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Measuring Energy Demands of Dynamic Remodeling Events During Embryogenesis in Live Drosophila Embryos

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Measuring Energy Demands of Dynamic Remodeling Events During Embryogenesis in Live *Drosophila* Embryos

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Abstract

Embryonic development is a complex process requiring dynamic cell movements to create complex tissue structures. We assume that these embryonic dynamic remodeling events are highly energy intensive and that this energy expenditure is mediated by mitochondria. However, this has been difficult to demonstrate in live embryos. Convergent extension of the *Drosophila* neuroectoderm is a promising system to study the bioenergetics of tissue remodeling because it changes from a static to a dynamically remodeling tissue with temporal precision. We hypothesize that the switch from static to dynamic development at the onset of convergent extension will be accompanied by an increase in metabolic energy production. Our method of measuring metabolic production is through the use of the Seahorse XF HS Mini Analyzer, which is capable of measuring bioenergetic processes in small numbers of cells. Notably, the Seahorse Analyzer measures Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR), which are indicators of energy production. I expect that an increase in metabolic production will be indicated by an increase in OCR and a drop in ECAR. By measuring these bioenergetic markers before and during convergent extension, the effects of dynamic tissue development on metabolism can be uncovered. All empty control wells resulted in OCR and ECAR readings near zero, and all wells with embryos measured between 100-300 pmol/min OCR. The Seahorse Analyzer could detect the presence of live embryos but was unable to detect consistent differences between one and multiple embryos in a well. These results were incapable of demonstrating whether metabolism increases or remains constant, as we did see an effect, but the data lacked consistency and the effect did not scale with the number of embryos.

Introduction

Embryonic development is a dynamic and complex process involving large groups of cells that move and change shape to form complex organisms. Therefore, we assume there must be significant changes in energy usage, mediated by mitochondria, to provide energy for these dynamic remodeling events (Yu and Pekkurnaz, 2018). However, it has been difficult to measure these processes in living embryos. One of these dynamic remodeling events is convergent extension. During convergent extension, the tissue narrows along one axis and extends along the perpendicular axis through dynamic cell movements (Ybot-Gonzalez et al., 2007). Convergent extension is well conserved throughout animal development and is seen in many contexts, notably the elongation of the head-to-tail axis in both vertebrate and invertebrate animals (Shindo, 2018). However, the function of mitochondria during convergent extension has not been well described. Mitochondria are very important in other epithelial remodeling events, such as epithelial wound closure, which shares many similarities with convergent extension (Arribat et al., 2019; Muliyil and Narasimha, 2014; Xu and Chisolm, 2014; Wegner et al., 2014; Hunter et al., 2018; Sood et al., 2015; Jones et al., 2018; Chowdhary et al., 2017). Therefore, due to the similarities to other epithelial remodeling events, we hypothesize that mitochondrial processes play a significant role in convergent extension as well.

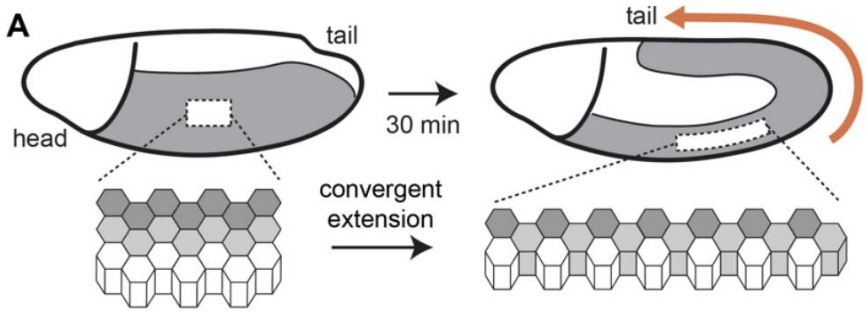


Figure 1 Legend: This diagram shows the embryo and tissue morphological changes resulting from convergent extension. The neuroectoderm (the gray area on the embryo) narrows along the dorsal-ventral axis and elongates on the head-tail axis. This elongation is mediated by cellular rearrangements.

The two main pathways of energy production in the cell are oxidative phosphorylation in the mitochondria and glycolysis in cytoplasm. Oxidative phosphorylation is much more efficient at creating ATP and involves the consumption of oxygen. We hypothesize that convergent extension is an energy intensive process, so we expect to see the amount of oxygen consumed by the embryos increase as they undergo oxidative phosphorylation. Conversely, we expect the embryos to undergo less glycolysis and thus less lactic acid fermentation, which would reduce the amount of acidic waste products excreted into the extracellular medium. The *Drosophila* embryo was chosen as the model organism in this study because the *Drosophila* neuroectoderm undergoes rapid convergent extension (~30 min) in a consistent, predictable time frame (**Figure 1**) (Irvine and Wieschaus1994). In theory, this temporal precision should allow us to measure Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) in a reproducible manner during convergent extension. We believe the OCR and ECAR will be interesting measurements because they are indicators of oxidative phosphorylation and lactic acid fermentation, respectively. Our method of measuring these metabolic indicators was to use a Seahorse XF HS Mini Analyzer. Seahorse Analyzers are designed to measure cultured cells, however, we wondered if the Seahorse Analyzer could be used to quantify OCR and ECAR in living *Drosophila* embryos, which has never been reported before.

Under high bioenergetic demand, I expect cells in the embryo to switch from glycolysis and lactic acid fermentation to oxidative phosphorylation in order to increase ATP production (Leulier and Lemaitre 2008; Abreu-Blanco et al. 2012). This switch should be indicated by a rise in oxygen consumption and a corresponding drop in extracellular acidification rate. To test this, I placed pre-convergent extension embryos into the Seahorse Analyzer and measured OCR and ECAR over the course of convergent extension to see if we could reliably quantify the metabolic processes in single *Drosophila* embryos.

Methods

Embryo Collection

For all runs of this experiment, OreR wild-type *Drosophila* embryos were used. Adult flies were kept in ventilated enclosures at 18°C and allowed to lay fertilized eggs (embryos) for approximately 20 hours. The embryos were collected on apple juice agar plates.

Embryo Dechorionation and Staging

Embryos were transferred from the apple juice agar plates to a mesh cell strainer and immersed in a 1:1 bleach: water solution for 2.5 minutes to strip the embryos of the chorion, or the outer eggshell. The embryos were then rinsed with tap water to remove the bleach solution. Dechorionated embryos were then dried and transferred to an agar plate and covered in halocarbon oil. This was necessary in order to better visualize the embryos by microscopy. Using a standard binocular dissecting microscope, stage 6 embryos (pre-convergent extension) were selected. To remove the halocarbon oil from the embryo, the embryos were rolled on parafilm using a paintbrush before being transferred into the Seahorse wells.

Seahorse Preparation and Run

To ensure the embryos stayed adhered to the bottom of the wells, 4 uL of scotch tape glue dissolved in heptane was pipetted into the Seahorse tray wells. The heptane evaporates quickly, leaving only the glue behind. The embryos, once adhered to the bottom of the wells, were immersed in 50 uL of phosphate - buffered saline (PBS). The tray was placed inside the Seahorse Analyzer for a run of either 1 or 3 hours at 25°C (in a cold room). To ensure accurate measurements, the Seahorse sensor cartridge was hydrated in a calibration plate in a CO2 free chamber overnight before the run. For each run, I used the standard XF Real Time ATP Rate Assay setup provided by the Seahorse Analyzer.

Results

Preliminary Runs: We encountered several issues in our preliminary runs. Notably, the OCRs were high but experienced a drop off after approximately 30 minutes in the wells, suggesting that the embryos were dying during the measurement process. These preliminary runs were done at 31°C, and we used heptane to remove halocarbon oil from the embryos after staging. We thought the heptane might be dessicating the embryos, making them fail to thrive once placed in the Seahorse Analyzer. To counter this problem, we devised a new method of halocarbon removal, which involved manually rolling the embryos along a parafilm sheet using a paintbrush. I found this to be effective in removing oil from the embryos while keeping the embryos alive. Also, our preliminary runs were performed in Dulbecco's Modified Eagle Medium (DMEM), which is a common cell medium for running Seahorse analyses. After these failed runs, we thought this medium may not be suitable for *Drosophila* embryo growth, so we decided to switch to a simple buffered salt solution, phosphate Buffer Saline (PBS), as *Drosophila* embryos do not need any outside growth factors or nutrients.

Run 1: In run 1, single embryos were placed into wells B through G, and we measured OCR and ECAR for around 50 minutes (**Figure 2**). Considering multiple embryos (Embryos C, D, and G) gave OCR and ECAR readings that were essentially zero, we were concerned there was some problem with the mounting or survival of the embryos during the analysis. Indeed, I discovered only Embryo B was still attached to the bottom of the well at the end of the run, and I could not find any other embryos in the wells at all. Therefore, I concluded that the poly-L-lysine glue we were using was not a strong enough adhesive to keep the embryos adhered to the wells throughout the process. After this run, I switched to standard scotch tape glue dissolved in heptane (this glue is known to be non-toxic to *Drosophila* embryos). In all further runs, I pipetted 4 uL of this glue into each well of a non-poly-L-lysine coated tray. The heptane would then evaporate, leaving the glue at the bottom of the well. Also, up to this point, all runs had been performed at 31°C. Considering the optimal temperature for raising *Drosophila* is 25°C, we thought the

embryos might be healthier at a lower temperature. After this point, we decided to perform the runs at 25°C by moving the Seahorse Analyzer to a cold room.

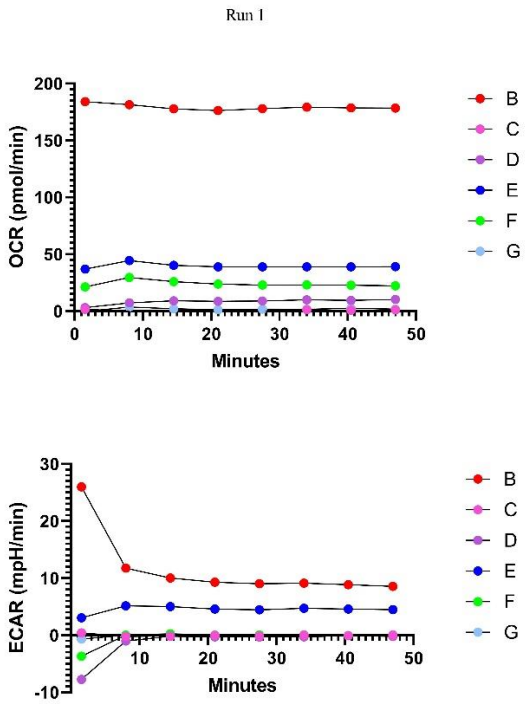
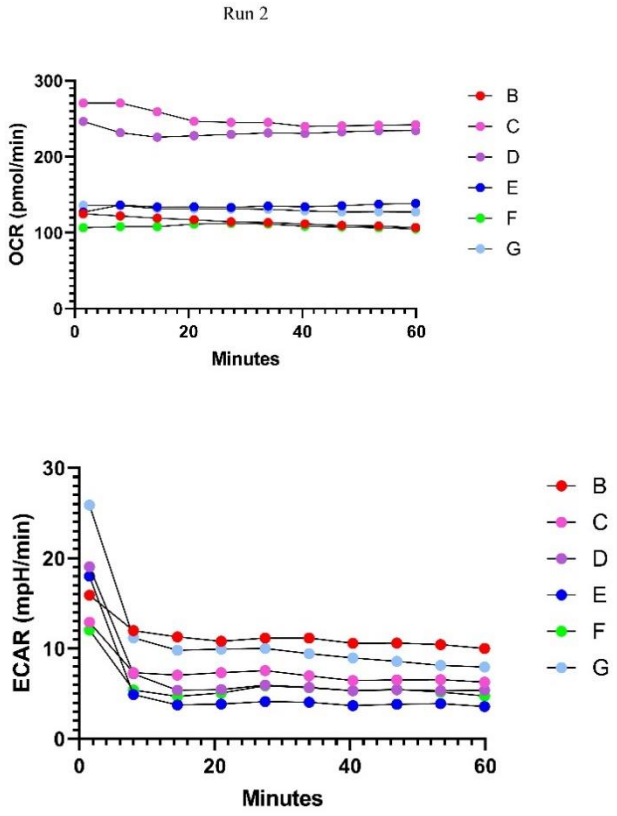


Figure 2 Legend: Run 1 measuring bioenergetic markers in live *Drosophila* embryos using PBS at 31°C. **Top**) Oxygen consumption rate (OCR) in single embryos per well. **Bottom**) Extra Cellular Acidification Rate (ECAR) in single embryos.

Run 2: During Run 2, all embryos stayed attached to the bottom of the wells. To test if all embryos survived the analysis, I examined them under a microscope after the Seahorse run, and I saw that they were all morphologically normal and appeared to be around stage 8, indicating that they had gone through convergent extension (stage 7) during the analysis, as expected. I noted the OCR readings from Run 2 were stable and well above zero, and the ECAR readings all began high before dropping off and stabilizing. However, there was no obvious connection between the OCR readings, as embryos C and D gave higher readings (around 250 pmol/min) compared with embryos B, E, F, and G (between 100-130 pmol/min). This led us to wonder whether these readings were accurately reflecting the metabolic processes within the embryos.



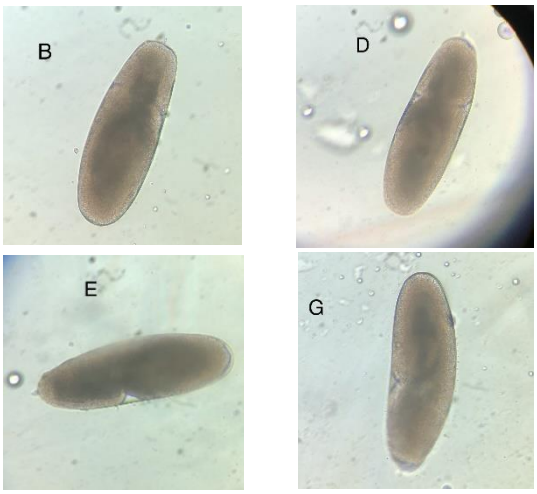


Figure 3 Legend: Run 2 using heptane suspended scotch tape glue at 25C. One embryo per well. **Top left**) OCR for individual embryos. **Bottom left**) ECAR for individual embryos. **Right**) Images of Embryos B, D, E, and G taken after the run was completed.

Runs 3 and 4: Next, I decided to test whether the effects we observed would scale based on the number of embryos in each well. Two separate runs were conducted with varying numbers of embryos per well. First, I conducted Run 3 with two wells each containing one, two, or three embryos per well. Data from this run showed no obvious correlation between number of embryos and OCR or ECAR (**Figure 4 left**). After consultation with Dr. Suresh Thallapuranam, head of the Bioenergetics Core facility, he suggested such small numbers of embryo may be insufficient to yield accurate measurements. For Run 4, I decided to test many more embryos per well, as well increasing the total number of measurement cycles. Staging, selecting, and placing the embryos is a labor-intensive process, so I could only collect 13 embryos before the embryos became too old. Therefore, I placed nine embryos in one well, three in another, and one embryo in another, leaving three wells empty; these empty wells were used as negative controls, and they received the same glue and PBS treatment as the experimental wells (**Figure 4 right**). Runs 3 and 4 both gave different readings between wells with the same number of embryos and the OCR readings did not consistently increase as numbers of embryos increased. Notably, the wells with multiple embryos did not yield measurements that were higher than previous runs with one embryo in each well (**Figures 2 and 3**). However, all the wells with zero embryos gave readings near zero (**Figure 4 right**).

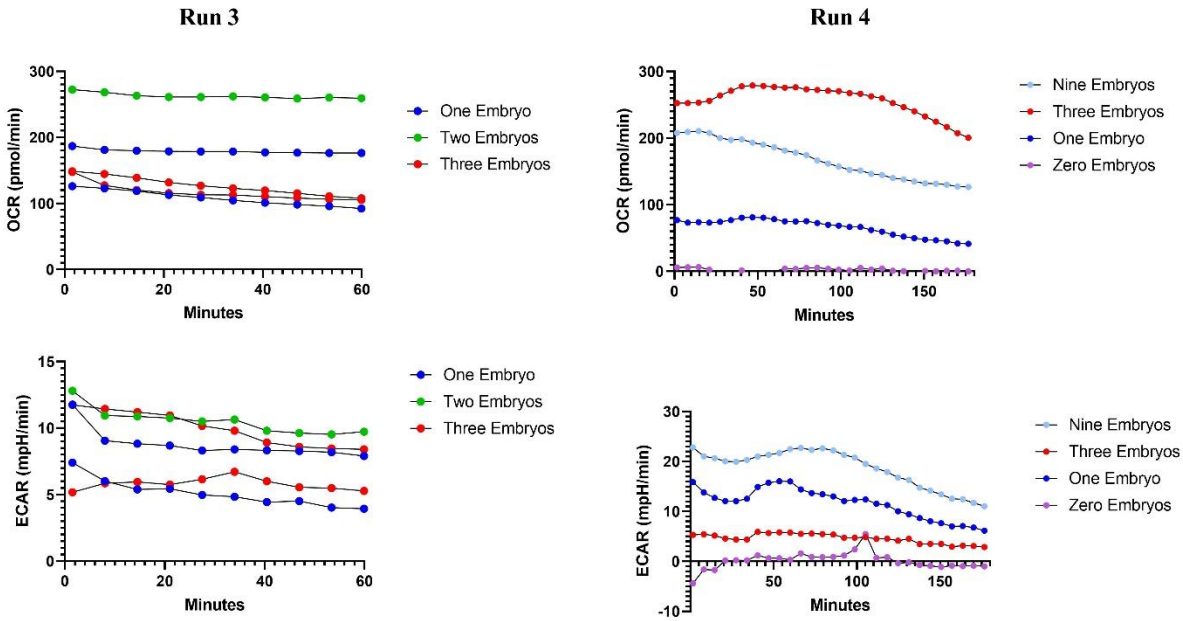


Figure 4 legend: Two separate runs (Runs 3 and 4) with multiple embryos in each well. **Left**) Run 3: OCR (top) and ECAR (bottom) data from a run with two wells containing one embryo, one well containing two embryos, and two wells containing three embryos. A well with two embryos was excluded after an embryo was found to be popped in post-run analysis. **Right**) Run 4: OCR (top) and ECAR (bottom) data from a run with wells containing zero, one, three, or nine embryos. This run was conducted for three hours (28 measurement cycles).

Discussion

During this experiment, I was able to collect pre-convergent extension embryos, adhere them to Seahorse Analyzer tray wells, and confirm the embryos survived the measurement. Using post-run imaging, I was able to confirm the embryos survived and continued development while inside the Seahorse Analyzer, so we have no reason to believe the inconsistency in the measurements we observed was due to development failure or death of the embryos. However, overall my results indicate that this experimental setup is unable to consistently quantify OCR or ECAR in individual *Drosophila* embryos.

My results demonstrate that the Seahorse Analyzer can differentiate between wells containing live embryos and empty wells. In particular, the empty control wells in Run 4 gave stable OCR and ECAR readings of effectively zero, while wells in the other runs with at least one embryo gave stable, non-zero readings. However, the Seahorse Analyzer measurements did not increase with increasing numbers of embryos. In Run 4, the well containing nine embryos gave OCR readings between 150-200 pmol/min. This measurement was actually lower than the Run-4 well containing three embryos (225-275 pmol/min), and it was not different than previous runs containing one embryo per well. For example, in Run 2, both Embryo C and Embryo D gave higher OCR readings than the nine-embryo well in Run 4. There was also no obvious difference between the wells with one, two, or three embryos in Run 3, with the OCR and ECAR readings showing no obvious relationship based on number of embryos. It is also important to note the differences in OCR and ECAR readings between wells with the same number of embryos. Run 2 included six wells containing one embryo each, and the readings gave no consensus result. Embryos C and D stabilized around 250 pmol/min while the other 4 embryos stabilized between 100-150 pmol/min. Furthermore, Runs 1, 3, and 4 included wells with one embryo, and these readings were not consistent with the readings from Run 2. In Run 4 the well with one embryo gave readings below 100 pmol/min, dipping as low as 50 pmol/min, while in Run 3 the single embryo wells stabilized around 100 pmol/min and 180 pmol/min. This wide range of measurements demonstrates that we cannot be confident in any one reading as an effective measurement of oxygen consumption or extracellular acidification. Overall,

our data indicates that the Seahorse Analyzer was not sensitive enough to quantify metabolic processes in single or small numbers of *Drosophila* embryos.

The Seahorse Analyzer manual suggests using a minimum of 5,000 cells per well. The *Drosophila* embryo contains 6,000 cells at the onset of convergent extension. However, we note that *Drosophila* cells are, in general, much smaller than typical mammalian cells, so it could be that the number of mitochondria in a single *Drosophila* embryo is not sufficient to be reliably measured by the Seahorse Mini Analyzer (Karaiskos et al., 2017). One publication used the Seahorse Analyzer to measure bioenergetic processes in *C. Elegans* in as few as 50 worms (Haroon and Vermulst, 2019). There are approximately 1,000 somatic cells per *C. Elegans*, equating to ~50,000 invertebrate cells per well in their experiments. Therefore, it is perhaps not surprising that one *Drosophila* embryo does not have sufficient mitochondrial mass to give reliable Seahorse measurements. A future challenge will be devise new methods to collect and place 20 or more similarly staged *Drosophila* embryos into individual wells and repeat these analyses.

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