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The validation of a sensitive, non-toxic *in vivo* metabolic assay applicable across zebrafish life stages

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Abstract

Energy expenditure and metabolism, is a well-studied field as it is linked to many diseases as dysregulation of metabolism is associated with cancer, neurodegeneration, and aging. Classical methods of studying metabolism *in vivo* are well established, but most are tedious and expensive, thus, finding methods of accurately measuring metabolism in living organisms that is quick and non-invasive is of strong interest. In this work, we validate the use of resazurin; a compound that is conformationally changed into fluorescent resorufin upon metabolic reduction by NADH₂, as a metabolic assay for adult zebrafish. This assay is based on the principle that increases in resorufin fluorescence intensity (FI) conveys relative changes in metabolic output of the organisms. We demonstrate the effectiveness of resazurin in measuring metabolic changes in zebrafish larvae and adults in relation to number of pooled fish, as well as temperature alteration. Moreover, we provide details on the appropriate and optimized diluents and concentrations of resazurin. Further, by using a novel sample collection technique, we can increase the temporal possibilities that were previously limited, as well as show that samples can be stored and measured at a later time point with no decrease in accuracy. Thus, the validation of this assay in adult zebrafish may increase the versatility and complexity of the types of experiments that can be performed and have many practical applications in the field.

Keywords

energy expenditure; NADH₂; metabolism; metabolic assay; zebrafish

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1. Introduction

Dysregulated metabolism has been linked to many diseases, such as cancer (reviewed in Simula et al., 2017), Alzheimer's disease (reviewed in Rojas-Gutierrez et al., 2017), and aging (reviewed in Finkel, 2015) and is therefore an important target of measurement. The definition of metabolism is the chemical processes that occur within a living organism to maintain life. *In vitro* methods of studying metabolism are useful, however limited, as it cannot fully capture the integration of all the systems involved in metabolism *in vivo*, thus finding methods of accurately measuring metabolism, or metabolic output, in a living organism is a target for these studies.

Zebrafish have recently been established as an excellent model for translational studies on metabolic disease for several reasons. The first one being that zebrafish have all the appropriate and key organs responsible for metabolism seen in humans. They have conserved hypothalamic circuitry, a known regulator of energy balance in vertebrates, as well as conserved insulin-sensitive tissues such as liver, muscle, and white adipose tissue (Seth et al., 2013). Further, the development of the pancreas is also well established, with the primary islet of the pancreas being visible at 24 hours post fertilization (Argenton et al., 1999), and the secondary islet at 5 days post fertilization (Hesselson et al., 2009). Zebrafish pancreas islet composition of insulin-secreting beta-cells and glucagon-secreting alpha cells is highly conserved.

Second, zebrafish share 90% genetic identity with humans (Barbazuk et al., 2000; Renquist et al., 2013), of which several genes have been shown to mirror human diseases upon dysregulation. For example, Song and colleagues (Song and Cone, 2007) validated a new model system to study obesity by creating a transgenic zebrafish overexpressing Agouti-related protein (AgRP) (endogenous melanocortin antagonist). These transgenic fish exhibited obesity, increased linear growth, and adipocyte hypertrophy, all hallmarks of the human condition. With the zebrafish genome fully sequenced and available (Howe et al., 2013), forward genetic approaches together with the recent technology of CRISPR can be used to generate more transgenic models to study human diseases in zebrafish. The use of zebrafish to study obesity, diabetes, non-alcoholic fatty liver disease (NAFLD), and atherosclerosis have all been validated (reviewed in Seth et al., 2013) thus far.

Finally, zebrafish offer both technical and practical advantages over terrestrial models due to their rapid developmental life stages, ease of husbandry, full genome sequence availability (Howe et al., 2013), and vast applications via experimental designs and available strains; all of which serve to further the understanding of metabolic processes.

Given these advantages, zebrafish make for an exceptional model for studying both short and long-term effects on metabolic processes. However, methods for measuring metabolic rates are often complicated, time consuming and expensive, which causes stressful conditions for the organism tested. For example, metabolic chambers are a well-established method of obtaining *in vivo* metabolic data. For this method, the fish is placed inside a respirometry system chamber with an oxygen sensor that records the oxygen levels in the sealed chamber which is connected to multiple machines to measure the metabolic output.

As the fish respire, oxygen levels decrease, revealing that those with higher metabolic rates will respire more, causing sharper drops in oxygen levels, which can be analyzed. Moreover, using the known parameters of the system, mass-specific oxygen consumption rates (also known as V_{O2} rates) can be calculated, which also provides insight into the metabolic state of the fish. Unfortunately, metabolic chambers can be very difficult to set-up, as well as difficult to analyze due to the overwhelming amount of data that is generated. In addition, metabolic chamber systems are very expensive and can only analyze a small number of organisms at a time, making it extremely time consuming. Thus, elucidating other accurate methods of metabolic markers would be of great interest.

Resazurin is a water-soluble, non-toxic sodium salt that has been well established as the main active ingredient in the alamarBlue® cell viability assay (ThermoFisher Scientific, Waltham, MA). Resazurin can detect *in vitro* metabolic changes in both animal and human cell lines, as well as, bacterial, plant, and fungal cells. The application for resazurin encompasses a multitude of organisms and can be utilized in cell to detect *in vitro* metabolism. Moreover, there are numerous assays that use resazurin to detect cell viability through growth and proliferation of cells (De Fries and Mitsunashi, 1995; Xiao et al., 2010; Riss et al., 2016; Prabst et al., 2017) and the cytotoxicity of chemicals on cells (Slaughter et al., 1999; Born et al., 2000; O'Brien et al., 2000; Walzl et al., 2014). When resazurin is solubilized in water it creates a dark blue non-fluorescent colored solution. Once the solution is added to the cells, it can permeabilize through the cell membrane where it becomes conformationally changed into resorufin under metabolic reduction. This reduction is a result of the oxidized blue resazurin solution accepting electrons from metabolic enzymes i.e., $NADH_2$, which causes the solution to become reduced into the resorufin solution, which is pink and fluorescent (O'Brien et al., 2000; Gonzalez and Tarloff, 2001, Rampersad, 2012; Riss et al., 2016; Prabst et al., 2017). Therefore, higher metabolic rates can be directly measured fluorometrically via increases in fluorescent intensity of the media (Rampersad, 2012).

In 2013, Renquist and colleagues bridged that gap for resazurin assays by applying an *in vitro* method to their *in vivo* studies. They were the first to demonstrate that resazurin could be used for energy and metabolism *in vivo* studies, by showing its effectiveness in assaying the metabolism of zebrafish larvae after drug application and genetic manipulation (Renquist et al., 2013; Williams and Renquist, 2016). Renquist and colleagues demonstrated that resazurin could enter larval zebrafish, become reduced to resorufin and exit the larvae, all while changing the fluorescent state of the media containing the larvae after 1 hour of exposure (Renquist et al., 2013). This change in the fluorescence of the media is a direct measurement for metabolic rate in the larval fish. As the resazurin has permeabilized itself in the zebrafish cell it will become reduced by metabolic metabolites, like $NADH_2$, which have been produced from oxidative phosphorylation (Renquist et al., 2013; Williams and Renquist, 2016). Thus, fish with more ATP production and a higher metabolic rate will produce more $NADH_2$, which in turn causes more resazurin to become reduced to the fluorescent pink resorufin. Moreover, Renquist and colleagues further demonstrated the efficacy of this method by increasing the metabolic rates of zebrafish treated with leptin and insulin (Renquist et al., 2013). However, these experiments were conducted only during larval stages and as metabolic phenotypes occur throughout all life stages, we wanted to

investigate if this technique could be applied across all stages of zebrafish development to better optimize this assay for metabolic studies.

In this work, we validate that resazurin is effective in measuring metabolic output *in vivo* in both larval and adult zebrafish. In addition, we demonstrate that the resazurin assay is non-toxic and sensitive enough to use over prolonged periods of time. Thus, the validation of this assay may increase the versatility and complexity of the types of experiments that can be performed.

2. Material and Methods

2.1 Ethical procedures

All *in vivo* experimentation involving larval and adult zebrafish was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and is consistent with the guidelines established by the Office of Laboratory Animal Welfare, National Institutes of Health of the U.S. Department of Health and Human Services.

2.2 Animals

Wild-type adult (>1 year of age) AB strain zebrafish (*Danio rerio*) were obtained from the Aquatic Animal Research Core (AARC) at the University of Alabama at Birmingham (UAB). Fish were maintained in a recirculating aquatic system (dechlorinated city water) at 25°C under a 14-hr light-10-hr dark photoperiod. Fish were fed once daily *ad libitum* with otohime (Pentair Aquatic Eco-System, Inc., Apopka, FL). Wild-type AB strain adult zebrafish were bred following the procedures of Westerfield and colleagues (Westerfield, 1995). Once the eggs were collected, they were pipetted into a 10-cm petri dish with a stocking density of 100–150 embryos. For the following days 1–5, embryos were maintained in a 28°C housing chamber with a 14-hr light-10-hr dark photoperiod. Any embryos that had died or were unfertilized were removed with a disposable pipette and water changes were performed daily with 4 mL of fresh tank water; ensuring not to disturb or pipette out the healthy embryos. Embryos were allowed to develop until 5 days post fertilization for all larvae experiments. Day 5 larvae developmental stage was determined according to Kimmel and colleagues (Kimmel et al., 1995; Parichy et al., 2009).

2.3 Resazurin Stock Solution Preparation

A resazurin stock solution was prepared by mixing 0.5 g of resazurin sodium salt (Sigma-Aldrich Corp., St. Louis, MO) with 10 mL of distilled water and 10 μ L of dimethyl sulfoxide (0.1% DMSO, Sigma-Aldrich Corp., St. Louis, MO) bringing the stock solution to a total concentration of 50 mg/mL.

2.4 Resazurin Dilution Preparation

1L of tank water was collected from the aquatic system and acclimated to room temperature (23°C) in the experimental room. Tank water was prepared by running municipal water first through an AquaFX RO filter, then through a micro UV, charcoal, and mechanical sterile filter (Pentair Aquatic Eco-System, Inc., Apopka, FL). The filtered H₂O was then brought to

a conductivity range of 800–1200 uS with instant ocean sea salt (Instant Ocean, Cincinnati, OH) and had a pH of 7.1–7.5. Resazurin stock solution was diluted with tank water according to Table 1.

2.5 Larvae and Diluent Assay Preparation

A. Diluent Preparation—To investigate if different diluents would have different effects on resazurin sensitivity, the resazurin stock was diluted to a final concentration of 0.02mg/mL using either tank water, autoclaved tank water, deionized water, or autoclaved deionized water. (For tank water properties refer to Material and Methods 2.4). 1 L of tank water was obtained from the aquatic system and acclimated to room temperature (23°C) in the experimental room. 500 mL of the acclimated tank water was aliquoted into a 1 L autoclavable glass container and placed into the STERIS AMSCO Lab-205 autoclave (STERIS Corp., Mentor, OH) on cycle L20 for 1 hour. Deionized water was obtained from the Millipore 30 L Storage Tank (MilliporeSigma, Burlington, MA) that was prepared by running municipal water through the Milli-Q Direct 8 Water Purification System (MilliporeSigma, Burlington, MA). Similar to tank water preparations, the 1 L of deionized water was acclimated to room temperature and 500 mL were aliquoted into a new 1 L autoclavable glass container, followed by the autoclave protocol. Water samples were prepared as outlined in Table 1-Dilution 2. 2 mL of the resazurin diluent solutions (0.02 mg/mL) were pipetted into one of the wells of the 35 mm 6-well plate. Then, the 6-well plate was covered with the lid and the edges were taped with masking tape.

B. Larvae Preparation—To analyze resazurin effectiveness in larval fish, 100 5 days post fertilization (dpf) larvae were combined into a single 100 mL sterile beaker with 20 mL of fresh tank water that had been acclimated to room temperature (23°C) in the experimental room. The larvae were then rinsed 2–3 times with a disposable transfer pipette, ensuring that for every 10 mL of tank water removed, it was replaced with another 10 mL of fresh tank water. After the final rinse, 10 mL of the tank water was removed from the beaker leaving a final volume of 10 mL. With the same disposable transfer pipette either 10, 20, or 40 larvae were transferred through gentle suctions from the 100 mL beaker and placed into one of the wells of the 35 mm 6-well plate (refer to Figure 1A). This setup can be modified for 1 larvae per well in a 96-well plate, following the protocol of Williams and Renquist (2016). One of the wells contained no larvae, serving as the “blank.” When 5 of the 6 wells had the same desired number of larvae per well (i.e., 10, 20, or 40), any extra tank water in the wells was aspirated out, without touching or aspirating the larvae. Following the aspiration, 2 mL of the Dilution 2: working stock resazurin (0.02 mg/mL) were added to all 6 wells. Then, the 6-well plate was covered with its lid and the edges were taped with masking tape. After the last plate was loaded with the larvae in the resazurin solution, larvae were fasted, as adding food to the solution could compromise the assay. In addition, the larvae were kept in a 14-hr light-10-hr dark photoperiod, which limited any circadian effects and were either placed into a chilled incubator (16°C) or kept at room temperature (23°C), to test the effects temperature had on metabolism. Moreover, if an experimental design had more than 6-time points, the amount of resazurin solution (0.02 mg/mL) per well was increased by 1 mL to compensate for the additional time points in the experimental design.

2.6 Adult Assay Preparation

Prior to the assay, 1 L of tank water had been collected and acclimated to room temperature (23°C) in the experimental room. The 1 L of tank water was used to make the working stock of resazurin at the Dilution 2 concentration (0.02 mg/mL) in Table 1. Next, 25 mL of the resazurin solution were pipetted into a 50-mL conical tube. Adult zebrafish were collected with a small net and individually weighed (grams) prior to their placements into the 50-mL conical tube filled with the 25 mL of resazurin solution (0.02 mg/mL). For the metabolic assays, either 1, 2, or 3 fish were placed into one 50-mL conical tube filled with the 25 mL of resazurin solution (0.02 mg/mL) (Figure 1B). One of the 50-mL conical tubes with the 25 mL resazurin solution (0.02 mg/mL) contained no adult zebrafish, serving as the “blank”. The lids of the 50-mL conical tubes were then placed back on the tubes, but not tightened to allow for oxygen flow. The conical tubes were then positioned at a 45° angle, which reduced the stress on the fish and spillage of the resazurin solution. Once the fish were in the resazurin solution, they were fasted, as adding food to the solution could compromise the assay. In addition, the fish were kept in a 14-hr light-10-hr dark photoperiod, which limited any circadian effects and were either placed into a chilled incubator (16°C) or kept at room temperature (23°C), to test the effects temperature had on metabolism. If an assay was designed to have more than 6-time points, the amount of resazurin solution (0.02 mg/mL) per conical tube was increased by 2.5–3 mL to compensate for the additional time points in the experimental design.

2.7 Larvae and Diluent Assay Sample Collection and Preparation

After the larval fish were placed in their appropriate wells (Figure 1A) and the resazurin diluents, the first samples were collected immediately, to represent the time point 0 (T_0). The resazurin diluents (0.02 mg/mL) and larvae solutions were collected by pipetting 180 uL of the solution out of the wells with the larvae or diluent, making sure that the larvae in the wells were not disturbed or pipetted out of the well. The 180 uL sample was transferred into a new 1.5 mL eppendorf tube and sealed with parafilm. Samples were collected at time points 0, 3, 6, 12, 24, and 48 hours post initial T_0 collection (Figure 1C). Once all the samples for that time point had been collected, they were placed in a cool, dark environment i.e., a refrigerator at 4°C. The sample collection method was repeated for all the designed time points and continued until the end of the experiment. 2 mL of resazurin solution (0.02 mg/mL) per well worked best with our 5-time points, 0, 3, 6, 12, 24, and 48-hour sample collections. For the larvae assay, samples from the “blank” wells were collected in conjunction with the samples collected at the designed time points. If there were multiple plates with samples, then a “blank” was collected from each plate to avoid interplate variation.

When all the 180 uL larvae and diluent samples were collected for their desired time points, a clear flat bottom 96-well plate was loaded with 85 uL of sample, loaded in duplicate. Once the 96-well plate(s) was loaded, the plate was then covered with its lid, wrapped in aluminum foil, protected from light, and temporarily stored (< 30 minutes) in a cool place, until the analysis.

2.8 Adult Zebrafish Assay Sample Collection and Preparation

A. Increasing number of organisms assay and temperature effect assay—After the adults had been placed in their 50-mL conical tubes (Figure 1B), the first sample was collected and represented time point 0. The resazurin (0.02 mg/mL) and fish solution was collected by pipetting 300 μ L of the solution out of the 50-mL conical tubes, making sure that the fish were not disturbed or touched in the tubes. The 300 μ L sample was transferred into a new 1.5 mL Eppendorf tube and sealed with parafilm. Samples were collected for the increasing number of fish and temperature assays at time points: 0, 3, 6, 12, 24, and 48 hours post initial T_0 collection (Figure 1C). A sample from the “blank” 50-mL conical tube was collected in conjunction with the other samples of the same time points. Once all the samples for each time point had been collected, they were placed in a cool, dark environment i.e., a refrigerator at 4°C, and temporarily stored (< 30 minutes) until they were prepared for analysis.

B. Toxicity Assessment and Long-term Sample Storage Assays—To test the toxicity of the resazurin assay and the long-term storage application, individual adult zebrafish were placed into their respective 50-mL conical tubes (Figure 1B) that contained 28 mL of resazurin solution (0.02 mg/mL). 550 μ L of the solution was pipetted out of the individual 50-mL conical tubes and transferred into a new 1.5 mL Eppendorf tube and sealed with parafilm. The toxicity assessment and storage assay required sample collections at time points: 0, 3, 6, 12, 24, 48, 72, 96, and 120 hours post initial T_0 collection. Also, a sample from the “blank” 50-mL conical tube was collected in conjunction with the other samples of the same time points. Once all the samples for the time point had been collected, they were placed in a cool, dark environment i.e., a refrigerator at 4°C, and temporarily stored (< 30 minutes). Half of the volume of the fresh collected sample were immediately analyzed following preparation for its toxicity assessment. The other half of the sample was wrapped with parafilm and stored at –20°C for 3 weeks. After 3 weeks, the frozen samples were allowed to acclimate to room temperature and then were immediately analyzed following preparation to determine if the samples maintained fluorescence intensity, by comparing them the values obtained from the toxicity assessment.

C. Adult Sample Preparation for Analysis—When all the adult samples were collected for their desired time points, a clear flat bottom 96-well plate was loaded with 85 μ L of sample, in triplicate. Once the 96-well plate(s) was loaded, the plate(s) was then covered with its lid, wrapped in aluminum foil, protected from light, and then temporarily stored (< 30 minutes) in a cool place, until further analysis.

2.9 Assay Analysis

After the last sample was pipetted into the 96-well plate and wrapped with aluminum foil, additional plate(s) were removed from their temporary storage. A fluorescent plate reader was required to analyze the samples (BioTek Synergy 2, BioTek Instruments Inc., Winooski, VT). Using the BioTek Gen 5 Microplate Reader and Imager Software (BioTek Synergy 2, BioTek Instruments Inc., Winooski, VT), the 96-well plates were read with an excitation wavelength at 530 nm, followed by an emission wavelength at 590 nm to read the resorufin fluorescence intensity (FI).

Total resorufin FI was calculated by taking the average total resorufin FI values of the wells for the samples of interest at a specific time point and subtracting them from the averaged total resorufin FI of the blank wells at the same time point and this was reported as arbitrary fluorescence units (AFU). For adult zebrafish, the total resorufin values were normalized to the weight of the individual adult zebrafish, by dividing the average total resorufin FI by the weight of the fish in grams and was reported as arbitrary fluorescence units per grams (AFU/g). Conceptually, the samples at time point 0 and the blank were equivalent and total resorufin FI resulted in extremely close values for the AFU. Thus, time point 0 was set to 0 for all the samples (Time point 0-blank = 0). Any differences were negligible and were attributed to plate reading variability and sample loading errors.

$$\text{AFU} = \bar{x}_{\text{Sample Tx}} - \bar{x}_{\text{Blank Tx}}$$

Equation Key:

Tx = Time point X (X = 0, 3, 6, 12, 24, and 48 hour.)

$\bar{x}_{\text{Sample Tx}}$ = Average Sample of Interest of total Resorufin FI at timepoint X

$\bar{x}_{\text{Blank Tx}}$ = Average Blank of total Resorufin FI at timepoint X

Additionally, resazurin response kinetics were analysed by setting the control number of organisms (for larvae = 10, adults = 1), to 1 and measuring relative change in resorufin fluorescence (modified from Renquist et al., 2013). Linear regression line was analysed and denoted by R^2 value (closer to $R^2 = 1$ indicates linearity).

2.10 Statistical Analyses

All graphs are represented as mean $\pm$ SEM. All data were analyzed by two-way repeated-measures (RM) ANOVA. Sidak's post-hoc test was used to determine P-values. An *a priori* hypothesis states that the asterisks represent the following: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. Graphpad Prism 7 was used to analyze each statistical test and for all graphing.

3. Results

3.1 Optimization of resazurin concentration in solution for in vivo experiments while maintaining sensitivity and non-toxicity

To elucidate the optimal resazurin assay concentration to ensure maximal sensitivity and lack of toxicity, individual adult zebrafish were placed in 50-mL conical tubes at 23°C containing serial concentrations (refer to Table 1) and samples were collected and analysed 24 hours later. Two resazurin concentrations, 0.01 and 0.02 mg/mL, exhibited the highest conversion of resazurin to resorufin (Figure 2A). Repeating the experiment at 16°C, to determine if this range was still the most effective even under colder temperatures, demonstrated that 0.01 mg/mL resazurin resulted in the greatest detection of resorufin (Figure 2B). However, this concentration (0.01 mg/mL) also exhibited the most variability. Lastly, to test the toxicity of the 0.02 mg/mL dose, adult zebrafish were placed in 50-mL conical tubes containing the resazurin solution and measured for 5 days where a continued

increase in resorufin concentration was detected (Figure 2C). After 5 days, fish were still lively and showed no obvious signs of distress (visual observations). Thus, a resazurin concentration range from 0.01–0.02 mg/mL should be used for *in vivo* studies, however, for less variable data the concentration of 0.02 mg/mL is recommended.

3.2 Tank water can be used as an appropriate diluent and blank solution for resazurin assay

To test if diluting the resazurin sodium salt in system tank water is effective, the differences in types of waters as a diluent, as well as a “blank” control, were analyzed. Samples of deionized water (diH₂O), autoclaved diH₂O, tank water, and autoclaved tank water were added to resazurin solution and measured over 48 hours. diH₂O and tank water had a significantly different baseline resorufin FI (****P<0.0001) (Figure 3A). Second, sterilization of the diH₂O water by autoclaving had no effect on AFU (Figure 2A). However, autoclaving the tank water resulted in a significant decrease at 48 hours (***P=0.0003) in total resorufin FI, when compared to non-sterile tank water (Figure 3A). More importantly, although the different types of water have some moderate variances between them as control blanks, these are insignificant changes when compared to changes induced by metabolic activity due to organism, such as in this case, larvae (Figure 3B). Only the wells containing larvae had significantly higher total AFU (****P<0.0001) (Figure 3B).

3.3 Resazurin assay can measure increases in metabolic activity in both larval and adult zebrafish due to increasing number of organisms

To demonstrate that the resazurin assay can measure increases in metabolic activity, the total resorufin FI in increasing numbers of both larvae and adult zebrafish was analyzed. This is based on the principle that as number of organisms increase, there should be a resulting increase resazurin conversion to fluorescent resorufin.

For the larval study, zebrafish larvae (5 days post-fertilization, dpf) were pooled in wells of a 6-well plate that contained resazurin solution. Wells contained either 10, 20 or 40 larvae, and solution samples were collected from the wells over 48 hours. The first differences were seen at 24 hours between wells that contained 10 larvae compared to 40 larvae, with the latter having higher AFU (P=0.033) (Figure 4A). Moreover, at 48 hours, wells that contained 40 larvae had significantly higher total resorufin FI compared to wells that contained 10 larvae and 20 larvae (**P=0.0015 and *P=0.02, respectively) (Figure 4A). Additionally, by measuring the relative change in resorufin fluorescence of 10 larvae compared to 20 and 40 larvae, we were able to assess the larval response kinetics of resazurin. The response was shown to be linear, as defined by an R² value of 0.9998 of the slope (Figure 4B).

To assess the effectiveness of the assay on adult zebrafish, conical tubes containing either 1, 2 or 3 fish with resazurin solution were sampled over 24 hours. After 6 hours, the total resorufin FI of 3 fish was greater than 1 fish (*P=0.04). At 12 hours, 2 fish and 3 fish had increased AFU compared to 1 fish (***P=0.0006 (2 fish) and ****P<0.0001 (3 fish)). At 24 hours, all fish resorufin FI were different from each other (****P<0.0001) (Figure 5A). Resazurin response kinetics was also measured in adult zebrafish by comparing the relative

change in AFU/g of 1 fish to 2 and 3 fish. The response was shown to be linear, as defined by an R^2 value of 0.9765 of the slope (Figure 5B).

3.4 Resazurin assay can measure decreases in metabolic activity in both larval and adult zebrafish due to cold temperature exposure

It is well established that cold temperatures decrease metabolic rates in terrestrial and aquatic animals. To demonstrate that the resazurin assay can measure decreases in metabolism, larval and adult zebrafish were placed in both warm (23°C) or cold (16°C) temperatures for 48 or 24 hours and total resazurin conversion to fluorescent resorufin was measured. Larvae that were exposed to the cold temperature had significantly lower AFU at 48 hours (**** $P < 0.0001$) (Figure 6). At 12 hours, adult zebrafish exposed to cold temperatures exhibited lower total resorufin FI, which is continuously suppressed through 24 hours later (*** $P = 0.0002$, 12 hours; **** $P < 0.0001$, 24 hours) (Figure 7).

3.5 Samples collected from resazurin assay can either be assayed immediately, or stored for prolonged periods of time with equal accuracy in total resorufin FI

As previously described, samples are collected from the wells or conical tubes over time, and then read on a plate-reader to measure the total resorufin FI (AFU). To determine if samples can be stored and read later with equal accuracy, samples were collected from adult zebrafish and either read immediately or were frozen (−20°C) and subsequently read 3 weeks later. No significant differences were observed between the fresh and frozen samples at any time point (Figure 8).

4. Discussion

In this work, we establish that resazurin is effective in measuring metabolic output *in vivo* in zebrafish across all life stages. As total resorufin fluorescence intensity is proportional to the amount of NADH₂ reduced in the organism, it gives evidence of changes in metabolism within the organism. This study builds upon previous reports from Renquist and colleagues that demonstrate the effectiveness of resazurin as an *in vivo* zebrafish larvae metabolic detector (Renquist et al., 2013; Williams and Renquist, 2016), whereas, here we highlight life course use by demonstrating its effectiveness in zebrafish adults. Moreover, Renquist and colleagues demonstrated that larvae adequately internalize resazurin by visualization of confocal image after 1 hour of exposure, indicating that changes seen in NADH₂ are reflecting global consumption, and are able to sense changes in the external environment by reduced resorufin fluorescence intensity. This work was the foundation of our interest into investigating the usage of resazurin metabolic assay in adult zebrafish.

First, we elucidated the optimal resazurin concentration for *in vivo* studies that maximizes sensitivity as well as ensuring it is a non-toxic dose. For zebrafish, we determined that the dose range from 0.01–0.02 mg/mL is appropriate for sensing changes in metabolism (Figure 2A), regardless of temperature conditions (Figure 2B). We chose to proceed with the dose of 0.02 mg/mL as it was less variable in its response and was non-toxic to the fish. Interestingly, the dose response curve of resazurin showed that high concentrations of resazurin had a lowered resorufin signal. This may be a result of a certain degree of toxicity

caused by too high of concentrations. This was shown in cell culture as after long term treatment (4–8 days) with resazurin resulted in lowered glucose uptake ability and cell proliferation (Pace & Burg, 2015). Alternatively, it could also be a result of its dark opaque color at this concentration causing the fish to falsely sense it is in dark cycle. Zebrafish pineal glands are photoreceptive and give cues to the body of the time cycle, such as to lower spontaneous locomotion during dark cycles (del Pozo et al., 2011; Vatiné et al., 2011). If the resazurin solution was creating too dark of an environment and caused decreased locomotor movement, this could explain the lowered resorufin fluorescence signal as well. In either case, this indicates the intermediate dosage range of resazurin is most optimal. Importantly, we further show that this dose is non-lethal to zebrafish, as they are lively and exhibit no obvious signs of distress for at least 5 days in the resazurin solution (Figure 2C). However, before using the resazurin assay in a different fish species, we recommend optimizing the resazurin concentration as outlined here, as some species may exhibit differences in sensitivity, depending on size and age.

For our studies, we diluted the resazurin sodium salt in tank water. Thus, we investigated if different diluents would have different effects on resazurin sensitivity. We compared samples from resazurin diluted with either deionized water, autoclaved deionized water, tank water, or autoclaved tank water (Figure 3A). Deionized water and tank water demonstrated significantly different baseline resorufin fluorescent intensities (Figure 3A), which may have to do with a difference in chemical composition of the tank water and its interaction with the resazurin compound itself. This indicates that although both can be used as diluents for resazurin, the blank solution must be kept consistent with whichever diluent used to properly control for difference in background fluorescence. Further, if using the bath drug application technique, you must also treat your blank wells to appropriately accommodate any potential differences as described by Renquist and colleagues (Renquist et al., 2013; Williams and Renquist, 2016).

Although sterilization of the water via autoclaving had no effect on the fluorescence of deionized water, it did have a significant impact on tank water (Figure 3A). This may be because deionized water is already presumably sterile, whereas tank water has naturally occurring microbes. This could indicate that the resazurin assay is sensitive enough to detect very minor changes in metabolic output induced by these microorganisms, which would be lost upon sterilization. However, previous reports from Williams and Renquist suggest that any increased signal response from these microorganisms would be rare and by having a blank, controls for any effects microbiota have on total resorufin fluorescence. Importantly however, these differences in total resorufin fluorescence intensities between the types of water or sterility are negligible when in the presence of organisms (Figure 3B). Thus, we have demonstrated that these experiments do not require special media or water, as this assay is equally effective using tank water, which is much less taxing on the fish and does not disrupt the fish environment, leading to much better temporal analysis.

Next, we determined that the resazurin assay can detect increases in metabolic output as the number of organisms increase within the well. This positive control test validates that resazurin is sensitive enough to detect changes in numbers of pooled larvae (Figure 4A) as well as in zebrafish adults (Figure 5A). This is consistent with reports that demonstrated

resazurin was equally sensitive to measuring metabolic output increase as larvae number increase in 96-well plates as the BD Biosensor assay for oxygen consumption (Renquist et al., 2013). Second, to demonstrate that resazurin can measure decreases in metabolic output, we exposed larvae (Figure 6) and adults (Figure 7) to colder temperatures, a known metabolic suppressor (Vergauwen et al., 2010). As expected, the resazurin assay accurately demonstrated that organisms exposed to cold temperatures had significantly lowered metabolism (Figure 6, Figure 7). The larvae results are corroborated by those presented in Renquist et al., which show cold temperature decreases metabolic output (2013). Our adult zebrafish results are corroborated by those presented in Uliano and colleagues (2010), where they used a closed respirometry system to measure oxygen consumption rate in adult *Danio rerio* under acute temperature stress. They demonstrated that a 7°C degree decrease in experimental temperature (27°C to 20°C) was sufficient to induce a significant decrease in metabolism as measured by routine oxygen consumption rate (rM_O₂). Our results showed a 2.5-fold decrease compared to their approximately 1.6-fold decrease, most likely due to our experimental temperature being relatively lower into the zebrafish temperature tolerance range (23°C to 16°C). This clearly demonstrates that the resazurin method of assessing cold temperature stress is corroborated by other standard methods of metabolism measurement. In addition, Renquist and colleagues showed dose-dependent increases in metabolic activity by the hormones, insulin and leptin, and that genetic manipulations affecting metabolism could also be measured by resazurin (Renquist et al., 2013). As our results in adults have been highly consistent with the experiments performed in larvae, based on these reports we hypothesize that the same experimental concepts of hormonal and genetic manipulation can be performed in adult zebrafish.

Another interesting aspect we wanted to investigate was the kinetics of the resazurin response. By measuring relative change in fluorescence, we could observe if the response was linear or non-linear (i.e. if number of fish is doubled, does the resorufin fluorescence also double). In the larval response, there is a very clear and strong linear response among the 10, 20 and 40 larvae resorufin FI, with an R² value of 0.9998 (Figure 4B). However, in the adult response, although the response is linear as denoted by an R² value of 0.9765 (Figure 5B), it is not directly proportional, indicating it is not increasing solely based on an increase in number of organisms in the tube. There are different factors that could influence this, however the most likely explanation is the compounded stress of having more than one animal in a confined space, which is known to increase metabolic rate.

Finally, we tested if samples taken from the experiments could be read at a later time while still maintaining fluorescence intensity accuracy. To do this, we took samples from adult zebrafish over 120 hours. We assayed half the volume of the fresh samples immediately following collection and froze the other half at -20°C. When comparing the AFU/g between fresh and frozen samples, we found no significant difference between the samples (Figure 8). Thus, our sample collection technique has a multitude of benefits. This has many practical applications for those who work in the field, where samples could be collected, frozen, and assayed at a later point with no impact on the accuracy of the experiment. Moreover, this sample collection technique is non-invasive or stressful on the organism, resulting in more possibilities for temporal analyses. This collection technique also allows for numerous samples to be collected at once, meaning you can test many organisms at the

same time, which is a limitation of other common metabolic experimental set ups, such as metabolic chambers. Lastly, by collecting the sample and thereby not reading the measurements with the fish in the 96-well plate, it leads to less errors or inaccuracies caused by non-specific refraction during plate-reading.

Although with any assay, there are some considerations to make before utilizing this method for your study. The first is that the fish must be fasted while in the resazurin solution, as the food could compromise the solution, which depending on your research question, could impact your experimental design. The larvae application of this method has been well established in Williams and Renquist methods, primarily because larvae rely on their yolk sacs for nutrition, and any influence from gut or organismal microbiota on total resorufin signals of this assay will be limited (Williams and Renquist, 2016). Thus, one aspect one must consider is the potential influence of the gut and mucosal microbiotas of the adult zebrafish on total resorufin FI. However, we argue that once the larvae hatch from the chorion around 3dpf, they are exposed to the microorganisms on the egg chorion and the microorganisms of the water, which are the same microorganisms that the adults are exposed to, given that they are hatched in the same tank water that the adults are housed (Vatsos, 2017). Moreover, we tested larvae that are 120 hours post fertilization (5dpf), however, zebrafish start to develop their gut tracts at 60 hours post fertilization (2.5dpf) and by 72 hours post fertilization (3dpf) the larvae have a wide-open mouth and are actively seeking food. This suggests that the tank water microbiota are able to penetrate the mouth, gut tract, gills, and skin in the larvae as it would in the adults (Kimmel et al., 1995). Therefore, the microbiota for the adults and larvae could increase total resorufin FI, however, this increase would be masked by the overall metabolic functions from the organism itself. In addition, studies have demonstrated that zebrafish and other teleost species develop large numbers of mitochondria rich cells on their gills as early as 5 days post fertilization, suggesting that resazurin would immediately permeabilize the gills and react with the mitochondria and be reduced here into the fluorescent resorufin, rather than being reduced by the microbiota (Rombough, 2002). Thus, this would further indicate that the changes in total resorufin FI are a result of metabolic functions within the fish. However, future studies should aim to elucidate where in the adult organisms the resazurin is primarily found and metabolized. One can speculate different tissues would have different metabolic rates and thus different amounts of resazurin/resorufin fluorescence. Thus, this assay would illustrate how different tissues in the adult zebrafish process resazurin and further elucidate what the roles of individual tissues are in overall organism metabolism.

In conclusion, we demonstrate in these works the validity of the resazurin assay in adult zebrafish as a metabolic assay that is inexpensive, non-invasive, less labor intensive, and accurate for quick identification of metabolic changes using fluorometric analyses. By utilizing the natural properties of the resazurin compound conformational change into resorufin upon reduction, the relative changes in total resorufin fluorescent intensities provide insight into the metabolic output of the organisms. We show here that this assay is utilizable across all zebrafish life stages, and predict that it will work well with other aquatic organisms.

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References

- Argenton F, Zecchin E, Bortolussi M. Early appearance of pancreatic hormone-expressing cells in the zebrafish embryo. *Mech Dev.* 1999; 87:217–221. [PubMed: 10495291]
- Barbazuk WB, Korf I, Kadavi C, Heyen J, Tate S, Wun E, Bedell JA, McPherson JD, Johnson SL. The syntenic relationship of the zebrafish and human genomes. *Genome research.* 2000; 10(9):1351–1358. [PubMed: 10984453]
- De Fries R, Mitsuhashi M. Quantification of mitogen induced human lymphocyte proliferation: Comparison of alamarbluetm assay to 3h- thymidine incorporation assay. *Journal of clinical laboratory analysis.* 1995; 9(2):89–95. [PubMed: 7714668]
- Del Pozo A, Sanchez-Ferez JA, Sanchez-Vazquez FJ. Circadian rhythms of self-feeding and locomotor activity in zebrafish (*Danio rerio*). *Chronobiology international.* 2011; 28(1):39–47. [PubMed: 21182403]
- Finkel T. The metabolic regulation of aging. *Nature medicine.* 2015; 21(12):1416.
- Gonzalez RJ, Tarloff JB. Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity. *Toxicology in vitro.* 2001; 15(3):257–259. [PubMed: 11377098]
- Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature.* 2013; 496:498–503. [PubMed: 23594743]
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Developmental dynamics.* 1995; 203(3):253–310. [PubMed: 8589427]
- Hesselson D, Anderson RM, Beinat M, Stainier DY. Distinct populations of quiescent and proliferative pancreatic β -cells identified by HOTcre mediated labeling. *Proceedings of the National Academy of Sciences.* 2009; 106(35):14896–14901.
- O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *The FEBS Journal.* 2000; 267(17):5421–5426.
- Pace RT, Leadbetter Burg KJ. Toxic effects of resazurin on cell cultures. *Cytotechnology.* 2015; 67:13–17. [PubMed: 24242827]
- Parichy DM, Elizondo MR, Mills MG, Gordon TN, Engeszer RE. Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. *Developmental dynamics.* 2009; 238(12):2975–3015. [PubMed: 19891001]
- Präbst K, Engelhardt H, Ringgeler S, Hübner H. Basic Colorimetric Proliferation Assays: MTT, WST, and Resazurin. *Cell Viability Assays: Methods and Protocols.* 2017:1–17.
- Rampersad SN. Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors.* 2012; 12(9):12347–12360. [PubMed: 23112716]
- Renquist BJ, Zhang C, Williams SY, Cone RD. Development of an assay for high-throughput energy expenditure monitoring in the zebrafish. *Zebrafish.* 2013; 10(3):343–352. [PubMed: 23705823]
- Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, Minor L. Cell viability assays. 2016
- Rojas-Gutierrez E, Muñoz- Arenas G, Treviño S, Espinosa B, Chavez R, Rojas K, Flores G, Díaz A, Guevara J. Alzheimer's disease and metabolic syndrome: A link from oxidative stress and inflammation to neurodegeneration. *Synapse.* 2017
- Rombough P. Gills are needed for ionoregulation before they are needed for O₂ uptake in developing zebrafish, *Danio rerio*. *Journal of Experimental Biology.* 2002; 205(12):1787–1794. [PubMed: 12042337]

- Slaughter MR, Bugelski PJ, O'Brien PJ. Evaluation of Alamar Blue reduction for the in vitro assay of hepatocyte toxicity. *Toxicology in vitro*. 1999; 13(4):567–569. [PubMed: 20654517]
- Seth A, Stemple DL, Barroso I. The emerging use of zebrafish to model metabolic disease. *Disease models & mechanisms*. 2013; 6(5):1080–1088. [PubMed: 24046387]
- Simula, L., Nazio, F., Campello, S. The Mitochondrial dynamics in cancer and immune-surveillance. *Seminars in Cancer Biology*; Academic Press. 2017 Jun.
- Song Y, Cone RD. Creation of a genetic model of obesity in a teleost. *The FASEB Journal*. 2007; 21:2042–2049. [PubMed: 17341684]
- Uliano E, Cataldi M, Carella F, Migliaccio O, Iaccarino D, Agnisola C. Effects of acute changes in salinity and temperature on routine metabolism and nitrogen excretion in gambusia (*Gambusia affinis*) and zebrafish (*Danio rerio*). *Comparative Biochemistry and Physiology: Part A*. 2010; 157:283–290.
- Vatine G, Vallone D, Gothilf Y, Foulkes N. It's time to swim! Zebrafish and the circadian clock. *FEBS letters*. 2011; 585:1485–1494. [PubMed: 21486566]
- Vatsos IN. Standardizing the microbiota of fish used in research. *Laboratory animals*. 2017; 51(4):353–364. [PubMed: 27932684]
- Vergauwen L, Benoot D, Blust R, Knapen D. Long-term warm or cold acclimation elicits a specific transcriptional response and affects energy metabolism in zebrafish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. 2010; 157(2):149–157.
- Walzl A, Unger C, Kramer N, Unterleuthner D, Scherzer M, Hengstschläger M, Schwanzer-Pfeiffer D, Dolznig H. The resazurin reduction assay can distinguish cytotoxic from cytostatic compounds in spheroid screening assays. *Journal of biomolecular screening*. 2014; 19(7):1047–1059. [PubMed: 24758920]
- Westerfield, M. *The zebrafish book: a guide for the laboratory use of zebrafish (Brachydanio rerio)*. University of Oregon Press; 1995.
- Williams SY, Renquist BJ. High Throughput Danio Rerio Energy Expenditure Assay. *JoVE (Journal of Visualized Experiments)*. 2016; (107):e53297–e53297. [PubMed: 26863590]
- Xiao J, Zhang Y, Wang J, Yu W, Wang W, Ma X. Monitoring of cell viability and proliferation in hydrogel-encapsulated system by resazurin assay. *Applied biochemistry and biotechnology*. 2010; 162(7):1996–2007. [PubMed: 20437208]

Figure 1. Larvae and Adult Zebrafish Assay Preparation and Collection Schedule

(A) Zebrafish larvae (5 dpf) were pipetted into 5 of the 6 wells of a 6-well plate containing 2 mL of the resazurin solution (0.02 mg/mL). (B) Adult zebrafish were individually placed in a 50-mL conical tube with 25 mL of the resazurin solution (0.02 mg/mL) and positioned at a 45° angle. (C) Timeline for adult and larvae sample collections of total resorufin at the designated time points (T₀ – T₄₈) with collections being at 3, 6, 12, 24, and 48 hours post initial T₀ collection.

Figure 2. Optimization of resazurin concentration for sensitivity and non-toxicity

(A) Individual adult zebrafish were placed in conical tubes containing serial dilutions of resazurin stock solution to determine optimal resazurin concentration for sensitivity for *in vivo* studies at 23°C. (B) Resazurin optimization experiment was repeated with individual adult zebrafish at 16°C [n=3 (warm), n=3 (cold)]. (C) This dose is non-toxic to fish for at least 5 days [n=5].

Figure 3. Tank water is an appropriate control for the 'blank' wells in the assay

(A) Samples of deionized water (diH₂O), autoclaved diH₂O, tank water and autoclaved tank water were added to resazurin solution and measured over 48 hours. This assay demonstrated that diH₂O and tank water have inherently different baseline fluorescence ($P < 0.0001$). There was no significant difference between diH₂O and autoclaved diH₂O, although there was a significant difference between tank water and autoclaved tank water at 48 hours ($P = 0.0003$). (B) Results of the control blank assay compared to wells with larvae, which had significantly higher AFU than blank wells ($P < 0.0001$). Statistical test: Two-way RM ANOVA, Sidak's post-hoc test. [$n = 3$ (blanks), $n = 4$ (larvae)]

Figure 4. Increasing the number of pooled larvae zebrafish significantly increases the total resorufin fluorescence intensity (AFU) in a linear response

(A) 6-well plate wells that contained resazurin solution housed either 10, 20 or 40 zebrafish larvae (5 dpf). Significant increases in total resorufin fluorescence intensity were seen at 24 hr ($P=0.033$) between 10 and 40 larvae. After 48 hr, 40 larvae had significantly higher total resorufin fluorescent intensity when compared to 10 larvae ($P=0.0015$) and 20 larvae ($P=0.02$). Statistical test: Two-way RM ANOVA, Sidak's post-hoc test. [$n=4$ (10 larvae), $n=5$ (20 larvae), $n=5$ (40 larvae)]. (B) Larval response kinetics were analysed by measuring the relative change in AFU/g of 20 and 40 larvae compared to 10 larvae, and it had an R^2 value of 0.9998.

Figure 5. Increasing the number of adult zebrafish significantly increases the total resorufin fluorescence intensity (AFU) in a linear response

(A) 1, 2 or 3 adult zebrafish were added to resazurin solution in conical tubes and samples were collected over 24 hours. At 24 hours, the metabolic rates of the number of fish are all significantly different ($P < 0.0001$), where the greater number of fish result in higher AFU. [Statistical test: Two-way RM ANOVA, Sidak's post-hoc test. [$n=5$ (1 fish), $n=4$ (2 fish), $n=4$ (3 fish)]]. (B) Adult response kinetics were analysed by measuring the relative change in AFU of 3 and 2 fish compared to 1 fish, and it had an R^2 value of 0.9765.

Figure 6. Pooled larvae zebrafish had significantly lowered metabolism after exposure to cold temperature for 48 hours

Larvae zebrafish that were pooled in 6-well plate (10 larvae/well) contained in resazurin solution were exposed to either 23°C (warm) or 16°C (cold) temperature for 48 hours. Larvae had significantly ($P < 0.0001$) lower metabolism at 48 hours as measured by total resorufin fluorescent intensity (AFU). Statistical test: Two-way RM ANOVA, Sidak's post-hoc test. [n=3 (cold), n=4 (warm)]

Figure 7. Adult zebrafish had significantly lowered metabolism after exposure to cold temperature for 24 hours

Individual adult zebrafish contained in resazurin solution were exposed to either 23°C (warm) or 16°C (cold) temperature for 24 hours. At 12 hours, the animals had significantly lower metabolic rate ($P=0.0002$), which was maintained until 24 hours ($P<0.0001$), as measured by total resorufin fluorescent intensity (AFU). Statistical test: Two-way RM ANOVA, Sidak's post-hoc test. [$n=8$ (cold), $n=5$ (warm)]

Figure 8. Fresh samples and frozen samples exhibit the same fluorescence intensity even after 3 weeks of storage

Samples were collected from individual adult zebrafish and assayed either immediately after collection or frozen (-20°C) and assayed 3 weeks later. This test shows no significant difference between samples at any time point. Statistical test: Two-way RM ANOVA, Sidak's post-hoc test. [n=5 (fresh), n=5 (frozen)]

Table 1
Preparation of resazurin sodium salt for optimization curve

Resazurin stock solution was diluted and prepared for use in optimization curve. This table shows the dilution steps to obtain the concentrations required for the curve.

	Volume of Resazurin Solution (mL)	Volume of Tank Water (mL)	Total Volume of Solution (mL)	Resazurin Concentration (mg/mL)
Working Stock	1 mL Stock (50mg/mL)	49 mL	50 mL	1 mg/mL
Dilution 1	5 mL (Working Stock)	45 mL	50 mL	0.1 mg/mL
Dilution 2	1 mL (Working Stock)	49 mL	50 mL	0.02 mg/mL
Dilution 3	0.5 mL (Working Stock)	49.5 mL	50 mL	0.01 mg/mL
Dilution 4	0.1 mL (Working Stock)	49.9 mL	50 mL	0.002 mg/mL
Dilution 5	0.05 mL (Working Stock)	49.95 mL	50 mL	0.001 mg/mL