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Measuring pH of the *Plasmodium falciparum* digestive vacuole by flow cytometry

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Abstract. Studies show that the pH of the malaria parasite's digestive vacuole (DV) plays a key role in the physiological functions of this organelle and antimalarial drug accumulation, and yet is technically difficult to measure. In this study, a flow cytometry-based technique was developed to measure the DV pH using a ratiometric pH indicator, FITC-dextran loaded into the DV of saponin-permeabilized parasites. To calculate the DV pH, a standard pH calibration curve was generated by incubating the saponin-permeabilized cells in buffers with different pH in the presence of an ionophore, CCCP. The measured average pH of the DV was 5.27 ± 0.03 that is approximately the same in the parasites observed microscopically by Hayward *et al.* (2006) (5.50 ± 0.14) using the same probe. The removal of glucose from the medium, causing a rapid depletion of parasite ATP, resulted in an alkalization of the DV. The DV was reacidified upon restoration of glucose to the medium. This technique provides a rapid, simple and quantitative measurement of the DV pH on a large number of cells. It will also be useful in future attempts to evaluate the effect of antimalarial drugs (i.e. chloroquine- and artemisinin-based drugs) in pH changes of the DV.

INTRODUCTION

The malaria parasite feeds on the host cell hemoglobin using cytostome-dependent invaginations. The hemoglobin is transferred to an acidic digestive vacuole (DV) and degraded by proteases providing a source of amino acids and of osmolytes (Goldberg, 2005) and generating space for growth (Allen & Kirk, 2004). The DV pH has long been thought to play a major role in the degradation of hemoglobin, the detoxification of heme (a toxic waste product of hemoglobin degradation) and in the event of antimalarial drug action and resistance (Homewood *et al.*, 1972). Several proteolytic enzymes of the parasite such as aspartic and cysteine proteases involved in hemoglobin degradation have pH optima in the range 4.5-5.0 suggesting that the DV maintains an acidic environment (Goldberg, 2005). The vacuolar-type proton-pumping ATPase (V-type H^+-ATPase) is thought to be responsible for maintaining an acidic DV (Saliba *et al.*, 2003). There is evidence for the presence of this proton pump on the parasite plasma membrane, DV and small vesicle compartments (Hayashi *et al.*, 2000).

Despite the importance of DV pH, its definitive measurement has been surprisingly challenging (Spiller *et al.*, 2002). A recent study investigated changes in the distribution of fluorescent tracers and pH measurements of parasite endocytic compartments and DVs by confocal microscopy (Abu Bakar *et al.*, 2010). Flow cytometry offers an alternative and/or complementary technique for evaluating DV pH on a larger cell population (Godfrey *et al.*, 2005). pH-sensitive fluorescent probes such as fluorescein and its derivatives and conjugates are widely used for estimating intracellular pH. Their popularity derives from the high extinction coefficient and

quantum yield properties (Geisow, 1984). A dextran-conjugated fluorescein derivative, fluorescein isothiocyanate-dextran (FITC-dextran) can be used as a dual-emission ratiometric pH indicator by flow cytometry, which means one excitation wavelength is used and the emission is analysed at two different wavelengths (Myers *et al.*, 1995). The ratio of the fluorescence intensities at two emission wavelengths can be used to estimate the pH of the cellular compartment where FITC-dextran is located.

Herein, we report a flow cytometry-based technique for measuring the DV pH of *P. falciparum*-infected erythrocytes that permitted quantitative analysis from much larger population of individual cells as well as quick and robust sample processing. FITC-dextran was used to label the DV to which the fluorophore-filled vesicles derived from the endocytic process are finally delivered.

MATERIALS AND METHODS

Culturing of malaria parasites

Parasites (D10 strain of *P. falciparum*) were cultured using human erythrocytes and pooled serum (Red Cross Blood Bank, Melbourne, Australia) as described previously (Frankland *et al.*, 2006). Parasite-infected erythrocytes were maintained in medium containing 0.25% Albumax in RPMI 1640 (GIBCO BRL, Invitrogen) supplemented with hypoxanthine (0.21 mM, Sigma) and Glutamax (4 mM, Invitrogen). For synchronization, cultures (~5% parasitemia, mainly ring stage) were treated with 5% D-sorbital (Sigma) at room temperature for 10 minutes and allowed to mature for 24 hours (Lambros & Vanderberg, 1979). Synchronized infected erythrocytes were harvested on a VarioMACS magnetic separation system (Miltenyi Biotec) to above 95% purity as assessed by counting Giemsa-stained thin blood smears.

Resealing of erythrocytes and culturing of malaria parasites in resealed erythrocytes containing FITC-dextran

Washed erythrocytes were lysed in 2.25 volumes of ice-cold 5 mM sodium phosphate (pH 7.5, supplemented with 1 mM Mg-ATP, Sigma) in the presence of 50 µM FITC-dextran (10 kDa, Invitrogen) using a protocol modified from that of Abu Bakar *et al.* (2010). The cells were then resealed by the addition of 220 ìL resealing buffer A (5 mM sodium phosphate, supplemented with 700 mM NaCl, 100 mM KCl, 27.5 mM glucose, pH 7.4) at 37°C. After 20 minutes incubation, 8.5 mL of resealing buffer B (10 mM sodium phosphate, 140 mM NaCl, 20 mM KCl, 5.5 mM glucose, pH 7.4) was added to the cell suspension and incubated for a further 20 minutes. To initiate infection, harvested mature stage parasites (3% parasitemia, 2% hematocrit) were cultured using FITC-dextran containing resealed erythrocytes and grown under normal culture conditions. After infection, the probe along with the host cell hemoglobin was ingested by the parasite that finally destined in the DV.

pH calibration of FITC-dextran and measurement of DV pH using saponin-permeabilized parasites

Prior to the assay, trophozoite stage parasites in which the DV was preloaded with FITC-dextran (see Figure 1) were released from the host erythrocytes using a protocol modified from that of Saliba *et al.* (1998) by brief exposure (approximately 10 seconds) to 0.025% saponin (an optimized final concentration, w/v sapogenin, pH 7.5, Sigma) at room temperature. The saponin-permeabilized parasites were suspended in a medium containing RPMI 1640, 25 mM HEPES, 0.125% Albumax, 0.02 mg/mL gentamycin and 0.4 mM and kept at 37°C until being used in experiments usually within 2 hours. For pH calibration of FITC-dextran, saponin-permeabilized parasites were suspended in solution containing 20 mM buffer and 150 mM NaCl. Buffers used were 2-[N-morpholino] ethane sulfonic acid (MES, pH 5.5 and 6.0), sodium phosphate (pH 6.5, 7.0, 7.5 and 8.0) and tris (hydroxymethyl) aminomethane (TRIS, pH 9.0). An ionophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma) was added to a final concentration of 10 ìM to equilibrate the DV pH to that of the pH of the buffer before analysis by flow cytometry. Data were

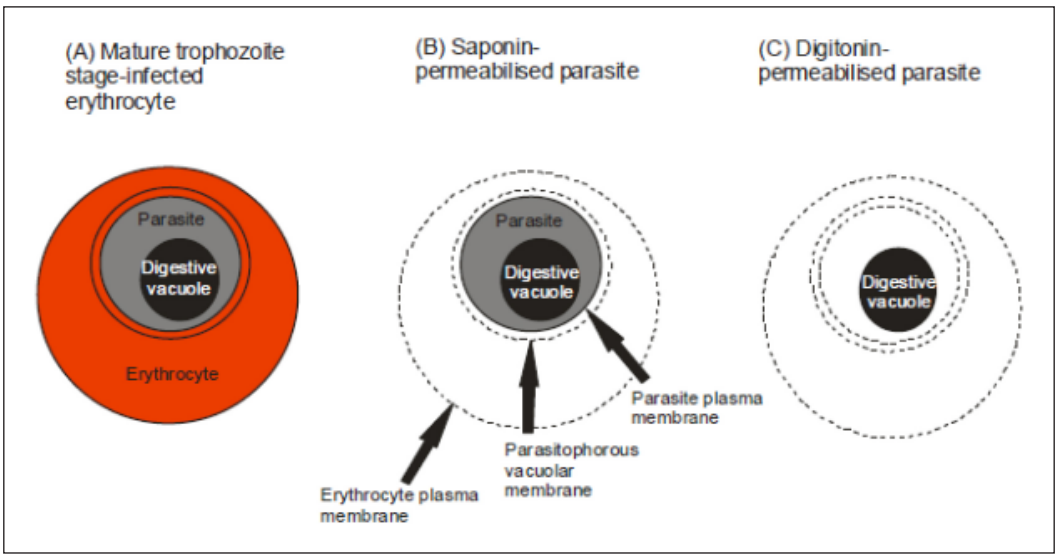

Figure 1. Schematic representation of the different parasite preparations
(A-B) Mature trophozoite stage parasites were released from their host cells by permeabilization of the erythrocyte plasma membrane and the parasitophorous vacuolar membrane by brief exposure to saponin (0.025%, w/v). (C) The treatment of the saponin-lysed parasites with digitonin (0.01%, w/v) permeabilizes the parasite plasma membrane to release the contents of the parasite cytosol.

analysed to generate a pH calibration curve of FITC-dextran. Kinetic analysis of re-acidification of the DV was also performed using the saponin-permeabilized parasites following the addition of glucose (20 mM) in the cell suspension and monitored by flow cytometry.

Flow cytometric analysis

Flow cytometric measurements were performed on a six-colour FACSCanto™ II cytometer (Becton Dickinson, La Trobe University, Melbourne, Australia) equipped with a High Throughput Sampler, enabling sampling from 96-well microtiter plates. A 488 nm argon ion laser was used as the excitation source for FITC-dextran. Green and yellow fluorescence signals of FITC-dextran were collected using 530 nm (30 nm bandpass) and 585 nm (42 nm bandpass) filters. Saponin-permeabilized parasites were selected based on their scatter profiles, and 10 000 events per sample were collected. FITC-dextran uptake by the parasites in resealed erythrocytes was monitored using an additional gate based on the green/yellow fluorescence ratio (R_{gy}) of FITC-dextran to select parasites that had the probe in an acidic DV (see Figure 2). Analysis was performed using FCS Express Version 3 (De Novo Software), FlowJo (Tree Star, Inc.) or Microsoft Excel spreadsheet (methods of analysis was developed by Dr. Nick Klonis, La Trobe University, Melbourne, Australia).

RESULTS AND DISCUSSION

Characterization of FITC-dextran as a pH indicator

The use of a dextran-conjugated FITC for measuring the DV pH by flow cytometry has not been reported previously. The measurement of two emission signals of FITC-dextran incorporated into resealed erythrocytes permits correction for non-specific experimental fluctuations that can compromise a single-wavelength intensity measurement (Muller-Borer *et al.*, 1998). Therefore, the feasibility of FITC-dextran as a pH indicator in saponin-permeabilized erythrocytes was assessed. Infected erythrocytes resealed to contain FITC-dextran were permeabilized by saponin to release any fluorescent marker from the host cell cytoplasm so that the parasite-associated

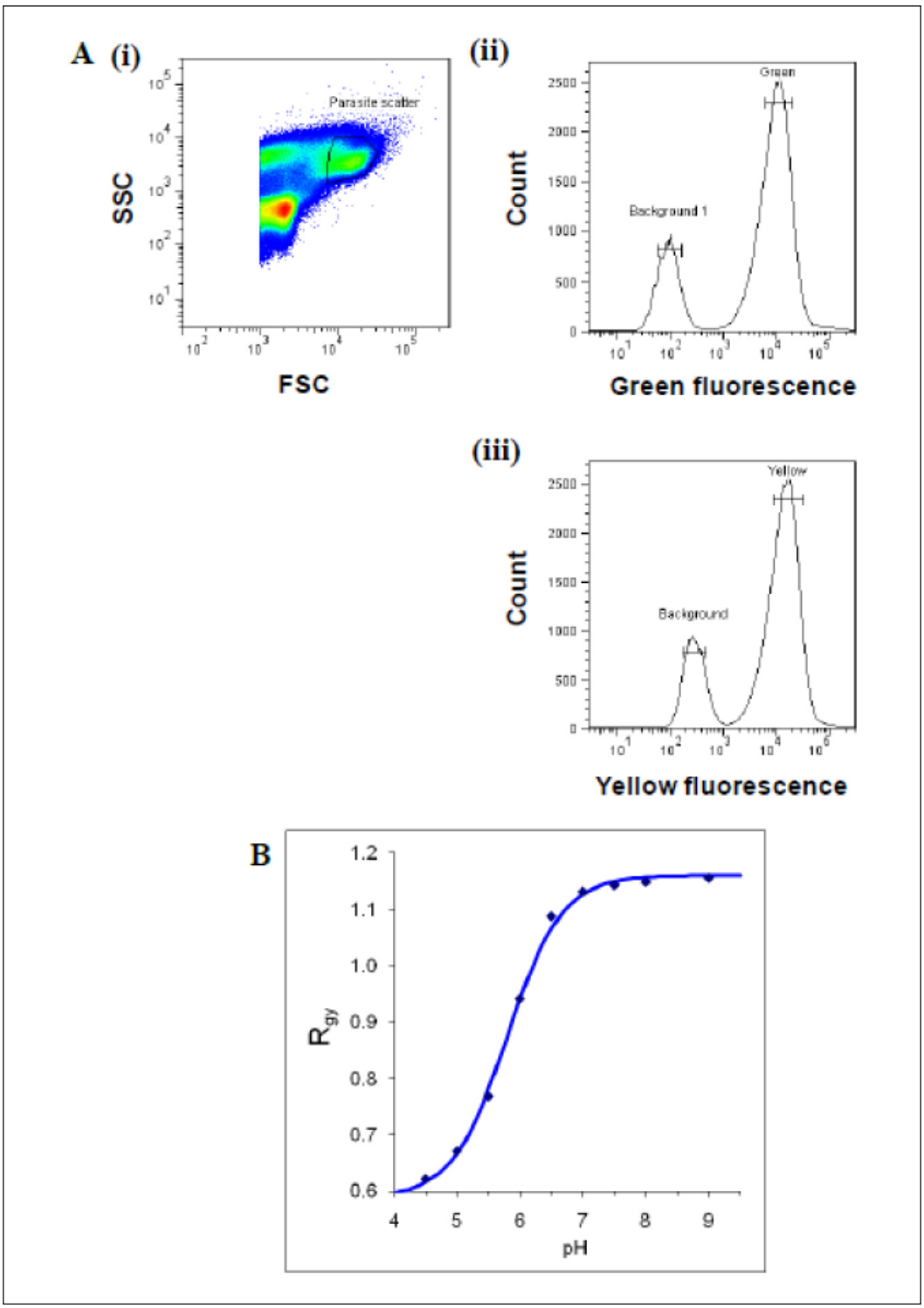

Figure 2. Characterization of FITC-dextran as a pH indicator in saponin-permeabilized cells

(A) Saponin-permeabilized cells in which the DV was preloaded with FITC-dextran were suspended in buffer (with CCCP) and analysed by flow cytometry. (Ai) The scatter profile of the cell population is shown. The parasitized population was gated as a 'parasite scatter'. (Aii) The green and (Aiii) yellow intensity distributions of the gated population are displayed in the histograms. (B) A pH calibration curve was generated using FITC-dextran in saponin-permeabilized cells.

FITC-dextran can be quantitated (Figure 1). Lengthy exposure of the parasites to saponin was avoided to minimize damage of the parasite plasma membrane. These cells were suspended in buffers of different pH in the presence of the ionophore, CCCP. This causes all parasite compartments to equilibrate with the pH of the external buffers. The fluorescence microscopic examination of the cell preparation was made and showed that the DV remained intact as indicated by the retention of FITC-dextran within the DV of parasites (data not shown).

The population of saponin-permeabilized cells (gated as "parasite scatter") was first identified based on the scatter profile shown in Figure 2Ai. The gate of the parasitized cells was established by comparing samples from uninfected cells (labelled or non-labelled) (data not shown). Figure 2Aii-iii show representative fluorescence histograms of the scatter-gated population of the saponin-permeabilized cells. The gated population exhibited peaks of fluorescence in both green and yellow channels. The higher signal represents fluorescence from the parasites with intact DVs. The lower signal (background) might represent debris or parasites with non-fluorescent DVs arising from parasites that had invaded intact erythrocytes during the inoculation procedure. The higher fluorescence peak from each of the channels was gated (Figure 2Aii-iii, double arrows) and the geometric mean of the fluorescence intensities was used to calculate the ratio and generate the pH calibration curve.

A calibration curve for FITC-dextran, based on the relationship between pH and ratio (R_{gy}) of the fluorescence intensities emitted at the green and yellow channels, were constructed (Figure 2B). The R_{gy} is I_g/I_y where I_g and I_y correspond to the fluorescence intensities in the green and yellow channels, respectively, which background intensities subtracted from each event. FITC-dextran displayed a characteristic sigmoidal response to changes in pH. The acid-base transition of FITC-dextran is depicted by a decrease in R_{gy} values with decreasing pH with the point of inflection corresponding to an apparent p*Ka* of ~5.8. The relationship between FITC fluorescence and pH is independent of the concentration or ionic environment of the fluorochrome (Klonis & Sawyer, 1996). The pH range over which the change in the FITC-dextran signal is linear was ~5.0-7.0. Given its response profile, FITC-dextran can be used to determine the DV pH at pH values <6, which cannot be measured by SNARF-1-dextran with p*Ka* of >7.0 (Abu Bakar *et al.*, 2010). A similar p*Ka* value was obtained from the pH calibration curve of FITC-dextran in digitonin-permeabilized erythrocytes (data not shown). Digitonin was used to release any fluorescent marker from the host cell and parasite cytoplasm so that the DV-associated FITC-dextran can be exclusively quantitated. This confirms that the R_{gy} measurement can comfortably distinguished between FITC-dextran located in the DV and that present in a neutral or alkaline environment and regardless of whether the fluorescence signal was measured in saponin- or digitonin-permeabilized parasites. Thus, FITC-dextran was employed to determine the DV pH in saponin-permeabilized parasites.

Measurement of the DV pH in saponin-permeabilized erythrocytes

The pH of the DV in saponin-permeabilized parasites was measured by flow cytometry. Normalized distributions of the R_{gy} values using saponin-permeabilized cells suspended in buffers of different pH in the presence of CCCP were used as pH calibration curves as shown in Figure 3A. When the parasites were suspended in a medium containing glucose (20 mM), the DV showed a R_{gy} value of 0.72 ± 0.02 (n = 6, three experiments) corresponding to an apparent pH value of 5.27 ± 0.03 (Figure 3B, solid curve) (using a loading concentration of 50 µM FITC-dextran). This was 0.23 pH unit lower than that estimated by Hayward *et al.* (2006) (5.50 ± 0.14; 40 ìM FITC-dextran). Other authors have reported different pH values of the DV in the range 4.3- 4.7 using the same probe (Geary *et al.*, 1986; Geary *et al.*, 1990). There are two alternative explanations of these findings. The variation might be due to the use of different loading concentrations of FITC-dextran (Hayward

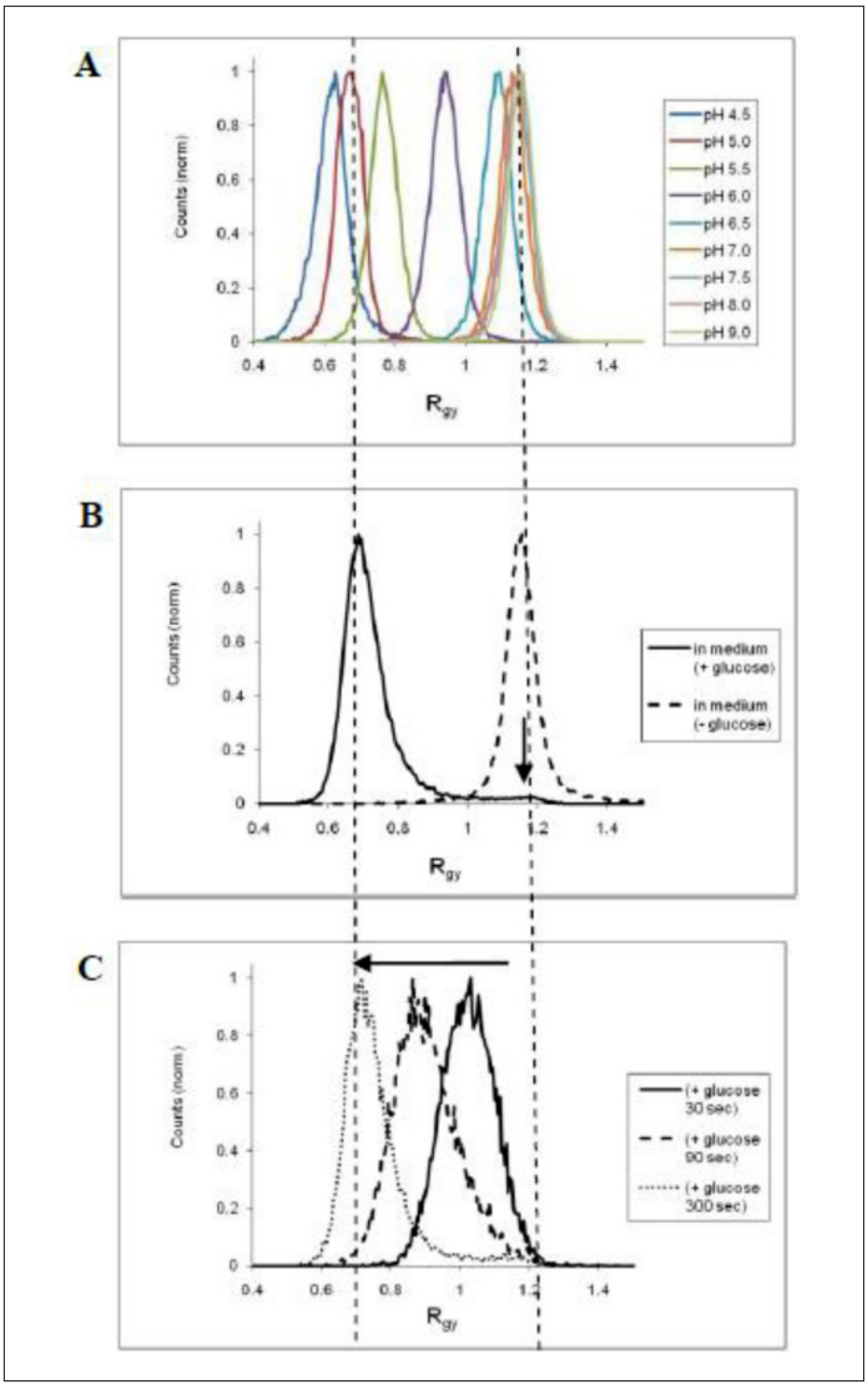

Figure 3. Distribution of the FITC-dextran fluorescence ratio (R_{gy}) of the saponin-permeabilized cell population

(A) Normalized R_{gy} distributions of the gated cell population, which were suspended in buffers of different pH (with CCCP), were used to generate pH calibration curves. A shift of the R_{gy} curve with pH was observed. The vertical dashed lines correspond to pH 5 and 7. (B) The alkalization of the DV in no glucose-containing medium and (C) the re-acidification of the DV upon the restoration of glucose (20 mM) as shown by the shift of the R_{gy} distribution of the cell population to the left (indicated by the arrow).

et al., 2005). An optimal level of FITC-dextran during the loading step is essential as overloading may result in the probe concentration being too close to the buffering capacity of the DV (Saliba *et al.*, 2003; Hayward *et al.*, 2006). A second possibility is that the variation might be due to fluorescence quenching of the dye resulting from resonance energy transfer between fluorescein and heme molecules (Stocks *et al.*, 2007). In this study, the characteristic of the fluorescence response of FITC-dextran obtained under cell-free conditions (data not shown) was very similar to those observed *in situ* with the point of inflection corresponding well with the given p*Ka* for the dye (i.e. 6.0-6.4, provided by Invitrogen).

When the cells were suspended in a medium without glucose, the R_{gy} distribution of the DV shifted to the right (Figure 3B, dashed curve) indicating an alkalinization of the DV (pH = 7.10 ± 0.10, R_{gy} = 1.13 ± 0.07) (n = 6, three experiments). This effect was rapidly reversed (within ~5 minutes) by the restoration of glucose to the medium (Figure 3C, indicated by the horizontal arrow towards the left). These observations may be explained by the lack of ATP supplied by glucose in the medium, which has the ability to acidify the DV in saponin-permeabilized parasites (Saliba *et al.*, 2003). These authors also demonstrated that the specific V-type H^+-ATPase inhibitors, concanamycin A and bafilomycin A_1 caused a pronounced alkalinization of the DV suggesting the presence of a V-type H^+-ATPase on the *P. falciparum* DV. The presence of this H^+ pump resembles the situation in the acidic tonoplasts of plant cells (Maeshima, 2001) and the acidocalcisome of a number of protozoan parasites (Schott & Docompo, 2000; Docampo & Moreno, 2001).

Overall, these data validate FITC-dextran as a pH indicator and is suitable for quantitative analysis of pH changes under the conditions employed. The methods that we have developed may represent useful tools for investigating the comparisons of the DV pH of CQ-sensitive and -resistant parasites. An increase in DV pH would be expected to decrease the level of CQ accumulation via the weak base trapping effect and could account for the development of CQ resistance. Surprisingly, however, some studies reported a decreased DV pH in CQ-resistant strains by which the heme solubility is probably decreased, thus decreasing CQ accumulation (Bennet *et al.*, 2004). Other studies failed to find any differences in the DV pH of CQ-sensitive and -resistant parasites (Kuhn *et al.*, 2007). The methods that we have developed may also represent useful tools for investigating the mechanisms by which the DV pH is maintained. Direct physiological evidence for the presence on the DV membrane of two discrete H^+ pumps, V-type H^+-ATPase and H^+-pyrophosphatase, can be performed. Moreover, the use of H^+ pump inhibitors can demonstrate the inhibition of ATP hydrolysis by isolated DVs. Furthermore, the flow cytometry-based system is more convenient than the fluorescence microscopic assays when processing large numbers of samples. The impact of fluorescence microscopy in research cannot be denied as it is well suited to the resolution of cell and tissue architecture, and to following kinetic responses in single cells.

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