HHS Public Access

Author manuscript

J Vis Exp. Author manuscript; available in PMC 2024 February 21.

Measuring Dynamic Glycosomal pH Changes in Living *Trypanosoma brucei*

Daniel Call[*,1], **Sabrina S. Pizarro**[*,2,3], **Erica Tovey**[1], **Emily Knight**[2,3], **Carrie Baumgardner**[2,4], **Kenneth A. Christensen**[1], **James C. Morris**[2]

[1]Department Chemistry and Biochemistry, Brigham Young University, Provo, UT, USA

[2]Eukaryotic Pathogens Innovation Center, Clemson University, Clemson, SC, USA

[3]Department of Genetics and Biochemistry, Clemson University, Clemson, SC, USA

[4]Department of Physics and Astronomy, Clemson University, Clemson, SC, USA

Abstract

Glucose metabolism is critical for the African trypanosome, *Trypanosoma brucei*, as an essential metabolic process and regulator of parasite development. Little is known about the cellular responses generated when environmental glucose levels change. In both bloodstream form and procyclic form (insect stage) parasites, glycosomes house most of the glycolysis. These organelles are rapidly acidified in response to glucose deprivation, which likely results in the allosteric regulation of glycolytic enzymes such as hexokinase. In previous work, localizing the chemical probe used to make measurements was challenging, limiting its utility in other applications.

This paper describes the development and use of parasites that express glycosomally localized pHluorin2, a heritable protein pH biosensor. pHluorin2 is a ratiometric pHluorin variant that displays a pH (acid)-dependent decrease in excitation at 395 nm while simultaneously yielding an increase in excitation at 475 nm. Transgenic parasites were generated by cloning the pHluorin2 open reading frame into the trypanosome expression vector pLEW100v5, enabling inducible protein expression in either lifecycle stage. Immunofluorescence was used to confirm glycosomal localization of the pHluorin2 biosensor, comparing the localization of the biosensor to the glycosomal resident protein aldolase. The sensor responsiveness was calibrated at differing pH levels by incubating cells in a series of buffers that ranged in pH from 4 to 8, an approach we have previously used to calibrate a fluorescein-based pH sensor. We then measured pHluorin2 fluorescence at 405 nm and 488 nm using flow cytometry to determine glycosomal pH. We validated the performance of the live transgenic pHluorin2-expressing parasites, monitoring pH over time in response to glucose deprivation, a known trigger of glycosomal acidification in PF parasites. This tool has a range of potential applications, including potentially being used in high-throughput drug screening. Beyond glycosomal pH, the sensor could be adapted to other organelles or used in other trypanosomatids to understand pH in the live cell setting.

Corresponding author: James C. Morris jmorri2@clemson.edu.
*These authors contributed equally to this work.

A complete version of this article that includes the video component is available at http://dx.doi.org/10.3791/66279.

DISCLOSURES:
The authors declare no conflicts of interest.

SUMMARY:

We describe a method to study how pH responds to environmental cues in the glycosomes of bloodstream form African trypanosomes. This approach involves a pH-sensitive heritable protein sensor in combination with flow cytometry to measure pH dynamics, both as a time-course assay and in a high-throughput screen format.

INTRODUCTION:

Parasitic kinetoplastids, like most living organisms, rely on glucose as a fundamental component of central carbon metabolism. This group includes medically important organisms, such as the African trypanosome, *Trypanosoma brucei;* the American trypanosome, *T. cruzi;* and parasites of the genus *Leishmania.* Glucose metabolism is critical to parasite growth in the pathogenic lifecycle stages. For example, when deprived glucose, the bloodstream form (BSF) of the African trypanosome dies rapidly. Notably, glycolysis serves as the sole source of ATP during this stage of infection [1]. Leishmania parasites are likewise dependent on glucose in the human host, with the amastigote lifecycle stage that resides in host macrophages reliant on this carbon source for growth [2].

While these parasites have distinct lifestyles involving different insect vectors, they share many commonalities in how they respond to and consume glucose. For example, these parasites localize most glycolytic pathways into modified peroxisomes called glycosomes. This kinetoplastid-specific organelle is related to mammalian peroxisomes based on conserved biosynthetic mechanisms and morphology [3–6].

The compartmentalization of most of the glycolytic pathway enzymes into the glycosome offers parasite-specific means of regulation of the pathway. Using a chemical pH probe, we established that nutrient deprivation triggers a rapid acidification of procyclic form (PF) parasite glycosomes that results in altered glycolytic enzyme activity through exposure of an allosteric regulator binding site on the key glycolytic enzyme hexoinase [7,8]. In our previous work, the chemical probe required constant delivery for use, limiting its utility in other applications. Additionally, challenges maintaining the probe distribution in the glycosome in the BSF limited the utility of the approach for investigating glycosomal pH in that life stage.

In this study, we have used the fluorescent protein biosensor pHluorin2 to monitor glycosomal pH change in BSF *T. brucei* in response to environmental cues including glucose starvation[9]. Results from this work suggest that BSF *T. brucei* acidifies glycosomes rapidly in response to starvation in a reversible fashion, similar to responses we have observed in PF parasites. We expect this biosensor will improve our understanding of glycolytic regulation in *T. brucei* and related parasites.

PROTOCOL:

1. Trypanosome culture and transfection

1.1. Culture *T. brucei brucei* 90–13 BSF trypanosomes, a monomorphic parasite line, in HMI-9 medium supplemented with 10% heat-inactivated FBS and 10%

Serum IV at 37 °C in 5% CO_2 [10]. Using these cells requires consideration of safety, as they are considered Risk Group 2 organisms that should be handled in biosafety level 2 facilities. NOTE: To keep the culture healthy, maintain the cell density between 2×10^4 and 5×10^6 cells/mL.

1.2. Cloning pHluorin2-PTS1 into pLEW100v5.

1.2.1 Synthesize the pHluorin2 open reading frame commercially to yield the gene with a 3' extension encoding a two glycine linker followed by the tripeptide AKL, a PTS1 localization tag. (We had this construct synthesized by Twist Bioscience which provided the construct in a high-copy cloning vector.)

1.2.2 Clone this construct into the inducible trypanosome expression vector pLEW100v5 (a gift from Dr. George Cross) by restriction digest. Double digest both the cloning vector containing pHluorin2-PTS1 and pLEW100v5 using HindIII and BamHI by following the "Restriction Digest V.2" protocol at Protocols.io and NEBCloner. Perform a clean-up step, preferably by agarose gel purification, to remove restriction enzymes, the undesired cloning vector backbone, and the excised luciferase gene-containing fragment.

1.2.3 Ligate the pHluorin2-bearing insert into digested pLEW100v5 with T4 DNA ligase. Use the "Ligation Protocol with T4 DNA Ligase (M0202) V.3" at Protocols.io. (See Supplemental Figure 1 for a scheme for cloning.)

1.3. Sequence the plasmid by next generation whole-plasmid sequencing to verify the insert and vector are ligated correctly and that no mutations are introduced within the pHluorin2-PTS1 gene during the cloning process. (The complete plasmid sequence has been submitted to Addgene.org and has been assigned #83680.)

1.4. Linearize 20 μg of the plasmid by digesting with 40 units of NotI; then, transfect into BSF 90–13 parasites via nucleofection using the Lonza Human T-cell Kit (see Table of Materials). Select for stable integration as described by Burkard et al. [11].

2. Immunofluorescence colocalization of pHlourin2-PTS1

2.1. Prepare microscopy slides by treating them with poly-L-lysine 0.1% (w/v) in H_2O for 10 min. After removal of the poly-L-lysine solution, wash the slide once with PBS.

2.2. To induce pHluorin2-PTS1 expression, treat cells at 2×10^5 cells/mL with tetracycline or doxycycline (1 μg/mL) 24 hours prior to harvest. Pellet 2×10^6 parental and induced pLEWpHluorin2-PTS1 (pHL) parasites by centrifugation (room temperature [RT], 10 min, 1,000 × *g*) and wash once with PBS.

2.3. Resuspend the cells in 200 μL of freshly prepared 2% paraformaldehyde in PBS made from commercially supplied 16% EM grade solution. Apply the cells in the fixative to the slide and allow the cells to settle for 30 min. Wash the adhered cells 2x with wash solution (0.1% normal goat serum in PBS).

2.4. Apply the permeabilization solution (0.5% Triton X-100 in PBS) to the cells and incubate for exactly 30 min. Remove the permeabilization solution and wash once with ample amounts of wash solution.

2.5. Apply the block solution (10% normal goat serum and 0.1% Triton X-100 in PBS) and incubate for 30 min.

2.6. Dilute the antisera raised against *T. brucei* aldolase 1:500 in block solution and apply to the cells [12]. (Note: antisera raised against *T. brucei* aldolase is available from Dr. Meredith T. Morris, Clemson University, upon request.) Incubate for 1 h at RT. Wash the slides for 5 × 3–5 min with wash solution.

2.7. Dilute goat anti-rabbit Alexa fluor 568 1:1,000 in block solution and apply to the cells. Incubate for 1 h at RT. Wash the slides for 5 × 3–5 min with wash solution.

2.8. Apply mounting medium and seal a coverslip to the slide.

2.9. Image the cells with a 100x objective (NA 1.4 – 0.7) and analyze the images using ImageJ. (See Supplemental Figure 2 for a representative field of cells.) Perform Pearson's colocalization analysis using the 'Coloc 2' plug-in for ImageJ (https://imagej.net/plugins/coloc-2). To complete this add the Leica Image File to the Fiji and select images. Adjust brightness and contrast for each channel to a point in which no background is visible. Change images from 16-bit to 8-bit, merge the images, and then crop to a single cell with split channels. Under Analyze and Colocalization, select the Coloc2 plug-in. Select aldolase (the red channel) as Channel 1 and pHL (the green channel) as Channel 2 and click "okay" to initiate calculation of the Pearson's correlation.

3. Sample preparation for flow cytometry

3.1. Induce pHL cells with either tetracycline or doxycycline (1 μg/mL) overnight.

3.2. Pellet the cells (~4×10^7 pHL and ~1×10^6 parental) by centrifugation (RT, 10 min, 1,000 × *g*). Remove the supernatant and resuspend the cells in 1 mL of PBS either with or without 10 mM glucose depending on whether the sample is starved or un-starved. For time-course assays, resuspend in PBS supplemented with 10 mM glucose to prevent the cells from starving till the washes are complete. For the high-throughput screen (HTS) assay, resuspend in PBS without glucose to minimize carryover of glucose. Repeat the washes two more times.

3.3. Centrifuge the cells for a fourth time, remove the supernatant, and resuspend the cell pellet in either PBS, PBS plus 5 mM glucose, or PBS plus 10 mM glucose depending on the treatment. Supplement the samples with either 1 μg/mL propidium iodide (PI) or 100 nM thiazole red (TR) for live/dead determination. Transfer each sample to 5 mL tubes compatible with the flow cytometer.

4. Flow cytometry

Note: Prepare the experiment on a flow cytometer containing the following lasers: 405 nm (violet), 488 nm (blue), and 561 nm (yellow) or 638 nm (red). (Our lab used an Attune

NxT flow cytometer with the CytKick Max Auto Sampler.) See Supplemental Table 1 for common names for channels discussed below.

4.1. To measure pHL fluorescence, use the channels KO525 (VL2-H, excitation 405 nm, emission 542/27 nm) and FITC (BL1-H, excitation 488 nm, emission 530/30 nm). To differentiate live cells from dead cells, use either PI or TR; measure PI on the YL2-H channel (excitation 561 nm, emission 620/25 nm). Measure TR on the RL1-H channel (638 nm excitation, 660/10 BP).

4.1.1 To set up the experiment on the flow cytometer software, create the following plots: 1) YL2-H channel histogram (if using PI) or RL1-H channel histogram (if using TR), 2) FSC-A vs SSC-A dot plot, 3) FSC-A vs FSC-H dot plot, and BL1-H vs VL2-H channel dot plot.

4.1.2 Place the unstained WT (parental cell-line) control onto the sample injection port (SIP) first and raise the stage into position. Begin acquiring data on the cytometer at the lowest flow rate to give time to make necessary adjustments. To avoid scoring spurious debris and dead cells, begin recording events 5 s after sample acquisition begins.

4.1.3 Adjust YL2 or RL1 voltage so the main peak is within 10^3–10^4 relative fluorescence intensity (RFI) units. Adjust FSC and SSC voltages so > 90% of events fit on the dot plot. Adjust the FSC threshold to exclude the debris population but not likely cells.

4.1.4 Adjust VL2 and BL1 channels so the primary peak is thin 10^3–10^4 RFI units for the unstained WT control.

4.1.5 Place the first sample containing induced pHL stained with either PI or TR onto the SIP and raise the stage into position. Begin acquiring data at the lowest flow rate and carefully observe events in each plot. Ensure > 90% of events are within each plot and ensure that no events are saturating the VL2-H and BL1-H channels.

4.2. Proceed with running samples. Be sure to record at least 10,000 events per sample.

4.3. Save the data from the samples in .fcs file format and export for analysis.

5. Data analysis of flow cytometry results

(Note: This data analysis workflow uses FlowJo software. If other flow cytometry analysis software is used, continue to follow the key steps described below, using software-appropriate tools. To visualize the plots and gating, see Supplemental Figures 3 and 4.)

5.1. Open a new FlowJo layout and open the .fcs files acquired in step 4.3. Drag and drop the .fcs files into the layout window.

5.2. Gate for live cells.

5.2.1 Double-click the unstained WT control to open a window for this sample.

5.2.2 View the data as a histogram on either the YL2-H channel (if using PI) or RL2-H (if using TR) channel. All events should be unstained since a viability dye was not added to this sample. Toggle between this and samples stained with the viability dye to identify the live and dead populations.

5.2.3 Create a bisector gate dividing the live and dead populations; name the left gate "Live" and the right gate "Dead". Apply this gate to all samples then toggle between samples to ensure this gate is drawn appropriately for all samples. Adjust the gate as needed.

5.3. Gate for the cells.

5.3.1 On the unstained WT control, double-click on the "Live" gate to view events in that gate. Change the dot plot x-axis to FSC-A and y-axis to SSC-A.

5.3.2 Use the polygon gate tool to draw a gate around the cell population and name it "Cells". Take care to exclude debris/dying cells (typically in the far left and bottom of the dot plot) and aggregates (far right and top of dot plot).

5.3.3 Apply this gate under the "Live" gate for all samples. Toggle between samples to ensure the gate encompasses the likely cell population for all samples and make necessary adjustments. Be sure to reapply the gate to all samples after altering it. (Note: The cell population distribution noticeably changes between starved and un-starved conditions on FSC vs SSC; ensure the "Cell" gate encompasses the cell population in all conditions.)

5.4. Gate for single cells to increase the quality of pH measurements.

5.4.1 On the unstained WT control, double-click on the "Cell" gate to view events in that gate. Change the dot plot x-axis to FSC-A and y-axis to FSC-H.

5.4.2 Single cells should appear as a diagonal distribution on this dot plot with doublets forming a secondary population to the lower right of the singlet population (see Supplemental Figure 3 third plot). Using the polygon gate tool, draw a gate around the singlet events excluding the doublet population. Name this gate "Singlets".

5.4.3 Apply the "Singlets" gate under the "Cell" gate for all samples. Again, toggle between samples to ensure the gate is properly excluding the doublet population while including the singlet population. Adjust as needed.

5.5. Gate for fluorescent pHL cells

5.5.1 On the unstained WT control sample, double-click on the "Singlets" gate to open a dot plot for that population. Change the x-axis to BL1-H and the y-axis to VL2-H.

5.5.2 The pH sensor pHluorin2 is fluorescent in both VL2 and BL1. Use the polygon gate tool to draw a diagonal-shaped gate extending to the top and right away from the autofluorescent population in the lower left of the dot plot. Name this gate "pHL+".

5.5.3 Apply the “pHL+” gate under the “Singlets” gate for all samples. Toggle to a pHL sample and adjust the gate to include events with greater fluorescence intensity in both VL2-H and BL1-H than the WT control. Ensure this gate encompasses this fluorescent population for all samples as the position of this population will shift as glycosomal pH changes. (Note: This small but visible shift in the pHL+ population is due to pH-dependent changes in the fluorophore’s excitation spectrum.)

5.6. Export the statistics.

5.6.1 - Click on the Table Editor then click on the Edit bar. Click on Add Column to add new statistics to export. (Note: For each statistic to export, choose the respective statistic and which population to export it from. Ensure to choose the appropriate parameter for applicable statistics such as Median. Leave the Sample unchanged.)

5.6.2 Add columns with the following statistics: Total (ungated) Count, pHL+ Count, Live Freq. of Total (percentage based on total events), pHL+ Freq. of Parent (percentage based on parent gate), pHL+ Median VL2-H, and pHL+ Median BL1-H.

5.6.3 Click on the Table Editor and change the following export settings: “Display” to “To File” and “Text” to “CSV” and then choose the file destination and name. Click “Create Table”.

5.7. Calculate the fluorescence ratio.

5.7.1 Save the exported .csv file as an Excel file.

5.7.2 Perform quality control analysis by comparing the following across all samples in the experiment: number of events per sample, percentage of live events, and percentage of pHL+ events. Compare these visually in bar or scatter plots depending on the experiment.

5.7.3 Label a new column as “pHL+ Median VL2/BL1”. For each sample, divide the median VL2-H value by the median BL1-H value as shown in equation (1)

$$Fluorescence\ Ratio = \frac{VL2\ MFI}{BL1\ MFI} \quad (1)$$

5.8. Use statistical analysis software, such as GraphPad Prism, to perform statistical analysis using the fluorescence ratio.

6. pH biosensor calibration

NOTE: To convert measured fluorescence ratios to pH units, calibrate pHL-expressing cells using nigericin and valinomycin. Nigericin is a K+/H+ antiporter, an ionophore that can equilibrate pH across membranes when there is sufficient K^+ in the buffer [13]. Nigericin has been commonly used to calibrate pHluorin and other pH sensors [14,15]. Peroxisomally-

localized pHluorin was previously calibrated using 10 μM nigericin [16], so we chose to treat with that concentration. Valinomycin is a potassium ionophore and has been used (at 4 μM) to equilibrate pH across mitochondrial membranes [17]. We used 10 μM valinomycin to assist the pH equilibration activity of nigericin by ensuring K+ ions were equilibrated across the membranes. While we used a nigericin-valinomycin combination, nigericin may be sufficient to equilibrate organellar pH.

6.1. Prepare eight solutions of universal calibration buffer (UCB; 15 mM MES, 15 mM HEPES, and 130 mM KCl) each at a different pH ranging from 5 to 8.5.

6.2. Centrifuge (RT, 10 min, 800–1,000 × *g*) eight separate tubes of 2 mL of pHL-expressing BSF culture (~4×10^6 cells each).

6.3. Remove the supernatant and then resuspend each cell pellet in UCB at different pH values.

6.4. Introduce nigericin and valinomycin, each to 10 μM. Spike in PI to 1 μg/mL.

6.5. Incubate the cells in each solution for 15 min.

6.6. Run each sample on a flow cytometer to measure the fluorescence ratio as described in steps 4–5.

6.7. Repeat this experiment twice more to obtain three biological replicates for each pH value. Export data in .fcs format.

6.8. Analyze the .fcs files as described in steps 5.1 through 5.8. Supplemental Figure 3 shows the dot plots and gating scheme. Our results can be found in Supplemental Table 2. Use the measured fluorescence ratio for each pH to interpolate glycosomal pH in future experiments using equation (2):

$$Fluorescence\ Ratio = Bottom + \frac{(Top - Bottom)}{(1 + 10^{(LogIC50 - pH) * HillSlope})} \quad (2)$$

Note: The following describes how to interpolate pH from fluorescence ratio using GraphPad Prism. If using other statistical software, follow the same key steps.

6.9.1. Open GraphPad Prism and create an XY table with 3 y-replicates. Paste the pH values in the x-column and their associated fluorescence ratio values in the y-replicate columns.

6.9.2. Click on the graph associated with the table. When viewing the graph, click on Analyze under the Analysis ribbon, then click “Interpolate a standard curve” under XY analyses.

6.9.3. Choose “Sigmoidal, 4PL, X is log(concentration)” since pH units are on a logarithmic scale. The Top and Bottom parameters are the estimated top and bottom plateaus. Choose “No special handling of outliers”.

6.9.4. The software will seek to fit the data to the model described in equation (2) and step 6.9.3. Look for evidence of lack of fit in the Table of results and the curve on the graph.

6.9.4. To interpolate pH from fluorescence ratios in other experiments, go to the table with the pHL calibration data. Paste in the fluorescence ratio values below the calibration data on **in the y-column(s)**. Give each y-value a title but leave the x-value (the pH) blank since it is unknown.

6.9.5. In the Results gallery, click on the interpolation analysis then go to the "Interpolated X replicates" tab. The interpolated pH values will be listed alongside the entered fluorescence ratio values. Note: The software uses the model and best-fit parameter values found from the calibration data to interpolate pH from fluorescence ratios for experiments where pH is unknown.

7. Glucose starvation and addback time-courses

7.1. Induce 15 mL of BSF pHL parasites overnight with 1 μg/mL doxycycline incubated at 37°C as described in step 1.1.

7.2. Wash 15 mL of induced pHL culture in PBS supplemented with 10 mM glucose. Repeat this step three times.

7.2.1. Concurrently, wash 3 mL of WT culture in PBS 3x as described in step 3.2.

7.2.2. After the first wash when the pHL sample is resuspended in 1 mL of PBS, remove a 0.1 mL aliquot of pHL cell solution to keep in 10 mM glucose as an un-starved control.

7.3. After the final wash, resuspend the pHL sample in PBS supplemented with 1 μg/mL PI.

7.4. Acquiring flow cytometry data.

7.4.1. Begin monitoring glycosomal pH for both the starved and un-starved samples by measuring each sample on a flow cytometer every 5, 10, 30, or 90 min, depending on the experiment and sample. Acquire flow cytometry data as described in steps 4.1 through 4.3, ensuring the voltages and plots are prepared beforehand.

7.4.2. Run the unstained WT control first at 0 min.

7.4.3. Run the un-starved control sample on the cytometer every 90 min starting at 0 min. For the Starvation time-course assay, run the starved sample every 10 min starting at 0 min. For the Glucose Addback assay, run the starved sample at 0, 5, 10, 20, 30, 60, and 90 min then repeat after introducing glucose.

7.4.4. Incubate the samples at room temperature during the 90 min starvation time-course and the 180 min Glucose Addback time-course.

7.5 Analyze the .fcs files as described in steps 5.1 through 5.8. Supplemental Figure 4 shos how to perform the gating and what the dot plots should look like. Supplemental Tables 3 and 4 show the results of these experiments.

8. Optimizing the assay for drug screening

8.1. Wash approximately 32 mL of pHL-expressing BSF parasites and 3 mL of WT cells, both at approximately 2×10^6 cells/mL, 2x in PBS as described previously.

8.1.1. Note: >10,000 events are needed to be recorded per well to minimize variability of measured fluorescence ratios and accurately measure the Z-factor statistic. The minimum culture necessary to accomplish this is approximately 26 mL, but we recommend 32 mL for ease of handling.

8.1.2. After the first wash, separate the pHL sample equally into two microcentrifuge tubes.

8.2. After the washes, resuspend one pHL sample in 18 mL of PBS containing 5 mM glucose, 0.1% DMSO, and TR. Resuspend the other pHL sample in 18 mL of PBS plus 0.1% DMSO and TR.

NOTE: The DMSO is used to mimic the buffer composition in a drug screen as compounds are typically dissolved in DMSO.

8.3. Transfer these two cell solutions into a 12-well reservoir, 9 mL per well.

8.4. Use a pipetting robot to pipet the cell solutions into separate halves of a 384-well plate, 80 μL per well.

8.5. Incubate the plate at room temperature for 1.5 h, gently shaking and wrapped in aluminum foil to protect the fluorophores from light.

8.6. Run the plate on a flow cytometer capable of running 384-well plates.

Note: The following workflow is adapted to an Attune NxT with Cytkick Max Auto Sampler. If another flow cytometer is used, continue to follow the key steps.

8.6.1. For the plate, run at the fastest flow rate (1000 uL/min) and enable boost mode. Acquire 20 uL/well. Include once mix cycle and one rinse cycle between each well.

8.6.2. Create plots as described in step 4.1.3.

8.6.3. Run an unstained WT tube control and optimize voltages as described in steps 4.1.3 and 4.1.4. Run a starved pHL tube sample and optimize the VL2 and BL1 voltages as described in step 4.1.

8.6.4. Begin acquiring the plate on the flow cytometer. Run the plate horizontally so there is not a significant acquisition time difference between the starved and un-starved sample wells. Plate acquisition should finish in about 1.5 hours.

8.7. Export the .fcs files and analyze the data as described in steps 5.1 through 5.8. Find the average (AVG) and standard deviation (SD) of the fluorescence ratios for the samples treated with either glucose (Glc) or no glucose (Starved). The data from our Z-factor experiments can be found in Supplemental Table 5.

8.8. Calculate the Z-factor [18] statistic using equation (3):

$$\mathrm{Z-\ Factor} = 1 - \frac{3\ SD_{Glc} + 3\ SD_{Starved}}{|AVG_{Glc} - AVG_{Starved}|} \quad (3)$$

Note: The Z-factor statistic is used to determine how suitable an assay is for HTS. Values between 0.5 and 1.0 generally mean the assay quality is acceptable for HTS.

REPRESENTATIVE RESULTS:

pHLuorin2-PTS1 localization to glycosomes in BSF *T. brucei*

To assess subcellular localization of the pHluorin2-PTS1, parasites were subjected to immunofluorescence assays. Signal from the transgene colocalized with anti-sera raised against a glycosome-resident protein, aldolase (TbAldolase) (Figure 2A) [Place Figure 2 here]. The average Pearson's correlation coefficient of colocalization between anti-TbAldolase and pHluorin2-PTS1 was 0.895, indicating pHluorin2-PTS1 was primarily localized to glycosomes. With pHluorin2-PTS1 localized to the glycosome, we proceeded to investigate BF glycosome pH.

pHluorin2-PTS1 calibration

Changes in pH alter the excitation spectrum of pHluorin2-PTS1. Under neutral pH, pHluorin2-PTS1 excitation at 405 nm is greater than at 488 nm; as pH falls, the reverse is true [19,20]. To measure the relative pH of the glycosome by flow cytometry, we measured emission when excited by the 405 nm laser (VL2 channel) and emission when excited by the 488 nm laser (BL1 channel), using the ratio of VL2/BL1 (fluorescence ratio) to measure the relative glycosomal pH. To convert fluorescence ratio to pH, we equilibrated intracellular and glycosomal pH with extracellular pH using the ionophores valinomycin and nigericin [15,21] followed by flow cytometry analysis to find the fluorescence ratio. As expected, the fluorescence ratio increased as extracellular pH increased with an intracellular K_d of pH 6.5 (Figure 2B). This K_d was slightly lower than the reported *in vitro* K_d of pHluorin2 [19]. Interestingly, the glycosomal pH in the presence of glucose was ~pH 8.0, which was more basic than PF glycosomes in the presence of glucose [21]. We used this calibration curve to convert fluorescence ratio to pH in subsequent experiments.

Glycosome acidification due to glucose starvation

While *T. brucei* PF parasites acidify their glycosomes when starved of glucose [21], how BSF glycosomes respond to glucose is unknown. To explore this, we washed BSF parasites expressing pHluorin2-PTS1 in PBS plus 10 mM glucose to remove culture medium and then resuspended the cells in PBS without glucose. The sensor response to this perturbation

was measured immediately and then every 10 min thereafter for 1.5 h by flow cytometry. Responses were compared to cells in PBS plus 10 mM glucose (un-starved).

In response to starvation, we observed a gradual mild acidification over time, which plateaued by ~90 min (Figure 3A) [Place Figure 3 here]. This change in glycosomal pH was statistically significant ($p < 0.0001$) and repeatable across three separate experiments. This suggests that BSF cells mildly acidify their glycosomes when starved of glucose, similar to the response observed in the PF life stage [9].

Reversible glycosomal acidification in response to glucose

We next tested if BSF glycosome acidification was reversible by starving the cells and then reintroducing glucose. Parasites were incubated in the absence of glucose for 90 min. Glucose (10 mM) was then added and the sensor response was measured by cytometry for another 90 min (Figure 3B). We observed that after glucose was reintroduced, glycosomal pH returned to pre-starvation levels in ~30 min. These results suggest that BSF glycosomal pH is dynamic and regulable in response to glucose, similar to the pH response observed in PF parasites.

Adaptation of the pHluorin2 assay for high-throughput drug screening

Glycosomes are essential organelles for the trypanosome, as they house key metabolic pathways. The importance of glycosomes suggests that inhibitors of their homeostasis could hold promise as potential therapeutic leads. Here, we have adapted the assay for glycosomal pH to a high-throughput format, which will allow adaptation to drug screens to identify inhibitors of glycosomal pH. We anticipate disruption of the regulation of this response could be detrimental to the parasite, given the known impact of pH on glycosome-resident protein function [5].

To establish the high-throughput format, we scored the assay robustness. To complete this, parasites induced to express the pHluorin2-PTS1 were plated in a 384-well microtiter plate in either 5 mM glucose (high controls) or no glucose (low controls) and then incubated for 90 min at room temperature. The plate was then analyzed by flow cytometry. As shown in Figure 3, there was low variability between replicate measurements and the high and low controls are well-separated, features that resulted in an acceptable Z-factor of 0.645 [Place Figure 4 here]. Assays with values > 0.5 are generally considered robust enough for adaptation to high-throughput screening campaigns. Given the success here, we anticipate that this sensor and approach will be used in future high-throughput drug screens (Figure 1).

DISCUSSION:

Environmental perception and response mechanisms in the African trypanosome are poorly understood. Changes in nutrient availability are known to trigger diverse responses in the parasite, including acidification of glycosomes. Here, we have described a method to study glycosomal pH response to environmental perturbations in living cells using a heritable protein sensor, pHluorin2, and flow cytometry.

There are several critical steps in the use of the sensor. First, characterization of transfected parasites that express the transgene is important, as there can be cell-to-cell variation in expression. Expression levels of some transgenes can fall over time. While that has not been observed with the pHluorin2-bearing cells to date, if sensor responsiveness becomes highly variable (e.g., after induction, sensor signal is not robust), it may be necessary to clone out the culture, as some members of the population may express the protein at higher levels than others. Alternatively, retransformation and selection has proven useful for generating new cell lines with higher (albeit possibly transient) expression. If re-transfection is required, we recommend cloning out individual lines, as expression of the transgene of interest could impart a subtle growth disadvantage leading to loss of high expressors in the population over time.

Additionally, it is essential to monitor parasite viability—both during the assay and while routinely maintaining cultures. We have included either PI or thiazole red for this purpose during the assay, and these reporters ensure that data only reflects responses in living cells. Ensuring that cell doubling time during normal culture is constant, particularly prior to initiation of large-scale assays (e.g., HTS-type assays) limits concern about initiating assays with cells that are not thriving, which could negatively alter high and low control signals and impact overall robustness of the screen.

The approach we have described here for measuring glycosomal pH is robust and sensitive. However, there are limitations to the approach. First, access to cytometers with appropriate capabilities, including samplers capable of accepting microtiter plates, is required. While ratiometric data can be collected by fluorescence microscopy, the limited number of samples that can be analyzed that way limits the utility for approaches such as screens (and time course-based assays).

A second potential limitation is that the assays involve an agent of biosafety concern. It is possible that core facilities that house the instruments needed for the work may not allow living parasites on their equipment. These limitations, along with the technical challenges of generating and maintaining transgenic trypanosomes, can be overcome through collaboration between groups with appropriate expertise in parasite biology and cell analyses.

The work described here focuses on using the glycosomal pH sensor in African trypanosomes, it is possible the tool could be adapted for use in other kineotplastid parasites. While it is unclear what role acidification of the organelle might have in the biology of *Leishmania spp.* or *Trypanosoma cruzi*, is it possible that pHLuorin2-PTS1 could be expressed as a transgene in the cells given that parasite-specific expression vectors are available and that the glycosomal import machinery is similar in the organisms [5,22,23].

One potential application of the sensor-bearing cells is identification of small molecules that alter the cellular capacity to acidify the glycosome. These would likely be noxious to the parasite, as altered pH has been found to be a possible means of regulation of hexokinase, a glycosome-resident protein that is essential in the pathogenic life cycle stage of the African

trypanosome[7]. The assay described here has been adapted to high throughput assays (Figure 4), allowing interrogation of large chemical collections for this activity.

In summary, this tool offers the opportunity to dissect mechanisms involved in dynamic responses in a parasite-specific organelle. Using the sensor lines in combination with forward and reverse genetics it is likely that unique parasite-specific pathways will be identified. Further, the sensor lines offer the opportunity for the identification of small molecule inhibitors of the response, opening the door to new drug target discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS:

Work from the JCM and KAC laboratories was partially supported by an award from the National Institutes of Health (R01AI156382). SSP was supported by NIH 3R01AI156382.

REFERENCES:

1. Coley AF, Dodson HC, Morris MT, Morris JC Glycolysis in the African trypanosome: Targeting enzymes and their subcellular compartments for therapeutic development. Molecular Biology International. 2011 123702 (2011). [PubMed: 22091393]
2. Mcconville MJ, Saunders EC, Kloehn J, Dagley MJ Leishmania carbon metabolism in the macrophage phagolysosome- feast or famine? F1000Research. 4 (F1000 Faculty Rev), 938 (2015). [PubMed: 26594352]
3. Parsons M Glycosomes: Parasites and the divergence of peroxisomal purpose. Molecular Microbiology. 53 (3), 717–724 (2004). [PubMed: 15255886]
4. Parsons M, Furuya T, Pal S, Kessler P Biogenesis and function of peroxisomes and glycosomes. Molecular and Biochemical Parasitology. 115 (1), 19–28 (2001). [PubMed: 11377736]
5. Haanstra JR, Gonzalez-Marcano EB, Gualdron-Lopez M, Michels PA Biogenesis, maintenance and dynamics of glycosomes in trypanosomatid parasites. Biochim Biophys Acta. 1863 (5), 1038–1048 (2016). [PubMed: 26384872]
6. Allmann S Bringaud, F. Glycosomes: A comprehensive view of their metabolic roles in *T. brucei*. International Journal of Biochemistry and Cell Biology. 85 85–90 (2017). [PubMed: 28179189]
7. Dodson HC, Morris MT, Morris JC Glycerol 3-phosphate alters *Trypanosoma brucei* hexokinase activity in response to environmental change. Journal of Biological Chemistry. 286 (38), 33150–33157 (2011). [PubMed: 21813651]
8. Lin S, Morris MT, Ackroyd PC, Morris JC, Christensen KA Peptide-targeted delivery of a pH sensor for quantitative measurements of intraglycosomal pH in live *Trypanosoma brucei*. Biochemistry. 52 (21), 3629–3637 (2013). [PubMed: 23651061]
9. Mahon MJ Phluorin2: An enhanced, ratiometric, pH-sensitive green florescent protein. Advances in Bioscience and Biotechnology. 2 (3), 132–137 (2011). [PubMed: 21841969]
10. Wirtz E, Leal S, Ochatt C, Cross GA A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Molecular and Biochemical Parasitology. 99 (1), 89–101 (1999). [PubMed: 10215027]
11. Burkard G, Fragoso CM, Roditi I Highly efficient stable transformation of bloodstream forms of *Trypanosoma brucei*. Molecular and Biochemical Parasitology. 153 (2), 220–223 (2007). [PubMed: 17408766]
12. Crowe LP, Wilkinson CL, Nicholson KR, Morris MT *Trypanosoma brucei* pex13.2 is an accessory peroxin that functions in the import of peroxisome targeting sequence type 2 proteins and localizes to subdomains of the glycosome. mSphere. 5 (1), (2020).

13. Kucejova B, Kucej M, Petrezselyova S, Abelovska L, Tomaska L A screen for nigericin-resistant yeast mutants revealed genes controlling mitochondrial volume and mitochondrial cation homeostasis. Genetics. 171 (2), 517–526 (2005). [PubMed: 16020778]
14. Huynh MH Carruthers VB. Toxoplasma gondii excretion of glycolytic products is associated with acidification of the parasitophorous vacuole during parasite egress. PLoS Pathog. 18 (5), e1010139 (2022). [PubMed: 35512005]
15. Lehoux S, Abe J, Florian JA, Berk BC 14–3-3 binding to Na+/H+ exchanger isoform-1 is associated with serum-dependent activation of Na+/H+ exchange. Journal of Biological Chemistry. 276 (19), 15794–15800 (2001). [PubMed: 11279064]
16. Jankowski A et al. In situ measurements of the ph of mammalian peroxisomes using the fluorescent protein pHluorin. Journal of Biological Chemistry. 276 (52), 48748–48753 (2001). [PubMed: 11641408]
17. Jankowski A Grinstein, S. A noninvasive fluorimetric procedure for measurement of membrane potential. Quantification of the NADPH oxidase-induced depolarization in activated neutrophils. Journal of Biological Chemistry. 274 (37), 26098–26104 (1999). [PubMed: 10473559]
18. Zhang JH, Chung TD, Oldenburg KR A simple statistical parameter for use in evaluation and validation of high throughput screening assays. Journal of Biomolecular Screening. 4 (2), 67–73 (1999). [PubMed: 10838414]
19. Mahon MJ Phluorin2: An enhanced, ratiometric, pH-sensitive green florescent protein. Advances in Bioscience and Biotechnology (Print). 2 (3), 132–137 (2011). [PubMed: 21841969]
20. Miesenböck G, De Angelis DA, Rothman JE Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature. 394 (6689), 192–195 (1998). [PubMed: 9671304]
21. Lin S et al. pH regulation in glycosomes of procyclic form T*rypanosoma brucei*. Journal of Biological Chemistry. 292 (19), 7795–7805 (2017). [PubMed: 28348078]
22. Ha DS, Schwarz JK, Turco SJ, Beverley SM Use of the green fluorescent protein as a marker in transfected Leishmania. Molecular and Biochemical Parasitology. 77 (1), 57–64 (1996). [PubMed: 8784772]
23. Kelly JM, Ward HM, Miles MA, Kendall G A shuttle vector which facilitates the expression of transfected genes in *Trypanosoma cruzi* and Leishmania. Nucleic Acids Research. 20 (15), 3963–3969 (1992). [PubMed: 1324472]

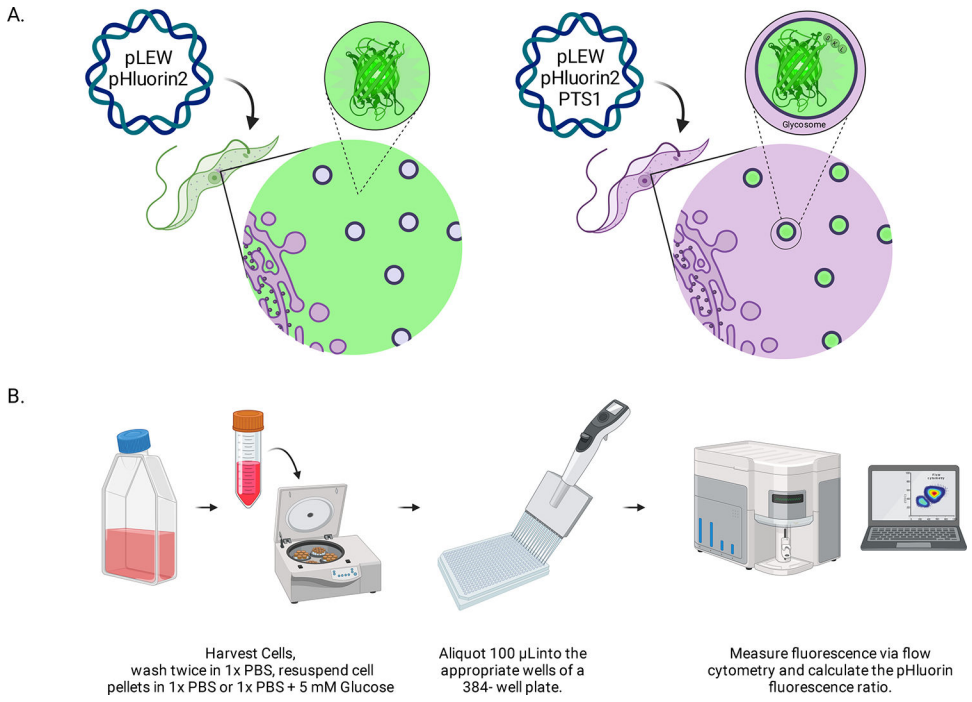

Figure 1: Diagram of method for scoring glycosomal pH in live BSF trypanosomes.

A: Depiction of cell lines expressing glycosomally located pHluorin2 sensor. The inclusion of a peroxisomal targeting sequence provides control over the localization. (Please note, we anticipate that elimination of the PTS-1 would lead to cytosolic localization, allowing future analysis of pH in that subcellular compartment. B: Depiction of the sensor validation assay. Abbreviation: BSF = bloodstream form.

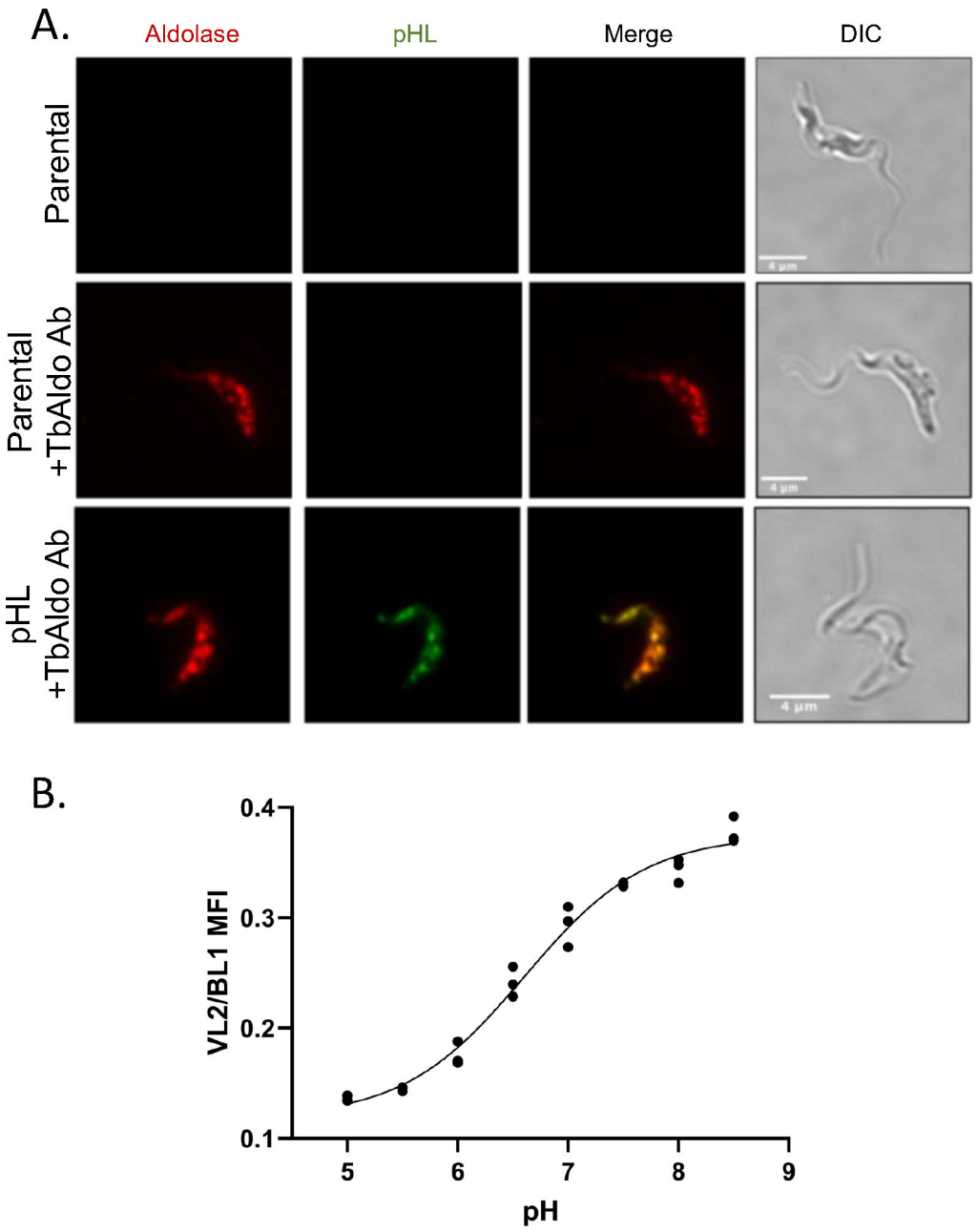

Figure 2: Localization of pHluorin2-PTS1 to glycosomes in BSF *Trypanosoma brucei*.

(**A**) Colocalization of pHluorin2-PTS1 with the glycosomal-resident protein TbAldolase. BF 90–13 parasites were transfected with pLEWpHluorin2-PTS1 and expression was induced with Doxycycline (1 µg/mL). TbAldolase was localized using anti-TbAldolase sera, followed by incubation with goat anti-rabbit Alexa fluor 568. The average Pearson's correlation coefficient was 0.895 (30 cells). (**B**) Calibration of pHluorin2-PTS1 in BSF *T. brucei*. Scale bars = 4 µm. Abbreviation: BSF = bloodstream form.

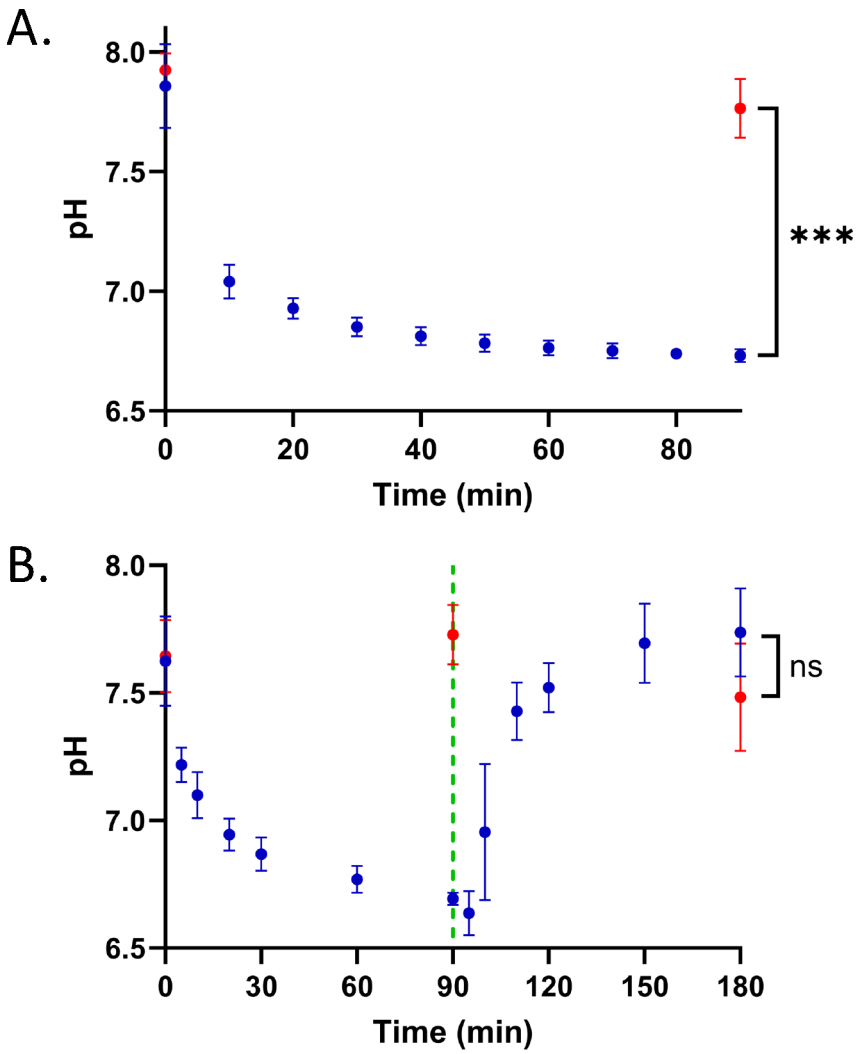

Figure 3: Reversible acidification of glycosomes of BSF *T. brucei* when deprived glucose.

(**A**) Glycosomal pH of cells grown in the absence (starved, blue) or presence (un-starved, red) of glucose. The starved cells were resuspended in PBS without glucose ~2 min prior to the first measurement on an Attune NxT flow cytometer. Three biological replicates of the time course were performed. Un-starved parasites were incubated in PBS plus 10 mM glucose. An unpaired two-tailed Student's *t*-test was performed comparing the starved and un-starved 90 min time points, ***p = 0.0001. (**B**) Time course of glycosomal pH change starved (blue) and un-starved (red) BSF parasites with 10 mM glucose reintroduced at 90 min (green dotted line). NS, not significant (p = 0.25, unpaired two-tailed Student's *t*-test).

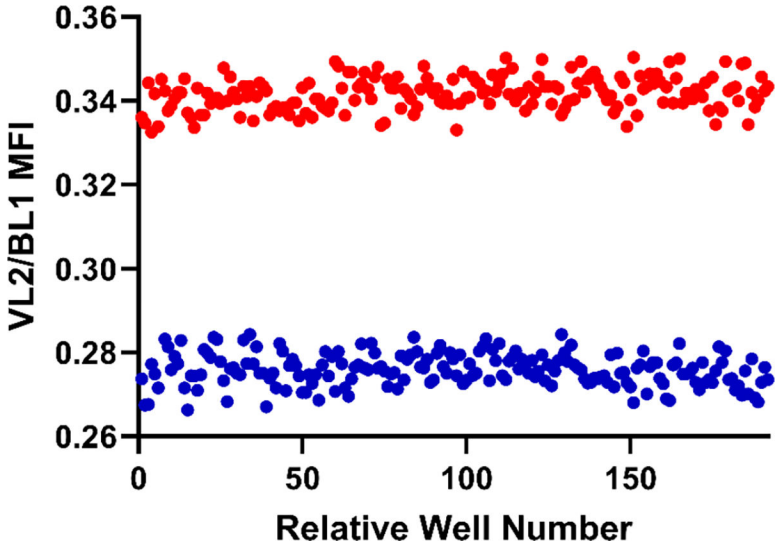

Figure 4: Assay to assess the suitability of the pHluorin2-PTS1 sensor-bearing BSF for future HTS campaigns.

Cells were incubated for 90 min with glucose (high control, red, 5 mM glucose) or without the hexose (low control, blue, no additional glucose). The calculated Z-factor was 0.645.

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Amaxa™ Human T Cell Nucleofector™ Kit	Lonza	VPA-1002	
Nucleofector™ 2b	Lonza	Discontinued Product	
50 mL Tissue Culture Flasks (Non-treated, sterile)	VWR	10861–572	
75 cm^2 Tissue Culture Flask (Non-Treated, sterile)	Corning	431464U	
BRANDplates® 96-Well, flat bottom plate	Millipore Sigma	BR781662	
Thiazole Red (TO-PRO®-3)	biotium	#40087	
CytoFLEX Flow Cytometer	Beckman-Coulter		
Attune NxT Flow Cytometer	invitrogen by Thermo Fisher Scientific	A24858	
CytKick Max Auto Sampler	invitrogen by Thermo Fisher Scientific	A42973	
80 µL flat-bottom 384-well plate	BrandTech	781620	We machined a custom acrylic plate stand so this brand of plate could be detected and used on our CytKick Max Auto Sampler
OP2 Liquid Handler	opentrons	OP2	Pipetting robot for HTS assay
Nigericin (sodium salt)	Cayman Chemical	11437	For pH calibration
valinomycin	Cayman Chemical	10009152	For pH calibration
Electron Microscopy Sciences 16% Paraformaldehyde Aqueous Solution, EM Grade, 10 mL Ampoule	Fisher Scientific	50–980-487	For IFA
poly-L-lysine, 0.1% (w/v) in H_2O	Sigma Life Science	CAS:25988–63-0	For IFA