Subcellular pH and Ca^{2+} in *Plasmodium falciparum*: Implications for understanding drug resistance mechanisms

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Live cell imaging plays an ever increasing role in the investigation of the human malaria parasite *Plasmodium falciparum*. For example, fluorescence microscopy and fluorescence spectroscopy, in combination with ion specific fluorochromes, have been used to determine steady-state and dynamic pH and Ca^{2+} concentrations within different compartments of the infected erythrocyte, including the parasite's digestive vacuole that is thought to be the target of several important antimalarial drugs. The data obtained, using live cell imaging, have led to the hypothesis that the digestive vacuole serves as a dynamic intracellular Ca^{2+} store or that changes in the pH gradient across the digestive vacuolar membrane are associated with certain drug resistance phenotypes. However, both these hypotheses and the underpinning data have been challenged on the grounds that alternative explanations are possible to account for the data obtained. Here we review the known problems that can arise when imaging live *P. falciparum*-infected erythrocytes.

Keywords: Chloroquine, live cell imaging, malaria.

LIGHT microscopy plays an important role in cell biology. At the turn of the nineteenth century, light microscopy was instrumental in the description of the living world and in the discovery of cellular organisms and subcellular structures. In recent decades, specific fluorescent probes and new advances in microscopic techniques, such as confocal laser scanning microscopy, have revolutionized the application, enabling the investigation of biological processes in living cells, in real time and under physiological conditions. While early microscopy has been descriptive, today's expanded application range allows for analytical, quantitative and three-dimensional investigation, with an emphasis on live cell imaging. Current applications of live cell imaging include quantification of ion concentrations in cellular compartments, monitoring ion fluxes and signalling events, protein sorting and trafficking processes.

Because of its potential, live cell imaging has been widely applied to study the biology and pathophysiology of the human malarial parasite *Plasmodium falciparum*. *P. falciparum*, being responsible for severe malaria, invades both human hepatocytes and erythrocytes, and it is in the latter where the pathogenesis of malaria develops. Live cell fluorescence microscopy has been used to study several processes, including pH homeostasis, Ca^{2+} signalling, protein trafficking and cell motility, in an effort to identify novel targets for rational intervention. While the data obtained, e.g. regarding subcellular pH and Ca^{2+} homeostasis, were initially considered breakthroughs in the field, concerns were soon raised regarding the interpretation of the data. This review focuses on the various complications known to arise when imaging *P. falciparum*-infected erythrocytes.

Subcellular pH determinations

pH homeostasis plays an important role in the pathophysiology of *falciparum* malaria, such as host cell exploitation and responses to antimalarial drugs. Accordingly, baseline pH values and the mechanisms underpinning pH homeostasis in different parasite compartments have been of interest for several decades.

During intraerythrocytic development, *P. falciparum* ingests large amounts of haemoglobin to meet its nutrient requirements[1] and to maintain osmotic stability within the host cell[2]. The haemoglobin taken up by endocytosis is hydrolysed in the parasite's digestive vacuole. The enzymes, cysteine and aspartic proteases, involved in haemoglobin proteolysis, have reported pH optima in the range 4.5–5.0 (ref. 3), suggesting that the digestive vacuole maintains an acidic environment. A low digestive vacuolar pH further promotes biomineralization of haem to haemozoin[4,5], which seems to constitute the primary pathway used by the parasite to detoxify the large quantities of haem released during haemoglobin degradation[6].

Several quinoline and alkylamine antimalarial drugs, including chloroquine and quinine, are thought to exert their antimalarial activity by interfering with haem detoxification[7–12], resulting in a build-up of toxic haem complexes that perforate membranes[13,14]. Since many of the quinoline drugs are amphiphilic weak bases, their uptake, and possibly their cytotoxicity, is determined in part

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by passive diffusion along the pH gradient between the intracellular and extracellular compartments[15]. Accordingly, it has been proposed that the observed accumulation (to toxic levels) of these amphiphilic drugs is a partitioning effect brought about by the steep pH gradient across the digestive vacuolar membrane[16]. Reports showing that increasing the intra-digestive vacuolar pH by blocking the digestive vacuole's inwardly-driving proton pump or overwhelming it (by adding permeable proton-binding weak bases) reduced chloroquine accumulation[17], supports this view. On the basis of these findings, some authors proposed a 'partitioning model' of chloroquine resistance to explain the reduced chloroquine accumulation observed in resistant parasites[17,18]. According to this model, chloroquine-resistant parasites have an elevated digestive vacuolar baseline pH value[17], which in turn would result in reduced chloroquine accumulation – below toxic levels.

Several efforts were made to obtain accurate readings of the steady-state digestive vacuolar (pH_{vac}) and cytoplasmic pH (pH_{cyt}) values in *P. falciparum*, knowing the importance pH plays in haemoglobin degradation and haem detoxification, as well as in drug partitioning and possibly drug resistance. However, the results obtained have been controversial and a consensus is yet to be reached.

The first estimates of pH_{vac} were based upon the experimentally determined distribution ratio of chloroquine and methylamine and provided a value of 4.9–5.0 (ref. 16), which was lowered to 4.2–4.5 in subsequent studies[19–21]. Direct photometric determinations were initially made on detergent-lysed cells using the membrane-impermeable pH sensitive fluorescein-dextran, which was ingested into the digestive vacuole by endocytosis from the cytoplasm of preloaded erythrocytes. Using this approach, pH_{vac} values ranging from 5.2 to 5.4 were reported[16,22]. In 1989, Ginsburg *et al.*[21] investigated populations of intact *P. falciparum*-infected erythrocytes, using acridine orange in a spectrofluorometric setting, and estimated pH_{vac} to be approximately 4.2. Acridine orange is a lipophilic, readily membrane-permeable weak base that can accumulate in acidic compartments, where the protonated molecule forms aggregates fluorescing above 655 nm, with the absolute fluorescence intensities inversely correlating with pH. Applying this approach to chloroquine-resistant (CQR) and chloroquine-sensitive (CQS) parasites, a significantly higher digestive vacuolar pH value was reported for CQR parasites[19]. These data seem to validate the partitioning model of chloroquine resistance.

Roepe and colleagues refined acridine orange fluorimetry and examined individual live cells under continuous perfusion, using an epifluorescence microscope[23,24]. Surprisingly, and contrary to the study mentioned above, a lower pH_{vac} was reported for CQR as compared to CQS parasites (5.21 ± 0.04 versus 5.64 ± 0.03). The allegedly less acid pH_{vac} value of CQR parasites is paradoxical, as it would predict increased chloroquine accumulation in these parasites, contrary to the actual finding[8,25,26]. The authors explain this discrepancy by suggesting that the decreased pH_{vac} would enhance haem turn over and biomineralization kinetics, thereby reducing the amount of haem available for chloroquine binding[23,24,27].

Adding further confusion to the field, two other studies failed to detect any significant differences in digestive vacuolar pH between CQS and CQR parasites[28,29]. Hayward *et al.*[28] used spectrofluorometric measurements on populations of parasites isolated from their host cells by saponin-permeabilization of the erythrocyte plasma membrane. Using a range of dextran-linked pH-sensitive fluorescent dyes (dextran-linked-fluorescein and Oregon Green), pH_{vac} values ranging from 4.5 to 4.9 were observed for both CQS and CQR parasites[28]. They further noted extreme fluctuations in pH_{vac} from 3.7 to 6.5, depending on the type and concentration of the fluorochrome used[28]. In a most recent development, Kuhn *et al.*[29] employed a non-invasive technique based on parasites expressing the ratiometric pH-sensitive green fluorescent protein pHluorin[30] in the digestive vacuole. Single cell confocal measurements suggested a pH_{vac} of 5.18 ± 0.05, with no significant differences observed between the CQS and CQR parasite.

A similar controversy exists regarding baseline cytoplasmic pH in *P. falciparum* and whether there are differences between CQS and CQR parasites. Some studies support this view[31], while other studies do not[32–34]. What is the basis of these discrepancies? Are there rational explanations?

P. falciparum is small

Studies on intracellular pH in *P. falciparum*-infected erythrocytes have been complicated by the small size of the specimen, the numerous subcellular compartments and the resulting difficulties in the spatial resolution. Depending on the stage of the parasite, its size varies from approximately 1 to 6 μm. Organelles, such as the nucleus and the digestive vacuole, hardly reach sizes larger than 1.5 μm, rendering the quantification of signals within these compartments difficult. The large amount of haemoglobin present within the infected erythrocyte is another complicating factor. Haemoglobin and its prosthetic group haem are highly photoactive and can cause interference with excitation and emission signals.

Several studies have addressed these problems by investigating parasites isolated from their host cells using detergent-lysis[28,35] or, using more extensive treatment with detergents by examining crude digestive vacuolar preparations[16,22]. While these approaches reduced the complexity of the system, the techniques create new problems of their own. Difficulties could arise from detergent treatment, lysis of subcellular compartments and

subsequent exposure of isolated parasites or digestive vacuoles to a potentially non-physiological ion and metabolite environment, with unpredictable consequences on intracellular pH.

Another factor that needs to be considered when determining ion concentrations using fluorimetric approaches is the spatial resolution of the detection device. Fluorochromes distribute throughout the cell, thus fluorescent signals from one compartment can potentially interfere with fluorescence signals from another subcellular compartment. It is difficult, if not impossible, to separate the specific fluorescence signals of loaded fluorochromes from, e.g. the parasite's digestive vacuole and surrounding cytoplasm, when using epifluorescence microscopy or spectrofluorometry. Only more sophisticated detection devices, such as confocal microscopes, can achieve this spatial separation. Nevertheless, previous determinations of cytoplasmic and digestive vacuolar pH in *P. falciparum* have employed epifluorescence microscopy or spectrofluorometry[16,21–23,27,28,31,35,36].

P. falciparum is light sensitive

P. falciparum-infected erythrocytes display an unexpected light sensitivity that results in irreversible damage to intracellular membranes[37]. Even moderate to low exposure to light, which readily occurs during standard fluorescence microscopy, can generate large quantities of hydroxyl radicals (Figure 1 *a*), which in turn induce lipid peroxidation (Figure 1 *b*)[37]. This chain reaction results in the lysis of the digestive vacuolar membrane and the subsequent release of its acid load into the parasite's cytoplasm[37,38]. The photosensitivity of the parasite is explained by the presence of a parasite-specific photo-sensitizer[37], the likely candidate being haem. Haeme is a highly photoactive compound that is present in large quantities within the parasite. Haeme contains iron, a transition metal ion, which – when present as ferrous iron (Fe^{2+}) – catalyses the Fenton reaction, thereby yielding hydroxyl radicals ($OH^{\bullet}$) as follows,

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}.$$

Ferric iron reacts with superoxide ion ($O_2^{-\cdot}$) to recycle ferrous iron. The parasite generates both hydrogen superoxide (H_2O_2) and ($O_2^{-\cdot}$) in substantial quantities as a result of haemoglobin degradation[39]. The hydroxyl radical is a highly reactive oxygen species that can attack all classes of biomolecules, but lipids are the most likely targets[40,41]. Particularly susceptible are polyunsaturated fatty acids, since reactions with hydroxyl radicals proceed as a self-perpetuating chain reaction (Figure 1 *b*)[40,41]. Polyunsaturated fatty acids are typically enriched in vacuolar membranes[42], which may explain the sensitivity of the digestive vacuole to light under conditions where the parasite's plasma membrane and the erythrocyte plasma membrane remain intact. Lipid peroxidation dramatically alters membrane fluidity and permeability and may eventually induce widespread membrane damage, including disruption[40,41]. Although the parasite possesses natural defence mechanisms against reactive oxygen species, such as a superoxide dismutase[43], glutathione peroxidase[44,45] and a catalytic activity originating from the host erythrocyte[46], these are apparently readily out-competed when the parasite is exposed to light (Figure 1).

Difficulties arising when using acridine orange

Special caution is due when interpreting acridine orange fluorescence data. Acridine orange has complex spectral properties, depending on concentration and interaction with cellular macromolecules. While monomeric acridine orange emits green fluorescence of approximately 530 nm, aggregates that form at higher concentrations emit fluorescence of approximately 655 nm[47]. Since acridine orange is a lipophilic weak base, it readily passes through membranes and accumulates in acidic compartments, where the protonated molecule forms aggregates fluorescing in the 655 nm range[47,48]. These properties have made acridine orange a useful tracker and lysosomes or other acidic organelles[47]. However, aggregation and, hence, the red fluorescence signal intensity, depends on pH, acridine orange concentration, temperature, and ionic composition of the compartment investigated[48]. Further complicating the issue, acridine orange also shows red fluorescence when bound to single-stranded RNA; when bound to double-stranded DNA it shows green fluorescence. Furthermore, acridine orange is a non-ratiometric fluorochrome, thus calibrating fluorescence signals is difficult, with the intensity of the fluorescence signal measured being affected by dye concentration, bleaching effects and focus shifts during measurement.

Adding to the high photosensitivity of *P. falciparum*, acridine orange acts as a photosensitizer of oxidative re-

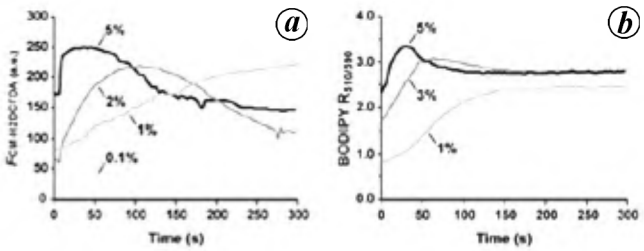

Figure 1. The digestive vacuole of *P. falciparum* is light sensitive. ***a***, Effect of light on the production of hydroxyl radicals. The temporal changes in fluorescence as a function of light transmission were plotted. The decline in fluorescence observed for 5% and 2% light transmission is due to the lysis of the digestive vacuole and the subsequent dilution of the dye within the cell. a.u. arbitrary units. ***b***, Effect of light on lipid peroxidation.

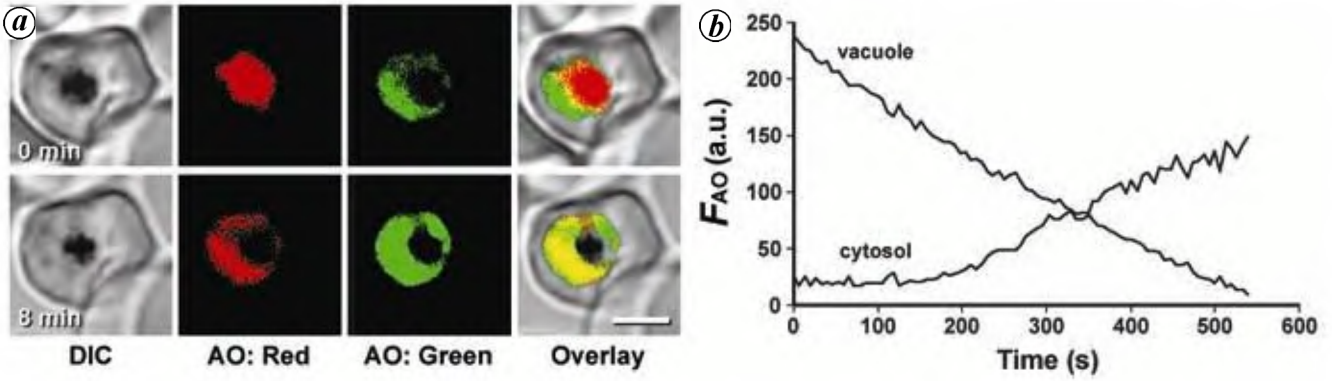

Figure 2. Acridine orange staining pattern in *P. falciparum*. ***a***, Confocal images of an infected erythrocyte stained with acridine orange are shown at the start and after 8 min. Excitation was at 488 nm and emission detected from 505 to 610 nm (green channel) and above 650 nm (red channel). Scale bar, 5 µm. ***b***, Quantification of the time course of the vacuolar and cytosolic red fluorescence intensities. a.u. arbitrary units.

actions[49]. Some images of acridine orange stained infected-erythrocytes, used for pH determination, show fluorescence patterns typical of a cell damaged by light exposure. The crescent-shaped acridine orange fluorescence pattern seen in these images was later identified as the parasite's cytoplasm[37,50], and not the digestive vacuole (Figure 2 *a*). This crescent-shaped fluorescence pattern typically arises after photolysis of the digestive vacuolar membrane, brought about by light-induced lipid peroxidation, and subsequent release of the digestive vacuole's acid load into the cytoplasm (Figure 2 *b*)[37].

Another factor to consider, when using acridine orange in live cell imaging, is the possibility of transport processes that act on the dye. Acridine orange is a known substrate of multi-drug resistance (MDR) transporters[51]. *P. falciparum* has an MDR1 homologue, termed Pgh-1, residing in the digestive vacuolar membrane, with its ATP-binding domain facing the cytoplasm[52,53]. A recent study has determined the direction in which Pgh-1 pumps and found it to transport substrates into the digestive vacuole[54]. Moreover, polymorphic variants of Pgh-1 that are associated with different responses to antimalarial drugs, including chloroquine[55,56], have altered substrate specificities[54]. Taking this into consideration, possible differences in digestive vacuolar acridine orange fluorescence[23,27] may result from different Pgh-1-mediated dye handling rather than changes in digestive vacuolar pH.

Other studies have implicated PfCRT, another digestive vacuolar transporter linked to drug-resistance[57] and a member of the drug/metabolite superfamily[58,59], in altered acridine orange transport. Acridine orange shares structural similarities with quinacrine, an antimalaria drug that exhibits cross-resistance with chloroquine[60,61] and which, like chloroquine, is a putative substrate of PfCRT[26,62].

Determining pH using green fluorescent proteins

Many of the difficulties mentioned above have been addressed using pHluorin, a ratiometric pH-sensitive green fluorescent protein[30]. pHluorin was expressed in genetically engineered parasites and targeted to the cytoplasm and digestive vacuole respectively (Figure 3), where the fluorescence signals were monitored in single live cells using confocal microscopy[29]. As far as we currently know, this is the least invasive approach to quantify intracellular pH. The integrity of the cell is preserved, facilitating measurements under physiological conditions. Since pHluorin is targeted to specific subcellular compartments, fluorescence signals are clearly correlated with a defined region with the cell.

A steady-state pH_{cyt} of approximately 7.15 ± 0.07 and a steady-state pH_{vac} of approximately 5.18 ± 0.05 were determined for trophozoites, with no significant differences in CQS and CQR parasites. These determinations are consistent with several previous reports[16,22,28,35,36]. The comparable pH_{vac} found for CQS and CQR parasites[28,29] supports the view that mechanisms other than altered drug partitioning and/or pH-dependent changes in haem solubility, turn-over or biomineralization rates are responsible for the chloroquine-resistant phenotype and the significantly reduced drug accumulation observed in CQR parasites. Current models of chloroquine resistance propose that CQR parasites have acquired either a carrier or a channel for chloroquine, the former actively expelling the drug[26,57,62–67], the latter facilitating its passive diffusion[61,67,68] out of the parasite's digestive vacuole.

Subcellular calcium determinations

In the malaria parasite, Ca^{2+} signalling is involved in numerous processes, such as invasion[69–71], maturation[72], synchronization[73], and exflagellation and gamete formation[74,75]. Its cytosolic concentration is regulated by the assembly of several transporters present in the plasma membrane, endoplasmic reticulum, mitochondria and acidocalcisomes. For example, a putative calcium channel resides in the plasma membrane of infected erythrocyte[76]. PMCA-type Ca^{2+}–ATPases (PfATP6 (ref. 77);

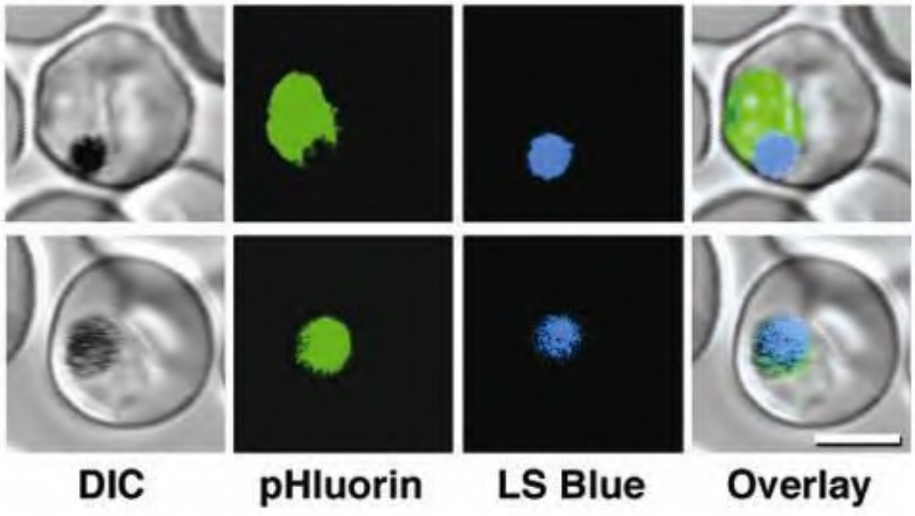

Figure 3. Confocal live cell images showing the expression of pHluorin in the cytosol (above) and digestive vacuole (below) of *P. falciparum*. The digestive vacuole was identified using the acidotropic fluorochrome LysoSensor Blue-DND 192 (LS Blue). Scale bar, 5 μm.

PfATP4 (ref. 78)) and a putative Ca^{2+}/H^{+} exchanger have been identified[79]. Furthermore, a family of six putative calcium-dependent protein kinases (CDPKs) and one CDPK-related kinase were found. *P. falciparum* calcium-dependent protein kinase 1 (PfCDPK1) is located in the membrane and organelle fraction of the asexual blood stages of the parasite[80]. PfCDPK4 was shown to play a key role in cell cycle regulation during male gamete formation[81]. PfCDPK3, originally suggested to be involved in sexual stage-specific events and possibly serving as a link between calcium and gametogenesis in *P. falciparum*[82] has recently been shown to be required for ookinete gliding motility and mosquito midgut invasion[83].

Calcium is a well-known second messenger in eukaryotic cells and its binding to various proteins is responsible for the activation of different cellular functions. Erythrocytes, where *P. falciparum* spends most of its asexual life cycle, are devoid of intracellular Ca^{2+} stores and have low cytosolic Ca^{2+} concentrations. The lack of extracellular Ca^{2+} poses a problem that the parasite must solve. Thus, calcium homeostasis of *P. falciparum*-infected erythrocytes has been the subject of intense research in the past. Since calcium regulation in the parasite may differ from processes occurring in other eukaryotic cells, this divergence may be used to find new target therapies.

To study the dynamics of Ca^{2+} in this system, optical techniques – in combination with Ca^{2+}-sensitive fluorescent dyes – have mainly been applied, with only one study making use of a GFP-aequorin construct to identify Ca^{2+} signalling in the parasite[81]. The various techniques and calcium indicator dyes used have given a variety of results, producing much confusion. This section investigates what is known about *P. falciparum* calcium homeostasis and tries to shed some light on the discrepancies found.

Identifying the Ca^{2+} stores of P. falciparum

The total calcium concentration of *P. falciparum*-infected erythrocytes increases with parasite maturation, with levels far exceeding those of uninfected erythrocytes[84]. Since high cytosolic concentrations of Ca^{2+} are toxic and could lead to cell death, eukaryotic cells have developed mechanisms for sequestering Ca^{2+} in intracellular compartments or stores. Parasitic compartments implicated in storing calcium include the parasitophorus vacuole, the endoplasmatic reticulum (ER), the mitochondria, and an acidic compartment. For the parasitophorus vacuole, a high Ca^{2+} concentration of approximately 40 μM was reported[72,85]. These determinations were made using populations of infected erythrocytes in a spectrofluorimetric set-up. Parasites were allowed to invade erythrocytes in the presence of the cell-impermeant Ca^{2+} indicator Mag-Fura-2, which was added to the culture medium, resulting in its accumulation in the parasitophorus vacuole.

Another reservoir for free Ca^{2+} is the ER. The parasite seems to maintain high Ca^{2+} concentrations within its ER[86], using a sarco/endoplasmic reticulum Ca^{2+}–ATPase (SERCA). The pharmacological properties of the SERCA are controversially discussed. One study suggests that both cyclopiazonic acid (CPA) and thapsigargin (TG) inhibit this enzyme[87], whereas other studies only see an inhibitory effect using CPA[78,86].

Mitochondria are also known to participate in intracellular Ca^{2+} storage. Using isolated *P. falciparum* parasites loaded with the mitochondrial Ca^{2+} indicator Rhod-2 and the cytosolic Ca^{2+} indicator Fluo-3, it was demonstrated that mitochondria were able to accumulate Ca^{2+} once cytosolic Ca^{2+} concentrations were increased[88].

Perhaps the most debated calcium storage is the acidic store. Several studies have identified an acidic calcium

store in *P. falciparum*[86,89], with properties similar to the acidocalcisome[90], an electron-dense organelle containing high levels of calcium, phosphorus, magnesium, and other elements, with most of the calcium bound to polyphosphates[91]. X-ray microanalysis detected an electron-dense organelle rich in phosphorus and calcium in *P. falciparum* merozoites[92] and was proposed to be the acidic calcium store. A later study, however, suggested the parasite's digestive vacuole to be the acidic Ca^{2+} store[93]. This conclusion was based on the preferential Fluo-4 staining of the digestive vacuole in the *P. falciparum* strain investigated.

Imaging the digestive vacuole using Fluo-4

Fluo-4 is a sensitive Ca^{2+} indicator. Its fluorescence properties are strongly pH dependent, with fluorescence intensity decreasing with increasing pH (Figure 4 *a* and *b*)[38]. The parasite's cytoplasm and the acidic digestive vacuole have pH values that differ by more than two orders of magnitude[29], which, in turn, affects Fluo-4 fluorescence intensity, indicating that fluorescence signals from the digestive vacuole and the cytoplasm cannot readily be compared.

Another disadvantage of Fluo-4 is its non-ratiometric nature, complicating the calibration of fluorescence signals. A further concern is that Fluo-4 belongs to a group of fluorochromes that are known substrates of P-glycoproteins (P-gp)[94]. Thus, a bright Fluo-4 staining of the digestive vacuole may not be an indication of a high Ca^{2+} concentration, but rather result from compartmentalization of the dye by the parasite's digestive vacuolar Pgh-1. Biagini *et al.* [93] took this into consideration and tested the effect of the P-gp inhibitor verapamil and observed no effect on Fluo-4 staining. However, a subsequent study demonstrated that the parasite's digestive vacuolar Pgh-1 was not affected by verapamil, in combination with Fluo-4 (ref. 54).

To complicate the situation, when various parasite strains were loaded with Fluo-4, two distinct staining phenotypes were observed[54]. Some parasites showed an intense Fluo-4 staining of the digestive vacuole, as previously seen[93] (Figure 5 *a*), while others showed a diffuse fluorescence

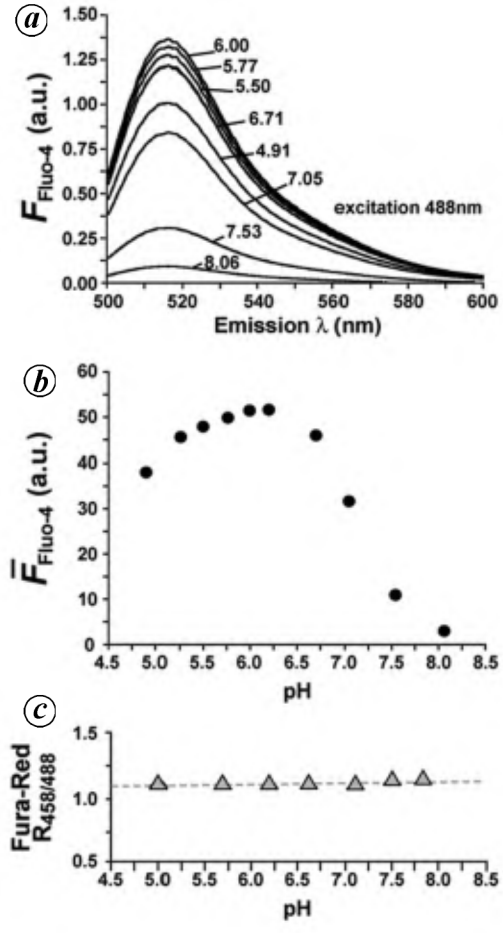

Figure 4. pH dependency of fluorochrome fluorescence. ***a***, Fluo-4 fluorescence is pH dependent. The emission spectra were recorded with a spectrofluorometer using fixed free Ca^{2+} concentrations and varying pH. ***b***, The integrated fluorescence-pH relationship (*y*-axis) for a long pass filter of 505 nm, as used when quantifying Fluo-4 fluorescence in *P. falciparum*. ***c***, Fura-red fluorescence is not pH dependent. The integrated fluorescence-pH relationship (*y*-axis) for a long pass filter of 570 nm, as used when quantifying Fura-Red fluorescence in *P. falciparum*. The ratio ($R_{458/488}$) is largely pH independent. a.u. arbitrary units.

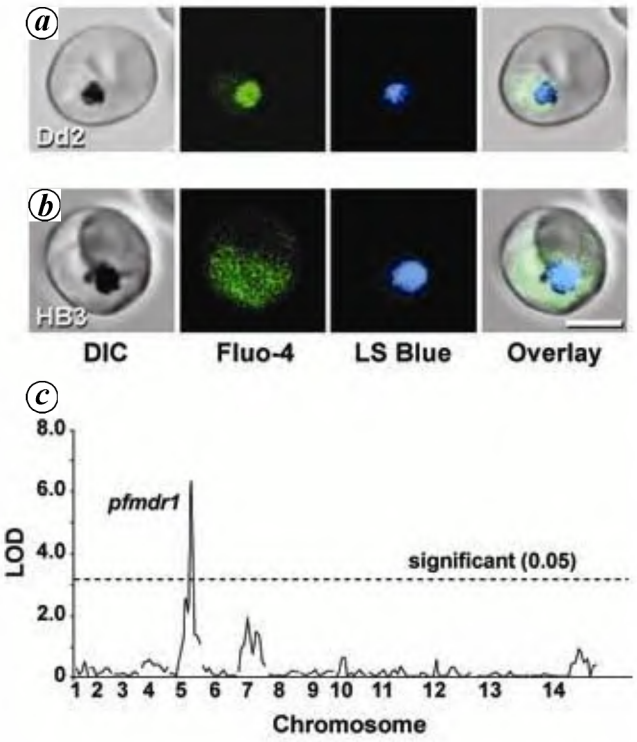

Figure 5. Differences in the Fluo-4 fluorescence staining pattern of *P. falciparum*-infected erythrocytes of (***a***) Dd2 and (***b***) HB3 parasites. The digestive vacuole was identified using the acidotropic fluorochrome LysoSensor Blue-DND 192 (LS Blue). Scale bar, 5 µm. ***c***, Linkage of the Fluo-4 staining pattern to *pfmdr1*. QTL analysis, using Fluo-4 $R_{vac/cyt}$, revealed a locus on chromosome 5, having a LOD score > 6, associated with the *pfmdr1* marker.

of the entire parasite (Figure 5 *b*). At first sight, this would suggest major clonal differences in Ca^{2+} homeostasis. Since most cells tightly regulate their Ca^{2+} homeostasis, the idea that some parasites use the digestive vacuole as a Ca^{2+} store while others do not seems doubtful. Indeed, no difference in digestive vacuolar-free Ca^{2+} concentrations exist between these parasites, as demonstrated using the ratiometric Ca^{2+} indicator Fura-Red[54], which is structurally distinct from Fluo-4.

An alternative explanation for the Fluo-4 phenotypes involves altered fluorochrome handling. In line with this hypothesis, the digestive vacuolar Fluo-4 staining pattern responded to several established P-gp inhibitors, providing evidence of dye transport into the digestive vacuole. Moreover, segregation of the Fluo-4 staining pattern with *pfmdr1* in a genetic cross yielded a LOD score of > 6, suggesting a strong linkage with *pfmdr1* (Figure 5 *c*). Investigating several genetically engineered *pfmdr1* mutants confirmed the linkage of the digestive vacuolar Fluo-4 staining phenotype with certain *pfmdr1* polymorphisms[54]. Thus, Pgh-1 acts on Fluo-4 in a variant-dependent manner, pumping it into the parasite's digestive vacuole. Considering its unfavourable pH-dependent spectral properties and it being a substrate of Pgh-1, Fluo-4 is not suited to quantify Ca^{2+} concentrations in *P. falciparum*.

The Pgh-1 variants mediating Fluo-4 transport into the digestive vacuole are those that have been implicated in altered responses to quinine, mefloquine, halofantrine and artemisinin[55,56]. Thus, Fluo-4 imaging can be used as an *in vivo* diagnostic tool to identify Pgh-1 variants involved in altered drug responses. The finding that some of these drugs compete with Pgh-1-mediated Fluo-4 transport[54] may suggest that these drugs are either substrates or inhibitors of Pgh-1. Transport of antimalarial drugs into the digestive vacuole may be advantageous for those drugs that target cytoplasmic factors, resulting in their deposition and sequestration in a subcellular compartment where they are less harmful.

Another Ca^{2+} indictor is Fura-2, a UV light-excitable ratiometric fluorochrome that has been mainly used in wide field fluorescence microscopy. Its chemical structure differs from that of Fluo-4. Photo-bleaching is eliminated due to the ratiometric nature of the dye. However, the isosbestic point of Fura-2 shifted from 360 nm to approximately 380 nm when loaded in the parasite, thus preventing its use as a ratiomentric dye in a confocal setting with the 351 and 364 nm laser lines[38]. The shifting of the isosbestic point to a longer wavelength is a well-known phenomenon that occurs in several cell-based systems[95].

The most reliable Ca^{2+} indicator is Fura-Red. Unlike Fluo-4, Fura-Red is a ratiometric dye that is not transported by P-gp or by the parasite's homologue Pgh-1. Furthermore, pH differences and photo-bleaching do not affect recordings due to the ratiometric nature of this dye. Fura-Red fluorescence ratios can be readily calibrated *in situ*. Using Fura-Red in a single live cell confocal set-up, Ca^{2+} concentrations of approximately 450 nM were measured in the digestive vacuole of trophozoite stage parasites[38].

Quantifying cytosolic Ca^{2+} concentrations

Perhaps the most challenging is the quantification of cytosolic free Ca^{2+} in the malaria parasite. Various techniques and fluorochromes have been used to determine calcium levels. In one study, isolated parasites that were released from their host cells by saponin permeabilization showed resting Ca^{2+} concentration in the cytosol in the range of 50–150 nM when these were incubated in Ca^{2+}-free medium[86]. The Ca^{2+} concentration increased to 740 nM when the parasites were incubated in a medium containing 1 mM Ca^{2+} (ref. 86), suggesting that the extracellular-free Ca^{2+} concentration markedly affects the intracellular Ca^{2+} homeostasis. Other studies estimated the cytosolic Ca^{2+} concentration to be approximately 50 nM and 350 nM, using Fluo-4 (see above)[93] and Fura-Red[38].

During intraerythrocytic development, *P. falciparum* builds up an extensive ER that fills most of the parasite's cytosol[38]. Since the resolution of the microscopes available today cannot differentiate between signals arising from the parasite's cytosol and the ER, quantifying Ca^{2+} in the cytosol of *P. falciparum* using Ca^{2+} indicator dyes is problematic. Superposition of fluorescence signal from both the cytosol and the ER are likely to occur, resulting in artificially high free cytosolic Ca^{2+} concentrations. Perhaps aequorin, pericam or other ratiometric Ca^{2+} sensitive GFP-derivatives[96,97] expressed in the parasite's cytosol can help overcome these restrictions and enable accurate determinations of free cytosolic Ca^{2+}.

Conclusion

Live cell microscopy, in combination with fluorescent indicators and ion-specific GFP-derivatives, has provided new insights into the physiology and transport mechanisms in *P. falciparum*. Further insight is expected using photoactivated GFPs (paGFP)[98], GFP timers (pTIMER)[99] and FRET-based sensors[100,101] in combination with live cell imaging. Improvement in imaging techniques, particularly a higher axial resolution, would be beneficial in detecting and quantifying signals coming from within the small subcellular compartments of *P. falciparum*. For example, 4Pi microscopes have shown a considerable improvement of the axial resolution by superposing two illumination beams and by adding coherently the two wave fronts emitted by the luminescent sample[102,103]. Noninvasive axial sections of 80–160 nm thickness deliver more faithful images of subcellular features, providing a new opportunity to significantly enhance our understanding of cellular structure and function. We can look forward to a multitude of new experimental oppor-

tunities for malaria researchers through the advances in live cell microscopy.

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