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**A FRET Flow Cytometry-Based High Throughput Screening Assay to
Identify Disrupters of Glucose Levels in *Trypanosoma brucei***

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Trypanosoma brucei, which causes human African trypanosomiasis (HAT), derives 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 have been described to identify and characterize potential inhibitors of glucose uptake and regulation. Here, we report development of a screening assay that identifies small molecule disrupters of glucose levels in 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 glucose level disrupters 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.

Keywords. *Trypanosoma brucei*, High Throughput Screen, Flow Cytometry, FRET biosensor, Glucose Metabolism, Glycosome

More than 400 million people live in areas where diseases caused by the kinetoplastid parasites *T. brucei*, *T. cruzi* and *Leishmania spp* are endemic.¹ Human African trypanosomiasis (HAT), which is caused by *T. brucei*, threatens the health of millions of people and leaves much of the impacted areas not suitable for rearing livestock.² Despite promising new drugs in development,³ common treatments for kinetoplastid diseases include compounds developed in the 1950s, which have noted toxicity to the host.⁴ Since these diseases affect some of the poorest regions on earth, the economic incentive for new drug development campaigns is limited.⁵

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.⁶ 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.⁷ 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, each of which has unique biochemical characteristics that differentiate them from mammalian glucose transporter homologs.⁸ Differences between parasite and human transporter include affinity for glucose, types of sugars transported, and sensitivity to inhibitors.^{9,8} Once inside the cell glucose must then be delivered to glycosomes to enter the glycolytic pathway, a process that may occur via facilitated diffusion. Importantly, each of these steps (glucose transport into the cytosol, translocation into the glycosome, and

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3 glucose consumption via metabolic enzymes) represent possible points for intervention via
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5 inhibitor compounds.¹⁰
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8 Compounds that inhibit kinetoplastid glucose homeostasis, thus altering metabolic
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10 function and viability, are attractive as a potential anti-trypanosome therapies. Given the
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12 biochemical differences between glucose transport in mammalian and kinetoplastid cells and
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14 the unusual subcellular organization of parasite glycolysis in the glycosome,⁸ a compound that
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16 specifically inhibits kinetoplastid glucose metabolism could be envisioned. For example, a small
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18 molecule inhibitor that compromised glucose uptake into glycosomes would be catastrophic to
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20 BSF parasite viability without impacting the mammalian host. While commonly used glucose
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22 measurement techniques are destructive, low throughput, and do not allow measurements of
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24 intraglycosomal glucose, we have recently expressed glucose-responsive biosensors in the
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26 cytosol and glycosomes of transgenic *T. brucei* and used them to quantify glucose
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28 concentration(s) in the different compartments of live parasites. Here we expand these
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30 measurements to screen for molecules that reduce intracellular glucose concentrations,
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32 presumably by altering glucose flux into *T. brucei* glycosomes or by impacting key steps in
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34 glycolysis.
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42 In yeast and mammalian cells, FRET biosensors have been used previously to track
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44 changes in cytosolic and organellar glucose that result from environmental perturbations.¹¹ We
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46 have expressed FRET biosensors in *T. brucei*, and have routed sensor proteins to the parasite
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48 glycosome by appending a peroxisomal targeting sequence to the polypeptide
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50 (unpublished).^{12,13} The presence or absence of the targeting sequence allows us to apply the
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52 biosensor and its resulting glucose measurements to either the cytosol or the glycosome. Using
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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.

T. brucei relies solely on glucose metabolism for its survival in the mammalian host bloodstream.¹⁴ Disruption of glucose metabolism and uptake have long been considered promising pathways for targeting with anti-parasitic compounds.¹⁵ Traditional analytical methods for measuring intracellular and glycosomal 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.

To monitor glucose concentrations in living cells, the fluorescent glucose biosensor FIII¹²PGLu-600μ was expressed using the pXS2 expression vector in PCF parasites. FIII¹²PGLu-600μ consists of an enhanced cyan fluorescent protein (ECFP) and mCitrine (an enhanced pH and chloride insensitive mutant of yellow fluorescent protein) FRET pair that flank a glucose binding domain derived from *E. coli*.¹⁶ Upon binding to glucose, a conformation change in the

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3 glucose recognition domain is initiated, which alters the spatial relationship of the
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5 ECFP/mCitrine FRET pair. Changes in ECFP/mCitrine orientation alter non-radiative energy
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7 transfer, yielding a measurable response in fluorescence. Importantly, the glucose sensor is
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9 specific for glucose and does not bind other hexose sugars including 2-deoxyglucose or glucose-
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11 6-phosphate (unpublished).¹⁷ The pXS2-FIII¹²PGlu-600μ construct yielded diffuse signal
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13 throughout the cytosol of cells (SI Figure 1). To deliver the biosensor to the glycosome, the
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15 transgene was expressed as a fusion with a C-terminal type-1 PTS signal sequence (AKL).
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17 Expression of this construct produced localization in vesicular organelle-like structures
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19 consistent with glycosomes (SI Figure 1). Monitoring changes in sensor fluorescence in the two
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21 compartments allows for measurement of glucose concentration in the cytosol and glycosomes
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23 of living parasites.
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33 Cells expressing biosensors are traditionally analyzed via fluorescence microscopy
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35 including high-content screening instruments. However, since trypanosomes are small, grow in
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37 suspension, and are highly motile, most imaging-based approaches lack the ability and
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39 throughput needed for rapid screening of candidate inhibitor compounds.¹⁸ To increase the
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41 throughput of our FRET-based screening assay, we utilized a two-laser flow cytometer to
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43 monitor changes in biosensor response (SI Figure 2). Using this approach, we could distinguish
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45 cells that were 10-100 times brighter in the FRET emission channel (ECFP excitation; mCitrine
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47 emission) and the directly excited acceptor (mCitrine) channel compared to untransfected
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49 controls, making them suitable for measurements (SI Figure 2, cells in the upper right
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51 quadrant). Incorporating a cytometer with an auto sampler into the assay enhanced the speed
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of sample analysis to a throughput rate of ~100-500 samples per hour depending on the cytometer used. Additionally, in flow cytometry, cells are analyzed individually and are only exposed to the laser for a brief moment (<1ms), which limits the impact of phototoxicity and sensor photobleaching when compared to microscopy. Although flow cytometers represent a powerful tool for high throughput screening, there are limited examples where they have been utilized for screening.¹⁹ 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.²⁰

Parasites expressing the glucose FRET sensor respond to external glucose and 2-DOG concentrations (SI Fig 3). To explore the temporal response of the biosensors as a result of changes in external environmental glucose concentrations, PCF cells expressing cytosolic (Fig. 1A) or glycosomal (Fig. 1B) sensors were incubated in buffer and then exchanged into different buffers of different glucose concentrations. When incubated in high glucose (5mM) the FRET/mCitrine ratio remained high, representing a high internal glucose concentration. Once cells were exchanged into low glucose (0.1mM), the 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 returned to the steady state levels observed in the beginning of the time course.

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 1). 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. These results, which reveal a rapid change in subcellular glucose concentrations, suggest that alternative glucose quantitation methods such as glucose oxidase assays may be too slow or low throughput to be suitable for measuring trypanosome intracellular glucose levels. Furthermore, characterization of glucose concentrations in subcellular compartments using standard approaches for glucose measurements would be challenging because it would require fractionation of compartments with compromising their integrity.

Following validation of sensor expression via microscopy and flow cytometry, we verified that the FIII¹²PGlu-600 μ FRET ratio responds to environmental conditions. The normalized FRET signal in parasites expressing FIII¹²PGlu-600 μ in the cytosol or glycosomes shows that intracellular glucose levels rose as external glucose was increased (SI Figure 3A). As expected, when parasites were incubated in sufficient quantities of the competitive uptake inhibitor and metabolic poison 2-DOG to compete with extracellular glucose, intracellular glucose levels decreased in a dose-dependent manner (SI Figure 3B).²¹

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 fell to dramatically lower levels; steady state was reached within 5 min (Figure 2a & Figure 2b). Notably (and unlike the response to glucose starvation), the glucose depletion of glycosomes and the cytosol in the presence of 2-DOG occurred at similar rates (Figure 2). 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.

Both the glucose depletion data and 2-DOG treatment data (Figures 1 and 2) 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.

To pursue novel inhibitors of intracellular glucose homeostasis, we developed a screening assay that used flow cytometry to monitor changes in biosensor 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 impacted intracellular glucose concentrations. Thus, we were able to avoid the liabilities of poor cell permeability, off-target

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3 effects, and general cytotoxicity. To maximize the sensitivity of the FIII¹²PGLu-600 μ glucose FRET
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5 assay, conditions were optimized to yield the highest and lowest signal of biosensors. Glucose
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7 at 5 mM was the optimal high signal control (Fig. 3A and 3B, in red) while reduced glucose (0.1
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9 mM, Fig. 3A and 3B, in blue) was used as the low signal control. Using a bivariate
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11 representation where mCitrine emission was plotted against normalized FRET emission, the x-
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13 axis was correlated to the FRET/mCitrine ratio and the distribution along the trend line was
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15 representative of the standard error in the assay (Fig. 3C and 3D). Test compounds that were
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17 between the trend lines of the high and low controls were considered for further analysis. Any
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19 compounds that fell outside of these constraints likely impacted absorbance or fluorescence
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21 resulting in artificially inflated FRET and/or mCitrine fluorescence. Optimization of screening
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23 controls yielded PCF cytosol and glycosome screens with Z' values of 0.75 and 0.70,
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25 respectively. Table 1 outlines the screening parameters, following guidelines suggested for
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27 universal HTS method communication.²²
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Table 1. Parameters for small molecule screen

Category	Parameter	Examples (see text for more detail)
Assay	Nature of assay	Cell based fluorescence flow cytometry assay
	Assay Strategy	Detection of intracellular and/or glycosomal glucose depletion using an endogenously expressed FRET biosensor that specifically binds glucose
	Assay protocol	Key steps are outlined in Table 2
Library Screened	Nature of Library	Drug like molecules that are active against a neglected tropical disease of interest
	Size of library	400 compounds arrayed in 96 well plates at 10mM in DMSO
	Source	Medicines for Malaria Venture (MMV)
	Concentration Tested	10µM concentration, 1% DMSO, 1:100 dilution
HTS process	Format	Sterile 384 well plate (Greiner Bio)
	Plate Controls	Positive control: 0.1mM glucose (A1-P1); negative control: 5mM glucose (A2-P2)
	Plate number and duration	5 384 well plates over 6 days
	Reagent dispensing system	Reagents and cell dispensed using a electronic 12 channel pipette (LabNet)
	Normalization	Normalized FRET response = (sample result – average low controls) / (average high controls – average low controls)
Post HTS analysis	Screen Robustness	Z-prime: Cytosol = 0.75, Glycosome = 0.70
	Selection of activities	Active compounds were selected using a threshold based off the statistics of the high controls
	Selection threshold	3 standard deviation below high controls; Cytosol <0.72 FRET ratio, Glycosome <0.70 FRET ratio
	Retesting of initial activities	Compounds with replicate positive activities were tested in a 12-point dose response
	Compound purification	Validated compounds were required from MMV for further testing

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 maintenance in the cytosol and glycosomes of PCF trypanosomes. The pathogen box library was provided by Medicines for Malaria Venture (MMV) and contains 70 well characterized anti-kinetoplastid drugs as well as reference anti-trypanosomal compounds including suramin and pentamidine. Each compound was tested in duplicate at 10 µM, with solvent (1% DMSO) used as a control (protocol outlined in Table 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 (Fig. 4) in both replicates without altering PF parasite viability. Three compounds reduced cytosolic glucose; five compounds reduced glycosomal glucose (Fig. 4B and 4D). One compound reduced glucose concentration in both the glycosome and cytosol, resulting in a total of seven compounds identified. Since the sensors are specific for glucose and do not bind phosphorylated glucose or other hexose sugars, identified compounds lower the concentration of glucose before it phosphorylated by hexokinase, most likely by inhibiting glucose transport and glycosome translocation mechanisms.

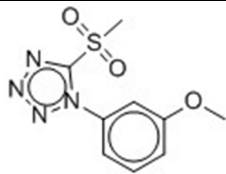
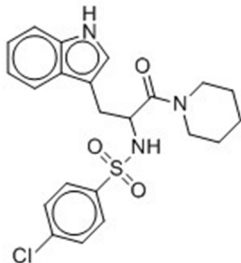
Table 2. FRET flow cytometry HTS protocol

Step	Parameter	Value	Description
1	Controls	1 μ l	DMSO
2	Library compounds	1 μ l	10 μ M; 10 μ M to 4.9nM for serial dilution
3	Addition of cells	100 μ l	5,000-10,000 PCF 2913 cells expressing glucose biosensor, PBS with 5mM glucose
4	Addition of low controls	100 μ l	5,000-10,000 PCF 2913 cells expressing glucose biosensor, PBS with 0.1mM glucose
5	Incubation time	1 hour	Ambient conditions
6	Assay Readout	405ex/530em, 488ex/530em	BD Attune flow cytometer
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 μ l 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 0.1mM 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 5). Additional amounts of these compounds were acquired from Pathogen Box for use in glucose dose response and bloodstream form killing downstream assays (Fig. 5 & Table. 3). One compound (MMV085210) was found to inhibit glucose homeostasis in both the cytosol and glycosomes with an $EC_{50} \approx 5 \mu M$. However, this compound did not appreciably impact BSF parasite viability at $10 \mu M$; due to poor solubility, higher concentrations were not tested. The second compound, MMV 272144, only acted on glucose homeostasis in the glycosome, with an $EC_{50} \approx 700 \text{ nM}$. Notably, this agent killed BSF parasites, causing a $41 \pm 7 \%$ reduction in parasite number at $10 \mu M$ (Table 3). This BSF toxicity assay likely underestimates the impact of identified compounds under physiological conditions, since it is performed in HMI-9 media, which has a glucose concentration of $\sim 22 \text{ mM}$, roughly four times that found in mammalian blood. Since effective glucose concentrations inside the glycosome and cytosol increase with increasing extracellular glucose concentrations (unpublished), 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, which 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 homeostasis, the compound appears to target machinery specific to glycosomal glucose uptake or regulation. It is important to note that none of the kinetoplastid specific drugs (including suramin and pentamidine) were hits in

our assay, indicating this assay can discriminate trypanocidal compounds that do not affect glucose homeostasis from those that impact glucose uptake.

Table 3. Pathogen box compounds effect on glucose homeostasis and BSF cell viability

MMV ID	Structure	PCF Glucose EC50	BSF Killing
272144		0.7 μ M	41 $\pm$ 7% @10 μ M
085210		$\sim$ 5 μ M	N/A

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 glycolysis enzyme inhibitors have been identified that were effective *in vitro*, but less so against live cells, presumably because of limited delivery of inhibitors to the glycosomes in live parasites.^{10,23} 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.

Methods.

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).

Parasite Culture, Sensor Transfection and Microscopy. BSF parasites were continuously cultured in HMI-9 media supplemented with 10% FBS.²⁴ PCF parasites were grown and maintained in SDM-79 media.²⁵ The glucose sensor FIII¹²PGlu-600μ was cloned into pXS2 for expression in PCF as described.²⁶ FIII¹²PGlu-600μ expressing parasites were imaged using a CFP/mCitrine FRET filter set with a 430/30 nm excitation filter and 480/30nm and 530/30nm bandpass filters for CFP and mCitrine emission.

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.; mCitrine emission) was obtained from the 405nm excitation channel using a 530/30nm bandpass filter. Direct excitation of mCitrine was measured in the 488nm excitation channel using a 530/30nm bandpass filter. FRET ratio (405nm ex. mCitrine emission/ 488nm ex. mCitrine emission) was calculated on a cell by cell basis using the cytometer software to calculate FRET ratio as a custom output parameter. FRET/mCitrine ratio is a score of intracellular glucose concentration, an increase in FRET/mCitrine is indicative of higher intracellular glucose. Dead cells were excluded from analysis using forward and side scatter characteristics; only live 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), FRET ratio data was further analyzed using Microsoft excel.

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 FIII¹²PGlu-600μ or PT-FIII¹²PGlu-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 μ L 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.

2-DOG Response. PCF parasites expressing FIII¹²PGLu-600 μ or PT-FIII¹²PGLu-600 μ were rinsed three times in PBS supplemented with 5 mM glucose and resuspended in PBS + 5 mM glucose. 200 μ L 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.

Library Components and Plate Preparation. The Pathogen Box library was supplied by (MMV; Switzerland) and consisted of 5 plates of 96 wells, each containing 10 μ L of 10 mM compound dissolved in dimethyl sulfoxide (DMSO). Drugs used for screening were diluted to 1 mM in DMSO before transferring 1 μ L 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 μ L of DMSO

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3 as a vehicle control. DMSO concentration was kept at or below 1% to reduce effects on cell
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5 viability and assay readout. Compounds identified as candidate glucose homeostasis disruptors
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7 were reacquired from MMV and dissolved to a final concentration of 10 mM in DMSO before
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9 being used for glucose inhibition dose response and trypanosome killing assays.
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15 **Glucose Depletion Assay.** Glucose uptake assays were based off a flow cytometry method that
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17 can be used to monitor changes in intracellular glucose in live trypanosome parasites
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19 (unpublished). Briefly, cells were washed three times with PBS, cells to be incubated with test
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21 compounds or high controls were incubated in PBS supplemented with 5 mM glucose; low
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23 controls were incubated in PBS alone. 100 μ L of the cell suspension ($\approx$ 10,000 cells per well)
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25 were pipetted into the wells containing the test compounds in 1 μ L DMSO to a final
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27 concentration of 10 μ M in 1% DMSO, positive controls contained 1% DMSO and 5 mM glucose,
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29 low controls contained 1% DMSO with 0.1mM glucose. The loaded plate was incubated at
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31 ambient conditions for one hour before analysis via flow cytometry as described above.
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33 Confirmed preliminary hits were retested using reacquired compounds; IC₅₀ values were
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35 determined from 2-fold serial dilution with 10 μ M compound as the highest concentration. All
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37 dose response curves and IC₅₀ curves and values were fit to a single site ligand binding curve
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39 using Sigmaplot 11.0 (Systat Software Inc.; San Jose, CA).
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50 **HIT Identification.** All primary screen data were analyzed using FlowJo flow cytometry analysis
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52 software and exported as a Microsoft Excel file for further analysis. Histograms were
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54 constructed with high and low controls used to determine Z' values. FRET/mCitrine ratios for
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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. Any compound that fell outside of the area in between the high and low controls were considered a fluorescent or highly absorbing compound and excluded from further analysis.

***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).

Abbreviations

PCF, procyclic form *Trypanosoma brucei*; FRET, Förster Resonance Energy Transfer; 2-DOG, 2-deoxyglucose; THT, trypanosome hexose transporter; ECFP, enhanced cyan fluorescent protein; PTS, peroxisomal targeting sequence

ASSOCIATED CONTENT

Supporting Information. Fluorescent and DIC images of PCF trypanosomes expressing FLII¹²PGLu-600 μ (Figure S1), Flow cytometry gating scheme (Figure S2), and *in vivo* glucose sensor response characterization (Figure S3)

Acknowledgment

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Figures

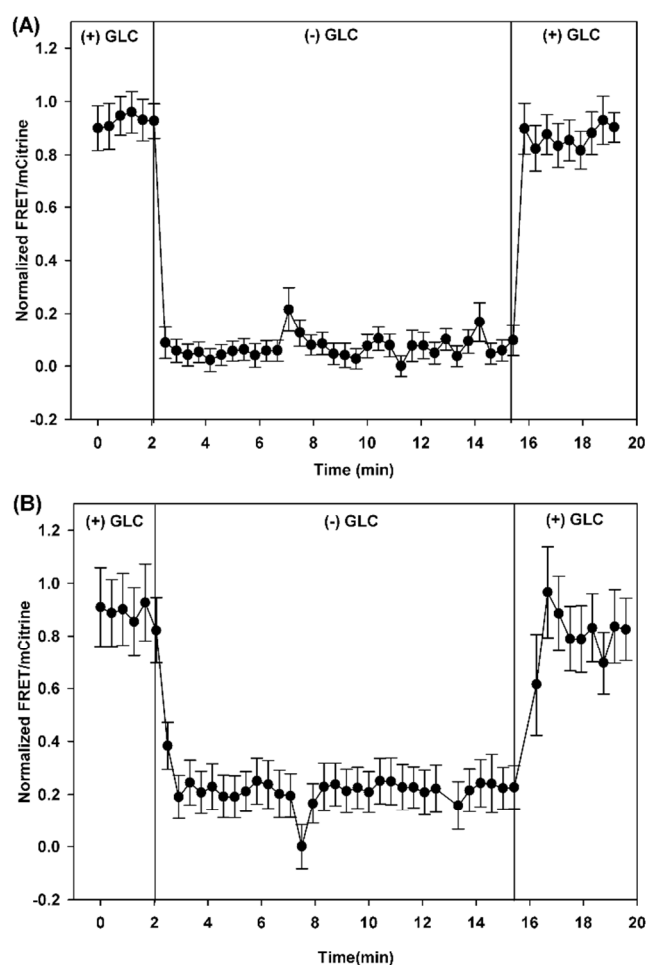


Figure 1. Normalized FRET/mCitrine ratios for time traces of PCF cells expressing the glucose sensor in cytosol (A) and glycosomes (B). Cells were allowed to incubate in high glucose (5 mM; + GLC) and exchanged into low glucose at the vertical line (0.25 mM; - GLC). Cells were then allowed to achieve 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.

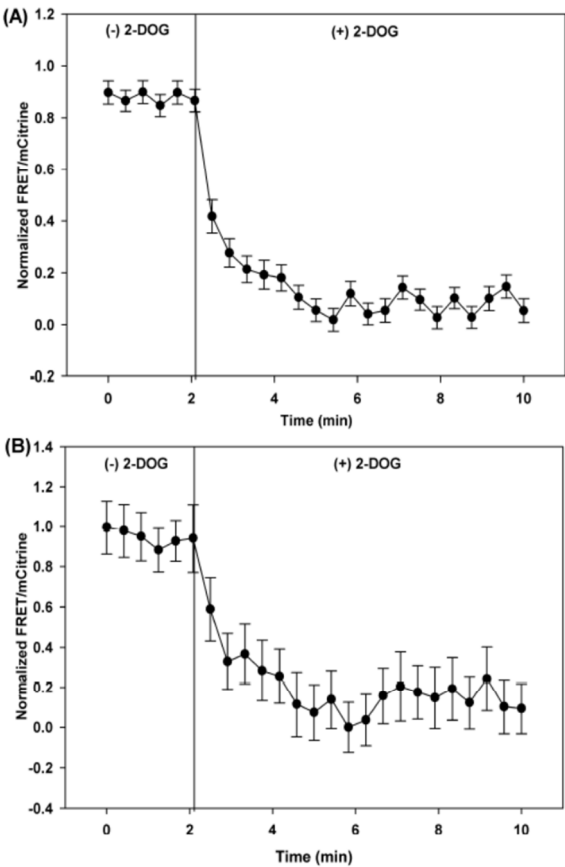


Figure 2. Normalized FRET/mCitrine time chase for PCF expressing glucose biosensor in cytosol (A) and glycosomes (B) in response to addition of 2-DOG. Cells were allowed to incubate in PBS with 5 mM glucose (- 2-DOG) and monitored using flow cytometry. 2-DOG was then added to a final concentration of 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.

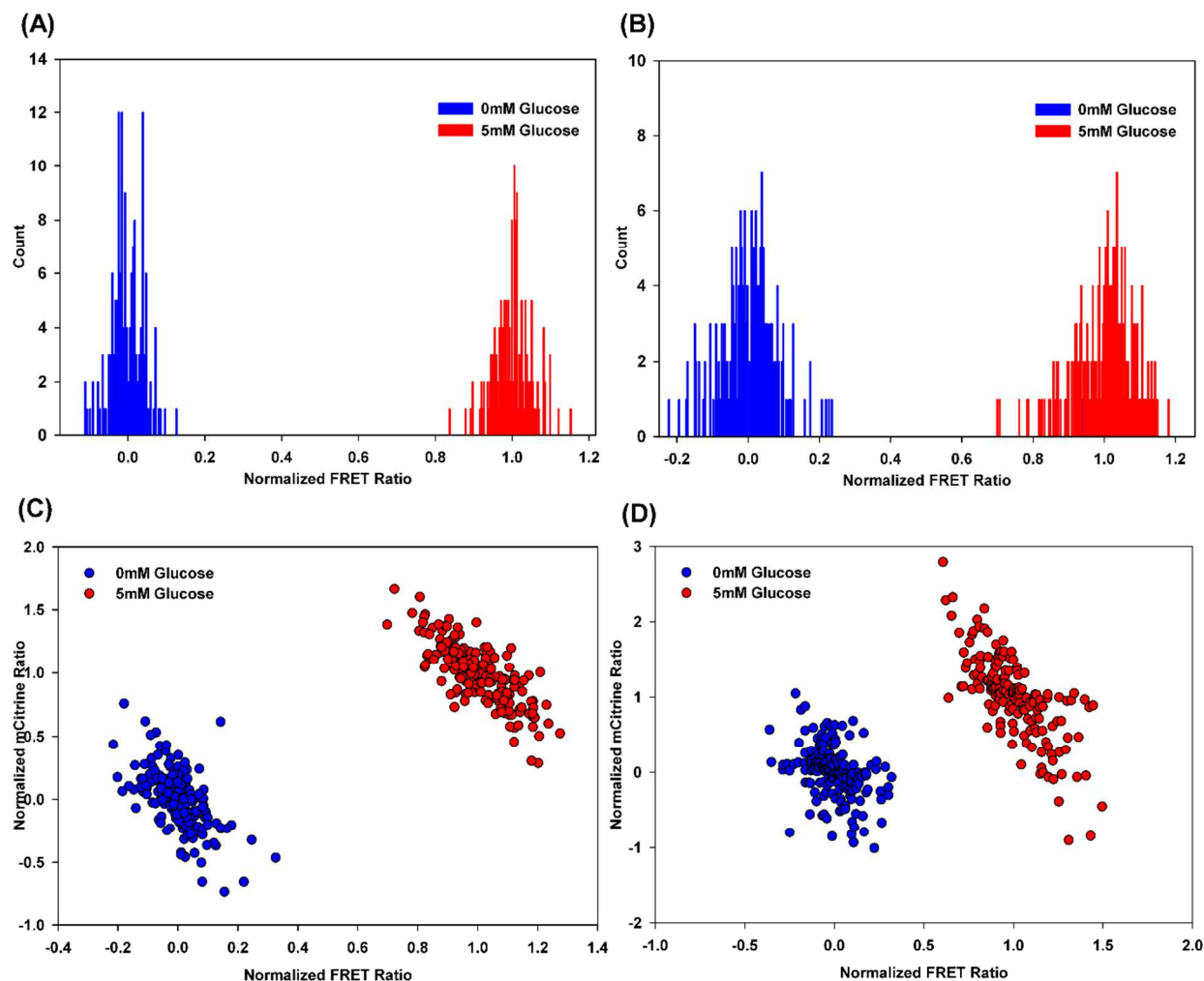


Figure 3. Histograms and plots representing the FRET/mCitrine ratio of the high and low controls in PCF cells expressing the glucose biosensor in cytosol (A and C) or glycosomes (B and D). These represent the same data presented in two different formats. Bivariate plots representing the normalized FRET emission (x-axis) and mCitrine emission (y-axis) of PCF cells expressing Flii¹²PGLu-600 μ 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 0.1. Z' values for cytosol and glycosome assay calculated as 0.7 and 0.75 respectively.

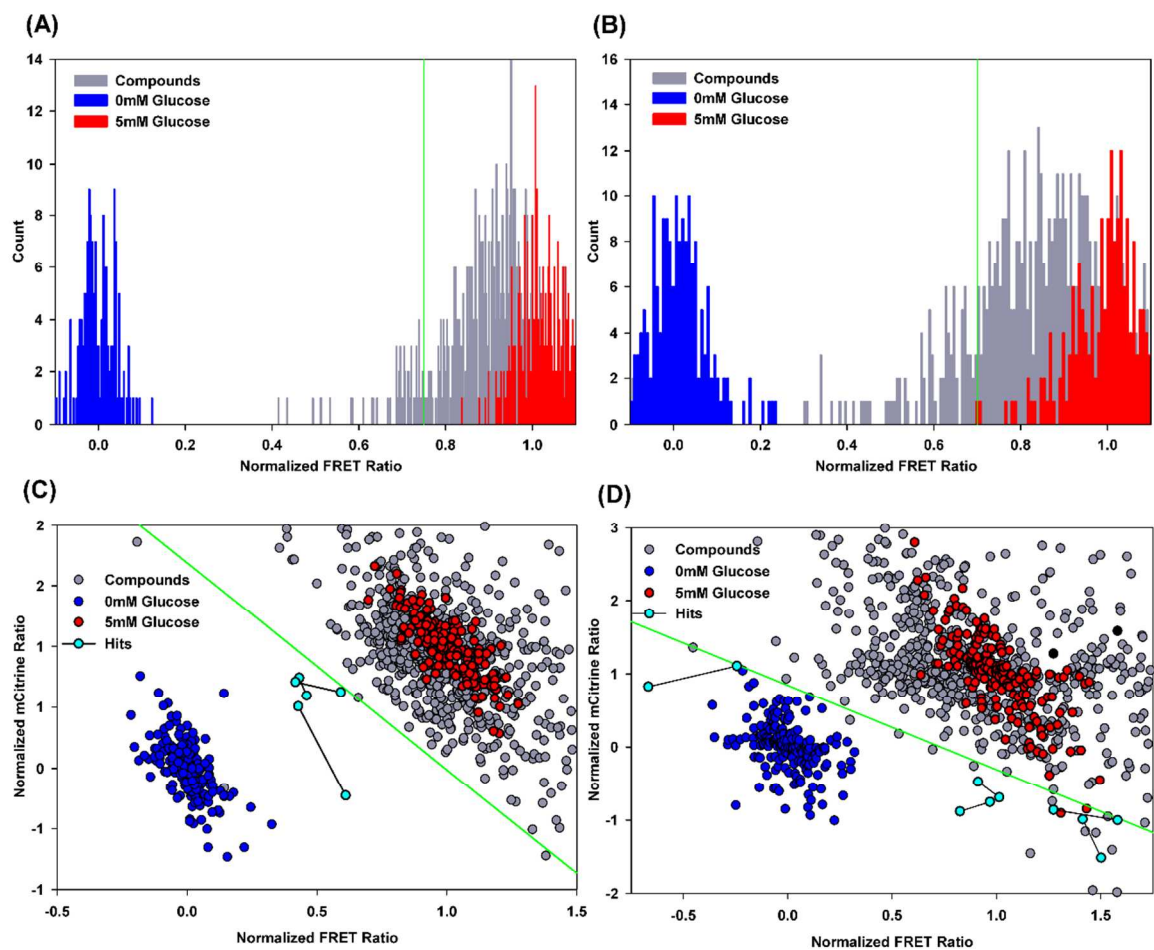


Figure 4. Histograms representing the FRET/mCitrine ratios from the glucose depletion screen in PCF cytosol (A) and glycosome (B). Bars represent the FRET/mCitrine ratio of 400 candidate inhibitor compounds (grey bars), high controls 5mM glucose (red bars) and low controls (blue bars). Hit compounds 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 0.1 mM glucose (blue, low controls). Fluorescent and highly absorbing compounds were characterized as falling outside the region between the trend lines of the high and low controls.

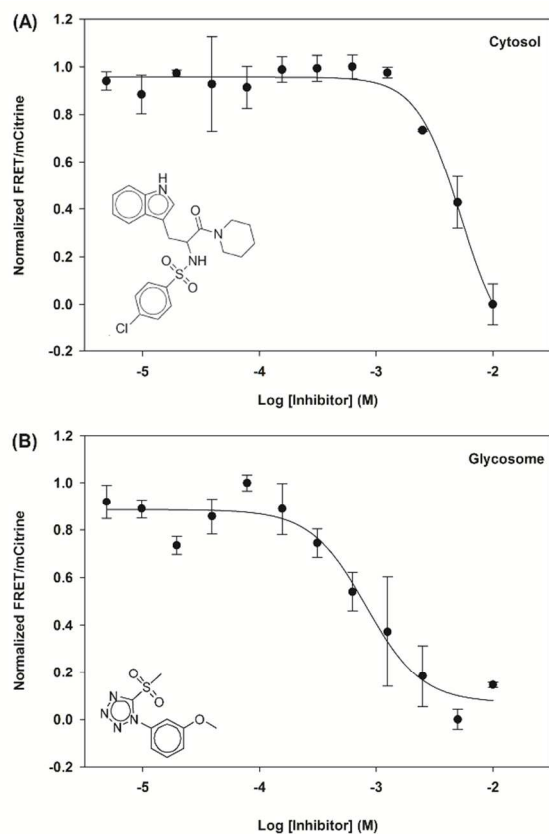


Figure 5. Dose response curves for two hits 085210 (A) and 272144 (B) are shown where FRET/mCitrine ratio is plotted as a function of inhibitor concentration. EC₅₀ values for 085210 and 272144 were calculated as 0.7 μ M and 5 μ M respectively. Trend lines were fitted using a single site isotherm and error bars represent the standard deviation (n=3).

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