the clean well of a spot plate containing 500 µL of 1x PBS.
5. Place the spot plate under the dissecting microscope.
6. Grasp the larva at its midsection with one pair of tweezers, while grasping the eye hooks with a second pair of tweezers.

7. Gently and smoothly pull the larva in opposite directions using the two sets of tweezers.
8. Visualize the brain, which typically stays attached to the eye hooks and will commonly have eye-antennal discs attached to it.
9. Carefully remove additional tissues from the brain, using the eye hooks to hold the brain in place. Lastly, separate the eye hooks from the brain.
10. Use tweezers to move the dissected brain to a new well on a spot plate containing 1x PBS.
11. Repeat steps 6.1–6.10 until 14 brains are dissected.
   NOTE: The total time from the start of the dissection to the assay run should not exceed 30 min.

7. Addition of the Dissected Brains to the 96-well Metabolic Assay Cell Plate (Day 2)

1. Add 50 µL of assay media to the wells of the 96-well metabolic assay plate (**Table of Materials**) being used in the experiment, including the experimental, control, restraint-only, and background wells.
2. Carefully place one brain in each well from A2-A8 and B2-B8 of the cell plate, using tweezers, a spatula, or a pipette.
   NOTE: This may be done away from the dissecting microscope.
3. Under the dissection microscope, use a bent needle micro-probe to sink the brains to the bottom of the well.
4. Gently position the brain in the middle of the three raised spheres using the probe.

8. Addition of the Micro-tissue Restraints to the 96-well Metabolic Assay Cell Plate (Day 2)

1. Using tweezers, place the micro-tissue restraint on the edge of the assay plate well, and use the microscope to inspect that it is oriented with the plastic ring facing down and the mesh on top.
2. Remove the plate from under the microscope and use tweezers to grasp the restraint on both sides and gently drop it into the well.
3. Use a bent needle micro-probe to push the restraint down into the well.
4. Under the microscope, verify that the brain can be seen through the tissue restraint and that it is centered in the well.
5. Repeat steps 8.1–8.4 for all the wells that will be in the assay — A2-A8, B2-B8, and C2-C8.
6. Carefully add 130 µL of assay media to each of the experimental, control, and restraint-only wells.
7. Verify that the brains and micro-tissue restraints have not moved while adding the media by visualizing them under the microscope.
8. Add 180 µL of assay media to the four corner wells for use as background control.

9. Addition of the Cell Plate to the Metabolic Analyzer and the Start of the Assay (Day 2)

1. Verify that the equilibration and calibration are complete by waiting for the software to prompt for the cell plate containing the sample.
2. Select "Open Tray" and wait for the instrument to eject the utility plate.
3. Remove the utility plate and add the cell plate, without the lid, in the same orientation.
4. Select "Load Cell Plate" for the instrument to take in the cell plate and close the tray, and then begin the assay.
5. View the measurements in real-time with error bars on the computer screen running the software.
6. Remove the ejected cell plate and cartridge when the assay is complete.
   NOTE: Paired cartridges and cell plates can be re-used 5x.

10. Post-measurement Analysis (Day 2)

1. Use a dissecting microscope to verify that the brains and the micro-tissue restraints are still positioned properly in the well after the assay has completed. Exclude any wells with abnormalities from the analysis.
2. Export the Oxygen Consumption Rate (OCR) and the Extracellular Acidification Rate (ECAR) data for further analysis.

Representative Results

The protocol presented here requires the use of micro-tissue restraints that meet the specifications described below. Using other methods to hold the brains in place at the bottom of the 96-well cell plate were unsuccessful (**Figure 2A** and **2B**). First, various agents to increase the tissue adherence to the plate were tested. A commercially available tissue adhesive (**Table of Materials**) is recommended for the use of cells and organoids with cell plates and spheroid plates, respectively. Initially, brains appeared to adhere to the well surface; however, the oxygen consumption (OCR) readings were low, and observing the wells after the assay revealed that the brains were no longer attached to the well surface (**Figure 2A**). The same results occurred with super glue (**Figure 2A**). Next, manufacturing small screens to hold the brains within a small chamber at the bottom of the well was attempted (**Figure 2B**).

Metal screens produced high OCR readings alone, as did metal screens with a polymer ring holding the screen in place (**Figure 2B**). Plastic mesh netting was used with the same polymer ring. This device caused a negative OCR reading to be obtained. Finally, micro-tissue restraints were designed using an inert polymer for the ring measuring an outer diameter of 0.146 in, an inner diameter of 0.1335 in, a thickness of 0.0125 in, and a height of 0.016 in (**Table of Materials**). Super glue (**Table of Materials**) was used to attach the ring to a nylon mesh with a specific pore size of 0.0039 in in diameter (**Table of Materials**). The pore size is critical, as it allows the media and metabolite exchange without the formation of air bubbles, yet still acts as a barrier to the brains. These restraints alone result in little to no OCR reading, and when used with brains, allow for reproducible readings (**Figure 2A** and **2B**). The verification after the assay showed that the brains were still positioned properly in the wells. These tissue restraints are currently not commercially available but can be manufactured by following the specification above. While this requires precise manufacturing, most machine shops will be able to reproduce this restraint using the materials referenced above.

The protocol was further optimized to account for the assay media, the time allowed before the assay run, and the temperature (**Figure 3**). Initially, assays were set up in the Schneider medium to model the larval *in vivo* environment. However, the media used cannot contain buffering agents since pH is used to measure the ECAR. This media, thus, has to be custom made, which is costly. To optimize this, a commercially available assay media designed for metabolic analysis (**Table of Materials**) was used in place of the Schneider media. The OCR levels were unchanged, even when increasing the glucose levels slightly to model assay media conditions (**Figure 3A**). All assays from this point on were conducted in the assay medium. The supplements to the media were optimized by observing whether the oxygen consumption and the extracellular acidification rates showed a response when treated with known mitochondrial inhibitors. Next, the waiting time between the dissections and the assay runs was analyzed. An incubation of 3 h significantly dropped the OCR levels (**Figure 3B**). **Figure 3D** demonstrates the difference at the sixth time point, which is when the OCR and ECAR levels stabilize for both adult and larval brains. Thus, the protocol indicates to begin the assay immediately following the dissections, unless the experiment requires a temperature equilibration, in which case an incubation for less than 30 min is suggested. The metabolic analyzer is stored in an incubator set to 11 °C to allow for a 25 °C temperature throughout the assay. If the temperatures are elevated due to the ambient temperature and the instrument operation, reduced OCR levels are observed (**Figure 3C**). This is hypothesized to be due to tissue death.

When the optimized conditions are followed, the assays with the larval and adult brains result in readings of OCR slightly exceeding 150 pmol/min at the stabilized sixth time point, ~25 min into the assay (**Figure 4B**). This rate is maintained for at least 30 min (**Figure 4A**) and up to 2 h (data not shown). The ECAR is slightly lower in adult brains than in larval brains at the sixth time point (**Figure 4D**) and is maintained for at least 30 min (**Figure 4C**). This finding corresponds to an increase in glycolysis during the larval stages to support growth. An injection with a mitochondrial inhibitor, such as oligomycin, lowers the OCR readings to reveal the ATP-dependent respiration, and further treatment with rotenone and antimycin A lowers the OCR to reveal non-mitochondrial oxygen consumption (**Figure 5A**). This data demonstrates that these inhibitors are able to penetrate the brain tissue and can be used to compare mitochondrial respiration in brains of varying genotypes. For this assay, the mix, wait, and measure times were optimized by observing the oxygen levels, not the consumption rate, and ensuring that after the mixing, the oxygen level returned to the level prior to the measurement.
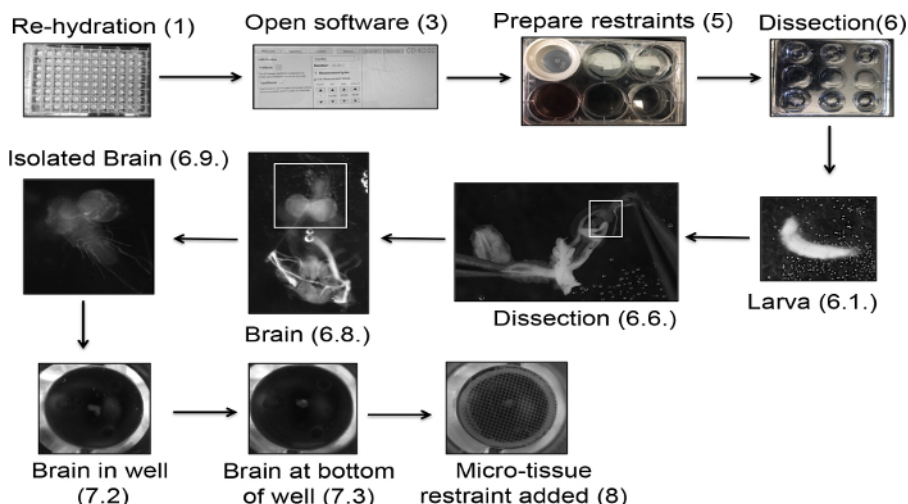

Figure 1: A schematic of larval brain OCR and ECAR measurements in the metabolic analyzer. The cartridge is prepared a day prior to running the assay. The next day, drugs are added to the injection ports of the cartridge. *D. melanogaster* larval brains are dissected, and micro-tissue restraints are added to secure the brain to the bottom of the well. The cell plate with the brains is assayed in the metabolic analyzer and the data is analyzed. Please click here to view a larger version of this figure.
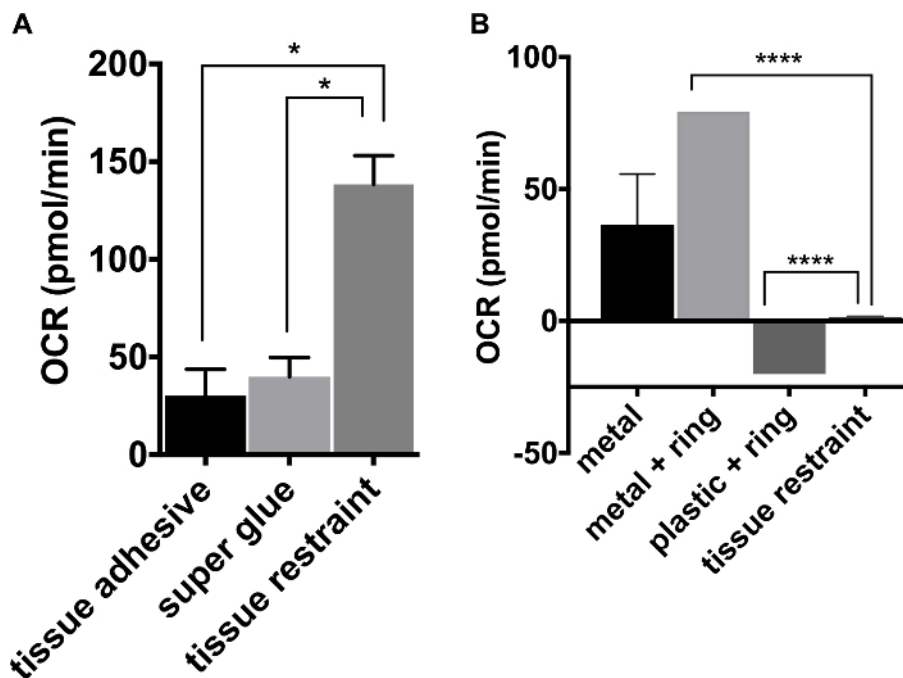
Figure 2: Micro-tissue restraints are not metabolically active and hold the brain in a chamber at the bottom of a plate well. (**A**) The OCR of *Oregon-R D. melanogaster* larval brains is measured in wells coated with tissue adhesive (**Table of Materials**) or when the brains are super glued to the bottom of the well. The results are compared to those of brains positioned at the bottom of the well using micro-tissue restraints. (**B**) The OCR is measured in wells containing assay media and metal, metal with a polymer ring, plastic mesh netting and a polymer ring, or micro-tissue restraints. * *p*-value <0.05, ** *p*-value <0.01, *** *p*-value <0.001, **** *p*-value <0.0001. Please click here to view a larger version of this figure.
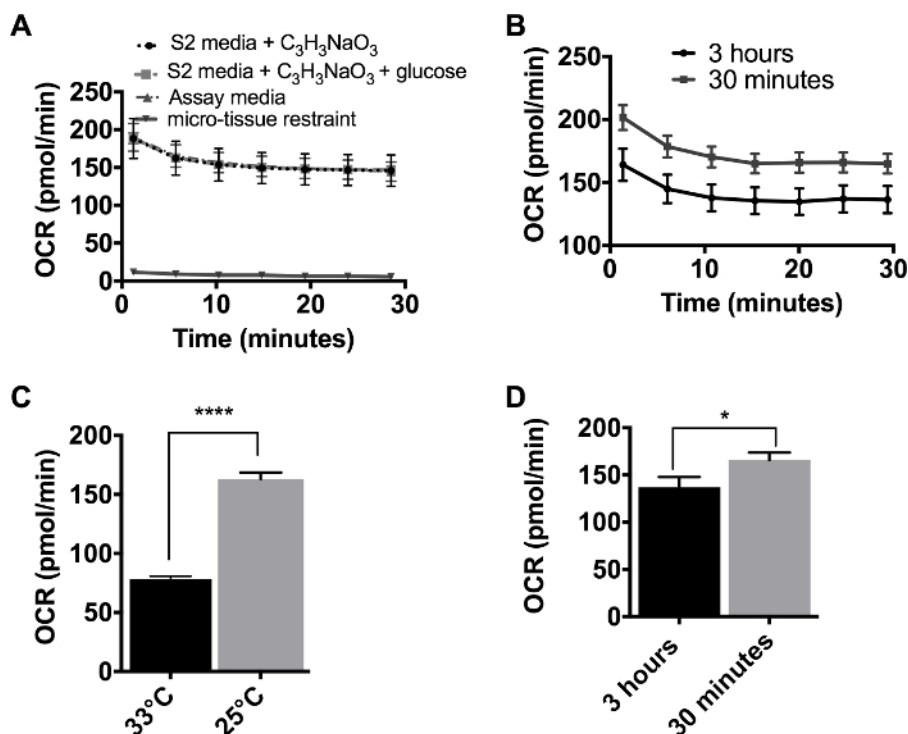

Figure 3: Optimization of the media, incubation time, and temperature. (**A**) The OCR is measured in *Oregon-R D. melanogaster* larval brains using Schneider media (S2) with sodium pyruvate added, S2 with glucose and sodium pyruvate added, and assay media with glucose and sodium pyruvate added. (**B**) The OCR is measured in larval brains after 3 h of incubation prior to the assay, or after 30 min of incubation prior to the assay. (**C**) The OCR is measured in larval brains at assay run temperatures of 33 °C and 25 °C. The sixth time point is graphed. (**D**) The OCR is measured in larval brains after 3 h of incubation prior to the assay, or after 30 min of incubation prior to the assay. The data is reported for the sixth time point. * *p*-value <0.05, ** *p*-value <0.01, *** *p*-value <0.001, **** *p*-value <0.0001. Please click here to view a larger version of this figure.
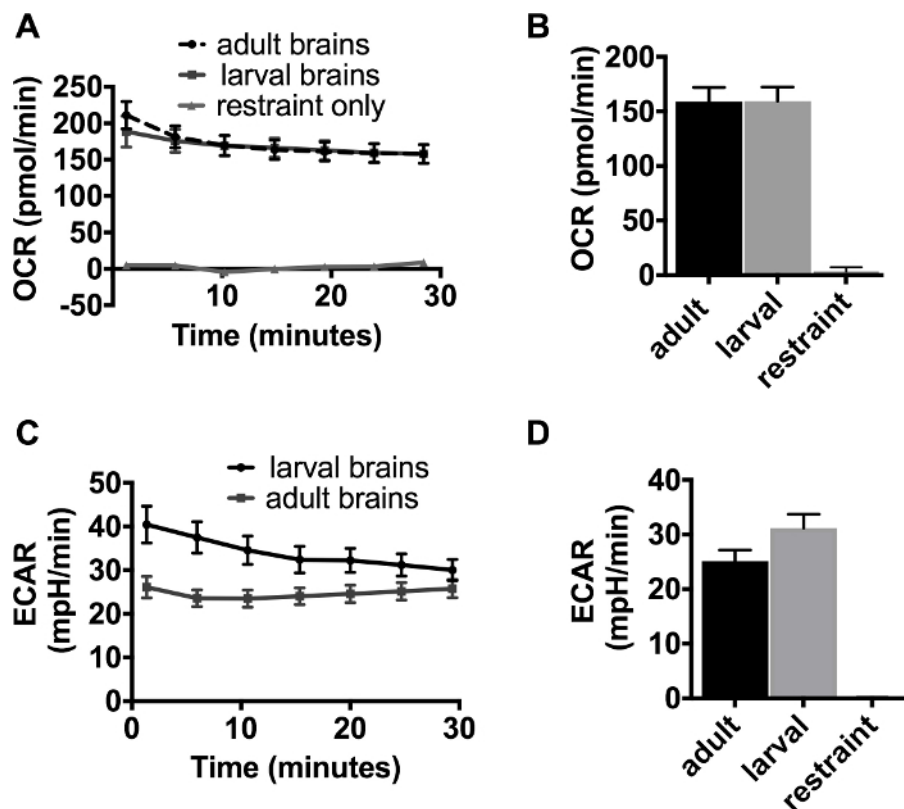
Figure 4: The OCR and ECAR are reproducibly measured in adult and larval *D. melanogaster* brains. (A) The OCR is measured in *Oregon-R D. melanogaster* adult and larval brains for 30 min. (B) The OCR data of the sixth time point from panel **A** is graphed. This is the time point at which the OCR readings stabilize. (C) The ECAR is measured in *D. melanogaster* adult and larval brains for 30 min. (D) The ECAR data of the sixth time point from panel **A** is graphed. This is the time point at which the ECAR readings stabilize. * *p*-value <0.05, ** *p*-value <0.01, *** *p*-value <0.001, **** *p*-value <0.0001. Please click here to view a larger version of this figure.
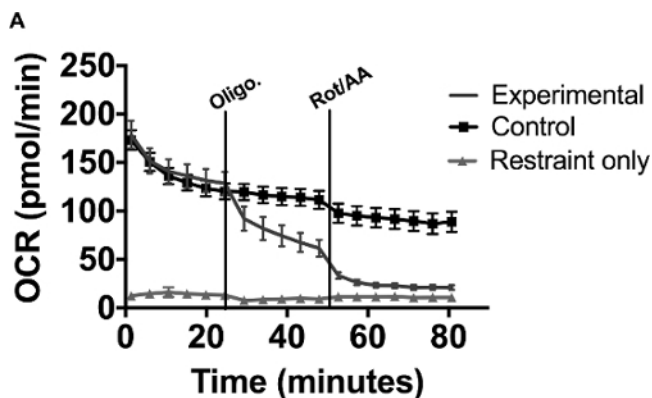

Figure 5: Oligomycin lowers the *D. melanogaster* larval brain OCR levels to reveal ATP-dependent respiration, and rotenone/antimycin A further lowers the OCR to reveal non-mitochondrial oxygen consumption. (A) The OCR is measured in *Oregon-RD. melanogaster* larval brains after a treatment with a 20 µM oligomycin and 5 µM rotenone/antimycin A mixture. The control and restraint-only wells were injected with assay media. Please click here to view a larger version of this figure.

Discussion

Here, a novel method for the metabolic analysis of *D. melanogaster* larval and adult brains *ex vivo* is described. Under basal conditions, oxygen consumption and extracellular acidification rates indicate how metabolically active the brain is and can determine whether a tissue has become more dependent on mitochondrial *versus* glycolytic respiration, respectively[11].

Treating the brains with inhibitors or therapeutic drugs can provide further information about the metabolic status of the tissue. Metabolic substrates can also be added to determine dependencies on specific metabolites, such as glucose to test for the Warburg effect[22] or glutamine to investigate glutaminolysis[23]—both common in metabolic reprogramming. For long-term studies, larvae or flies could also be fed drugs instead of using injection ports, and brains could be assayed at intervals or at the end-point, depending on the experimental design. This method can be optimized for numerous applications.

This method has been optimized for brains but can be used to analyze other larval[18] and adult tissues. Additionally, other small model systems can utilize this method to measure whole organisms[18] or tissues. The only limitation to optimizing this method for other systems is the size of the organ, tissue, or organism. The chamber created by the micro-tissue restraint is the size barrier to running the assay. If the metabolic output is too low for the brain or tissue being tested, and the sample size is not a constraint, pooling samples can be used to increase the assay measurements and sensitivity. Modifying this protocol for other applications should only require minor optimizations, including 1) choosing the appropriate measure and mix cycle numbers and timing, and 2) determining effective treatment concentrations for the tissue. The measure and mixing times were determined by the monitoring oxygen concentration measured by the metabolic analyzer during each cycle. This can be viewed by selecting **O₂** in the Y2 axis button in the overview window after the run is complete. During each cycle, there should be a drop-in oxygen concentration reflecting the tissue's oxygen consumption during the measured time, followed by a return to the initial oxygen concentration during the mixing cycle. The measure and mix times should be tested until this pattern is observed. Using this method, other insect brains have been studied. These brains were larger than the fly brains used here but still fit within the chamber generated by the tissue restraint. The OCR readings from these brains were as high as 400 pmol/min (data not shown). The results from these studies, as well as OCR rates that align with other studies measuring whole-fly oxygen consumption[18,24], suggest that the *D. melanogaster* brains were not oxygen-limited. For both *D. melanogaster* brains and tissues from other organisms, applying this method requires special attention to four critical steps.

To successfully achieve reproducible and biologically relevant measurements, there are critical steps of the protocol that must be followed. First, the use of a micro-tissue restraint is required for this method. Manufacturing these restraints using the specifications and materials listed is essential. The materials were selected due to their inert chemical composition, and their ability to allow proper media exchange during the mixing steps without the creation of air bubbles. Second, a timely dissection and plate preparation is required to maximize the metabolic output of the tissue. Short dissection times should not significantly impair the brain. Other studies have shown that fly brains can be kept alive for hours to days after dissection if properly stored in a perfusion chamber[25]. However, as seen in **Figure 3B** and **3D**, if brains are left sitting in assay media for long periods of time prior to the assay, the oxygen consumption rates are decreased. During the assay, samples undergo a mixing step during every cycle. This mixing not only aids with the injections of chemical compounds but also acts to recirculate the media and provide oxygen. The brains are able to stay metabolically active for longer periods during these measure-mix cycles than they would be incubating in media on the bench top. Therefore, the dissection of larval brains should be mastered before beginning to set up this assay. When setting up this assay, analyze a small number of genotypes at a time, to minimize the number of brains needed and wells utilized. This will prevent delays between the dissecting and the assay measurements. Third, maintaining a stable temperature is required to analyze the metabolic rates in physiologically relevant conditions. Increased assay temperatures can cause tissue death, observed by lower oxygen consumption rates (**Figure 3C**). If flies are reared at a different temperature for experimental purposes, the measurements should also be performed at this temperature. While dissections will most likely be performed at room temperature, if the assay will be conducted at a different temperature, the plated and restrained brains should be incubated at the required temperature for 15 min prior to running the assay. Lastly, observing the wells post-assay is critical to determine if tissue positioning affected the measurements. Occasionally, there will be one outlier well that, after observing the plate under the dissecting microscope, can be eliminated from the data analysis due to either loss of tissue or mispositioning, where the brain has slipped out of the center of the well and is stuck under the polymer ring of the restraint. Tissue can be lost if the restraint was not secured properly to the well and was disturbed during the mixing cycles. While neither of these events happens often, if they are not excluded from the analysis, they will lower the rate values substantially.

The measurement of whole-tissue metabolic flux by monitoring oxygen consumption and extracellular acidification provides a biologically relevant method for understanding how metabolism is altered by the genetic landscape or other experimental conditions. This method is sensitive enough to detect metabolic changes in a single *D. melanogaster* brain, which is not possible using stop-flow respirometry or indirect calorimetry. It is also less technically challenging than using a Clark electrode to measure oxygen consumption. An additional advantage of this protocol is the ability to analyze one whole brain per well. The 96-well format allows for more sensitive readings, and thus, more than one genotype can be assayed at a time due to the smaller number of samples required per assay. While measuring metabolism in tissue is still challenging and requires carefully reared and synchronized animals, this protocol describes a fast, relatively simplistic method, and has the potential to analyze numerous metabolic sensitivities in *D. melanogaster* larval and adult brains.

Disclosures

M. Tipping holds a provisional patent for the micro-tissue restraints described in this protocol[17].

Acknowledgements

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