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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 $NADH_2$, 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.

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 h 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.

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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 respires, 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 V02 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 Mitsuhashi, 1995; Xiao et al., 2010; Riss et al., 2016; Präbst et al., 2017) and the cytotoxicity of chemicals on cells (Slaughter et al., 1999; 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; Präbst 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 h 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-h light-10-h 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-h light-10-h 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 µL 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

1 L 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 H_2O was then brought to a conductivity range of 800–1200 µS 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.