5-2018

Novel Methods for Monitoring Glucose Metabolism in *Trypanosoma brucei* Using Fluorescent Biosensors

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NOVEL METHODS FOR MONITORING GLUCOSE METABOLISM IN TRYPANOSOMA BRUCEI USING FLUORESCENT BIOSENSORS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Chemistry

by
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May 2018

Accepted by:
Dr. George Chumanov, Committee Chair
Dr. Kenneth Christensen
Dr. James Morris
Dr. Daniel Whitehead
ABSTRACT

Members of the class Kinetoplastea including *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* spp. cause diseases endemic in rural regions of South America, Sub-Saharan African and the Eastern Asian continent, effecting hundreds of millions of people and livestock. Existing treatments are associated with high toxicity and rates of resistance, are expensive to produce, and are difficult to administer in rural areas. To develop additional treatment strategies, we must better illuminate the pathways amenable for anti kinetoplastid treatments. One pathway susceptible to drug intervention is glucose metabolism, which in kinetoplasts takes place in glycosomes that are specialized organelles related to mammalian peroxisomes. Disruption of glycosome function is hypothesized to lead to cell death in the pathological bloodstream form of *T. brucei* as they obtain all cellular ATP via glycosome metabolism.

To explore glucose import, and consumption mechanisms in *T. brucei* we deployed a series of recombinant fluorescent protein biosensors that specifically detect glucose moieties. Biosensors were expressed in *T. brucei*, and targeted to the cytosol or glycosomes allowing for real time monitoring of intracellular and intraglycosomal glucose concentrations. Using flow cytometry to monitor changes in sensor fluorescence, bloodstream form *T. brucei* cytosolic and glycosomal glucose were measured as $1.9 \pm 0.6$ mM and $3.5 \pm 0.5$ mM respectively in response to glucose levels similar to blood ($\sim 5$ mM). Higher glycosomal glucose versus the surrounding cytosol suggests active transport of glucose across the glycosomal membrane, a process that was assumed to occur via passive transport.

Monitoring biosensor response in trypanosomes accurately via microscopy is very difficult due to high motility and flagellar undulation. To monitor dynamics in intracellular...
biosensor response we adapted a microfluidic device which mechanically traps parasites, allowing for continuous imaging of cells under constant perfusion conditions. We found that single trypanosome glucose responses were consistent with bulk glucose measurements, cells also responded in a dose dependent manner when perfused against different glucose concentrations. Microfluidic trapping of *T. brucei* allows continuous imaging of single cellular dynamics which were previously not possible to image.

To identify small molecules that inhibit glucose uptake into parasites we adapted a high throughput screening assay utilizing the fluorescent glucose sensor as a score of glucose uptake inhibition. A pilot screen of 400 compounds identified two novel compounds that inhibit glucose uptake in trypanosome parasites with EC50s of 700nM and 5000nM respectively, one compound exhibited good killing (IC50 5uM) against infectious form parasites. To build upon the success of the pilot screen, 25,000 compounds were analyzed, from this library 57 compounds were identified, 40 of which kill infectious form trypanosomes with an IC50 value lower than 5uM. As predicted, trypanosome specific glucose uptake inhibitors identified in our screen exhibit potent anti-trypanosome killing activity.

The findings herein describe methods that help fill in the gaps in kinetoplastid glycosome glucose transport and metabolism. Using these methods, we have characterized glycosomal glucose transport, monitored single trypanosomes via fluorescent microscopy, and identified glucose uptake inhibitors specific to *T. brucei*. Although the methods here are limited to glucose measurements, they are amenable to studying a wide range of biologically relevant analytes with the broad pallet available fluorescent biosensors, in organelles of the parasitic kinetoplasts.
ACKNOWLEDGMENTS

I would like to first thank Dr. Kenneth Christensen for his guidance and assistance over my time as his student. Without his continuing support none of this would have been possible.

I would also like to thank my committee members who have been immensely helpful and accommodating during this unique experience, Dr. George Chumanov, Dr. James Morris and Dr. Daniel Whitehead.

I would also like to thank all of my collaborators and colleagues at Clemson and BYU that have made significant contributions, including Dr. Christine Ackroyd for all of her help and advice in manuscript construction over the years. All current and previous members of the Christensen Lab including Jeremy Tsang, Jordan Finnell, Sheng Lin, and the cohort of undergrads that have kept me busy. Members of the Morris’ lab for all of their continued help in all things trypanosome; including Meredith, Jim, Evan Qiu, Logan Crowe, and Jimmy Suryadi.

Finally, I would like to thank my family and friends for supporting me during this endeavor. In particular, I would like to thank my parents for imparting me with the unique skillset which allowed me to achieve what I have. To my brothers, Dave and Joe, thanks for being my first friends. Last I would like to thank my wife and partner in crime, Marissa, for agreeing to join me on this journey and all journeys to come.
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CHAPTER 1: Literature Review

Fluorescent Protein Biosensors for Detection of Analytes in Eukaryotic Parasites: Current Applications and Future Perspectives
Abstract

Kinetoplastids are a group of parasitic protozoa that infect millions worldwide with hundreds of millions more living in affected areas. One kinetoplastid of interest is *Trypanosoma brucei* which causes African sleeping sickness in a mammalian host. In biological systems, including that of the kinetoplastid parasites, small molecules play critical roles in energy storage and production, signal transduction, and are responsible for driving adaptations to different external environments for survival. The concentrations of these small molecules can change quickly as the metabolic demands of the cells shift, as the cells are introduced to different environments, or in response to internal cell signaling cascades. Tracking changes in small molecule concentration can provide a strong basis for understanding the complex metabolic and host evasion mechanisms in kinetoplastid parasites. Currently, genetically encoded biosensors serve as a powerful tool capable of continuously monitoring biological processes with subcellular resolution. This review summarizes the development of fluorescent proteins into biosensors, indexes the currently available sensors and their uses in monitoring (sub)cellular analyte concentrations, and also reviews the limited use of biosensors in kinetoplasts and other eukaryotic parasites, demonstrating how these fluorescent protein biosensors could have a significant impact on Kinetoplast biology.
1.2 Introduction

Organisms of the class Kinetoplastid and the diseases caused by these parasites are often denoted as neglected tropical diseases by the World Health Organization (WHO) [1]. Members of this class include *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania spp.* cause Human African Trypanosomiasis (HAT), Chagas disease and visceral Leishmaniasis respectively [2]. These diseases are endemic to tropical climates primarily in the rural communities of South America, the Indian continent, and sub-Saharan Africa [3]. Together, these diseases affect millions of people a year leading to approximately 100,000 deaths, with nearly half a billion people living in endemic regions.

Kinetoplastid parasites are distinguished from other protozoa by the presence of circular DNA called kinetoplast DNA (kDNA) that is found in their single large mitochondrion (Figure 1-1) [4]. The Kinetoplasstids also handle glucose consumption in a somewhat unusual manner, unlike most other eukaryotes they do not metabolize glucose in the cytosol. Instead, the first seven enzymes used in glycolysis are housed in specialized organelles called glycosomes [5]. Glycosomes are similar in protein composition to mammalian peroxisomes, but the exact protein composition is species and lifecycle dependent [6]. In bloodstream form (BSF) *T. brucei*, glucose is required for survival as the parasites do not possess an active mitochondrion and must obtain all cellular ATP from glucose metabolism in the glycosome. The insect life stage or procyclic form (PCF) *T.
*brucei* parasites contain a more complete mitochondrial protein repertoire and can produce ATP via oxidative phosphorylation [7].
Despite unique glucose metabolism and intricate metabolic shifts in the different life stages, the deployed analytical methods to monitor these phenomena in Kinetoplasts are limited. For decades, genetically encoded fluorescent biosensors have offered a powerful method for real time measurement of key analytes including glucose, calcium, pH and ATP [8]. There are dozens of cases demonstrating the usefulness of sensors in illuminating key biology in mammalian, yeast, and plant cells, but the literature is all but devoid of uses in *T. brucei* and other eukaryotic parasites [9],[10],[11]. Below, we briefly outline previous analytical methods used to monitor biologically relevant analytes. Next, we present the use of fluorescent proteins in biosensors, and highlight common structural motifs as well as common methods used for monitoring sensor fluorescence. We then summarize the current suite of available protein sensors and discuss their use in monitoring cytosolic and organellar analyte concentrations. Finally, we discuss current applications in eukaryotic parasites.

1.3 Monitoring Small Molecules in Biology

1.3.1 Monitoring ATP and glucose. Monitoring small molecules is important in biochemistry to be able to monitor key biological processes. Typically, small metabolic molecules including ATP and glucose can be measured in cell lysates with enzymatic or luciferase assays. Glucose uptake rates are traditionally studied with the use of radiolabeled glucose or 2-DOG analogs. To study metabolism, mass spectrometry can be
used to monitor the intermediate metabolites produced when the cells are introduced to different carbon sources [12]. These methods are all destructive and cannot measure the uptake of glucose into single live cells. A fluorescent glucose analog (2-NBDG) is available and enables exploration of glucose uptake mechanisms is live cells via flow cytometry [13]. These mechanisms are all costly, laborious, and can only make end point measurements [11].

1.3.2 Chemical biosensors. Imaging of analyte concentration in living cells was developed using chemical indicator dyes. One of the first and perhaps most studied of these chemical indicators are the calcium indicator dyes introduced by the Tsien lab in the 1970s and 1980s [14]. These chemical calcium dyes consist of a cation chelating group (BAPTA or EGTA) that is covalently attached to a reporter molecule. The excitation and emission spectra of the fluorescence indicator shifts in response to cation binding. Chemical probes for Na⁺, K⁺ and redox potential are also available [15][16]. Intracellular pH is critical for biological systems and plays a critical role in enzyme activity, ion and small molecule transport, and endocytosis [17]. Monitoring pH in living cells helps to illuminate the mechanisms of these key processes. To monitor pH in living cells, historically chemical probes have been deployed that possess pH dependent excitation or emission spectrum. By monitoring the shift in fluorescence due to known pH concentrations, intracellular pH can be quantified [17].
Although chemical biosensors have served as a powerful means for biochemical analysis they have limitations. The cells to be analyzed must be loaded with the sensor by microinjection, via cell membrane permeabilization, or synthesis of membrane permeable sensors that can be sequestered by the cell [18][19]. Dye loading protocols need to be optimized for each cell line to be studied because excess sensor can be cytotoxic and introduce artifacts into the experiment. Chemical sensing dyes may leak from the cell or be pumped out via the organic anion transporters that results in a gradual loss in fluorescence intensity limiting the length of experiments that can be run [20]. Chemical dyes are also unable to be targeted to organelles and are primarily limited to monitoring species in the cytosol.

1.3.3 **Fluorescent proteins history and perspective.** Many of the disadvantages of chemical sensors were alleviated with the advent of genetically-encoded fluorescent proteins and biosensors in the 1990s [21]. Analyzing endogenously expressed sensors is typically faster and easier, primarily because the cells are actively expressing the biosensor and do not require a lengthy loading step, although they require a lengthy transfection (24 hours). Using current expression systems, the levels of expression can be modulated to reduce any possible cytotoxic effects. Fluorescent protein biosensors are also amenable to alteration via molecular biology techniques, allowing easy addition of signaling sequences. Upon signal sequence addition genetically, encoded biosensors can
be localized to a number of organelles including the nucleus, peroxisomes, endoplasmic reticulum and mitochondria [22].

The first fluorescent protein Green Fluorescent Protein (GFP) was discovered in the 1960s, and its widespread influence on biochemistry and biomedical fields warranted the 2008 Nobel prize nomination in chemistry (Figure 1-2) [23]. Following the popularity of GFP as a means of gene regulation analysis as well as a tool for monitoring protein localization a number of related fluorescent proteins of different colors have been introduced [24]. To achieve an expanded pallet of fluorescent proteins, mutation were made in the protein’s chromophore region to yield different colored variants, primarily Cyan Fluorescent Protein (CFP), and Yellow Fluorescent Protein (YFP) [25][26]. The search for red shifted fluorescent proteins led to the discovery of the mFruit line of sensors which include mCherry and mApple which are popularly used in tandem with GFP mutants due to their lack of spectral overlap [27].
Figure 1-2: Cartoon of the structure of GFP. (Figure From: Daya RN, Davidson MW. The fluorescent protein palette: tools for cellular imaging. Chem Soc Rev. 2009;38: 2887–2921. doi:10.1039/b901966a.)
1.4 **Fluorescent Protein Biosensor Design**

1.4.1 **FRET based biosensors.** Following the widespread use of fluorescent proteins, they have been widely implemented as the fluorescent reporter species in a wide range of endogenously expressed biosensors. Perhaps the most popular sensor type relies on non-radiative transfer of energy between two spectrally compatible fluorescent proteins, this phenomenon is referred to as Forster Resonance energy transfer (FRET) [28]. By monitoring the amount of energy transfer between the two fluorophores, the relative distance between the two fluorophores can be inferred. FRET phenomena are utilized in analysis of protein-protein interactions, FRET substrate based enzyme assays and in intramolecular FRET constructs including FRET biosensors [22]. Distance is an important parameter that dictates the efficiency of FRET energy transfer (~4-10nm), but a number of other parameters must be optimized to enhance energy transfer. Fluorophore orientation is also critical, the dipoles of the donor and acceptor must be aligned for optimal FRET transfer [29]. The extinction coefficients and quantum yields of the two fluorophores also play a small role in FRET efficiency but distance and dipole orientation play the biggest role in biosensor optimization.

Biosensors that utilize FRET transfer as a means of sensor readout most often consist of a fluorescent protein FRET pair that flank an protein with an analyte binding domain (Figure 1-3). Upon analyte binding the recognition protein should undergo a conformational change that alters the spatial orientation of the two fluorescent proteins.
The change in spatial relationship alters the efficiency of FRET which is read out as a fluorescence ratio. By calibrating the resultant ratio in response to known analyte concentrations, intracellular analyte concentrations can be measured [30]. Due to their spectral overlap and early adoption, the most common fluorescent protein FRET pair consists of cyan fluorescent proteins (ECFP) and yellow fluorescent proteins (EYFP). ECFP and EYFP however are plagued with low photo stability and are sensitive to pH and chloride ions and have subsequently been replaced with brighter and more stable mutants. Common ECFP and EYFP mutants are mCerulean and mTurquoise for ECFP and mVenus and mCitrine for EYFP [31]. Although ECFP/EYFP FRET has been the most common other colored FRET pairs have been developed as multi-parameter imaging has become prevalent. The most popular FRET pair alternative are based off of variants of GFP/mCherry because of their relative brightness, pH stability, distinct emission profiles. As the fluorescent protein color palette is ever expanding, so does the number of available FRET pairs with mOrange/RFP and ECFP/mCherry pairs becoming available [32].
Figure 1-3: Sample FRET biosensor diagram. (Image from: Newman RH, Fosbrink MD, Zhang J. Genetically-encodable Fluorescent Biosensors for Tracking Signaling Dynamics in Living Cells. Chem Rev. 2011;111: 3614–3666. doi:10.1021/cr100002u.)
1.4.2 **Single fluorescent protein sensors.** Additionally, genetically encoded biosensors that utilize a single fluorescent protein as a fluorescence reporter have been developed. Single FP biosensors are typically composed of a circularly permutated fluorescent protein (GFP, EYFP, RFP) that has had an analyte binding protein inserted into it (Figure 1-4). Upon analyte binding the conformation of the binding domain changes and is allosterically transferred to the fluorescent protein. The conformation change alters the microenvironment of the fluorescent proteins chromophore, allowing interaction with the surrounding solution, which most commonly resulting in a decrease in protein quantum yield [33]. Analyte binding is measured increase/decrease in total fluorescence. Single protein biosensors have the added benefit of lower molecular weight due to the single reporter fluorophore. Single fluorophore sensors are typically used when sensor multiplexing is essential as they only inhabit one “spectral region” whereas FRET probes require two regions. Taking up a smaller spectral footprint allow for multiple single protein biosensors to be utilized simultaneously.
1.5 **Measuring Sensor Response *in vivo***

There are three primary means of monitoring the changes in fluorescence spectra of the biosensors in response to analyte binding. Measuring the fluorescence change with a plate reader is common practice as plate readers are readily available in most facilities. Plate readers come with the benefit of speed, as they can measure the fluorescence of samples in a 96 well plate in less than a minute, and can measure a 384 well plate in less than 5 minutes. This speed is advantageous when a large number of samples need to be analyzed, as in a high throughput screen where tens or hundreds of thousands of individual samples need to be run. High end plate readers can also be equipped with liquid handling features that enable different assay components to be added in real time. Plate readers do come with a number of disadvantages; primarily they are relatively insensitive compared to other fluorescence-based methods, and may require a large number of cells (>10^8) for adequate sensitivity, which in some systems may not be feasible due to cell density concerns or limitations. Plate readers also rely on measuring the bulk fluorescence of a population and cannot monitor changes in individual cells.

Fluorescence microscopy is an attractive alternative to plate readers that has been used extensively to monitor fluorescent sensor response since their introduction. Microscopes with appropriate optics to monitor FRET and single protein sensors have become common as manufacturers have standardized production of the filters and other components necessary for analysis. Microscopy also allows for single cell resolution and
can be used to monitor inter and intra-organellar events. One of the main drawbacks of microscopy, is the relatively low throughput that is achievable on common setups. Moderate-high throughput screening is all but infeasible without the addition of specialized automation hardware and software, like in a high-content cell analysis platform [34]. Imaging small motile organisms including the flagellated Kinetoplasts, is also difficult with the current technology as the cells high motility affects image acquisition.

Flow cytometry can serve as an intermediate between plate readers combining some of the best characteristics of both. A workhorse in immunology, flow cytometry, as a means of measuring genetically encoded biosensors, has become popular and accessible perhaps in part due to the rise in compatible cytometers for analyses. Current flow cytometers have relatively high throughput averaging 20-30 seconds a sample with newer and more specialized cytometers capable of 2-10 seconds a sample [35]. Flow cytometers also analyze the fluorescence of each cell individually, which increases the statistical power of your results, analysis of 10,000 cells per sample being the norm. Common cytometers do lack intracellular resolution but imaging flow cytometers capable of imaging 1000 cells a second are now available. We believe that each method of measurements has its own merits and should be used in concert to produce data most effectively and efficiently.
1.6 Biosensors for metabolic molecules

There are dozens if not hundreds of available genetically encoded fluorescent biosensors that are specific for dozens of prospective analytes [36][37]. It was not our goal to identify all available sensors, but rather to outline the newest and most improved sensors in current use. Early sensors may be discussed to put the direction of the field in perspective, but in general we aimed to highlight the optimized sensors that have been released in the later portion of the last decade.

1.6.1 Biosensors for pH. pH is a critical parameter in biological systems, pH regulation is critical as protein structure and function are often pH dependent. Chemical probes for pH have been utilized for decades, but chemical pH sensors suffer from the same limitations of chemical sensors and probes generally [38][39]. Wild type GFP contains a tyrosine residue in its chromophore core that is susceptible to protonation and deprotonation. The two different protonated states of the chromophore exhibit distinct excitation and emission profiles, which allows its use as a pH sensor [40]. Building upon the pH sensitivity of GFP mutants, the pHlourin series of pH sensors were developed in 1998 [41].

Since the introduction of the initial GFP based pH sensor, a range of improved sensors have been developed. Improvements to sensor linear range, sensor brightness and changes to FP color are now available. An EYFP based sensor E²GFP was developed
with a good dynamic range (~6-fold change) and a pKa of 7.5 [42]. To create a brighter GFP pH sensor pHluorin 2 was produced that exhibits brighter fluorescence at both excitation wavelengths used for analysis [43][44]. Red shifted pH sensors are required for multi sensor imaging where distinct spectra are required. Red shifted sensors also have the added benefit of requiring longer wavelengths for excitation that results in reduced phototoxicity in living systems. pHred, a red shifted pH sensor, is based on the red fluorescent protein mKeima [45]. The ratio is insensitive to salts, cations, oxidative stress and temperature making it optimal for monitoring pH in living cells responding to different environmental stresses. pHred was the first RFP based pH sensor that is ratiometric making it ideal for sensor multiplexing [46].

1.6.2 Biosensors for intracellular glucose. Glucose, and other hexose FRET sensors, have been available since the early 2000s. The majority of these sensors utilize well characterized periplasmic binding proteins (PBPs) as their recognition element [47]. PBPs contain two globular domains that engulf the hexose substrate upon binding, leading to a large shift in protein conformation. To construct a FRET sensor using these recognition domains, early sensors utilized a ECFP/EYFP FRET pair appended to both the N- and C-terminus of the PBP binding domain [48]. Sensors constructed with the wild type periplasmic glucose binding protein exhibited a \( K_d \) for glucose of 170 nM which is ~30,000 times lower than the glucose concentrations found in the mammalian bloodstream (~5
mM in healthy adults) limiting its usefulness in common model organisms [48]. By making mutations in the glucose binding domain, a sensor with a $K_d$ of 600 µM (FIIPGlu-600u) was produced, making it better suited for measuring glucose in the physiological range. The most sensitive sensor FLII12Pglu-700µδ6 exhibits the highest in vivo change in FRET ratio in response to glucose binding showing a 5-10 fold increase in observed FRET change in response to glucose binding [49]. To expand upon the available range of glucose concentrations able to be quantified, a series of other mutations to the glucose binding domain yielded sensors with a $K_d$ of 4.6 mM, 2.2 mM, 13.2 mM and 2 µM [50].

1.6.3 Pyruvate and lactate. Relatively new additions to the available biosensor palette are lactate and pyruvate sensors. Lactate and pyruvate serve roles in metabolism and cell signaling, but until 2013, there were no reliable ways to monitor intracellular concentrations in live cells. In 2013 and 2014, FRET biosensors were developed for lactate and pyruvate to enable real time imaging in cytosol and organelles in live cells [51],[52]. The FRET lactate sensor (Laconic) utilizes a monomeric Turquoise fluorescent protein (mTFP)/mVenus FRET pair flanking a bacterial transcription regulator (LldR) derived from *E. coli* that consists of two binding domains that change conformation upon binding to lactate. Laconic has a linear range of 1 µM to 10 mM and does not bind to other carbon sources including glucose, pyruvate, acetate, citrate and a range of metabolites and other biologically relevant small molecules [51]. Following the design rationale for the Laconic
lactate sensor, a pyruvate sensor was constructed using a pyruvate controlled transcription factor (PdhR) also derived from *E. coli*. As with the lactate sensor, mTFP and mVenus were used as a FRET pair due to their increased brightness and pH stability in comparison to ECFP and EYFP [52]. The resultant pyruvate sensor (Pyronic) exhibits similar dynamic range as the lactate sensor with a linear range of 10 μM to 1 mM with a *K*<sub>d</sub> for pyruvate of 107 μM. Pyronic was also not susceptible to changes in intracellular pH or the addition of metabolic acids or other small molecules making it ideal for pyruvate monitoring in living cells.

### 1.7 Nucleotide Specific Biosensors

ATP use is conserved as a means of energy storage and transfer in living systems [53]. ATP is critical metabolic molecule responsible for a number of cellular tasks including transfer of energy, signal transduction and phosphate transfer. The ratio of ATP/ADP in the cells can serve as an accurate measure of the metabolic potential of the cell. In mammalian cells, ATP/ADP regulates glycolysis and can stimulate other means of ATP production [54]. ATP/ADP may also be critical in parasites given their changes in host organisms and the environments that they inhabit. ATP is the major energy storage molecule, but there were no current ways to monitor the levels inside of living cells in real time. Two main varieties of ATP sensors have now been optimized for imaging in living
cells; ECFP/EYFP FRET sensors that measure free ATP, and FP-chimeric sensors that monitor the ATP/ADP ratio and ATP in live cells.

1.7.1 FRET based ATP sensors. The ATeam biosensor class were some of the first ATP biosensors allowing for organelle specific monitoring [55]. The ATeam sensors utilize an ECFP and a pH and chloride stable EYFP variant mVenus. Binding domains were derived from ε subunit of the F₀F₁-ATP synthase. Two different ATeam families were produced sourcing their binding domain from *Bacillus subtilis* for AT1.03 and higher affinity *Bacillus* sp. PS3 for the AT3.10 class sensors. AT1.03 and AT3.10 have binding constants of 1.2 mM and 7.4 µM respectively. By modifying the binding domains, two additional mutants were produced with lower ATP affinity, AT1.03YEMK (Kd = 14 µM) and AT3.10MGK (Kd = 14 µM). In the mammalian cells used as a model in this study, ATP levels were in the sensitive range of AT1.03 and 1.03YEMK sensors [56]. This would suggest that the higher affinity versions are 10-100 times too far out of the linear range for analysis.

1.7.2 Chimeric single protein ATP sensors. In 2014, a single fluorescent protein biosensor for monitoring ATP, deemed QUEEN, was developed. The QUEEN sensor family consists of circularly permuted chimeric protein utilizing cpGFP as the fluorescent molecule and the ε subunit of the F₀F₁-ATP synthases of different Bacillus species [57]. Two different variants were produced, QUEEN-2m and QUEEN-7µ, and were used to
measure the distribution of cellular ATP in a population of *E. coli* cells. The QUEEN biosensors are superior to the ATeam sensors as the single fluorescent protein matures homogenously; whereas, the FRET based system FPs mature at different times [58]. Different fluorophore maturation rates result in a heterogeneous population of intracellular sensor within a dividing population leading to unexpected variances on a cell to cell basis, this limits the efficacy of FRET sensors in fast dividing cell lines. The QUEEN biosensors are more sensitive than previous sensors and are ideal when monitoring differences in cell to cell ATP concentration is wanted.

1.7.3 ATP/ADP ratio sensors. To measure ATP/ADP ratio in live cells, the Perceval sensors were developed. Perceval sensors are constructed from a circularly permutated yellow protein variant cpmVenus, which has the bacterial ATP binding domain GlnK1 inserted as a recognition element [59]. The sensor binds Mg-ATP with relatively high affinity ($K_d = 100$ nM), and at physiological conditions, the sensor is fully saturated. Under biological conditions, the sensor is saturated with ATP, but ADP can compete for binding, which alters the spectral properties of the sensor. ADP binding can be read out ratiometrically as the fluorescence spectra of the sensor changes proportionally with the ATP/ADP bound ratio. The original Perceval sensor was tuned to be able to measure ATP/ADP ratios that are relevant in biological systems, which is estimated to be in the range of 10:1 but could range as high as 100:1[60][54][61]. The sensor is pH sensitive and
pH must be measured in tandem with a pH sensor to correct for shifts in binding constants. In this report the chemical pH sensor SNARF-5F was employed to measure changes in intracellular pH.

To improve upon the relatively low dynamic range Perceval, PercevalHR was designed. By mutating the binding pocket of the original Perceval sensor [62], a new sensor able to bind a greater range of ATP:ADP ratios was created. ATP/ADP ratios in living cells are up to 100-fold, but with Perceval was only sensitive to a 5-10-fold difference. The change in excitation ratio for PercevalHR is >2 fold greater than the original sensor that yields an 8-fold change in excitation and emission ratios when sensor is bound to different ATP:ADP ratios. The affinity for Mg-ATP is 3 µM versus 100 nM for the original Perceval sensor. Like the original sensor, PercevalHR is still pH sensitive and requires pH correction to make accurate measurements.

1.7.4 cAMP FRET sensors. First generation FRET-based cAMP sensors were first developed in 2004 using a ECFP/EYFP FRET pair flanking one of two cAMP binding domains, the Epac domain (exchange protein directly activated) or PKA (protein kinase A) domains, which serve as the cAMP recognition element [63]. These early sensors exhibit low sensitivity, are sensitive to pH, and susceptible to photobleaching.

Since the first generation cAMP sensors, there have been many improvements by optimizing fluorophore location and linker length, as well as the FRET pair utilized in the
sensor [64]. Second generation Cyan/Yellow, Cyan/Green, Green/Red and Green/Orange FRET pairs were all analyzed, CFP/mVenus and GFP/RFP FRET pairs were found to be optimal when sensitivity and photo stability were considered. Fourth generation sensors optimize the ECFP/EYFP FRET pair with brighter mutants that increase brightness, photostability, pH sensitivity, and FRET emission. The fourth generation sensors employ mTourquise2 an enhanced mutant of ECFP and mVenus-mVenus tandem dimers [65][66].

1.7.5 Single protein cAMP sensors. The common observed trend to move from dual fluorescent protein FRET based biosensors, to single FP chimeric constructs continues in cAMP sensors. Yellow and red FP variants are found in the literature utilizing circularly permutated Citrine or mA1pple proteins as the fluorescence reporter using PKA or Epac1 binding domains for cAMP recognition. The resultant sensors are called Flamindo and Pink Flamindo respectively [67][68]. These Flamindo sensors exhibit binding constants for camp in the 1-10 µM ranges and exhibit a 4-10-fold change in fluorescence in response to analyte binding. These single protein sensors allow for simultaneously monitoring of multiple FP sensors but cannot be measured ratiometrically, meaning that cell to cell variation in protein expression will lead to irregularities in measurements.

1.8 Cationic Metals
1.8.1 **FRET based Zinc protein biosensors.** Early genetically encoded zinc sensors (GEZIs) called Zap1 utilize zinc finger binding domains flanked by the common ECFP/EYFP FRET pair. By altering the binding domain, sensors with $K_d$ of 0.2 nM and 4 nM were obtained [69]. Further studies aimed to improve Zap1 by altering the linker length between the ECFP and EYFP fluorophores and exchanged the EYFP for the pH insensitive mCitrine. The resultant sensor ZapCY1 exhibits a $K_d$ of 2.5 pM and was selective for zinc ions. Further optimizations to the zinc binding domain yielded a sensor with $K_d$ of 800 pM (ZapCY2) [70]. To enable multiple organelle zinc measurements, a suite of different colored FRET zinc sensors were developed. Sapphire/RFP (ZapSR2), mOrange/mCherry (ZapOC2), and Clover/mRuby2 (ZapCmR) FRET pairs were developed with a zinc finger domain as the zinc recognition domain. Using the expanded color pallet of zinc sensors, cytosolic and nuclear zinc concentration were monitored simultaneously for the first time [71]. Alternatives to the Zinc finger binding domain FRET sensors are the ATOX1, and WD4 based CALWY sensors [72][73].

1.8.2 **Chimeric single FP Zinc biosensors.** To expand the repertoire of sensors for multiparameter imaging, a suite of single FP zinc biosensors were developed. ZnRed and ZnGreen are two zinc binding sensors that utilize mApple or mTurquoise as their fluorescence reporter molecule and the same Zap1 zinc finger used in the FRET biosensors. Basing their structure off of the Camgaroo calcium sensors, the Zap1 recognition element
was inserted into a circularly permuted fluorescent protein [74]. ZnRed and ZnGreen show 8 and 4 fold changes in fluorescence when bound to zinc ions.

1.8.3 Calcium FRET based sensors. Like the other FRET sensors, the calcium sensor relies on a calcium bind domain for recognition of calcium. There are two primary domains used for calcium sensing, the calmodulin/M13 peptide design and the tropin C binding domain. The binding kinetics of the calmodulin/M13 peptide are somewhat slow and unsuitable for fast calcium pulse measurements. For fast measurements, the sensor TN-XXL was designed that uses a tropin C binding domain from zebrafish and has fast kinetics and a binding constant of 800 nM, which is optimal for organelle targeted studies [75]. The Twitch series of calcium sensors are designed using different tropin C binding domains, and were subsequently optimized in E. coli to identify the most sensitive sensors. Twitch 3 and 2b were the most sensitive with binding constants that are comparable to the commonly used Fura and Indo chemical sensors [76]. Monitoring the calcium concentration in the endoplasmic reticulum can be difficult as endogenous calmodulin effects the binding characteristics of the sensors. To overcome this issue, a sensor with a modified binding domain derived from smooth muscle myosin light chain kinase (m-smMLCK) allows for monitoring ER calcium without any interference [77].

Single FP sensor variants are also very popular with one of the recent versions being designed around a new calmodulin binding domain derived from fungi. As
previously discussed, the calmodulin binding domain is prone to interference with intracellular calmodulin and utilization of divergent proteins reduces interference. This single FP sensor utilized a circularly permutated EGFP with the calmodulin inserted. The sensor changes its observed ratio ~100 fold when binding calcium [78].

1.9 Targeting Sensors to Organelles

One of the distinct advantages in using endogenously expressed protein probes, is that they can be easily altered via molecular biology allowing the addition of targeting sequences [79]. Compared to chemical sensors, this allows for directed targeting to specific organelles to make intraorganellar measurements. Examples of sensor trafficking to organelles is limited in eukaryotic parasites, but in other eukaryotes sensors have been successfully localized to organelles without any noticeable degradation in sensor response [79]. Here we will outline all relevant targeting strategies as well as highlighting the uses of targeted sensors in eukaryotic parasites.

1.9.1 Mitochondria. Eukaryotic cells have contained mitochondria for nearly 2 billion years when it is hypothesized that an ancient eukaryotic cell engulfed a proteobacteria [80]. Mitochondria are the powerhouses of eukaryotic cells, and they host the pathways necessary for efficient ATP production via electron transport chain and oxidative phosphorylation. Transport of calcium into mitochondria also plays a critical role in
maintaining the potential across the mitochondrial membrane needed for optimal ATP synthesis [81]. The mitochondria’s importance in energy production, calcium homeostasis and signaling make it an interesting target for targeted biosensors.

Proteins destined for the mitochondrial matrix are tagged with N-terminal mitochondrial targeting sequences (MTS) that are then processed and cleaved once the protein enters the mitochondria [82]. MTS sequences vary depending on the protein of interest ranging from 9-90 N-terminal residues with a high concentration of positively charged arginine residues [83]. The high diversity of possible MTS peptides makes choosing a relatively short and optimal signal sequence difficult. Very common is the use of MTS sequences found on the mitochondrially localized cytochrome oxidase IV or VIII [84] [85][86]. The ATeam ATP sensors were trafficked to the mitochondrial matrix (mit-ATeam) using a tandem repeat of the cytochrome VIII oxidase subunit, that showed mitochondrial ATP was unexpectedly lower than that of the cytosol [56]. The zinc sensors eCALWY-4 and eZinCh-2 were both targeted to mitochondria with the N-terminal targeting sequence from the cytochrome oxidase subunit VIII (COX VIII)[87][88]. As calcium influx is critical for mitochondrial function, a suite of calcium sensors have also been sent to the mitochondria including GEM-GECO1 [89], Camgaroo-2 [90], and Pericam all using the COX VIII targeting sequence [91] [92]. The pH gradient across the mitochondrial membrane is the main driving force behind ATP production making monitoring pH flux in the mitochondria interesting for live cells. The pH sensors SyPher
[44], mtAlpHi [90], Rosella [93], pHred [45], were all targeted into the mitochondria via COX VIII MTS sequences.

1.9.2 Endoplasmic reticulum. The endoplasmic reticulum (ER) is responsible for protein synthesis, lipid synthesis, and steroid production [94]. Much like the other organelles in the cell, the ER has recently been suggested to act in concert with other organelles including the mitochondria and nucleus to coordinate cellular function [95]. It is also a store if cellular zinc and calcium. Proteins destined for the ER need a C-terminal targeting peptide, as well as an N-terminal retention peptide [96].

The glucose sensor FLIPglu-600u-ER [97], calcium sensors GCaMP3 [98], GCaMPer [98], and mCaM/msmMLCKp [77], among others, were targeted to the inner endoplasmic reticulum lumen with a C-terminal retention signal from BiP (TAEKDEL) and an N-terminal targeting sequence from calreticulin (MLLSVPLLGLGLAVL). The zinc sensors ZapCY (ER-ZapCY-1)[70][71], eZinCh-2[87] [99], and CALWYs (e CALWYs) [88] were all localized to the ER by the addition of an N-terminal preproinsulin (PPI) sequence as a C-terminal (KDEL) retention sequence. To monitor intra-ER pH, pHluorin-KDEL [100], was targeted using the N-terminal peptide from GluR-D and a KDEL retention signal.

1.9.3 Peroxisomes. Peroxisomes were originally named for their function in H₂O₂ production and degradation, but since then, their roles in fatty acids metabolism have
become apparent [101][102]. More recently, the peroxisome’s function in intraorganellar signaling has become an area of study [103][104]. Peroxisomes are ideal for targeting protein biosensor probes as their import mechanisms are well studied [105]. Proteins destined for peroxisomal transport are tagged with a peroxisomal targeting sequence (PTS) and are transported across the peroxisome membrane in their fully folded and functional form [106][107]. Targeting fluorescent probes via a C-terminal tripeptide PTS type 1 sequence (SKL) has been used commonly to study the function of the peroxisomal import machinery (PEX proteins) [108] [109]. Targeting fluorescent biosensors to the peroxisomal lumen also appends a PTS1 sequence to the C-terminus of the biosensor. The pH sensor pHluorin [110], redox sensors: roGFP (roGFP-SKL) [110], redoxfluor C [111], as well as the calcium sensor Cameleon D3cpv ((D3cpv-KVK-SKL) [86] were all targeted to peroxisomes via addition of a PTS.

1.9.4 Nucleus. The nucleus is partitioned from the cytosol via a double layered membrane and cargo destined for nuclear localization must pass through pores constructed of nuclear pore complexes (NPCs) [112]. Nuclear transport signals were first characterized in simian virus 40 (SV40) large t-antigen [113]. These classical nuclear localization signals (cNLS) are recognized by the protein importin-alpha [114]. Biosensors are localized to the nucleus by appending an NLS to the N-terminus of the protein. The glucose sensors FLIPglu-600µ and Flipglu-170nM were cloned in to the expression vector
pCMV/myc/nuc which imparts a NLS sequence for nuclear localization [115]. The cAMP sensor (Epac2-NLS) [116], ATeam ATP sensor (nuc-AT1.03) [56], pH sensor pHluorin (pHluorin-NLS) [117][118], and the Zinc sensors ZnRed (pNuc-ZnRed)[74], and calcium sensor GECIs[89] were all trafficked to the nucleus using the common nuclear localization sequence (DPKKKRKV).

1.10 Current uses in Eukaryotic Parasites

1.10.1 cAMP in T. brucei. Surface induced social behavior is critical in T. brucei and is used to coordinate group movement throughout the number of environments encountered during their life cycles. cAMP is an important signaling molecule in the cell and has been attributed to signaling social motility behavior in the parasites. To this end, the cAMP sensors have been deployed in the cells to monitor changes in intracellular cAMP in response to stimuli. T. brucei and other single-cell microbes are not found isolated in nature. They are often inhabiting environments with tens or hundreds of millions of other related organisms exhibiting group behaviors. Using Epac1 cAMP FRET probes, intracellular cAMP levels were monitored in response to phosphodiesterase inhibitors which have been previously shown to abolish social motility in T. brucei [119]. The cAMP FRET sensors were also used in T. brucei to confirm the in vivo effects of candidate phosphodiesterase inhibitors [120].
1.10.2 Calcium in Plasmodium and Toxoplasma. Calcium signaling is conserved in eukaryotes including the pathogenic eukaryotes plasmodium, toxoplasma, and the Kinetoplastid parasites [121][122]. In *Plasmodium falciparum*, the causative agent of malaria, the calcium sensor Yellow-Cameleon was utilized to monitor intracellular calcium concentration in the different life stages of the parasite [123]. Using flow cytometry to monitor the intracellular calcium levels, it was found that in intraerythrocytic trophozoite stage calcium is low (~30nM) but is roughly ten-fold greater in the other life stages. Intracellular calcium release was not induced by the mammalian SERCA inhibitor thapsigargin, suggesting that the ER is not a calcium store in the trophozoite stage of *P. falciparum*. The FRET flow cytometry method for calcium measurements is suggested as a possible application for high throughput screening.

*Toxoplasma gondii* is predicted to infect nearly 30% of the world population and causes birth defects and blindness (particularly in the immune compromised) [124]. The parasites rely on environmental cues to signal changes in its lytic cycle. Calcium is one of the signaling molecules that plays a critical role in driving forward the parasite’s lytic cycle [125]. To investigate intracellular calcium during different stages in the lytic cycle, the genetically encoded Ca^{2+} sensor GCaMP6 was deployed. With these endogenously expressed calcium sensors, calcium concentrations in intracellular parasites were able to be measured. Previous chemical probes were unable to accomplish this as there was no way to load the sensor into the cells when they are intracellularly localized. Using the
sensors, calcium concentrations were monitored during intracellular steady state and parasite egress [126]. Disruption via inhibitors or knockdown of phosphatidylthreonine synthase depletes calcium stores and leads to dysregulation of calcium release during parasite egress [127]. Calcium increases during cell egress, but was diminished in the phosphatidylthreonine synthase knock down mutants. These results suggest the importance of calcium release into the cytosol plays on parasite mobilization and egress.

1.10.3 pH in Plasmodium. Plasmodium parasites constantly ingest large amounts of hemoglobin to meet its ATP requirements [128]. Once taken up, hemoglobin is hydrolyzed in digestive vacuoles, which are assumed to be acidic, given that the enzymes housed therein work optimally at pH 4.5-5.0. To monitor the pH of the digestive vacuole, the GFP based pH sensor pHluorin was endogenously expressed in plasmodium parasites [129]. pHluorin was targeted to the digestive vacuole by appending the first 70 amino acids of plamepsin, a protein known to localize to digestive vacuoles. Steady state cytosolic pH was measured at 7.15 and the digestive vacuole was found to maintain a pH of 5.18.

1.11 Future Prospects for protein biosensors

As the suite of available biosensors expands and improves, the number of biological processes that can be monitored also increases. Genetically encoded biosensors have been used extensively in mammalian and yeast systems but have yet to
be deployed to their full potential in the Kinetoplasts and in other parasite families.
Protein Biosensors can be deployed to expand upon the current literature that utilized
chemical probes for measurements. Many measurements of cytosolic pH, and calcium
have been made in Kinetoplasts [130],[131],[132],[133], but monitoring the conditions
inside critical organelles is difficult with chemical probes. Glycosomal pH was monitored
in *T. brucei* using a FITC chemical probe for pH appended with a PTS1 signal [134],[135].
Expanding upon this research with the use of peptide targeted sensors to the glycosome,
mitochondria, nucleus, ER and other parasite specific organelle can help illuminate the
dynamics between these organelles and the cytosol and extracellular environments.
Deploying fluorescent biosensors for drug discovery screening is also advantageous with
examples existing in mammalian cells [136], and in trypanosomes as a validation of novel
inhibitors. By deploying a wide range of sensors targeted to different organelles, we
believe we will be able to explore and illuminate Kinetoplastid biology. Multiplexing with
flow cytometry can allow for simultaneous interrogation of a number of analytes in a
range of organelles, this allows for simultaneous measuring of different analytes. These
multiplexing experiments can serve as a strong basis for multiparameter high throughput
screening where upwards of a dozen different parameters could be monitored in
response to candidate screening compounds. This could allow for simultaneous drug
discovery for a specific phenotype as well as characterizing the mechanism of action via
the other sensors in the cell.
CHAPTER 2: A FRET Flow Cytometry Method for Monitoring Cytosolic and Glycosomal Glucose in Living Kinetoplastid Parasites

This manuscript was originally compiled for submission to PLOS Neglected Tropical Diseases. The paper has been through one cycle of review and is currently being resubmitted. It has been reformatted to remain consistent with other chapters.
2.1 Abstract

The bloodstream lifecycle stage of the kinetoplastid parasite *Trypanosoma brucei* relies solely on glucose metabolism for ATP production, which occurs in peroxisome-like organelles (glycosomes). Many studies have been conducted on glucose uptake and metabolism, but none thus far have been able to monitor real-time changes in cellular and organellar glucose concentration in live parasites. We have developed a non-destructive technique for monitoring changes in cytosolic and glycosomal glucose levels in *T. brucei* using a fluorescent protein biosensor (FLII12Pglu-700µδ6) in combination with flow cytometry. *T. brucei* parasites harboring the biosensor allowed for observation of cytosolic glucose levels. Appending a type 1 peroxisomal targeting sequence caused biosensors to localize to glycosomes, which enabled observation of glycosomal glucose levels. Using this approach, we investigated cytosolic and glycosomal glucose levels in response to changes in external glucose or 2-deoxyglucose concentration. These data show that procyclic form and bloodstream form parasites maintain different glucose concentrations in their cytosol and glycosomes. In procyclic form parasites, the cytosol and glycosomes maintain indistinguishable glucose levels (3.4 ± 0.4mM and 3.4 ± 0.5mM glucose respectively) at a 6.25mM external glucose concentration. In contrast, bloodstream form parasites maintain glycosomal glucose levels that are ≈1.8 fold higher than the surrounding cytosol, equating to 1.9 ± 0.6mM in cytosol and 3.5 ± 0.5mM in glycosomes. These observations suggest active glucose transport mechanisms operating
in the glycosomes of bloodstream form *T. brucei*. The methods described here will aid in the illumination of the mechanisms of glucose transport into the glycosome, which may help identify novel targets for anti-trypanosome treatments.
2.2 **Author summary**

African sleeping sickness is caused by *Trypanosoma brucei*. Tens of millions of people living in endemic areas are at risk for the disease. Within the mammalian bloodstream, *T. brucei* parasites sustain all their energy needs by metabolizing glucose present in the host’s blood within specialized organelles known as glycosomes. *In vitro*, bloodstream parasites rapidly die if glucose is removed from their environment. This reliance on glucose for survival has made glucose metabolism in *T. brucei* an important area of study with the aim to develop targeted therapeutics that disrupt glucose metabolism. However, there have previously been no reported methods to study glucose uptake and distribution dynamics in intact glycosomes in live *T. brucei*. Here we describe development of approaches for observing changes in glucose concentration in glycosomes in live *T. brucei*. Results obtained using these methods provide new insights into how *T. brucei* takes up and transports glucose to sustain cell survival.

2.3 **Introduction**

Glucose is an important metabolite for members of the class Kinetoplastea that includes *Trypanosoma brucei*, *T. cruzi*, and parasites of the genus *Leishmania*. Human African Trypanosomiasis (HAT) is caused by a bloodstream infection with *T. brucei*. The disease is endemic to sub-Saharan Africa where it is estimated that 60 million people live
at risk for contracting the disease [137]. Despite the millions of people at risk, effective treatment options, especially for late stage disease, are lacking, and can be accompanied by severe drug toxicity [138]. In addition, resistance to existing drugs has been reported [139], [140]. For these reasons, we are expanding the repertoire of analytical methods used to study *T. brucei* metabolism to advance our understanding of HAT and enable development of new and more effective anti-trypanosomal treatment(s).

The parasitic Kinetoplastea differ from one another in several key ways. First, *T. brucei* and *Leishmania* parasites spread by biting flies (the tsetse fly and sandfly, respectively) during bloodmeals, while *T. cruzi* is deposited in the feces of blood feeding Triatominae bugs. While *T. brucei* is exclusively an extracellular parasite, *T. cruzi* and *Leishmania* spp. can inhabit intracellular niches in the amastigote life cycle stage. Nevertheless, the mechanisms of acquisition, subcellular distribution and metabolism of glucose are largely conserved in the kinetoplastid parasites when compared to the complementing machinery of their mammalian hosts, suggesting commonalities that may yield potential therapeutic targets that are pan-kinetoplastid.

The bloodstream form (BSF) of *T. brucei* relies on glucose metabolism in the mammalian host’s blood for ATP production and does not use other means of ATP production such as mitochondrial respiration or the Krebs cycle. When denied glucose, BSF parasites die rapidly [141]. The procyclic or insect stage (PCF) parasite harbors a more complete mitochondrial protein repertoire and is capable of metabolizing other carbon
sources like amino acids and fatty acids, in addition to glucose [142]. However, PCF parasites will metabolize glucose preferentially over amino acids and other carbon sources, even if the glucose is present in much lower concentrations [143]. The parasite's reliance on glucose for survival make glycolysis, glucose transport, and the enzymes involved therein an important topic of study.

Importantly, kinetoplastid parasites house the majority of glycolytic enzymes in a unique kinetoplastid-specific organelle, the glycosome. Glycosomes are related to mammalian peroxisomes based on conserved biosynthetic mechanisms and general morphology [144] [145]. While related, the functions of these organelles are not entirely overlapping; the glycosome is unusual in that it harbors the first seven glycolytic enzymes. As a result, glucose metabolism requires glucose transport from the external environment through the cell membrane into the cytosol, and subsequently through the glycosomal membrane into the glycosome before glucose metabolism can occur. The glycosomal localization of glucose metabolism means that glucose transport into the glycosome is inextricably linked to glucose metabolism in both BSF and PCF parasites. Plasma membrane glucose transporter proteins known as trypanosome hexose transporters (THTs) are responsible for movement of glucose across the plasma membrane into the cytosol. However, these molecules have not been detected in glycosomal membranes [146], and the existence and identity of glucose transporter proteins responsible for movement of the hexose into glycosomes remains an unresolved question [147][144].
With the advent of green fluorescent protein (GFP) variants in the 1990s, Förster Resonance Energy Transfer (FRET) technologies using these proteins have been useful [148] in the development of recombinant protein biosensors that can detect biologically relevant analytes, including glucose, ATP, calcium and pH [11][149]. These biosensors commonly consist of a FRET pair flanking a protein binding domain specific for a particular analyte. In the case of the glucose FRET sensor FLII12Pglu-700μδ6, used here, an ECFP/mCitrine FRET pair flanks an E. coli periplasmic glucose binding protein [150]. Upon glucose binding, this domain undergoes a conformational change that alters the spatial relationship and orientation of the fluorescent proteins to cause a measurable change in energy transfer between the two fluorophores. This change can be observed in living cells proportional to analyte concentration, allowing glucose to be monitored in real time.

In this study, we have used ratiometric fluorescent protein biosensors to monitor glucose concentration in the cytosol and glycosomes of T. brucei and to measure glycosomal glucose changes in response to changes in environmental glucose levels. Results from this work suggest BSF T. brucei glycosomes can maintain glucose levels higher than those present in the cytosol. Given the findings, we anticipate active mechanisms are at work to support glucose uptake and distribution – some of which may be kinetoplastid-specific and therefore potential therapeutic targets.
2.4 Methods

2.4.1 Chemicals and reagents. 2-deoxyglucose (2-DOG), \( \beta \)-escin, Triton X-100, Digitonin and all buffer and cell culture media components were purchased from Sigma (St. Louis, MO). The Q5 mutagenesis kit, all restriction enzymes, and other enzymes and reagents used for cloning were purchased from New England Biolabs (Ipswich, MA). Human T Cell nucleofector kits used for BSF expression vector transfection were purchased from Lonza (Basel, Switzerland). IPTG, carbenicillin and all other drugs used for selection were purchased from Gold Bio (Olivette, MO). To normalize experiments between PCF and BSF cells and to mitigate media effects on cells, studies were carried out in phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 10mM Na\(_2\)HPO\(_4\), and 1.8 mM KH\(_2\)PO\(_4\)) adjusted to pH 7.4, and containing glucose at the concentration required for the experiment. pH calibration buffer used for intracellular FLI\(^{12}\)Pglu-700\(\mu\)d6 calibration was carried out in HEPES and MOPS buffered saline (HM-PBS; 137mM NaCl, 2.7mM KCl, 25mM MOPS, 25mM HEPES, 10mM Na\(_2\)HPO\(_4\), and 1.8 mM KH\(_2\)PO\(_4\)) adjusted to the desired pH, and titrated with glucose to the desired concentration for calibration.

2.4.2 Trypanosome culture. BSF parasites were cultured in HMI-9 media at 37°C in 5% CO\(_2\) and maintained fastidiously at cell densities between 5\(\times\)10\(^4\) and 5\(\times\)10\(^6\) cell/mL [151].
PCF form parasites were maintained at 29°C in 5% CO₂ in SDM-79 media and maintained at cell densities between 5x10⁵ and 1x10⁷ [152].

2.4.3 Sensor cloning and bacterial expression. The fluorescent biosensor pRSET-FLII₁²Pglu-700μδ6 [150] was purchased from Addgene (Addgene plasmid # 13568). For bacterial expression, chemically competent NEB T7 Express (BL21 strain) cells were transformed with pRSET-FLII₁²Pglu-700μδ6. Protein was expressed using methods adapted from [9]; briefly, cells were grown in LB broth at 30°C to density of OD₆₀₀~0.6 and then induced with 0.7mM IPTG and grown at 18°C for 24 hours. Cells were then harvested and lysed via French press and protein purified using Ni-NTA chromatography (GE; Pittsburg, PA). Purified protein was exchanged into PBS with 20%v/v glycerol using a PD-10 desalting column (GE; Pittsburg, PA) and then stored at -80°C until further use. FLII₁²Pglu-700μδ6 was cloned into the trypanosome expression vectors pXS2 and pXS6 using BamHI and HindIII restriction sites.

2.4.4 Trypanosome transfection. To monitor cytosolic glucose concentration, an endogenously expressed fluorescent glucose sensor FLII₁²Pglu-700μδ6 was transfected and expressed in PCF and BSF parasites using the expression vectors pXS2 or pXS6 respectively. FLII₁²Pglu-700μδ6 with a type one peroxisomal targeting sequence (PTS-1)
added to the C-terminus (GGAKL) via site directed mutagenesis was used to monitor glycosomal glucose levels herein referred as FLII^{12}Pglu-700μδ6-PTS (Forward: cgcgaaactgTAAAAGCTTGATCCGGCTG, Reverse: cgcccttcgcCTTGTACAGCTCGTCCATG). Constructs were transected into parasites as described [153][154].

2.4.5 **Microscopy.** All live cell DIC and fluorescence microscopy was carried out on an inverted epifluorescence microscope (Olympus IX73; Tokyo, Japan). FRET images were taken using a high speed DG-4 light source (Sutter; Novato, CA) and a sensitive CMOS camera (Hamamatsu Orca-Flash 4; Japan) following excitation 433/30 nm for ECFP and mCitrine or ECFP emissions collected using 530/30 and 480/30 nm filters, respectively. All microscope components were controlled and images collected and analyzed using Slidebook 6.0 (Intelligent Imaging Innovation; Denver, CO). To minimize background fluorescence caused by media components, cells were imaged in PBS supplemented with 10 mM glucose. Fluorescent intensities were calculated by subtracting the background fluorescence from a cell free field for each image field taken.

Immunofluorescence (IF) slides were prepared using a protocol modified from a previous report [155]. In short, trypanosomes in log phase were harvested by centrifugation (2000 rpm, 10 min), washed with PBS, and resuspended in 1:1 PBS and fix solution (4% formaldehyde in PBS, 100 μL per sample). Cells were loaded to slides and
allowed to settle for 30 minutes. Cells were then washed with wash solution (0.1% normal goat serum in PBS), permeabilized with 0.5% triton in PBS for 30 minutes, and blocked with blocking buffer (10% normal goat serum and 0.1% triton in PBS) for 30 minutes. Primary and secondary antibodies in blocking buffer were applied to the cells for 1 hour respectively with washes in between. Anti-Aldolase (1:2,000) and anti-GFP (M3E6) (1:200) antiserum were used to detect glycosome and PT-FLII12Pglu-700μδ66 respectively. Primary antibodies were detected with Alexa Fluor 488-conjugated anti-rabbit (1:1,000) or Alexa Fluor 568-conjugate anti-mouse (1:1,000) antisera. The nucleus and kDNA were stained using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories; Burlingame, CA). IF slides were visualized using Zeiss Axiovert 200M microscope.

2.4.6 Protease protection assay. PCF cells (1 x 10^6) expressing the FLII12Pglu-700μδ6-PTS glucose sensor were harvested by centrifugation (800g, 10 minutes), washed with PBS, and then washed again with STE buffer (250mM sucrose, 25mM Tris-HCl, pH7.4, 1mM EDTA). Pellets were resuspended in STEN buffer (STE with 150mM NaCl) and 0.1mM PMSF and cells permeabilized with digitonin (0.02mg/mL final concentration) by incubation at room temperature (4 minutes). The sample was then centrifuged (20,000 x g, 2 minutes) and resuspended in 85ul STEN buffer with either water or Triton-X100 (1% v/v final concentration) added before addition of water or proteinase K (0.1 ug/uL final concentration). Samples were incubated on ice (30 minutes) and the reaction halted by
addition of TCA (10% w/v) followed by centrifugation (20,000 x g, 2 minutes). The pellet was then washed with cold acetone and solubilized in SDS PAGE running buffer. Following resolution by SDS-PAGE and transfer to nitrocellulose, FLII12Pglu-700μδ6-PTS fragments were identified using anti-eYFP antisera.

2.4.7 In vitro characterization of biosensors. Emission spectra of purified FLII12Pglu-700μδ6 and FLII12Pglu-700μδ6-PTS and glucose titration curves were obtained using a fluorometer (PTI; Edison, NJ) with ECFP excitation at 433 (5-nm bandpass) and emission (5-nm bandpass) of ECFP (450-470nm) and mCitrine (520-540nm) collected. All measurements were performed in PBS and glucose calibration curves obtained for 1-50,000 μM glucose. The FRET ratio was calculated from the sensitized emission of mCitrine (520-540nm) and donor emission of ECFP (450-470nm) emission and plots of glucose FRET ratios relative to glucose concentration were generated using Sigmaplot with values for sensors determined using nonlinear regression for single-site ligand binding. To explore the specificity of FLII12Pglu-700μδ6 for glucose and assess its sensitivity toward glucose-6-phosphate, the product of hexokinase which is the first glucose modifying enzyme in glycolysis, purified sensor was incubated with varying concentrations of glucose or glucose-6-phosphate and the resulting change in FRET ratio was obtained using a fluorometer as described above.
2.4.8 FRET flow cytometry. All flow cytometry experiments were performed on a BD Attune flow cytometer (BD; Walthman, MA) equipped with violet (405nm) and blue (488nm) excitation lasers. mCitrine (530/30) and sensitized FRET (530/30) emission were excited with a 488nm and 405nm laser respectively. The FRET ratio was calculated on a cell by cell basis by programming a custom parameter (FRET/mCitrine ratio) using the BD attune cytometry software. Data was analyzed using FlowJo (FlowJo LLC, Ashland, OR) flow cytometry analysis software. To ensure accurate FRET measurements, dead cells and doublets were excluded from analysis using the forward and side scattering characteristics of the cells. With dead cells and doublets removed, cells were then gated based on their fluorescent intensity and only cells that were significantly brighter than non-transfected controls were used for analysis.

2.4.9 PCF trypanosome selective permeabilization to glucose and In vivo FLII^{12}Pglu-700µδ6 glucose calibration. In vivo glucose calibration of FLII^{12}Pglu-700µδ6 was performed on permeabilized cells incubated with varying glucose concentrations to ensure that the whole dynamic range of the sensor was assessed (0.025 – 50 mM glucose). In these experiments, PCF cells were harvested, washed three times in PBS, and then incubated with glucose in PBS in the presence of 45 µM β-escin for 30 minutes followed
by FRET analysis. Permeabilized cells were identified in the analysis as being positive for both propidium iodide and mCitrine. Dissociation constants were obtained from non-linear regression as described above. Permeabilized cells were identified in the analysis as being positive for both propidium iodide and mCitrine. Glucose dose response curves were generated at different pH values by incubating cells in HM-PBS with 45μM β-escin with varying glucose. Dissociation constants were obtained as described above.

2.4.10 Hexokinase inhibition assay. In a 96-well microplate, recombinant trypanosome hexokinase 1 (TbHK1) and recombinant glucose sensor were mixed in a buffer containing 50 mM TEA-HCl pH 7.4, 33 mM MgCl₂, and 10 mM Glucose and incubated for 15 minutes at room temperature. The TbHK1 reaction was then initiated by addition of reaction buffer (50 mM TEA-HCl pH 7.4, 33 mM MgCl₂, 2U G6PDH (Yeast, Alfa Aesar), 4 mM ATP, 1.5 mM NADP⁺. The activity of TbHK1 was determined from the change in absorbance at 340 nm using Synergy H1 Hybrid Multi-Mode Reader (Biotek).

2.4.11 2-DOG inhibition of glucose uptake. To determine the effect of glucose uptake inhibitors on intracellular glucose, parasites expressing biosensors were exposed to buffers containing varying 2-DOG concentrations. In short, parasites were harvested during mid log phase by centrifugation at 1250 x g for 5 minutes and washed 3 times with
PBS. Cells were then incubated in PBS supplemented with 10mM glucose with the addition of 2-DOG concentrations between 0.05-50 mM. Parasites were incubated with 2-DOG (29°C 5% CO₂ for PCF, 37°C 5% CO₂ for BSF) for 30 minutes to allow intracellular glucose concentration to stabilize. Parasites were then analyzed via flow cytometry as described herein to determine the resultant FRET/Citrine ratio. FRET/mCitrine ratios were then plotted and fitted as described above.

**2.4.12 Glucose deprivation response.** Comparison of glucose uptake under varying environmental glucose concentrations was carried out by incubating cells in glucose concentrations that would allow for the full dynamic range of the biosensor to be interrogated (0.025 and 50mM glucose). Briefly, parasites were harvested while in mid log phase growth and then washed 3 times with PBS to remove media components and normalize glucose concentrations. Washing steps were conducted as quickly as possible (≈2 minutes per wash) to attempt to maintain the cell viability of BSF parasites. Washed cells were exchanged into buffer containing glucose at a desired concentration (0.025 and 50mM), followed by a 30-minute incubation to allow intracellular glucose levels to reach a steady state. The resultant FRET/mCitrine ratios were then collected using flow cytometry and correlated to external glucose concentration. Dose response curves were then fitted assuming a variable Hill slope to yield an apparent Kₐ for glucose binding to sensor expressed in the cytosol.
The apparent in vivo $K_d$ for sensor glucose binding determined above was determined under conditions of cell permeability and was used as the basis for relating the sensor response to intracellular glucose concentration. Using the calculated apparent $K_d$, intracellular glucose concentrations were calculated in response to external glucose concentration using equation 1, where $K_d$ is the calculated intracellular $K_d$ at pH 7, $R$ is the FRET/mCitrine ratio of the experimental point, and $RH$ and $RL$ were the highest and lowest FRET/mCitrine observed intracellular ratio under high and low glucose conditions.

$$[\text{Glucose}] = K_d \frac{(R-RL)}{(RH-R)} \quad (1)$$

Values within one order of magnitude of the intracellular $K_d$ were considered in the linear range of the biosensor; hence, points below 0.195mM and points above 6.25mM extracellular glucose were not considered for calculation. Significance between cytosolic and glycosomal glucose concentration were calculated using one-way ANOVA analysis, with significance threshold $p < 0.05$ for $n=3$ replicates.

2.5 **Results**

2.5.1 **Biosensor expression, localization, and characterization in T. brucei.** To monitor glucose dynamics in the cytosol of living trypanosomes, a trypanosome expression vector (either pXS2 or pXS6, for PCF and BSF, respectively [156]) bearing the fluorescent protein biosensor FLII$^{12}\text{Pglu-700µδ6}$ was transfected into parasites and stable transformants
were selected. Figure: Relative abundances of identified proteins in PCF parasites demonstrates that this sensor is expressed throughout the cytosol. In kinetoplastids, including *Trypanosoma spp.* and *Leishmania spp.*, proteins tagged with a peroxisomal targeting sequence (PTS) are trafficked to the glycosome [157]. To deliver the biosensor to the glycosome, we appended a C-terminal PTS-1 signal sequence to the FLII$^{12}$Pglu-700µδ6 (designated as FLII$^{12}$Pglu-700µδ6-PTS). As shown in Figure: Relative abundances of identified proteins in PCF parasites, FLII$^{12}$Pglu-700µδ6-PTS localizes to small vesicular organelles, consistent with glycosome morphology. To confirm glycosomal localization of FLII$^{12}$Pglu-700µδ6-PTS, parasites were fixed and stained with antisera to the glycosome-resident protein aldolase [158]. Data shown in Figure 2-2 suggest that FLII$^{12}$Pglu-700µδ6-PTS is localized in glycosomes.
Figure 2-1: In vivo expression of FLII12Pglu-700μδ6 and FLII12Pglu-700μδ6-PTS in PCF and BSF *T. brucei*. DIC (LEFT), and FRET emission (440/20nm ex. 530/30nm em.) (RIGHT) are shown for BSF and PCF *T. brucei* cells expressing FLII12Pglu-700μδ6 cytosolically, and FLII12Pglu-700μδ6-PTS localized in glycosomes. Cells shown were grown under standard growth conditions in normal SDM-79 or HMI-9.
Figure 2-2: FLII12Pglu-700μδ6-PTS colocalizes with aldolase in glycosomes. Immunofluorescence (IF) microscopy of BSF and PCF T. brucei parasites harboring FLII12Pglu-700μδ6-PTS. IF microscopy was performed using α Aldolase and α GFP (M3E6) antisera. DAPI was added to stain the nucleus and kinetoplast DNA. Scale bar = 5 μm. (Figure was provided by Evan Qiu; Clemson University)
An additional control experiment was performed to ensure that the glucose sensor was delivered to the glycosomal lumen and not simply associating with glycosomal import machinery. A protease protection assay was performed (Figure 2-3). Proteolytic fragments of the sensor were only found in the intraglycosomal fraction with none being found associated with the external glycosomal membrane. These results are in agreement with literature showing that large fully folded proteins and protein complexes are imported into the organelle[159].
Figure 2-3: Glycosome bound protease protection assay. Western blot of purified glycosomes from PCF parasites expressing the glycosomal glucose sensor FLIL12Pglu-700μδ6-PTS. The sensor was probed with an anti-EYFP antibody. (Figure was provided by Logan Crowe; Clemson University)
2.5.2 **PTS-1 biosensor in vitro characterization.** To ensure that appending a PTS-1 to the biosensor did not impact glucose binding, we measured the affinity of bacterially-expressed and purified FLII\(^{12}\)Pglu-700\(\mu\)δ6-PTS for glucose via the FRET response of the sensor *in vitro*. Changes in biosensor response were recorded as a function of varied glucose concentrations (Figure 2-4). The observed \(K_d\) value (880 ± 50\(\mu\)M) was in good agreement with previously published values [150], verifying that PTS addition to the C-terminus does not affect sensor response.
Figure 2-4: *In vitro* glucose response curve of purified FLII12Pglu-700μδ6-PTS. *In vitro* calibration curve of bacterially expressed and purified FLII12Pglu-700μδ6-PTS in PBS titrated with glucose 1-50,000 μM glucose. FRET/mCitrine ratio was collected via fluorimeter (430nm excitation 533nm emission for FRET, 433nm excitation 480 nm emission for ECFP) for each glucose concentration. An increase in FRET/mCitrine indicates an increase in glucose concentration. Data were fitted to a single site binding isotherm, calculated $K_d$ for glucose was calculated as $880 \pm 50 \mu M$; error bars represent standard deviation from $n=3$ samples.
FLII$^{12}$Pglu-700μδ6 has previously been shown to not interact with other hexose sugars but binding to glucose-6-phosphate, following the first enzymatic step of glycolysis, has not been reported. Figure 2-5 shows that the FRET/mCitrine ratio observed when FLII$^{12}$Pglu-700μδ6 was incubated with glucose-6-phosphate is not dose dependent. The lack of response to glucose-6-phosphate, which is likely a consequence of the large size and high charge density of the 6-substituted phosphate moiety, ensures that changes in sensor response only result from glucose binding.
**Figure 2-5: Glucose-6-Phosphate does not affect FLII12Pglu-700μδ6-PTS.** Purified FLII12Pglu-700μδ6-PTS incubated with varying glucose (black bars) and glucose-6-phosphate (red) in PBS. FRET/mCitrine ratio was collected via fluorimeter (430nm excitation 533nm emission for FRET, 433nm excitation 480 nm emission for ECFP) for each glucose concentration. An increase in FRET/mCitrine indicates an increase in sensor binding. Error bars represent standard deviation for n=3 replicates.
2.5.3 **Biosensor characterization in *T. brucei***. Flow cytometry is ideal for making FRET measurements because dead and dividing cells can easily be eliminated from analysis. Figure 2-6 outlines the gating scheme used to identify cells suitable for analysis with dividing doublets being removed by forward scatter characteristics and dead cells by forward and side scatter signal. Live/dead discrimination using forward and side scatter was confirmed using propidium iodide (PI) staining (data not shown); PI was not used in further experiments to reduce interference with FRET biosensor emission.
Figure 2-6: Gating scheme used for flow cytometry analysis. Cells used for analysis were gated for three criteria. Bivariate plot of FSC:H versus FSC:A (A) was used to exclude double and dividing cells. Dead cells were then removed using a SSC:A versus FSC:A bivariate plot (B). Once doublets and dead cells were excluded, cells were then gated off of their fluorescence intensities in the FRET and mCitrine channels (C).
Flow cytometry was used to measure the mCitrine and FRET fluorescence of parasites expressing FLII^{12}Pglu-700μδ6 or FLII^{12}Pglu-700μδ6-PTS. To make FRET biosensor measurements it is necessary to excite the donor (ECFP) and acceptor (mCitrine) independently while monitoring the resultant fluorescence of the acceptor at each excitation wavelength [160]. Using the 405nm and 488nm laser of a flow cytometer to excite ECFP and mCitrine respectively, we were able to monitor the FRET and mCitrine fluorescent intensity of parasites expressing FLII^{12}Pglu-700μδ6 in cytosol and glycosomes. When analyzed by flow cytometry, PCF and BSF parasites expressing the sensors yielded fluorescent profiles distinct from the parental (untransformed) line (Figure 2-7a). The observed emission intensity of mCitrine was roughly 2 times higher than the sensitized FRET emission in cells incubated in 10 mM glucose (Figure 2-7b). This result was in part anticipated because FRET is less efficient than direct excitation due to the inefficiency of dipole-dipole energy transfer of fluorescent protein fusions. It should be noted that while the FRET intensity from BSF parasites was ~10 times lower than that from PCF cells, BSF parasites were still sufficiently bright to allow measurement of changes in FRET ratio.
Figure 2-7: Spectral properties of FLII^{12}Pglu-700μδ6 and FLII^{12}Pglu-700μδ6-PTS expressed in BSF and PCF parasites can be measured with flow cytometry. (A) Bivariate flow cytometry dot plots representing BSF and PCF *T. brucei* expressing FLII^{12}Pglu-700μδ6 and FLII^{12}Pglu-700μδ6-PTS. X-axis represents FRET emission (405nm excitation 530/30nm emission); Y-axis represents mCitrine emission (488nm excitation 530/30nm emission).
Wild Type cells not expressing biosensor are used for placing the quadrant gate (Left column). Cells in quadrant 2 (Top right quadrant) are expressing high levels of FLI12Pglu-700μδ6 (Middle Column) or FLI12Pglu-700μδ6-PTS (Right Column) and are used for FRET analysis. Dead cells, doublets, and the population of cells not robustly expressing biosensor were excluded from FRET analysis. (B) The emission intensities of FRET emission (405nm ex 530/300nm em) and mCitrine (488nm ex 530/30nm em) of FLI12Pglu-700μδ6 and FLI12Pglu-700μδ6-PTS expressed in PCF and BSF T. brucei cytosol and glycosomes. Fluorescent emission values were collected via flow cytometry for cells incubated in PBS supplemented with 5mM glucose. Black bars represent FRET emission and red bars represent mCitrine emission, error bars represent the standard deviation of n=5 replicates.
Intracellular calibration of FRET sensors is necessary to account for possible changes in sensor response in the intracellular environment, as well as to determine if autofluorescence from cellular components impacts observed FRET ratios. Though intracellular calibration is common practice when using calcium and pH biosensors, the challenge of calibration when glucose FRET sensors are used for intracellular measurements has limited its practice [161],[92]. This is primarily because, unlike calcium and pH, there are no known compounds that allow glucose to equilibrate across the plasma membrane. Most commonly, in vitro calibration curves are used to interpolate the FRET ratio obtained from a sensor expressed in cells [11].

To measure the $K_d$ of the biosensor in the cytosol, parasites were first incubated with varying concentrations of β-escin, a mild detergent that gently permeabilizes cell membranes [162] and then stained with propidium iodide (PI) to assess cell permeability to small molecules. A treatment with 45 µM β-escin allowed small molecules (glucose and PI) to enter the cell freely while larger molecules (FLII12Pglu-700μδ6, other proteins) remained inside the cell (Figure 2-8). Using these optimized permeabilization conditions, we carried out an intracellular glucose calibration that yielded a sensor $K_d = 1000 \pm 90 \mu M$ (Figure 2-9). This $K_d$ value, which differs only slightly from previously reported values generated in vitro [150] (including our own), indicated that the intracellular environment had little impact on FLII12Pglu-700μδ6 binding affinity, consistent with the behavior of other intracellular sensors [11]. Ideally, a glycosomal calibration would have been
conducted as well, but methods used for glycosomal permeabilization (i.e. digitonin or β-escin) quench the fluorescence of the sensor at needed concentrations. Given that the environment of the sensor has little effect on the cytosolic intracellular and in vitro $K_d$, the difference between glycosomal and cytosolic composition is anticipated to have little impact on the binding constant of the sensor. Consequently, the intracellular $K_d$ was used as the basis for comparison for all subsequent intracellular experiments. In addition, inclusion of the glucose sensor in an in vitro assay of recombinant trypanosome hexokinase (TbHK1) did not significantly alter enzyme activity (Figure 2-10), suggesting that expression of the sensor in the glycosome would not interfere with the activity of the glycolytic enzymes therein [163].
Figure 2-8: β-escin selectively permeabilizes cell membranes to small molecules.

Bivariate plots of WT PCF cells (A) and PCF cells expressing FLI\textsuperscript{12}Pglu-700μδ6 (B). Cells partially permeabilized with β-escin (C) are represented by the double positive population for mCitrine and Propidium Iodide, representing cells that allow small molecules (i.e. PI or glucose) into the cell, but do not allow FLI\textsuperscript{12}Pglu-700μδ6 (and other larger molecules)
out of the cell. These cells were used for *in vivo* calibration of endogenously expressed FLII$^{12}$Pglu-700µδ6. Cells permeabilized with high concentrations of β-escin (D), were devoid of all protein sensor due to more complete permeabilization.
Figure 2-9: *In vivo* FLII$^{12}$Pglu-700μδ6 glucose calibration curves. Glucose dose response of PCF parasites expressing FLII$^{12}$Pglu-700μδ6. PCF cells expressing FLII$^{12}$Pglu-700μδ6 were permeabilized to glucose using 45μM β-escin and titrated with different glucose concentrations. After intracellular glucose equilibrates with external concentration (30 minutes), the resultant FRET/mCitrine ratio was measured using flow cytometry. Sigmoid curves were fitted using single site ligand binding equations, $K_d$ values were calculated as 1000 ± 90 μM. Error bars represent the standard deviation for n=3 replicates.
Figure 2-10: TbHK1 activity is not impacted by the presence of FLII\textsuperscript{12}Pglu-700\textmu d6.

Recombinant TbHK1 activity was monitored in vitro in the presence of increasing amounts of FLII\textsuperscript{12}Pglu-700\textmu d6 in order to achieve TbHK1 to sensor molar ratios of 1 to 0.5, 1, 2, 4, 8, and 10, respectively. K2354, a known benzamidobenzoic acid inhibitor of TbHK1 [164], was used at 10 \mu{M} as a control. (Figure was provided by Jimmy Suryadi; Clemson University)
PCF parasite glycosomes acidify in response to nutrient deprivation [135]. Additionally, fluorescent proteins can have pH dependent shifts in their excitation and emission spectra [165], which could affect glycosome measurements. To mitigate pH sensitivity, the glucose sensor FLII^{12}\text{Pglu}-700\mu\delta6 was generated with the pH- and chloride-insensitive yellow fluorescent protein mCitrine [165] [150]. To further demonstrate that changes in pH do not cause an artificial response in the glucose sensor, we measured the sensitivity of the intracellular glucose sensor to pH, over the range of pH that could be induced by glucose deprivation [135]. Cells expressing FLII^{12}\text{Pglu}-700\mu\delta6 were permeabilized and an intracellular calibration for glucose was assessed at relevant pH concentrations (Table 2-1). No pH sensitivity was observed, suggesting that mild acidification of glycosomes during glucose starvation does not impact observed FRET ratio.
Table 2-1: Binding constants for FLII12Pglu-700μδ6 biosensor in different environments

<table>
<thead>
<tr>
<th>Sensor Calibration</th>
<th>Calculated Kd (μM)</th>
</tr>
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<tbody>
<tr>
<td>FLII12Pglu-700μδ6 in vitro; pH 7</td>
<td>880 ± 50</td>
</tr>
<tr>
<td>FLII12Pglu-700μδ6 intracellular; pH 7</td>
<td>1000 ± 90</td>
</tr>
<tr>
<td>FLII12Pglu-700μδ6 intracellular; pH 6</td>
<td>1000 ± 110</td>
</tr>
</tbody>
</table>
2.5.4 FRET biosensor response to external environment To demonstrate that the FRET sensor reports changes in intracellular glucose concentration, BSF and PCF T. brucei were treated with 2-DOG, a small molecule that competitively inhibits glucose binding to glucose transporters and competes for uptake, thereby decreasing intracellular glucose concentrations. As shown in Figure 2-11, both cytosolic and glycosomal FRET/mCitrine ratios were diminished in the presence of 2-DOG in PCF (Figure 2-11A) and BSF (Figure 2-11B) parasites, consistent with the idea that reductions in FRET ratios reflect decreased glucose concentrations in the cell.
**Figure 2-11:** FLII\textsuperscript{12}Pglu-700μδ6 and FLII\textsuperscript{12}Pglu-700μδ6-PTS expressed \textit{in vivo} responds to changes in internal glucose concentration. PCF (A) and BSF (B) parasites expressing FLII\textsuperscript{12}Pglu-700μδ6 in cytosol (Black) or FLII\textsuperscript{12}Pglu-700μδ6-PTS in glycosomes (Red) were incubated with 10mM glucose in PBS, 2-DOG concentration was titrated (.025-25,000 μM). Intracellular glucose was allowed to reach a steady state (45 minutes) before being analyzed via flow cytometry. FRET/mCitrine ratios resulting from glucose depletion were fitted using a single site binding isotherm to obtain apparent K\textsubscript{d} values. Glucose dose response curves for PCF (C) and BSF (D) \textit{T. brucei} expressing FLII\textsuperscript{12}Pglu-700μδ6 (Cytosol,
Black) or FLII$^{12}$Pglu-700\(\mu\delta6\)-PTS (Glycosomes, Red). Cells were incubated in varied extracellular glucose concentrations (0.05-50,000\(\mu\)M) and allowed to reach a steady state for 30 minutes before measurement of intracellular FRET ratios. FRET/mCitrine ratios were fitted to a single site binding isotherm and the \(K_{app}\) was calculated. Error bars represent symmetric standard deviation of \(n=3\) replicates.
The glucose sensor signal of PCF and BSF parasites expressing FLII\textsuperscript{12}Pglu-700μδ6 were monitored as a function of external glucose to score cytosolic and glycosomal glucose levels (Figure 2-11C & 2-11D). Increasing extracellular glucose increases the observed FRET ratio in a dose dependent manner, indicating that the glucose levels in the biosensor microenvironment also increase under these conditions. Apparent $K_d$ ($K_{app}$) for cytosol and glycosomes in PCF parasites were $1500 \pm 150\mu M$ and $1100 \pm 200\mu M$, BSF parasites $K_{app}$ were calculated as $1700 \pm 300\mu M$ and $900 \pm 200\mu M$ respectively. As we have shown, intracellular environment has little effect on the glucose response of the FLII12Pglu-700μδ6 biosensor. Therefore, $K_{app}$ values can be used to make estimates about relative cytosolic and glycosomal glucose levels at external concentrations that are similar to the intracellular $K_d$ of the biosensor. $K_{app}$ values higher (or lower) than the calibrated biosensor $K_d$ would indicate that different external glucose levels are required for intracellular glucose levels to reach glucose levels sufficient to reach the midpoint of biosensor response (i.e. 1000 μM). For example, the $K_{app}$ for sensor response inside the glycosome of PCF and BSF parasites is different than the intracellular sensor response in permeabilized parasites ($K_{app} = 1500 \pm 150 \mu M$ and $1700 \pm 300\mu M$, respectively vs. 1000 ± 90 μM), an observation that suggests that glucose concentrations in the cytosol are lower than external values. However, sensor calibration is required to make more quantitative comparisons between external and intracellular glucose levels. Interestingly, cytosolic and glycosomal $K_{app}$ are different in both life stages, indicating that glucose levels
in the two compartments respond independently to changes in external glucose concentration.

To quantitatively measure intracellular glucose concentration, FRET responses from cytosolic and glycosomal sensors were calibrated to reflect corresponding intracellular glucose concentration(s). Biosensors with a single analyte binding site (including FLII12Pglu-700μδ6), have a two log linear response centered around the $K_d$ of the sensor [160]. A full sensor dynamic range in vivo indicates that resultant FRET responses can be calibrated using the in vivo biosensor $K_d$, together with the maximum and minimum biosensor response, using established sensor calibration protocols (Equation 1) [160]. Figure 2-11C and 2-11D show that the FLII12Pglu-700μδ6 responds over two logs of external glucose concentration, meaning that the full dynamic range of the sensor is observed over the external glucose concentrations probed. Using these methodologies, the FRET ratio in response to external glucose were converted into intracellular glucose concentrations (Figure 2-12).
Figure 2-12: Calculated cytosolic and glycosomal glucose concentration in response to external glucose. PCF and BSF *T. brucei* expressing FLII^{12}Pglu-700μδ6 in cytosol (Black) or FLII^{12}Pglu-700μδ6-PTS in glycosomes (Red) were incubated in PBS and glucose was titrated (.025-25,000 μM). FRET signal in response to external glucose concentrations were transformed into corresponding glucose concentrations and plotted versus environmental glucose. Resultant intracellular glucose concentrations for PCF (C) and BSF (D) were plotted versus the external glucose concentrations. Error bars represent symmetric standard deviation of n=3 replicates. Cytosolic and glycosomal glucose concentrations were compared using One Way ANOVA, p < 0.05 were considered significant.
**2.5.5 Cytosolic and glycosomal response to extracellular glucose.** Figure 2-12A shows the resultant calibrated cytosolic and glycosomal glucose concentration in response to external glucose concentration in PCF trypanosomes. At external glucose concentrations below 1 mM, glycosomal and cytosolic glucose concentrations are not statistically different from each other, suggesting that at these conditions the cytosol and glycosome do not regulate glucose dependently. Above external glucose concentrations of 1mM, glycosomal glucose concentrations maintain concentrations that are slightly (1.2-1.3 fold) higher than the surrounding cytosol. The increase in glycosomal glucose might be slight but this increase in intra-glycosomal suggests that glucose is being concentrated across the glycosomal membrane. The cytosol and glycosomes of PCF maintains glucose concentration roughly 50-70% of the external glucose, as external glucose increases the differences between intracellular and extracellular glucose increases. This suggests that as external glucose increases that glucose uptake into the cell becomes saturated and starts to approach a maximum rate. At concentrations, similar to physiological conditions in the host (6.25mM), cytosolic and glycosomal concentrations in PCF parasites are near saturated and maintain indistinguishable glucose concentrations of 3.4 ± 0.4 mM and 3.4 ± 0.5 mM respectively.

BSF parasites maintain intracellular glucose concentrations that are different than their PCF parasite counterparts, as shown in Figure 2-12B. As for the PCF data shown above, intracellular glucose concentrations in BSF parasites were quantified based on
observed calibrated biosensor response. We then compared cytosolic and glycosomal glucose concentrations to external glucose concentrations. BSF parasites maintain cytosolic glucose concentration between 30-40% of the external glucose concentration. At 6.25mM external glucose, this correspondence equates to 1.9 ± 0.6 mM cytosolic glucose. Notably, this value is significantly lower than that observed in the cytosol of PCF parasites.

Under most extracellular glucose concentrations, PCF parasites and BSF parasites maintain higher glucose concentrations in the glycosome than in the cytosol; quantified concentrations range from 50-80% of the external environment, depending on the external glucose concentration. At external glucose concentrations of 6.25mM, the glucose concentration of the BSF intraglycosomal fluid is 3.5 ± 0.5 mM. Notably, over the range of external glucose concentrations tested, glycosomal glucose concentration was significantly higher in the glycosome versus the cytosol. At lower external glucose concentrations, glycosomal glucose is maintained 1-2 fold higher than the adjacent cytosolic levels. At physiological glucose conditions (6.25mM), glucose concentration in glycosomes maintain glucose concentrations that are ~1.8 times that of the cytosol. These data suggest that glucose transport is not freely diffusing across the glycosomal membrane and glucose in concentrated in the glycosome of BSF parasites.

Our measurements show that PCF and BSF maintain glucose concentrations differently at the same external glucose concentrations. At glucose concentrations,
similar to physiological conditions (6.25mM), cytosolic glucose for PCF and BSF parasites were 3.4 ± 0.4 mM and 1.9 ± 0.6 mM respectively (Table 2-2). This observation indicates that cytosolic glucose concentrations in PCF parasites are maintained ~ 1.8-fold higher in the cytosol of PCF parasites versus BSF parasites at the same external glucose concentration. In contrast, glycosomal glucose levels are maintained at similar levels in PCF and BSF parasites, each roughly ~50% of the external glucose concentration, 3.4 ± 0.5 mM and 3.5 ± 0.5 mM respectively (Table 2-2).
Table 2-2: Intracellular glucose concentration in PCF and BSF parasites.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Glucose Concentration&lt;sup&gt;1&lt;/sup&gt;</th>
<th>% External Glucose&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Fold Increase&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procyclic Cytosol</td>
<td>3.4 ± 0.4 mM</td>
<td>54%</td>
<td>N/A</td>
</tr>
<tr>
<td>Procyclic Glycosome</td>
<td>3.4 ± 0.5 mM</td>
<td>54%</td>
<td>1</td>
</tr>
<tr>
<td>Bloodstream Cytosol</td>
<td>1.9 ± 0.6 mM</td>
<td>30%</td>
<td>N/A</td>
</tr>
<tr>
<td>Bloodstream Glycosome</td>
<td>3.5 ± 0.5 mM</td>
<td>56%</td>
<td>1.8</td>
</tr>
</tbody>
</table>

<sup>1</sup>Intracellular glucose concentration at an external glucose concentration of 6.25 mM.

<sup>2</sup>Compared to 6.25mM external glucose.

<sup>3</sup>Fold increase in glycosomal glucose versus cytosolic concentration.
2.6 Discussion

By measuring intracellular glucose with FRET-based sensors we have, for the first time, characterized the relationship between environmental, cytosolic, and glycosomal glucose concentrations in living trypanosomes. Our measurements show that PCF and BSF parasites maintain cytosolic and glycosomal glucose concentrations differently. Interestingly, our observations show that PCF *T. brucei* maintain cytosolic glucose levels 1.8 times higher than those measured in the cytosol of PCF parasites. The observed differences between PCF and BSF cytosolic glucose distribution likely reflects the differences in how the two life stages transport and process glucose. Such differences suggest active transport and/or control of glucose concentrations in the two compartments that are different between the two life stages.

The *T. brucei* genome encodes two hexose transporters, THT1 and THT2, both of which are members of the facilitated glucose transporter GLUT1 family [25] and are known to localize to the cell membrane. BSF parasites primarily express THT1 in the cell membrane, which has lower affinity for substrate than THT2, making it suitable for glucose acquisition in the glucose rich environment of the mammalian bloodstream. PCF parasites predominantly express the higher affinity, more efficient THT2 [166][167], a transport system optimized to acquire the hexose from the relatively glucose-depleted environs of the tsetse fly [166]. THT isoform expression differences could explain the
lower cytosolic glucose concentrations in BSF parasites versus PCF cells at equivalent extracellular glucose concentrations. Expression of a lower affinity glucose transporter in the BSF parasites would be expected to cause lower rates of glucose uptake, resulting in lower intracellular glucose levels.

Glucose consumption rates may also explain the discrepancies in cytosolic glucose concentrations found in the two life stages. BSF parasites rely entirely on glucose metabolism for ATP production, and excrete pyruvate as the major end product [168][12]. PCF trypanosome can metabolize other carbon sources for energy, primarily proline and threonine, and chiefly excrete acetate and succinate instead of pyruvate [169]. These differences in metabolism results in BSF parasites exhibiting dramatically higher glucose consumption rates than that of PCF trypanosomes [160]. In BSF cells, lower cytosolic levels suggest that the rate of glucose removal (i.e. the transport of glucose across the glycosomal membrane followed by metabolism in the glycosome), is greater than the rate of glucose transport into the cell via THT1. In sharp contrast, PCF cells maintains a cytosolic glucose concentration that is two-fold higher than that in BSF cells, and similar to that in the glycosome. This observation suggests that in PCF cells, the rate of glucose uptake into the cytosol via THT2 is similar to the rate of glucose removal into the glycosome. Hence, differences in glucose levels in the two life stages hint at a complex glucose homeostasis derived from the different glucose transport and metabolism characteristics to help adapt trypanosome cells to their occupied niches.
In BSF parasites, glycosomal glucose is roughly two-fold concentrated in the glycosome compared to the surrounding cytosol. This suggests that glucose transport into the glycosome is not a passive process; rather, glucose is actively transported, which results in the observed concentration gradient. The identity of potential transport machinery involved in both glucose uptake and glycolytic intermediate efflux to the cytoplasm for completion of glycolysis remains unknown. In peroxisomes, pores that selectively allow inorganic ions and hydrophilic metabolites to pass while blocking other molecules including ATP have been described [170]. Glycosomes also harbor pores with behavior that suggests that they are water-filled in the membrane and potentially "non-selective" channels [147], but the role of such channels in glucose homeostasis has not been resolved.

Once glucose is shuttled into the glycosome, it is processed via glycolysis, beginning with phosphorylation by hexokinase. Since the biosensor will not bind to phosphorylated glucose, our measurements exclusively reflect concentrations of unphosphorylated glucose. This observation has interesting implications. We observe significant concentrations of glucose in the glycosomes of both BSF and PCF cells, indicating that the rate of transport of glucose into the glycosome is faster than the rate of phosphorylation. Since hexokinase is catalytically efficient in in vitro assays [163] [171], it has generally been assumed that incoming glucose will be phosphorylated almost immediately, such that intraglycosomal concentrations of unphosphorylated glucose will
be negligible[163]. Our measurements suggest, instead, that a buildup of unphosphorylated glucose can occur, particularly in BSF parasites. If the number hexokinase molecules in the glycosome is small, it is possible that the enzyme may be saturated with glucose, leading to a situation in which glucose phosphorylation may be rate limiting. Further kinetic experiments are required to investigate this possibility.

We are uniquely equipped with methods capable of identifying the cellular machinery behind the uptake and distribution of glucose. We believe that the components of glycosomal glucose uptake will be revealed using the probes described here in concert with forward molecular genetics and chemical inhibition tools. A glycosomal glucose transporter would be an interesting target for anti-trypanosome therapies, given the parasites reliance on glucose metabolism in the glycosome, and the unique nature of glycosomal metabolism [138][172].
CHAPTER 3: A Microfluidic-Based Microscopy Platform Allows for Continuous Interrogation of Single *Trypanosoma brucei* parasites
3.1 Abstract

The African trypanosome, *Trypanosoma brucei* is the causative agent of a neglected tropical disease that together with related kinetoplasts affect some of the most impoverished regions on earth. *T. brucei* parasites are extracellular parasites that possess a single cell-length flagellum that imparts a high degree of motility to the cells. Microscopic imaging live parasites is difficult due to their high motility, requiring fixation or immobilization in a gel substrate that can compromise viability. Microfluidic platforms for trapping small motile cells have become a popular area of research, although these devices are cell specific, require specialized manufacturing facilities, and are difficult to produce uniformly. However, commercial microfluidic platforms have now emerged in recent years, one of which is the CellASIC ONIX2 self-contained microfluidic platform. Here we demonstrate that available bacterial trapping microfluidic devices can trap bloodstream and procyclic form trypanosomes allowing for acquisition of time-lapse microscopy images of motile parasites. As a case study to exhibit the utility of this trapping approach, we analyzed the intracellular glucose concentration in procyclic-form parasites using an endogenously expressed fluorescent protein biosensor. Using the perfusion capabilities of the system, we show that cytosolic glucose equilibrates with external conditions as fast as or faster than the rate of buffer exchange. The methods described herein allow for mechanical trapping of trypanosome parasites, perfusion-based experiments, and single cell transient tracking. In addition, these data allowed analysis of
key metabolic and biologic processes in living parasites in response to changing environment that may be key to understanding the adaptation of kinetoplasts to their unique biological niches.
3.2 **Introduction**

Kinetoplastids, including the African trypanosome, *Trypanosoma brucei*, exert immense stress on human and livestock health worldwide and are causative agents of neglected tropical diseases [173]. Kinetoplastid parasites deploy unique metabolic and biologic mechanisms to best suite their inhabited biological niches [174]. Given their unique biology, *T. brucei* has long served as a model organism for investigating key biochemical processes [175]. Many of the kinetoplastid life stages are highly motile making them difficult to image using traditional microscopy methods employing environmental control and perfusion with adherent cells [176]. Most commonly, parasites are fixed using formaldehyde, methanol or some other agent to fix the cells for imaging. Fixation techniques lack the ability for tracking cell dynamics in live cells with high temporal resolution. Alternatively, gel matrices have been employed which encase the cells to slow them enough for imaging, or totally impede movement of the parasite in 3-D space [177]. These gel immobilization methods are not ideal, as they do not allow cells to move, which may affect some of the biological processes in interest and cell viability. These gels are also lack the ability to exchange buffers that is critical in monitoring the dynamic cellular response to external environment cues.

In the last decade, construction of microfluidic devices designed for use in biological applications has been rising [178]. Increases in biologically specific microfluidic devices is largely due to the explosion of improved and streamlined lithography methods
developed for use in the microprocessor industry [179]. Many devices have been
developed to trap a various cell types including bacteria, yeast, and mammalian cells for
diverse types of analysis. Cell trapping microfluidic devices are most commonly
constructed by soft lithography and are composed of a PDMS mold covalently fused to a
microscopy slide [180]. Because of the small feature size, these devices require
specialized clean rooms for lithography and device assembly, which limits the accessibility
of these devices for many researchers. In 2009, Millipore released their CellASIC ONIX2
system, which is a self-contained microfluidic device that utilizes ready-made plates that
are available in a variety of plate configurations. The system includes pneumatic-based
fluidics accompanied with software allowing for precise monitoring and control of the
pressure and flow rates through the system. The pneumatic microfluidic system allows
for changing buffer conditions allowing for unique exchange experiments which are
difficult to conduct on small non-adherent or motile cell lines. Plates designed specifically
to trap bacteria, yeast, and flagellated chlamydomonas cells for continuous analysis via
microscopy are available. Here we describe methods that outline the repurposing of the
bacterial trapping plates to trap living procyclic and bloodstream form trypanosomes
allowing for continuous monitoring via microscopy during perfusion.

3.3 Methods
3.3.1 Materials and reagents. All buffer and media components used for analysis were purchased from Sigma-Aldrich (Sigma-Aldrich; Saint Louis, Missouri). Fluorescein powder (40% Fluorescein) was purchased from Fisher (Fisher Scientific; Pittsburgh, Pennsylvania). Hygromycin, blasticidin, and G418 were purchased from Gold Bio (Gold Biotechnology; Saint Louis, Missouri). The CellASIC ONIX2 system was purchased from Millipore (Merck Millipore; Burlington, Massachusetts, part number CAX2-S0000) along with the temperature controlled CellASIC ONIX2 Manifold XT (Merck Millipore; Burlington, Massachusetts, part number CAX2-MBC20). CellASIC ONIX plates for bacterial cells were purchased from Millipore (part number B04A-03-5PK). To reduce background fluorescence due to media components all imaging experiments were carried out in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄) supplemented with 0-10 mM glucose.

3.3.2 Trypanosome culture and transfection. Procyclic form (PCF) trypanosomes were continuously cultured in SDM-79 media supplemented with porcine hemin and 15% FBS at 29°C and 5% CO₂.[152] Bloodstream from (BSF) trypanosomes were cultured in HMI-9 media supplemented with 10% FBS.[181] To maintain cell viability and ensure a uniform population, PCF and BSF trypanosomes were maintained in log phase (5 x 10⁵-5 x 10⁶ cells/mL for PCF and 5 x 10⁴-5 x 10⁵ for BSF parasites). The fluorescent glucose sensor
FLI\textsuperscript{12}Pglu-700μδ was cloned into pXS2 and pXS6 allowing for transfection and expression in PCF and BSF parasites respectively [153]-[154].

3.3.3 Microscopy. All fluorescence and bright field images were taken using an Olympus IX73 epifluorescent microscope (Olympus Corporation; Tokyo, Japan). DIC images were captured images using a Sutter TLED for bright filed illumination (Sutter; Novato, California) and an Orca Flash V4.0 CMOS camera (Hamamatsu; Shizuoka, Japan). Parasites expressing the fluorescent biosensor were excited using a fast switching DG-4 fluorescent light source (Sutter; Novato, California) using a 430/30 filter for excitation of the ECFP and FRET emission. ECFP (480/30) and FRET (530/30) emission spectra were separated using an Andor Tucam (Andor Technology; Belfast, Northern Ireland) equipped with a 500-nm long pass filter. ECFP and sensitized FRET emission were captured simultaneously on a pair of Orca Flash V4.0 CMOS cameras (Hamamatsu; Shizuoka, Japan). Light source switching and camera exposure were all synchronized using a Sutter TTL control box using Slidebook 6.0 (Intelligent Imaging Innovation; Denver, Colorado) for synchronization control. All images were captured and analyzed using the same Slidebook 6.0 software used for system synchronization, the FRET module was used for calculating FRET ratios on a pixel per pixel basis for cell movies and data export.
3.3.4 **Plate preparation.** CellASIC bacterial plates were opened aseptically in a biosafety cabinet. The 0.05% azide in PBS solution in all channels (1-8) for one experimental row (A-D) were removed using a sterile vacuum aspiration tip. Buffers used for the experiment were loaded into wells 1-5 of the plate, channel 6 was loaded with 10mM glucose in PBS for all experiments. Channel 7 is designated as the waste well and is left empty in all experiments. Channel 8 contains the cell suspension that is to be used for microscopy experiments. The remaining channels were filled with 0.05% azide in PBS to ensure that the channels do not become contaminated. Figure 3-1A shows the plate layout and locations of the channel numbers and overall arrangement.
Figure 3-1: Representation of a CellASIC bacteria microfluidic plate. (A). Expanded view of the cell-trapping portion of the microfluidic plate (B). The different colored strips represent different heights of the trapping compartment represented by the labels to the right. Cells enter the device from the 2.3 µm side and are pushed through the device until they are trapped between the glass bottom (blue bottom) and the different ceiling heights (C). Cell are trapped in different portions of the device, in general they occupy 1-3 different height compartments (D).
3.3.5 Parasite plate loading. PCF or BSF parasites were washed three times in PBS and resuspended in PBS supplemented with 5mM glucose to a final cell density of $1 \times 10^6$ cells/mL. The cell suspension was then pipetted into channel 8 of the desired row on the microfluidic plate. Once the cell suspension and the desired experimental buffers were loaded into the plate as described above, the plate was sealed to the fluidics manifold following the manufactures instructions. To purge the channels of remaining azide the channel-flushing program was used (channels 1-6; 5 kPa for 5 minutes) prior to cell loading. To load cells, a modified cell-loading module was utilized. Briefly, channel 8 containing the cells was pressurized to 15 kPa for 2 minutes, then channel 6 and channel 8 were pressurized to 15 kPa for 10 minutes to lodge the cells into the capture area of the device, followed by depressurization of channel 7 and 8. All cells analyzed were trapped using the parasite loading method described here. After the device was loaded with parasites, 10 mM glucose was perfused from channel 1 by pressurizing the channel with 10 kPa. Cells were continuously perfused with 10 mM glucose in PBS and monitored using microscopy for 1 hour to ensure that cell viability was not effected by being trapped in the device.

3.3.6 Fluorescein perfusion. To explore solution mixing using the microfluidic platform, we perfused different concentrations of Fluorescein through devices by mixing the buffer channels at varying ratios at a constant total system pressure. Fluorescein stocks were
prepared in PBS at 1 mg/mL and sterilized by filtration through a 0.22 µm filter. The 1 mg/mL stock was diluted 100-fold to make a Fluorescein working solution. This Fluorescein working solution was pipetted into channel 1 of a prepared plate as described above. Channel 2 was filled with PBS and the remaining channels were filled with 0.05% azide in PBS. The plate was then attached to the pneumatic manifold and channels 1 and 2 were purged for five minutes using 10 kPa. Time-lapse fluorescence microscopy was then used to monitor the Fluorescein fluorescence (495/20 ex, 530/30 em) for the duration of the perfusion experiment. After system priming was complete, 100% PBS was perfused by pressurizing channel 2 to 20 kPa for five minutes. To perfuse the different Fluorescein concentrations, channel 1 and 2 were perfused at different pressures with a total pressure of 20 kPa, (25% Fluorescein; 5 kPa Fluorescein channel, 15 kPa PBS channel. 50% Fluorescein; 10 kPa Fluorescein channel, 10 kPa PBS channel. 75% Fluorescein; 15 kPa Fluorescein channel, 5 kPa PBS channel. 100% fluorescein; 20 kPa Fluorescein channel, 0 kPa PBS channel.). Different percentages (25, 50, 75, 100) of Fluorescein were then perfused for five minutes followed by 5 minutes of 100% PBS wash. A representative mask was then drawn using Slidebook 6.0 software, mean intensity and standard deviation were then exported for every frame of the fluorescence time-lapse. The perfusion mixing protocol is outlined in Table 3-1.
Table 3-1: Fluorescein perfusion protocol.

<table>
<thead>
<tr>
<th>Plate Preparation</th>
<th>Channel Number</th>
<th>Channel Contents</th>
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<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10 ug/mL Fluorescein</td>
</tr>
<tr>
<td>2</td>
<td>PBS</td>
<td>0 mM glucose</td>
</tr>
<tr>
<td>3,4,5,6,7,8</td>
<td>0.05% azide in PBS</td>
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</table>

<table>
<thead>
<tr>
<th>Perfusion Protocol</th>
<th>Protocol Step</th>
<th>Length (min)</th>
<th>Channels</th>
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<tr>
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<td>10</td>
<td>5</td>
<td>2</td>
<td></td>
<td>20 kPa</td>
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3.3.7 **Glucose mixing dose response.** To see how device mixing could be applied to live cell experiments, PCF parasites were perfused with varying glucose concentrations that were obtained by mixing multiple reagent channels to yield diluted concentrations. PCF parasites expressing the fluorescent glucose sensor in the cytosol were rinsed three times with PBS to remove media components and resuspended in PBS supplemented with 10 mM glucose to a final density of 1 x 10^6 cells/mL. Cells were then added into channel 8 of a plate that was already prepared as described above. Channels 1 and 2 were filled with PBS with 0 mM and 10 mM glucose respectively. All other channels (3-5) were filled with 0.05% azide in PBS. PCF cells were loaded onto the plate as described above and then the pneumatic and temperature control manifold was allowed to equilibrate at 29°C for 30 minutes before perfusion experiments were started.

Once the cells were loaded, they were exposed to various glucose concentrations using the mixing method described previously for the Fluorescein perfusion experiments. Cells were allowed to incubate in low glucose for five minutes in PBS with 0 mM glucose by pressuring channel 1 to 20 kPa. After incubation, various glucose concentrations were perfused (10 mM, 7.5mM, 5mM, and 2.5mM glucose) by mixing channels 1 and 2 at different pressures (Table 3-2) with a total pressure of 20 kPa (2.5mM glucose; 5 kPa 10mM glucose, 15 kPa PBS channel. 5mM glucose; 10 kPa 10mM glucose, 10 kPa PBS channel. 7.5mM glucose; 15 kPa 10mM glucose channel, 5 kPa PBS channel. 10mM glucose; 20 kPa 10mM glucose channel, 0 kPa PBS channel.). During perfusion, FRET and
ECFP were continuously monitored via fluorescence microscopy (430/30 ex, 480/30 and 530/30 em). The FRET/ECFP ratio was extracted for each cell and background was subtracted from a cell free region. FRET/ECFP ratio movies were made using Slidebook 6.0 software’s ratio module and exported as a series movie.
### Table 3-2: Glucose mixing protocol

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<th>Channel Contents</th>
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<tr>
<td>1,6</td>
<td>10 mM Glucose</td>
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</tr>
<tr>
<td>2</td>
<td>PBS 0mM glucose</td>
<td></td>
</tr>
<tr>
<td>3,4,5</td>
<td>0.05% Azide in PBS</td>
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</tr>
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<td>8</td>
<td>1 x 10^6 cells/mL</td>
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<td>5</td>
<td>2</td>
<td>20 kPa</td>
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3.3.8 **Single cell glucose response time-lapse.** To demonstrate the capabilities of the microfluidic device to continuously monitor single trypanosomes, we analyzed the resultant FRET/ECFP ratio in response to changes in external glucose concentrations. PCF cells expressing glucose sensor were rinsed and prepared as described above. Once cells were loaded into the microfluidic device, they were subjected to different glucose concentrations via perfusion. Cells were then allowed to incubate in 10 mM glucose for 15 minutes and the device manifold was allowed to equilibrate to 29°C for the next 15 minutes. The cells were then continuously monitored via fluorescence microscopy as the buffers were perfused. After measuring the FRET/ECFP ratio for 7.5 minutes the cells were perfused into 0 mM glucose for 12.5 minutes followed by perfusion into 10 mM glucose for 12.5 minutes. This cycle was then repeated again to determine the temporal response to the perfusion conditions. FRET/ECFP ratio movies were extracted from the ECFP and FRET emission data using the Slidebook 6.0 FRET ratio module and pseudocolor images were exported where high FRET/ECFP is red and low FRET/ECFP is blue. FRET/ECFP ratios for five representative cells were exported as well as the average and error for the FRET/ECFP ratio of a population of 25 cells.

3.4 **Results and Discussion**
3.4.1 Device functionality and design. The CellASIC ONIX2 system represents an off the shelf microfluidics platform that has been used to investigate many biological processes in mammalian, yeast and bacterial cells [182]. The platform allows for continuous temperature and environmental control for long-term microscopy experiments. In this study, we utilize microfluidic plates that are designed to trap small organisms of different sizes in a single focal plane allowing for continuous monitoring via transmitted light or fluorescence microscopy. The general plate layout for a bacterial cell-trapping device is represented in Figure 3-1A. Each plate contains buffer channels 1-5 which can be used for different buffers or media that can be used for perfusion experiments. Channel 6 and 8 are used to load cells into the device and channel 7 is the waste reservoir. The fluids are perfused through the device by pressurizing the different channels, which moves the buffers through the cell-trapping portion of the device (Figure 3-1B). The cell-trapping portion of the microfluidic plate consists of a glass bottom with a PDMS “ceiling” of different heights that is used to trap different sized cells. Cells are passed through the device under flow until they are pinched between the glass bottom and the PDMS ceiling in a spot that is small enough to mechanically squeeze the cells (Figure 3-1C). Once the cells are mechanically trapped they are maintained in X-Y position as well as being constantly maintained in the focal plane (Figure 3-1D). Mechanically trapping cells in a constant field of view is ideal for making fluorescence time-lapse movies of living trypanosomes, which is difficult to achieve using other sample methods.
3.4.2 **Trapping cells in device.** To explore the capabilities of the microfluidic plates to trap PCF and BSF parasites, we optimized parameters to trap and/or immobilize cells. Manufacturer software comes pre-loaded with protocols (Channel 6 27.6 kPa, Channel 8 13.8 kPa) for loading bacterial cells into the devices. These preprogrammed protocols perfuse trypanosome parasites too quickly though the device. We found that running the loading protocol at half the recommended pressures maintained cell viability while trapping cells in the device at sufficient densities. Once the device was pressurized, parasites are passed though the fluidics and are trapped inside of the device as shown in Figure 3-2. Cells enter the device from the bottom of the frame until they reach a region that is small enough to mechanically compress the cell. Once loaded into the device cells remain in a small field of view that allows continuously imaging. BSF and PCF parasites remain motile and viable for upwards of two hours under perfusion of 10 mM glucose in PBS. Representative images of trapped cells are shown in Figure 3-2A and 3-2B respectively.
Figure 3-2: Images of trapped trypanosomes. Representative DIC image of BSF (A) and PCF (B) trypanosomes tapped in the bacterial microfluidic plate. The line across the center of the image shows the barrier between two different height capture regions, the dots in the image are pillars that are used to maintain the uniform height of the device. Images were extracted from time-lapse movies found in Supplemental Information. Scale is 10 µm.
3.4.3 Fluorescein perfusion and mixing in device. The microfluidic devices used in this study are designed to hold up to five different buffers allowing multiple perfusion conditions. When a low number of conditions are needed or when step style perfusions are adequate, this microfluidic device is simply configured for an experiment. However, this device configuration is not conducive to generating a chemotactic gradient although a different plate design is available from the manufacturer that can produce a gradient. To increase the number of buffer conditions past the five that are available on this device, the platform is capable of pressurizing multiple wells at different pressures allowing for perfusion of various buffers at differential rates. To explore the buffer mixing and perfusion potential of this device and platform, we conducted experiments where we perfused different concentrations of a fluorescent dye solution by pressurizing channels of Fluorescein and PBS at different pressures. As each pulse of higher concentration dye passes though the device, the fluorescence intensity increases in a concentration dependent manner (Figure 3-3A). The delay, from buffer perfusion initiation, was two minutes until buffer conditions reequilibrated. This perfusion delay should be sufficient for most experiments where the expected biological effect takes place on a multiple minute or hour time scale.
Figure 3-3: Fluorescein and glucose perfusion profiles. Fluorescein perfusion experiment where different dye concentrations were achieved by mixing different microfluidic plate channels (A). Vertical lines represent the point where the solution was changed via software. Dye fluorescence was tracked via fluorescence microscopy (490/20 ex. 530/30 em). Mean fluorescence intensity from a region of interest is plotted versus time. Error bars represent the standard deviation of the measurement. Intracellular
FLII12PGlu700 response to different glucose concentrations (B). Vertical lines represent changes in the glucose concentrations perfused through the device. Error bars represent the standard deviation of n=25-50 cells.
3.4.4 **Cell response using on device mixing.** We have shown that the device can perfuse different analyte concentrations by mixing different buffer channels. Next, we wanted to explore the type of cellular responses you could obtain using this mixing method. Fluorescent glucose biosensors represent an exciting avenue to explore metabolite concentration flux in living parasites. Fluorescent glucose sensors are expressed in the cytosol and glycosomes of PCF and BSF parasites (unpublished). FRET/ECFP ratio imaging is difficult in motile organisms such as African trypanosomes, to make meaningful measurements it is important to have temporally overlapping ECFP and FRET emission images. In more traditional ratiometric microscopy setups ECFP and FRET emission is obtained by acquiring an image of each channel in rapid succession using a filter wheel or similar hardware. Unfortunately, in highly motile cells like these trypanosomes this is not ideal since cells move between the image acquisition leading to low image overlap. To combat overlap issues, we have implemented a Andor Tucam fluorescent emission wavelength splitter (Figure 3-4). The Andor Tucam separates the ECFP and FRET emission with a long pass dichroic mirror directing it to two cameras allowing for simultaneous ECFP and FRET emission capture [183]. Simultaneous capture ensures that the ECFP and FRET images are perfectly overlapping which enhances the quality of the resultant FRET ratio measurements.
Figure 3-4: Representative diagram of the Andor Tucam dual imaging microscopy system. Fluorescent emission from the microscope enters from the bottom before it reaches a long pass (500LP) dichroic mirror to split the light. Light is then directed to two bandpass filters for YFP and CFP emission which then are detected via two different CMOS cameras simultaneously.
Glucose transport and metabolism is critical for survival in trypanosome parasites, monitoring changes in intracellular glucose is key to informing glucose homeostasis mechanics. To explore how trypanosomes expressing biosensor respond to different external glucose concentrations we monitored the FRET/ECFP ratio of the expressed glucose sensor while perfusing different glucose concentrations using the microfluidic device to mix different concentrations of glucose. Figure 3-3B shows the cytosolic FRET response of FLi12PGlu700 in response to 0 mM, 2.5 mM, 5 mM, 7.5 mM, and 10 mM glucose concentrations. Cytosolic FRET/ECFP ratio increases as the external glucose concentration increases, which agrees with previous reports in mammalian, yeast, plant, and trypanosome cells [182][184][11]. Interestingly, cytosolic glucose approaches a steady state with external glucose concentration very quickly. From Figure 3-3B it was determined that glucose concentrations change as fast as the rate of perfusion shown in Figure 3-3A.

3.4.5 Cell response video and single cell traces. Analysis of single cells can often illuminate unique biological functions that are averaged out in bulk population analysis [185]. Without cell trapping, it is hard to keep single trypanosome cells in the field of view that is required for single-cell time course experiments. The trapping protocol outlined here allows for trypanosomes to be trapped in a way that they remain viable and trapped in the field of view for microscopy experiments. As a proof-of-principle experiment, we
sought to compare the single cell transient response to glucose to the bulk analysis under the same conditions. Representative FRET/ECFP ratio images (Figure 3-5A) were extracted from a FRET/ECFP ratio time-lapse for PCF trypanosome cells expressing FLII12PGLu700. The images are shown as a pseudocolor representation of the resultant FRET/ECFP ratio where red denotes high glucose concentration and blue denotes low glucose concentration. Extracted FRET/ECFP ratios are shown in Figure 3-5B and the response from the individual cells are shown in Figure 3-5C. Single cell response is very similar to the response of the bulk population. Once glucose is removed, cells take approximately 5 minutes to deplete intracellular glucose. Once glucose is reperfused, the cells internal glucose returns to high levels much faster.
Figure 3-5: Single trypanosome glucose response. (A) Representative FRET/ECFP ratio images extracted from a time-lapse fluorescence movie of PCF cells expressing the glucose sensor. Cells were perfused from high to low glucose as denoted by the label in the upper left corner of each image. Pseudocolor images were constructed from the
intracellular FRET/ECFP ratio, red cells represent high cytosolic glucose and blue represent low glucose. (B) Cytosolic FRET
In this experiment, single cells respond very similarly to the bulk population as expected due to a uniform population of monomorphic cells. We anticipate that the power of monitoring single trypanosomes using these methods would be enhanced using pleomorphic trypanosome cell lines where the population is less uniform and capable of differentiating into different life stages [185].

3.5 Conclusion

Here we outline a microfluidic method to trap trypanosome cell allowing for live cell imaging perfusion experiments. We utilized the commercially available CellASIC ONIX2 microfluidic system equipped with their bacterial tapping plate. We have demonstrated that this microfluidic device and platform can trap PCF and BSF parasites during perfusion experiments while maintaining viability. We have shown that trapped single cells expressing a fluorescent glucose sensor could report internal glucose concentration in response to dynamic changes in external glucose concentrations. The intracellular FRET/ECFP response is similar to the perfusion profile demonstrating that the internal glucose concentration changes very rapidly in response to external conditions. Using this trapping method, time-lapse measurement of single cells can be acquired and individually analyzed separate from the bulk population to identify the distribution of responses within a population of cells. Overall, this represents a significant improvement for single cell analysis in trypanosomes and kinetoplasts generally and expands the analytical repertoire available for these and similar cells.
CHAPTER 4: A FRET Flow Cytometry-Based High Throughput Screening Assay to Identify Glucose Uptake Inhibitors in

Trypanosoma brucei

The following manuscript was originally written and formatted for submission to ACS Infectious Diseases. Edits are concluded and the manuscript is currently being submitted for review. The original manuscript was reformatted to maintain format with the remainder of chapter.
4.1 Abstract

Trypanosoma brucei, which causes Human African Trypanosomiasis (HAT), derives all its cellular ATP from glucose metabolism while in the mammalian host. Targeting glucose uptake or regulation in the parasite has been proposed as a potential therapeutic strategy. However, few methods can identify and characterize potential inhibitors of glucose uptake. Here, we describe development of a screening assay that identifies small molecule inhibitors of glucose uptake into the cytosol and glycosomes. Using an endogenously expressed fluorescent protein glucose sensor expressed in cytosol or glycosomes, we monitored intracellular glucose depletion in the different cellular compartments. Two novel glucose uptake inhibitors were identified, one of which only exhibited inhibition of glycosomal glucose and did not affect cytosolic levels. In addition to inhibiting glucose uptake with relatively high potency (EC$_{50}$ = 700 nM), the compound also showed modest bloodstream form parasite killing activity. Expanding this assay will allow for identification of candidate compounds that disrupt parasite glucose metabolism.
4.2 Introduction

More than 400 million people live in areas where diseases caused by the kinetoplastid parasites *T. brucei*, *T. cruzi* and *Leishmania spp* are endemic [186]. Human African Trypanosomiasis (HAT), which is caused by *T. brucei* results in approximately 70,000 deaths per year, and leaves much of the impacted areas not suitable for rearing livestock [187]. Common treatments for kinetoplastid diseases include compounds developed in the 1950s, which have noted toxicity to the host [138]. Global economics and politics play a role in the lack of therapeutic development, since the diseases affect some of the poorest regions on earth, including sub-Saharan Africa, South America, and large areas east Asian subcontinent [188].

*T. brucei* inhabits the bloodstream of a mammalian host where the parasite metabolizes glucose as their lone carbon source; without this sugar, parasites die quickly [189]. Impairment of glucose uptake is therefore an attractive target for anti-kinetoplastid therapies. Glucose metabolism in kinetoplastid parasites is spatially localized in specialized peroxisome-like organelles known as glycosomes [6]. The existence of this distinct metabolic organelle creates kinetoplastid-specific glucose uptake/acquisition, and flux mechanisms that may be targeted for parasite-specific therapies. Glucose apparently enters the cell via the parasite-specific glucose transporters THT1 or THT2, and must then be delivered to glycosomes to enter the glycolytic pathway. This process has
previously been assumed to occur via facilitated diffusion across the glycosomal membrane; however, we have observed glucose concentrations in the glycosome that are higher than those in the cytosol, suggesting active glucose transport into glycosomes (unpublished). Hence, mechanism(s) that control glycolysis in kinetoplastids, including *T. brucei*, may be unique to glycosomes. Importantly, each of these steps (glucose transport into the cytosol, relocation into the glycosome, and glucose consumption via metabolic enzymes) all represent possible points for intervention via inhibitor compounds [190].

Compounds that inhibit kinetoplastid glucose homeostasis, thus altering metabolic function and viability are attractive as a potential anti-trypanosome therapy. Given the differences between glucose transport and localization of metabolism in mammalian and kinetoplastid cells,[191] such a compound could specifically inhibit kinetoplastid metabolism. For example, a small molecule inhibitor that compromised glucose uptake into glycosomes could be catastrophic to BSF parasite viability, without impacting the mammalian host. While commonly used glucose measurement techniques are destructive, low throughput, and do not allow measurements of intraglycosomal glucose, we have recently expressed glucose-responsive biosensors in the cytosol and glycosomes of transgenic *T. brucei* and used them to quantify glucose concentration(s) in the different compartments of live parasites. Here we expand these measurements to screen for molecules that reduce intracellular glucose concentrations, presumably by altering glucose flux into *T. brucei* glycosomes or impact key steps in glycolysis.
In yeast and mammalian cells, FRET biosensors have been used previously to track changes in cytosolic and organellar glucose that result from environmental perturbations [192]. We have expressed FRET biosensors in *T. brucei*, and have routed sensor proteins to the parasite glycosome by appending a peroxisomal targeting sequence to the polypeptide. The presence or absence of the targeting sequence allows us to apply the biosensor and its resulting glucose measurements to either the cytosol or the glycosome. Using the readout of these sensors to monitor intracellular glucose, we constructed a screening assay to identify compounds that inhibit glucose transport into the parasite cytosol or glycosomes. Given the importance of glucose to parasite viability, we anticipated that these compounds could have anti-parasitic activity.

4.3 Methods

4.3.1 Chemicals and reagents. Clear 384-well plates were purchased from Greiner (Monroe, NC) and used for all screening experiments. All cell culture media and components were purchased from Sigma (St. Louis, MO). Hygromycin, G418, and Blasticidin used for trypanosome selection were purchased from GoldBio. Serum used for media supplementation was purchased from Rocky Mountain Biologicals (Missoula, MT).
4.3.2 Parasite culture, sensor transfection and microscopy. BSF parasites were continuously cultured in HMI-9 media supplemented with 10% FBS [151]. PCF parasites were grown and maintained in SDM-79 media [152]. The glucose sensor FlII±PGlu-600µ was cloned into pXS2 for expression in PCF as described [153]. FlII±PGlu-600µ expressing parasites were imaged using a CFP/YFP FRET filter set with a 430/30 nm excitation filter and 480/30nm and 530/30nm bandpass filters for CFP and YFP emission.

4.3.3 FRET cytometry. All flow cytometry was carried out on a BD Attune Acoustic Focusing Flow Cytometer (BD Biosciences; Franklin Lakes, NJ) equipped with violet (405nm) and blue (488nm) lasers. FRET emission (405nm ex. YFP emission) was obtained from the 405nm excited channel using a 530/30nm bandpass filter. Direct excitation of YFP was measured in the 488nm channel using a 530/30nm bandpass filter. FRET ratio (405nm ex. YFP emission/ 488nm ex. YFP emission) was calculated on a cell by cell basis using the cytometer software to calculate FRET ratio as a custom output parameter. Dead cells were excluded from analysis using forward and side scatter characteristics; only cells expressing high amount of sensor were determined using non-transfected cells for gating. Raw data was exported as FCS files and analyzed using FlowJo software (FlowJo LLC; Ashland, OR), data was further analyzed using Microsoft excel.
4.3.4 **Glucose time chase flow cytometry.** To explore the temporal resolution of glucose response in PCF parasites, cytosolic and glycosomal FRET/mCitrine ratios were monitored in response to changes in external glucose concentration. Briefly, PCF parasites expressing FlII12Pglu-600µ or PT-FlII12Pglu-600µ were rinsed 3 times with PBS to remove media components. Cells were then allowed to incubate in PBS (137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄) supplemented with 5 mM glucose for 30 minutes. 10µL of the cell suspension was then added to the wells of a 96 well plate; 200 µL of PBS with 5 mM glucose was then added to the first row of the 96 well plate, which was measured using FRET cytometry methods described above. Upon completion of the first row, 200 µL of PBS with 0 mM glucose was added to the remaining wells of the plate, which decreased buffer glucose levels to 0.25 mM. The next 3 rows of the plate were immediately measured via flow cytometry, followed by spiking of remaining wells to a final concentration of 5mM glucose, using a 250mM glucose/PBS solution, and measurement of remaining wells for resultant FRET/mCitrine ratio. Wells were measured sequentially, representing different time points. FRET ratios were calculated as above, normalized versus high and low controls, and plotted versus collection time.

4.3.5 **2-DOG response.** PCF parasites expressing FlII12Pglu-600µ or PT-FlII12Pglu-600µ were rinsed three times in PBS supplemented with 5 mM glucose and resuspended in PBS + 5 mM glucose. 200 µL of the cell suspension was then added to wells of a 96 well plate,
and the first row of the plate was monitored via flow cytometry. Upon completion of reading the first row of cells with flow cytometry the remaining cells were spiked with 250 mM 2-DOG to a final concentration of 25 mM 2-DOG and 5mM glucose and then immediately monitored using flow cytometry. FRET/mCitrine ratios were then extracted for each well and normalized to the high and low FRET/mCitrine ratio over the experiment.

4.3.6 Library components and plate preparation. The Pathogen Box library was supplied by (MMV) and consisted of 5 plates of 96 wells, each containing 10 µL of 10 mM compound dissolved in dimethyl sulfoxide (DMSO). Drugs used for screening were diluted to 1 mM in DMSO before transferring 1 µL of the diluted compound into a 384 well plate in replicate. Pathogen Box compounds were flanked by three rows of high and low controls in 1 µL of DMSO as a vehicle control. DMSO concentration was kept at or below 1% to reduce effects on cell viability and assay readout. Compounds identified as candidate glucose uptake inhibitors were reacquired from MMV and dissolved to a final concentration of 10 mM in DMSO before being used for glucose inhibition dose response and trypanosome killing assays.
4.3.7 Glucose depletion assay. Glucose uptake assays were based off a flow cytometry method that can be used to monitor changes in intracellular glucose in live trypanosome parasites (Chapter 2). Briefly, cells were washed three times with PBS, cells to be incubated with test compounds or high controls were incubated in PBS supplemented with 5 mM glucose; low controls were incubated in PBS alone. 100 µL of the cell suspension (≈10,000 cells per well) were pipetted into the wells containing the test compounds in 1 µL DMSO to a final concentration of 10 µM in 1% DMSO, positive controls contained 1% DMSO and 5 mM glucose, low controls contained 1% DMSO with 0 mM glucose. The loaded plate was incubated at ambient conditions for one hour before analysis via flow cytometry as described above. Confirmed preliminary hits were retested using reacquired compounds; IC₅₀ values were determined from 2-fold serial dilution with 10 µM compound as the highest concentration. All dose response curves and IC₅₀ curves and values were fit to a single site ligand binding curve using Sigmaplot 11.0 (Systat Software Inc; San Jose, CA).

4.3.8 HIT identification. All primary screen data was analyzed using FlowJo flow cytometry analysis software and exported as a Microsoft Excel file for further analysis. Histograms were constructed with high and low controls used to determine Z’ values. FRET/mCitrine ratios for each compound were plotted versus high and low controls. Compounds were considered hits if the FRET/mCitrine ratio fell three standard deviations
below the average FRET/mCitrine ratio of the high controls for both replicates. As a secondary measure to exclude false positives the hit compounds were plotted on a bivariate plot with FRET fluorescence (X-axis) vs. mCitrine (Y-axis) fluorescence. A region was then drawn between the area that is outlined by the trend lines of the high and low controls. Any compound that fall outside of the area in between the high and low controls was considered a fluorescent or highly absorbing compound and excluded from further analysis.

4.3.9 T. brucei viability assay. To determine the efficacy of inhibitors on trypanosome viability, 5 x 10³ BSF cells were seeded into 96-well clear-bottom plates in 200 μL HMI-9 media, supplemented with compound or an equal volume of vehicle followed by incubation at 37°C at 5% CO₂ for three days. The Cell Titer Blue reagent (Promega; Madison, WI) was added (20 μL) to each well and the plates were incubated at 37°C at 5% CO₂ for three hours. Fluorescence at 585 nm was measured at 546 nm excitation using a GENios plate reader (Phoenix Research Products; Hayward, CA).

4.4 Results and Discussion.

4.4.1 Overview. T. brucei relies solely on glucose metabolism for its survival in the mammalian host bloodstream [193]. Disruption of glucose metabolism and uptake have
long been considered promising pathways for targeting with anti-parasitic compounds [194]. Traditional analytical methods for measuring intracellular glucose are not amenable to high throughput screening, but the development of genetically-encoded FRET biosensors have allowed for monitoring of analytes in living cells. We have adapted a FRET-based biosensor that specifically detects glucose to monitor cytosolic and glycosomal glucose levels in living kinetoplastid parasites. We used flow cytometry to monitor changes in sensor FRET signal upon treatment with a series of potential inhibitors. The result is a screening method capable of identifying small molecule inhibitors of intracellular glucose delivery and redistribution into glycosomes.

### 4.4.2 Sensor transfection & microscopy.
To monitor glucose concentration in living cells, the fluorescent glucose biosensor FlIII\textsuperscript{12}PGlu-600µ consisting of an ECFP/mCitrine fluorescent protein FRET pair flanking a glucose binding domain was expressed using the pXS2 expression vector in PCF parasites [50]. This construct yielded diffuse signal throughout the cytosol of cells (Figure 4-1). To deliver the biosensor to the glycosome, the transgene was expressed as a fusion with a C-terminal type-1 PTS signal sequence. Expression of this construct produced localization in vesicular organelle-like structures consistent with glycosomes (Figure 4-1).
Figure 4-1: Expression and localization of fluorescent glucose biosensor. DIC (LEFT) and FRET (RIGHT) images of PCF trypanosomes expressing FLII12-Glu600u in cytosol (TOP) or glycosomes (BOTTOM). Error bars represent 10um.
4.4.3 FRET cytometry. Cells expressing biosensors are traditionally analyzed via fluorescence microscopy including high-content screening instruments. However, since trypanosomes are small, grow in suspension, and are highly motile, most imaging-based approaches lack the ability and throughput rate needed for rapid screening of candidate inhibitor compounds [22]. To increase the throughput of our FRET-based screening assay, we utilized a two-laser flow cytometer to monitor changes in biosensor response. Using this approach, we could distinguish cells that were 10-100 times brighter in the FRET emission channel and the directly excited acceptor (mCitrine) channel compared to untransfected controls, making them suitable for measurements. Incorporating a cytometer with an auto sampler into the assay enhanced the speed of sample analysis to a throughput rate of ~100-500 samples per hour depending on the cytometer used. Although flow cytometers represent a powerful tool for high throughput screening there are few examples where they have been utilized for screening [195]. Similarly, FRET biosensors are rarely analyzed with flow cytometry even though flow cytometers are advantageous for analyzing a large number of cells expressing FRET sensors [196].

4.4.4 Temporal glucose response. Parasites expressing the glucose FRET sensor respond to external glucose and 2-DOG concentrations (Figure 4-2). To explore the temporal response of the biosensors as a result of changes in external environmental glucose concentrations, PCF cells expressing cytosolic (Figure 4-3A) or glycosomal (Figure
4-3B) sensors were incubated in buffer and then exchanged into different buffers of different glucose concentrations. When incubated in high glucose (5mM; + GLC) the FRET/mCitrine ratio remained high, representing a high internal glucose concentration. Once cells were exchanged into low glucose (0mM; - GLC) FRET/mCitrine ratio decreased rapidly, coming to a steady state within one minute. The low intracellular glucose concentration was maintained until cells were returned to a high glucose environment. Once exchanged back into high glucose, FRET/mCitrine ratios return to the steady state levels observed in the beginning of the time course.
Figure 4-2: In vivo sensor response to glucose and 2-DOG concentration.

(A) PCF parasites expressing FLII_{12}P-glu-600μ in cytosol (Black) or FLII_{12}P-glu-600μ-PTS in glycosomes (Red) were incubated in PBS, glucose concentration was titrated (.025-25,000 μM). Intracellular glucose was allowed to reach a steady state (45 minutes) before being analyzed via flow cytometry. FRET/mCitrine ratios were then plotted as a function of extracellular glucose and the data was fit using SigmaPlot software. (B) PCF parasites expressing FLII_{12}P-glu-600μ in cytosol (Black) or FLII_{12}P-glu-600μ-PTS in glycosomes (Red) were incubated with 10mM glucose in PBS, 2-DOG concentration was titrated 0, 5,
10mM). Resultant FRET/mCitrine was plotted for each 2-DOG concentration. Error bars represent standard deviation for n=3.
Figure 4-3: Normalized FRET/mCitrine ratios for time traces of PCF cells expressing the glucose sensor in cytosol. (A) and glycosomes (B). Cells were allowed to incubate in high glucose (5 mM; + GLC) and exchanged into low glucose at the vertical line (0.25 mM; -GLC). Cells were then allowed to achieve a steady state at the low glucose conditions for 13 minutes before being exchanged back into high glucose at the second solid vertical line.
All FRET/mCitrine ratios were collected using flow cytometry, error bars represent the standard deviation of n=1000 cells.
Interestingly, the glucose concentration in the cytosol changed very rapidly once cells were placed in low glucose solutions, with no data points in the transition between high and low intracellular glucose (Figure 4-3). This observation suggests that glucose equilibration was faster than we could currently measure (=30 seconds). In contrast, glycosomal glucose equilibration was slower, taking at least 90 seconds to equilibrate and reach a steady state. This difference between cytosol and glycosome response to external stimulus could be explained because glucose must first pass into the cytosol before it can enter the glycosome, which potentially creates a lag in glycosomal response to the external glucose.
4.4.5 Cellular response to 2-DOG. Following validation of sensor expression via microscopy and flow cytometry we verified that the FlIII\textsuperscript{12}\textsuperscript{PGlu-600µ FRET ratio changes in response to environmental conditions. The normalized FRET signal in parasites expressing FlIII\textsuperscript{12}\textsuperscript{PGlu-600µ in cytosol or glycosomes shows that intracellular glucose levels rise as external glucose increased (Figure 4-2A). As expected, when parasites were incubated in sufficient quantities of the competitive uptake inhibitor and metabolic poison 2-DOG that competes with extracellular glucose, intracellular glucose levels decreased in a dose dependent manner (Figure 4-2B) [197].

The measurements described above reflect an endpoint-like steady state measurement under conditions of constant extracellular glucose and/or 2-DOG. However, such measurements do not provide information about the kinetics of glucose transport into the cell. To investigate an approximate time frame for glucose transport into the cell, we carried out a time-course measurement in which cells expressing the biosensor were treated with a high concentration of 2-DOG. Within three minutes of 2-DOG addition (denoted as a vertical line), both cytosolic and glycosomal glucose concentrations fall to dramatically lower levels; steady state was reached within 5 min (Figure 4-4A & Figure 4-4B). Notably, unlike the response to glucose starvation, the glucose depletion of glycosomes and the cytosol to 2-DOG occur at similar rates. This observation likely reflects the mechanism of action of 2-DOG, which occupies glucose
binding sites in the relevant glucose transporter proteins and thus slows glucose uptake into the cytosol to rates approaching those for glucose entry into the glycosome.
Figure 4-4: Normalized FRET/mCitrine time chase for PCF expressing glucose biosensor in cytosol. (A) and glycosomes (B) in response to addition of 2-DOG. Cells were allowed to incubate in PBS with 5 mM glucose (- 2-DOG) and monitored using flow cytometry. 2-DOG was then added to a final concentration of 25 mM 2-DOG (+25 mM) at the vertical solid black line. All FRET/mCitrine ratios were collected using flow cytometry, error bars represent the standard deviation of n=1000 cells.
Both the glucose depletion data and 2-DOG treatment data (Figures 4-3 and 4-4) indicate that hexose transport processes into the cell are rapid, and that intracellular glucose responds to the external environment within minutes. Hence, during the time frame of our assay (1-3 hours) glucose would be depleted in response to candidate inhibitor compounds.

4.4.6 Screen controls and Z-prime. To pursue novel inhibitors of glucose uptake, we developed a screening assay that uses flow cytometry to monitor FRET response to small molecules. The screen design allowed us to probe glucose uptake and distribution in live cells in tandem with cell viability. This approach allowed us to exclude non-specific cytotoxic compounds while identifying bioactive molecules that specifically impact intracellular glucose concentrations. Thus, we were able to avoid the liabilities of poor cell permeability and off-target effects. To maximize the sensitivity of the FlII12PGlu-600µ glucose FRET assay, conditions were optimized to yield the highest and lowest signal of biosensors. Glucose at 5 mM was the optimal high signal control (Figure 4-5A and 4-54-5B, in red) while reduced glucose (~ 0.1 mM, Figure 4-5A and 4-5B, in blue) was used as the low signal control, serving to limit the negative impact of the complete absence of glucose on cell viability. Using a bivariate representation where mCitrine emission was plotted against normalized FRET emission, the x-axis was correlated to the FRET/mCitrine ratio and the distribution along the trend line was representative of the standard error in
the assay (Figure 4-5C and 4-5D). This approach yielded PCF cytosol and glycosome screens with Z’ values of 0.75 and 0.70, respectively. These assay robustness factors suggest the we can identify novel glucose uptake inhibitors with few false positives [198]. Table 4-1 outlines the screening parameters, following guidelines suggested for universal HTS method communication [199].
Figure 4-5: Screening controls. Histograms and plots representing the FRET/mCitrine ratio of the high and low controls in PCF cells expressing the glucose biosensor in cytosol (A and C) or glycosomes (B and D). These represent the same data presented in two different formats. Bivariate plots representing the normalized FRET emission (x-axis) and mCitrine emission (y-axis) of PCF cells expressing FlI12PGLu-600µ in cytosol (A and C) or glycosomes (B and D). High controls represent cells that are incubated in 5 mM glucose and low control represent cells incubates in 0 mM glucose plus 20 mM 2-DOG. Z’ values for cytosol and glycosome assay calculated as 0.7 and 0.75 respectively.
Table 4-1: Parameters for small molecule screen.

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<thead>
<tr>
<th>Category</th>
<th>Parameter</th>
<th>Examples (see text for more detail)</th>
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<tbody>
<tr>
<td>Assay</td>
<td>Nature of assay</td>
<td>Cell based fluorescence flow cytometry assay</td>
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<tr>
<td></td>
<td>Assay Strategy</td>
<td>Detection of intracellular and/or glycosomal glucose depletion using an endogenously expressed FRET biosensor that specifically binds glucose</td>
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<td></td>
<td>Assay protocol</td>
<td>Key steps are outlined in Table 4-2</td>
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<td>Library Screened</td>
<td>Nature of Library</td>
<td>Drug like molecules that are active against a neglected tropical disease of interest</td>
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<tr>
<td></td>
<td>Size of library</td>
<td>400 compounds arrayed in 96 well plates at 10mM in DMSO</td>
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<tr>
<td></td>
<td>Source</td>
<td>Medicines for Malaria Venture (MMV)</td>
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<td></td>
<td>Concentration Tested</td>
<td>10µM concentration, 1% DMSO, 1:100 dilution</td>
</tr>
<tr>
<td>HTS process</td>
<td>Format</td>
<td>Sterile 384 well plate (Greiner Bio)</td>
</tr>
<tr>
<td></td>
<td>Plate Controls</td>
<td>Positive control: 0mM glucose (A1-P1); negative control: 5mM glucose (A2-P2)</td>
</tr>
<tr>
<td></td>
<td>Plate number and duration</td>
<td>5 384 well plates over 6 days</td>
</tr>
<tr>
<td></td>
<td>Reagent dispensing system</td>
<td>Reagents and cell dispensed using an electronic 12 channel pipette (LabNet)</td>
</tr>
<tr>
<td></td>
<td>Normalization</td>
<td>Normalized FRET response = (sample result – average low controls) / (average high controls – average low controls)</td>
</tr>
<tr>
<td>Post HTS analysis</td>
<td>Selection of activities</td>
<td>Active compounds were selected using a threshold based off the statistics of the high controls</td>
</tr>
<tr>
<td></td>
<td>Retesting of initial activities</td>
<td>Compounds with replicate positive activities were tested in a 12-point dose response</td>
</tr>
<tr>
<td></td>
<td>Compound purification</td>
<td>Validated compounds were required from MMV for further testing</td>
</tr>
</tbody>
</table>
4.4.7 **Library screening and validation.** To identify novel inhibitors of glucose uptake and regulation in the African trypanosome, we screened the small molecule Pathogen Box library (a curated collection of approximately 400 compounds) for inhibitors of glucose uptake into the cytosol and glycosomes of PCF trypanosomes. The pathogen box library was provided by Medicines for Malaria Venture (MMV). Each compound was tested in duplicate at 10 μM, with solvent (1% DMSO) used as a control (protocol outlined in Table 4-2). Compounds were initially scored as active if they inhibited the FRET response at least three standard deviations below the average of high (5mM glucose) controls (Figure 4-6) in both replicates. Three compounds reduced cytosolic glucose; five compounds reduced glycosomal glucose (Fig. 4-6C and 4-6D). One compound reduced glucose concentration in both the glycosome and cytosol, resulting in a total of seven compounds identified. Since the sensors do not bind phosphorylated glucose, identified compounds lower the concentration of glucose before it phosphorylated by hexokinase, most likely by inhibiting glucose transport mechanisms.
Figure 4-6: Pathogen Box screening results. Histograms representing the FRET/mCitrine ratios from the glucose depletion screen in PCF cytosol (A) and glycosome (B). Bars represent the FRET/mCitrine ratio of 400 candidate inhibitor compounds (grey bars), high controls 7.5mM glucose (red bars) and low controls (blue bars). Hit compounds were identified as being three standard deviations below the average of the high controls (red). To exclude fluorescent and highly absorbent compounds, bivariate plots of cytosol screen (C) and glycosome screen (D) were plotted as FRET emission (x-axis) versus mCitrine (y-axis) for candidate compounds (grey), 5 mM glucose (red, high controls), and 0 mM glucose plus 20 mM 2-DOG (blue, low controls). Fluorescent and highly absorbing
compounds were excluded as being outside the region drawn that represents the area formed from the trend lines of the high and low controls.
**Table 4-2: FRET flow cytometry HTS protocol.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Controls</td>
<td>1 µl</td>
<td>DMSO</td>
</tr>
<tr>
<td>2</td>
<td>Library compounds</td>
<td>1 µl</td>
<td>10 µM; 10 µM to 4.9nM for serial dilution</td>
</tr>
<tr>
<td>3</td>
<td>Addition of cells</td>
<td>100 µl</td>
<td>5,000-10,000 PCF 2913 cells expressing glucose biosensor, PBS with 5mM glucose</td>
</tr>
<tr>
<td>4</td>
<td>Addition of low controls</td>
<td>100 µl</td>
<td>5,000-10,000 PCF 2913 cells expressing glucose biosensor, PBS with 0mM glucose</td>
</tr>
<tr>
<td>5</td>
<td>Incubation time</td>
<td>1 hour</td>
<td>Ambient conditions</td>
</tr>
<tr>
<td>6</td>
<td>Assay Readout</td>
<td>405ex/530em, 488ex/530em</td>
<td>BD Attune flow cytometer</td>
</tr>
</tbody>
</table>

**Step Notes**

1. Clear 384 sterile cell culture plates (Greiner Bio), DMSO control pipetted in columns 1-2
2. Compounds diluted in DMSO to 1mM and then 1 µl was pipetted into the plate, tips were exchanged between steps
3. Rinsed PCF cells resuspended in PBS with 5mM glucose were pipetted into well, tips were changed between compounds (rows 2-16)
4. Rinsed PCF cells resuspended in PBS with 0mM glucose were pipetted into well, tips were changed between compounds (row 1)
5. Plates cover with supplied plastic lid and allowed to incubate in ambient conditions
6. Cells were analyzed using a BD attune flow cytometer equipped with a 405nm and 488 laser and a plate reader. FRET emission (405nm ex, 530nm em) and mCitrine (488nm ex, 530nm em) were collected for each cell simultaneously. A custom FRET/mCitrine parameter was created using the flow cytometry software.
The seven active compounds were tested in a 12-point dose response assay, with two verified as lowering glucose in a dose-dependent manner (Figure 4-7). Additional amounts of these compounds were acquired from Pathogen Box for use in downstream assays (Fig. 4-7). One compound (MMV085210) was found to inhibit glucose uptake into both cytosol and glycosomes with an EC50 ≈ 5 uM. However, this compound did not appreciably impact BSF parasite viability at 10 uM. The second compound, MMV 272114, only acted on glucose uptake into the glycosome, with an EC50 ≈ 700 nM. Notably, this agent killed BSF parasites, causing a 41 ± 7 % reduction in parasite number at 10 uM (Table 4-3). This BSF toxicity assay is likely to underestimate the impact of identified compounds under physiological conditions, since it is carried out in HMI-9 media, which has a glucose concentration of ~22mM, roughly four times that found in mammalian blood. Since effective glucose concentrations inside the glycosome and cytosol increase with increasing extracellular glucose concentrations (Chapter 2), this larger than physiological glucose concentration will tend to compensate for decreased glucose flux into the glycosome caused by the small molecule inhibitor. Hence, 41% killing, as observed here, represents substantial inhibition and may be enhanced if the assay could be conducted at physiological glucose concentrations. Since the identified inhibitor compound affects glucose concentration inside the glycosome but does not impact cytosolic glucose uptake, the compound appears to target machinery specific to glycosomal glucose uptake or regulation.
Figure 4-7: Identified compound glucose dose response curves. Dose response curves for two hits 085210 (A) and 272114 (B) are shown where FRET/mCitrine ratio is plotted as a function of inhibitor concentration. EC50 values for 085210 and 272114 were calculated as 0.7 µM and 5 µM.
### Table 4-3: Pathogen box compounds effect on glucose uptake and BSF cell viability.

<table>
<thead>
<tr>
<th>MMV ID</th>
<th>Structure</th>
<th>PCF Glucose EC50</th>
<th>BSF Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td>272114</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>0.7 μM</td>
<td>41 ± 7% @10 μM</td>
</tr>
<tr>
<td>085210</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>~5 μM</td>
<td>N/A</td>
</tr>
</tbody>
</table>

150
4.5 Conclusions

Historically, one of the key challenges in identification of anti-trypanosomal therapies that target glucose metabolism has been glycosomal segregation of relevant enzymes. A series of small molecule enzyme inhibitors have been identified that were effective in vitro, but not in live cells, presumably because inhibitors were not delivered to the glycosomes of live parasites,[190] and activity is enhanced when specifically targeted to glycosomes [200]. Our screening strategy bypasses the difficulty of glycosomal drug delivery, because it identifies only molecules that effectively alter intraglycosomal glucose concentration. While many questions about the mechanisms of glucose delivery to the glycosome remain, the screening assay design will detect inhibitors without direct knowledge about individual transport mechanisms.

The pilot screen described here was able to identify a compound that both lowered intracellular glucose concentration and killed bloodstream T. brucei, out of a library of about 400 molecules. This observation suggests that screening larger libraries could yield additional classes of inhibitor(s) with potentially higher activity.

The FRET flow cytometry assay described here is applicable to a range of biosensors specific for ATP, pH, calcium, redox potential and many biologically relevant analytes. The large palette of available biosensors could allow for the probing of key metabolic processes against small molecule libraries to discover novel therapeutic lead compounds.
CHAPTER 5: Deploying a Novel FRET-Cytometry Glucose Uptake Assay to Identify Anti-Kinetoplastid Drug Scaffolds
5.1 Introduction

A bloodstream infection with the unicellular protozoan parasite Trypanosoma brucei causes Human African Trypanosomiasis (HAT), commonly known as sleeping sickness in humans, and nagana in cattle [201]. The diseases caused by T. brucei are endemic in Sub-Saharan Africa and cause ~10,000 deaths a year and hundreds of millions of dollars in damages due to livestock loss [202]. Current treatments are expensive, difficult to administer, and are prone to resistance via parasite mutation [203]. To develop alternative and more effective targeted therapies, compounds must be identified that arrest trypanosome specific pathways. One pathway of importance is glucose metabolism, which occurs in kinetoplastid specific organelles known as glycosomes [144].

The unique nature of glucose metabolism in T. brucei makes it attractive for small molecule intervention [190]. However, there are currently no methods capable of screening compounds with the throughput needed for high throughput screening. FRET flow cytometry can serve as a medium to high throughput analysis method. When paired with genetically encoded glucose sensors FRET cytometry has been employed using a previously validated screening assay (See Chapter 4) to identify and validate hit compounds that may ultimately be trypanocidal by targeting glucose uptake and/or metabolism in a specific fashion such that host glycolysis is unaffected.
We have now screened 25,000 compounds from the ChemLife library in both a cytosol- and glycosome-targeted assay to identify compounds that affect glucose uptake and/or metabolism. Following follow up screening assays and bloodstream from killing assays, 40 compounds are currently being validated by repurchase or resynthesis. Several compound classes have been identified in the hit compounds and the prospect for identification of highly active compounds is promising.
5.2 Materials and Methods

5.2.1 Materials and reagents. Black bottom assay plates for trypanosome glucose uptake assays were purchased from Grenier Bio (Monroe NC). For the mammalian toxicity assay, clear bottom white tissue culture treated plates were purchased from Sigma Aldrich (Saint Louis, MO). Black bottom 384 well plates for the bloodstream form trypanosome toxicity assay were purchased from Brandtech. CellTiter Blue cell viability assay reagent was purchased from Promega Scientific (Madison, WI). All buffer and media components were purchased from Sigma Aldrich (Saint Louis, MO). A 384 plate loading liquid handling manifold was purchased from V&P Scientific. All serial dilution preparation and compound validation plates were constructed using a Mosquito liquid handler (TTP Labtech).

5.2.2 Cell culture and transfection. Bloodstream form (BSF) parasites were cultured in HMI-9 media supplemented with 10% Serum Plus at 37°C and 5% CO₂ [151]. Procyclic form (PCF) parasites were grown and maintained in SDM-79 media supplemented with 10% v/v heat-inactivated calf serum and porcine hemin 35μg [152]. All cells used for glucose uptake assays were rinsed in PBS (137mM NaCl, 2.7mM KCl, Na₂HPO₄, KH₂PO₄) to reduce auto fluorescence due to media composition. The glucose sensor FLII¹²Pglu-700µδ6 with or without a peroxisomal targeting sequence (PTS-1) were cloned into pXS2
for expression in PCF described in Chapter 2. The mammalian cell line HEK293T cells were cultured in DMEM supplemented with glutamate and pyruvate plus 10% fetal bovine serum at 37°C and 5% CO₂. Cells were split prior to achieving 90% confluency to maintain uniform populations for experiments.

5.2.3 Screening compound and plate composition. All compounds screened were purchased from the University of Wisconsin Small Molecule Screening Facility and were part of the Chem Life commercially available library. The 25,000 compound library subset was selected in such a way that the number of highly conjugated and possibly fluorescent compounds, as well as suspected PAINS compounds were minimized to decrease the potential false positives due to fluorescence or non-specific effects. 80 nL of 5-10 mM compound stock solutions in DMSO were distributed into columns 2-23 and rows A-P. Columns 1 and 24 were dispensed with 80 nL DMSO as a vehicle control. A total of 25,000 individual compounds were tested in both the cytosolic and glycosomal targeted assays.

5.2.4 Dose response plate preparation. 3 µL of 5-10 mM initial hit compounds were repurchased from the University of Wisconsin Small Molecule Screening Facility for dose response analysis. An 11 point, 2-fold, dose response curve dilution was prepared for each of the compounds and 32 dose response experiments were consolidated onto one 384
well plate with 2 columns reserved for controls. Dose response serial dilutions and dispensing were performed using a Mosquito liquid handler; 1000 nL, 2-fold, dose response with the highest concentration at 5 mM using a custom prepared macro. 100 nL of the dilutions were then transferred to a clean and sterile black 384 well plates; 100 nL of DMSO was dispensed into column 1 and 24 as vehicle controls in further assays. Plates were then sealed and stored at -80°C until further analysis.

5.2.5 Flow cytometry glucose uptake assay. Glucose uptake assays were run following the protocol previously described in Chapter 2. Briefly, ~5 x 10^6 PCF or BSF parasites expressing the glucose sensor in the cytosol or glycosomes were washed three times with PBS. ~10,000 cells were then resuspended in 100 µL of PBS with 5 mM glucose and 80 µL of cell suspension were dispensed into 384-well screening plates with 80 nL of compound or DMSO vehicle controls. Parasites were then allowed to incubate at ambient conditions for 1 hour before being analyzed via flow cytometry. All flow cytometry analysis was performed on a Thermo Fisher Attune focusing cytometer equipped with a 405 nm and 488 nm laser. FRET emission and mCitrine emission were measured using 405nm ex 530/30 em and 488nm ex and 530/30nm em respectively. The FRET/mCitrine ratio was then calculated on a cell by cell basis using FlowJo (???, ??) cytometry software.
5.2.6  **Glucose assay data analysis.** A FRET/mCitrine ratio was exported from FlowJo for each compound in an Excel sheet format. Data was then normalized to the high and low plate controls using a custom programmed macro. Compounds were considered hits if they fell below three standard deviations of the on-plate controls. Hits were determined on a compound-by-compound basis. Hits from the PCF cytosol and glycosome screen were repurchased for hit validation via result replication. Compounds that validated in the cherry-picked screen were then purchased in a volume of 3 µL from the University of Wisconsin Small Molecule Screening Facility. To measure glucose uptake inhibition EC₅₀, an 11-point dose response for the repurchased compounds were constructed as described above. A total of 13 dose response plates were then analyzed using the PCF glycosomal localized sensor expressing parasites and analyzed via flow cytometry. All data was then exported and analyzed in FlowJo as described above and sigmoidal dose-response cures were fit using SigmaPlot software to extract Kᵋ and Hill slope from the non-linear least-squares regression analysis.

5.2.7  **Mammalian killing assay.** HEK293T cells (~90% confluency) were harvested via trypsin digestion followed by three rinses with PBS and resuspended in full DMEM media supplemented with 10% FBS. Approximately 5000 cells were then seeded into 384 well plates that were prepared as described above. Briefly, the 384 well plates contained either 100 nL of compound in DMSO or 100 nL DMSO as a vehicle control. High controls
were 100 nL DMSO and killing controls were 0.05% triton X-100. 100 µL of cell suspension was dispensed using a V&P Scientific plate loading manifold. Cells were then incubated for 48 hours at 37°C and 5% CO₂ and 100% humidity. 25 µL of CellTiter blue reagent was then pipetted with a 12-channel electronic pipette and allowed to incubate for 6 hours. The fluorescence intensity at 560/20 ex and 590/10 em was measured using a BioTek H4 plate reader. Dose response curves were fit and EC₅₀ values were calculated using SigmaPlot software.

5.2.8 Trypanosome killing assay. All validated compounds from the primary screen were run in BSF cells to test cell toxicity. To determine the efficacy of glucose uptake inhibitors on reducing trypanosome viability 5 x 10³ BSF cells were seeded into 96-well clear bottom plates in 200 µL HMI-9 media supplemented with inhibitor or an equal volume of vehicle followed by incubation at 37°C at 5% CO₂ for two days. Cell titer blue reagent (Promega, Madison WI) was added (20 µL) and the plates were incubated at 37°C and 5% CO₂ for three hours. Fluorescence at 585 nm was measured at 546 nm excitation using a GENios plate reader (Phoenix Research Products, Hayward CA).

5.3 Results and Discussion
5.3.1 Glucose uptake screening assay rationale and design. The relevant BSF parasites rely on glucose metabolism for all of their ATP production needs. Without a constant supply of glucose, BSF parasites die within a short time [204]. The glucose that BSF parasites use for parasite survival is acquired from the host’s bloodstream via a transmembrane glucose transporter related to the human glucose transporters known as trypanosome hexose transporters (THTs) [167]. Although THTs are somewhat like human GLUTs, they show low sequence homology, different glucose binding characteristics, and glucose binding efficiencies. In addition, they are not susceptible to inhibition using known GLUT inhibitors [191]. Once the glucose enters the cell it is then transported to the glycosomal lumen via an unknown mechanism. Given the importance of glucose transport and metabolism on parasite survival it is hypothesized that inhibiting these pathways would lead to cell death. We also hypothesize that since trypanosome hexose transporters have different binding properties, identified inhibitors of trypanosome glucose uptake would not affect the glucose transporters of the human host. We propose two points of chemical intervention. First, the cell membrane glucose transporters (THTs) and the second is at the glycosomal membrane (Figure 5-1).
Figure 5-1: Screening rationale. Schematic of FRET-based glucose measurements in cells expressing cytosolic (left) or glycosomally targeted probe (right). Glycosomal distribution for the probe occurs due to the inclusion of a PTS2 sequence on the N-terminus of the probe. Glucose binding will yield an increase in CIT (mCitrine) fluorescence due to an increase in non-radiative transfer from eCFP. (Figure provided by Dr. James Morris and Evan Qiu)
Current methods to quantify intracellular glucose primarily rely on enzymatic assays and radiolabeled analog uptake. Both methods are destructive and do not readily offer information about the glucose concentration in intracellular organelles [205][206]. To bypass these limitations, we deployed the genetically encoded glucose sensor FLII12P-Glu-700u to monitor glucose concentration in the cytosol and glycosomes of living PCF and BSF parasites. Using this sensor as a readout of intracellular glucose, we can infer the effect of small molecule compounds on glucose uptake and metabolism. If a small molecule inhibits glucose uptake and/or metabolism, the intracellular glucose sensor FRET/mCitrine decreases resulting in a “blue” parasite (Figure 5-2). The FRET cytometry screening method is also more specific allowing for cytotoxic compounds to be removed from analysis. This is an improvement versus other cell based screening protocols which primarily rely on parasite killing as their readout and cannot easily identify inhibitors of specific pathways [207].
Figure 5-2: Simplified representation of assay readout. *T. brucei* cells expressing the fluorescent glucose sensor FLI12PGLu700u emit different fluorescent profiles upon inhibition of glucose uptake by potential uptake inhibitors. When glucose is high in the cell they appear more “yellow” as a result of higher FRET transfer due to high intracellular glucose. If a compound inhibits glucose uptake the biosensor changes conformation and there is less FRET transfer and the cells appear more “blue”.
To build upon our pilot FRET cytometry screening assay, we screened and validated a larger compound library to identify compounds that affected the glucose uptake or metabolism in cells as well as identifying chemical scaffolds for further optimization. A total of 25,000 compounds were analyzed in two different screening assays. The library composition, screening rationale, and overview of the HTS process and analysis is shown in Table 5-1. The entire 25,000 compound library was run in two parallel screens designed to identify compounds that affected cytosolic and glycosomal glucose respectively. We hypothesized that the cytosol targeted screen would identify inhibitors of glucose uptake or metabolism via a THT-dependent mechanism as well as any other non-identified components of cellular glucose uptake [208]. We also hypothesized that the glycosomal screen will be able to identify inhibitors of the glycosomal glucose uptake pathway and that these inhibitors would be inhibiting the unknown mechanisms of glycosomal glucose uptake. The glycosomal uptake inhibitors should be specific for trypanosomes since mammalian cells do not possess such a pathway [172].
Table 5-1: Assay Conditions.

<table>
<thead>
<tr>
<th>Category</th>
<th>Parameter</th>
<th>Examples (see text for more detail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay</td>
<td>Nature of assay</td>
<td>Cell based fluorescent flow cytometry assay</td>
</tr>
<tr>
<td></td>
<td>Assay Strategy</td>
<td>Detection of intracellular and/or glycosomal glucose depletion using an endogenously expressed FRET biosensor that specifically binds glucose Key steps are outlined in Table 5-2</td>
</tr>
<tr>
<td></td>
<td>Assay protocol</td>
<td></td>
</tr>
<tr>
<td>Library Screened</td>
<td>Nature of Library</td>
<td>Drug like molecules, probable fluorescent and PAINS compounds removed from consideration</td>
</tr>
<tr>
<td></td>
<td>Size of library</td>
<td>25,000 compounds arrayed in 384 well plates at 5-10mM in DMSO</td>
</tr>
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<td></td>
<td>Source</td>
<td>Wisconsin Screening Facility</td>
</tr>
<tr>
<td></td>
<td>Concentration Tested</td>
<td>10µM concentration, 0.1% DMSO, 1:1000 dilution</td>
</tr>
<tr>
<td>HTS process</td>
<td>Format</td>
<td>Sterile 384 well plate (Greiner Bio)</td>
</tr>
<tr>
<td></td>
<td>Plate Controls</td>
<td>Positive control: 0mM glucose (Columns 1); negative control: 5mM glucose (Columns 16)</td>
</tr>
<tr>
<td></td>
<td>Plate number and duration</td>
<td>150 384 well plates over 60days</td>
</tr>
<tr>
<td></td>
<td>Reagent dispensing system</td>
<td>Cells dispensed using a 384 well dispensing manifold (V&amp;P Scientific)</td>
</tr>
<tr>
<td></td>
<td>Normalization</td>
<td>Normalize FRET response = (sample result – average low controls) / (average high controls – average low controls)</td>
</tr>
<tr>
<td>Post HTS analysis</td>
<td>Selection of activities</td>
<td>Active compounds were selected using a threshold based off the statistics of the high controls</td>
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<tr>
<td></td>
<td>Retesting of initial activities</td>
<td>Compounds with replicate positive activities were tested in a 11-point dose response</td>
</tr>
<tr>
<td></td>
<td>Compound purification</td>
<td>Validated compounds were required from MMV for further testing</td>
</tr>
</tbody>
</table>
5.3.2 Glucose uptake assay validation. Improvements had been made to the screening assays since running the pilot screen. The improved glucose sensor FLII12Pglu-700μ-δ6 was deployed in place of FLII12Pglu-600μ since it increases sensitivity for glucose [50][209]. To validate the new high (5 mM glucose) and low (0 mM; 20 mM 2-deoxy glucose) were analyzed to determine the assay reproducibility or Z’. Figure 5-3A and B show the histogram and scatter graph representation of the screening controls for the cytosol targeted screen and Figure 5-3C and D represent the histograms and scatter graphs for the glycosomal targeted screen. The histogram data show that the high and low peaks are narrow and well defined, scatter graph data show that the controls remain consistent during the duration of the library screening process (40 days). The Z’ for the cytosolic and glycosome targeted screens were 0.84 and 0.77 respectively. A Z’ above 0.5 is often considered sufficient for screening, but a score above 0.70 is greatly preferred to limit the number of false positives that the screen produces. To help standardize the HTS assay protocols, Table 5-2 outlines the protocol used for all FRET cytometry glucose targeted [199].
### Table 5-2: Outlined assay protocol.

<table>
<thead>
<tr>
<th>Step</th>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Controls</td>
<td>80nL</td>
<td>DMSO</td>
</tr>
<tr>
<td>2</td>
<td>Library compounds</td>
<td>80nL</td>
<td>10 μM; 5 μM to 2.45nM for serial dilution</td>
</tr>
<tr>
<td>3</td>
<td>Addition of cells</td>
<td>80 μl</td>
<td>5,000-10,000 PCF 2913 cells expressing glucose biosensor, PBS with 5mM glucose</td>
</tr>
<tr>
<td>4</td>
<td>Addition of low controls</td>
<td>80 μl</td>
<td>5,000-10,000 PCF 2913 cells expressing glucose biosensor, PBS with 0mM glucose</td>
</tr>
<tr>
<td>5</td>
<td>Incubation time</td>
<td>1 hour</td>
<td>Ambient conditions</td>
</tr>
<tr>
<td>6</td>
<td>Assay Readout</td>
<td>405ex/530em, 488ex/530em</td>
<td>BD Attune flow cytometer</td>
</tr>
</tbody>
</table>

### Step Notes

1. Black 384 sterile cell culture plates (Greiner Bio), DMSO control pipetted in columns 1,14-16
2. Compounds diluted in DMSO to 1mM and then 1 μl was pipetted into the plate, tips were exchanged between steps
3. Rinsed PCF cells resuspended in PBS with 5mM glucose were pipetted into well, tips were changed between compounds (column 16)
4. Rinsed PCF cells resuspended in PBS with 0mM glucose were pipetted into well, tips were changed between compounds (column 1)
5. Plates cover with supplied plastic lid and allowed to incubate in ambient conditions
6. Cells were analyzed using a BD attune flow cytometer equipped with a 405nm and 488 lasers and a plate reader. FRET emission (405nm ex, 530nm em) and mCitrine (488nm ex, 530nm em) were collected for each cell simultaneously. A custom FRET/mCitrine parameter was created using the flow cytometry software.
Figure 5-3: Screening controls. Histogram (A & C) and scatter-graph (B & D) representations of high (Red: 5mM glucose) and low (Blue: 0mM glucose, 20mM 2-DOG) on plate screening controls. Histograms for cytosol (A) and glycosomes (C) show the distribution or normalized FRET ratios (x-axis) for the high and low glucose controls. Scatter-graphs of the cytosol and glycosome controls allow for day to day control comparisons as the x-axis represents replicate well number in chronological order. Z’ values for cytosol and glycosomes were calculated as 0.84 and 0.78 respectively.
5.3.3 **Library screening and hit validation.** Initial hits were identified as having a FRET/mCitrine ratio lower than 3 standard deviations of the high (5 mM glucose) controls. Figure 5-4A and B show the results of the cytosol screen and Figure 5-4C and D represent the screening results for all 25,000 compounds in the primary screen. The green line in the figures denotes three standard deviations below the average of the high controls. From the 25,000-compound primary screen, 1057 cytosol and 705 glycosomal hits were identified respectively correlating to a hit rate of 4.2% and 2.25% (Figure 5-5). Of the original 1057 cytosolic and 705 glycosomal hits 196 and 225 compounds were validated respectively. To validate, the repurchased compound must have produced a FRET/mCitrine response lower than 3 standard deviations below the high controls (Figure 5-5). 61 compounds were identified in both screens which resulted in 360 compounds that were then repurchased and dose-response measured.
**Figure 5-4: Screening results.** Histograms representing the normalized FRET/mCitrine ratios from the high (Red) and low (Blue) controls as well as the 25,000 compounds analyzed against the cytosol (A) and glycosome (C) sensor. To monitor plate to plate consistency scatter graphs were constructed of normalized FRET/mCitrine versus compound number for cytosol (B) and glycosome (D) screen. Hit compounds were identified as being three standard deviations below the high (Red) controls (denoted as green line). In histogram representation (A & C) hits fall to the left of the green line (3 STDEV) and in the scatter graph representation (B & D) hit compounds fall below the green line.
Figure 5-5: Flow chart of work flow and selection criteria. Flow chart representing the screening workflow (Left). Number of compounds analyzed in subsequent screen are shown with no borders. All screening steps are shown in boxes. Criteria used to select compounds for the next step in analysis are briefly outlined (Right). 25,000 compounds were originally tested and 56 candidate compounds are scheduled for medicinal chemistry and Leishmania testing.
5.3.4 Secondary screening and structure activity relationship. To verify that identified compounds inhibit glucose uptake or metabolism, kill BSF parasites, and do not show mammalian cytotoxicity, three secondary screens were run. To validate the compounds effect on glucose uptake or metabolism in trypanosomes, PCF expressing the glycosomal localized sensor were analyzed in a 11-point dose response for each of the 360 identified hit compounds. From the 360 compounds screened, 28 showed dose dependent glucose concentration effects in response to the inhibitors (Figure 5-5). Representative EC$_{50}$ curves for the identified compounds are shown in Figure 5-6 for compounds that have EC$_{50}$ values lower than 5000 nM and a Hill slope close to 1 (+/- 1). The dose response data here represent the response in glycosomes and does not show effects on cytosolic glucose. In further experiments, we plan to test the cytosolic targeted assay as it may increase the number of compounds that have a measurable EC$_{50}$. 

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**Figure 5-6: Identified hit EC50 curves.** Representative glucose uptake inhibition curves from compounds identified in the screen. Dose response consist of 11 point 2 fold serial dilutions with 5000nM drug as the high concentration. Resultant FRET/mCitrine ratio were plotted as a function of drug concentration, curves were fitted and EC50 values were calculated.
The 360 identified candidate compounds were also tested for their efficacy in killing BSF parasites. 7 compounds that have glucose EC50s also inhibit BSF growth at least 25% at 5000 nM (Table 5-3). 40 compounds inhibit BSF growth >50% at 5000 nM, with only one of the compounds showing appreciable mammalian killing (Table 5-4). The high percentage of identified compounds that inhibit BSF viability (11%) and the low occurrence of mammalian toxicity show that the FRET cytometry screen identified BSF toxic compounds at a high rate with side effects in mammalian cells. It should be noted that BSF toxicity could also be increased if the killing assay were optimized. The current assay uses HMI-9 media which contains 22 mM glucose, which is roughly four-fold higher than found in the mammalian host. By decreasing the amount of glucose in the media we expect that the killing effect on the cells will be enhanced. The 57 compounds that exhibit glucose uptake inhibition and BSF killing were then clustered into structural families. 12 distinct clusters were identified with at least three members per cluster. Clusters with many members (> 5) were then identified. Three clusters with 5, 9, and 11 members were identified (Figure 5-7).
Table 5-3: Identified compounds with confirmed glucose EC50 and BSF IC50 values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Glucose Uptake EC50 (nM)</th>
<th>Mammalian IC50 (nM)</th>
<th>T. brucei % BSF growth inhibition (5µM)</th>
<th>Bloodstream IC50 (nM)</th>
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Table 5-4: List of BSF toxic identified compounds.

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<tr>
<th>Compound</th>
<th>PCF Glucose Uptake EC50 (nM)</th>
<th>Mammalian Killing EC50 (nM)</th>
<th>T. brucei % BSF growth inhibition (5µM)</th>
<th>Estimated IC50 (nM)</th>
<th>BSF</th>
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Figure 5-7: Identified structural families. Three structural families with at least 5 members identified from the screen as having glucose uptake and trypanosome killing capabilities. These will serve as possible scaffolds for potency improvement.
5.4 Conclusion

The FRET flow cytometry screen reported here has been shown to identify glucose uptake or metabolism inhibitors that affect BSF parasite viability. Of the 25,000 compounds analyzed 360 compounds were identified as having activity and 40 of which exhibit good BSF killing (>50% at 5 µM). The high percentage of BSF killing compounds demonstrates the specificity of the assay for BSF potent compounds. Of these 40 BSF killing compounds, at least three distinct scaffolds appear that are amenable for improvement for directed medicinal chemistry.

The FRET flow cytometry assay here is applicable to the wide number of available genetically encoded biosensors. By multiplexing the available sensors into a single assay, up to a dozen distinct intracellular parameters can be analyzed at one assay. These multiplexed assays could serve as a new generation of high throughput screening that can identify novel inhibitors while excluding compounds that negatively affect other biological processes. We believe that the scaffolds identified here can help serve as a new basis for anti-trypanosome therapies.
CHAPTER 6: CONCLUDING REMARKS
The number of deaths caused per year by sleeping sickness is on the decline, in part due to the increased management of the tsetse fly population, and increased drug implementation [210]. New and improved anti-trypanosome drugs are also currently in clinical trials that effectively treat early stage HAT, and are easier to administer (oral dose), as well as addressing the toxicity concerns of previous chemotherapies [211][201]. Although the number of HAT cases is on a downtrend, increase in global temperature is expanding the inhabitable domain of *Triatomine spp.* which are responsible for transmission of *Trypanosoma cruzi* the causative agent of Chagas disease [212]. Previously these organism’s habitat were limited to South America and the southern US, but in recent years Triatomine bugs and Chagas disease have been reported as far north as Utah and Pennsylvania [213]. The constant threat of parasite resistance and the increased reach of the other kinetoplastid diseases makes studying trypanosome biology as important as ever.

Techniques for monitoring intracellular metabolic dynamics and remodeling are constantly advancing as the instrumentation required for these analyses become more sensitive, compact, and less expensive. Fluorescent sensors capable of measuring intracellular analyte concentration have been available since the 1980s, but in the last decade the number of available fluorescent protein biosensors capable of measuring analytes in organelles has exploded. Despite the successful use of protein biosensors in mammalian, yeast, and plant systems the sensors are woefully underutilized in
kinetoplasts including *T. brucei* and eukaryotic parasites generally. This dissertation reports the development of methods enabling fluorescent protein biosensors to be utilized in *T. brucei* and other kinetoplasts, using a range of available methods to measure sensor fluorescence response.

Major outcomes of this work include deploying fluorescent protein glucose sensors to monitor dynamics in live cells (*T. brucei*) using fluorescence microscopy and flow cytometry. Using these approaches, we have for the first time, measured the glucose concentration in glycosomes and cytosol of living *T. brucei*. Furthermore, we have expanded these methodologies for use in high throughput drug discovery, which has yielded novel compounds suitable for further development as anti-trypanosome treatments.

By expressing the genetically encoded glucose biosensors FLII12Pglu-700μδ6 and FLII12Pglu-600μ, we were able to measure the glucose concentrations in the cytosol, and glycosomes, which are metabolically significant organelles [169]. Previous work in *T. brucei* has been unable to identify or characterize the glucose transporter responsible for glucose uptake into the glycosomal lumen, where all glycolysis occurs [214]. Using a glycosomally expressed glucose sensor, in tandem with flow cytometry, we were able to provide the first data suggesting the existence of an increased glycosome glucose concentration compared to the surrounding cytosol. This suggests the presence of an active transport mechanism; until now it was presumed that glucose was transported into
the glycosome via passive diffusion [144]. The existence of a glycosome specific glucose transporter also indicates a novel mechanism suitable for trypanosome targeted therapies for HAT.

Making accurate FRET/mCitrine ratio measurements on fast moving motile species such as *T. brucei* is difficult, partially due to rapid cell movement during image capture. We were able to adapt a microfluidics based cell-trapping device, coupled with dual-channel fluorescence microscopy to monitor glucose dynamics during perfusion experiments. Bulk population and single cells were analyzed showing a homogenous glucose concentration among the single cell of the population.

In the world of drug discovery, hundreds of thousands of compounds are routinely tested for activity is biochemical, and cell viability assays. To expand upon the available anti-kinetoplastid hit and lead compounds, we adapted out glucose monitoring methodologies into a high throughput screen capable of identifying glucose uptake or metabolism inhibitors. In a pilot screen of 400 compounds, we identified 2 novel molecules that inhibited glucose uptake or metabolism in PCF parasites, as well as affecting BSF trypanosomes viability. Expanding upon the small molecule pilot screen, a 25,000 compound library was analyzed, yielding 40 compounds that specifically kill trypanosome parasites with no effect on mammalian viability. These 40 compounds will serve as scaffolds to develop potent, trypanosome specific drugs.
The work here serves as an initial study for the use of genetically encoded biosensors in kinetoplasts as a means of investigating unique parasite biology. We are especially excited by the future implementation of the suite of applicable biosensors for ATP, pH and ATP/ADP, that we believe will serve to expand the knowledge of metabolic adaptation that the parasites undergo during differentiation. Multiplexing of sensors is also an exciting avenue as this can allow for tens of different analytes, in different organelles to be monitored simultaneously.
APPENDICES
APPENDIX A: Measuring Protein Synthesis in Trypanosoma brucei Utilizing Heavy Water Incorporation and Protein Mass Spectrometry
A.1 Introduction

Analysis of protein dynamics in biological systems is an incredibly powerful method allowing for large portions of the proteome to be analyze in a single experiment. For bottom up proteomics workflows Q-TOF and Orbitrap mass spectrometers are most common with the latter being more sensitive, capable of identifying more proteins in a single chromatographic run. Gene regulation can occur at the level of transcription, translation and via protein degradation and recycling. Current methods to monitor protein synthesis and degradation rely on incorporation heavy amino acids into newly made proteins. The media and reagents used in these methods are expensive and must be custom made for each model organism. Monitoring protein turnover with stable incorporation of deuterium serves as a cheaper alternative to the use of heavy amino acids. In this method deuterated water D2O is added to the culture media to a final concentration (5%) and subsequently covalently incorporated into amino acids through transaminase and other amino acid synthesis pathways. Here we briefly discuss the application of deuterium incorporation for monitoring proteome dynamics in procyclic and bloodstream form *T. brucei*. Of particular interest is the modulation of protein expression and half-life as the parasites adjust to changes external glucose levels and differentiate into different life stages.
A.2 Rationale
Figure A-1: Deuterium incorporation scheme. (A) Transaminase reactions showing the incorporation of the Deuterium from D2O being incorporated into alanine, glycine, and leucine amino acids. (B) The “Heavy” deuterium incorporated enzymes are then
Figure A-2: Turnover quantification via LC-MS. Incorporation of heavy amino acids shifts the mass distribution of the peptides and is proportional to the amount of newly synthesized protein. By monitoring these peak shifts over time you can calculate the half-life of the protein and rate of synthesis and degradation. (Figure from: De Riva, A.; Deery, M. J.; McDonald, S.; Lund, T.; Busch, R. Anal. Biochem. 2010, 403 (1–2), 1–12.)
Figure A-3: Proteomics workflow. Cells are incubated in standard culture media with the addition of 5% sterile D2O for different times ranging from 0.1 doubling time to 2-5 doubling times. Cells are then lysed in 6M guanidine and prepared for LC-MS analysis. At this stage the peptides can be fractionated secondary chromatography (for LC-QTOF) or run directly in the LC-MS (for LC-Orbitrap). Peptides are then identified and half lifes are calculated using a custom written python script called DeuteRater.
A.3 Peptide Identification Optimization Via Fractionation

Optimization of LC-QTOF sample preparation. The amount of identified proteins from a simple cell lysis of PCF and BSF trypanosomes was suboptimal <200 proteins of the >7000 protein proteome. A large background was observed from tubulin and VSG which are proteins that compose ~50-70% of the total cell protein. To help diminish the signal from tubulin and VSG peptides were fractionated in two different methods. Fractionating peptides essentially expands the separation of your lower abundance proteins from the high background. Fractionation using an orthogonal chromatography method is common but this dramatically increases the time require for analysis. Alternatively, proteins can be preferentially released from the cell via titration with digitonin a detergent known to selectively permeabilize different cellular membranes. Digitonin fractionation is fast and offers isolation of proteins bound to different organelles.

HPLC fractionation method. Peptides were acquired from trypsin digests on BSF trypanosomes. Peptides were then run in high pH reversed phase chromatography, 24 fractions were eluted. Fractions were then recombined for a final number of 8. The 8 fractions were then dried using a speedvac and resuspended in HPLC buffer (5% CAN 0.1%
formic acid). The fractions were then analyzed via LC-QTOF and the resultant peptides were identified.

**Digitonin fractionation.** BSF parasites were permeabilized with varying concentrations of digitonin (0.05mg/mg protein – 1mg/mg protein) followed by 8M urea. Between each condition the cells were pelleted and the supernatant was collected. The supernatants from all conditions were then prepared for mass spec analysis via on filter cleanup method followed by trypsin digest and resuspension in HPLC buffer. Peptides were analyzed and identified using PEAks mass spec software.
Figure A-4: Unique peptides identified using HPLC fractionation. Two different cell lysis conditions were tested, 8M urea and RIPA lysis buffer. Using 8M urea 1200 proteins were identified. With RIPA lysis 700 proteins were identified. 581 proteins were identified using both methods. UREA lysis is optimal.
Figure A-5: Digitonin Fractionation. Profile of identified proteins under the different lysis conditions. With digitonin fractionation >500 unique proteins are analyzed. This is an increase over non fractionated samples but less than that of HPLC fractionation.
A.4 Protein Half Life analysis.

Figure A-6: Representative Protein Turnover Rates. PCF parasites were exchanged from low glucose media into normal PCF media with the addition of 5% D2O. Time points were collected every hour for 4 hours followed by 8 and 12 and 24 hours. To collect a time point 5E7 cells (~50 ug total protein) were collected and rinsed three times with PBS via centrifugation. The cell pellets were then immediately frozen at -80C until further analysis. Cells were then lysed, the proteins were digested and analyzed on LC-MS. The
isotope patterns were extracted using DeuteRater software and the fraction new protein was plotted versus incorporation time for each protein. Half lives were then calculated from the fits.
Figure A-7: Relative abundances of identified proteins in PCF parasites. Distribution of the half lifes of 400 identified proteins. The majority of proteins have a half-life less than 20 hours which is consistent with the estimated doubling time of PCF parasites (~12-15 hours). There are a number of proteins with half-lifes multiple times the expected half-life of a parasite. Longer half-life proteins are most likely being transferred to daughter cells during cell division.

A.5  Conclusion
With the recent advances in mass spectrometer resolution and robustness proteomic analysis has become an incredibly powerful technique to analyze the resident proteome in organisms. The use of heavy water for labeling newly synthesized proteins has been used extensively in mammalian cell culture and even in human and mouse models to study protein turnover rates. Despite the power of Heavy water labeling there are little to no mentions of its application in kinetoplastid parasites. Kinetoplasts undergo drastic metabolic and morphological changes as they adapt to different environments during different life stages. These adaptations are accomplished by complex regulation of the expressed protein pool. We believe that using heavy water incorporation to analyze protein turnover during parasite differentiation will enlighten the biochemical pathways therein.
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