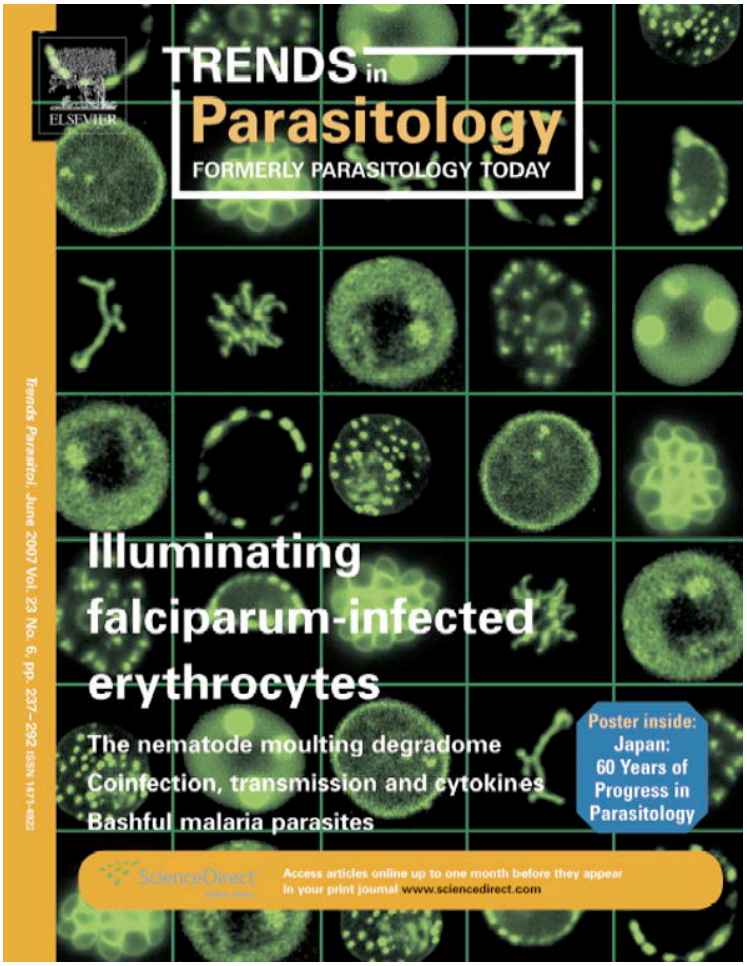

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Illuminating *Plasmodium falciparum*-infected red blood cells

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The malaria parasite undergoes a remarkable series of morphological transformations, which underpin its life in both human and mosquito hosts. The advent of molecular transfection technology coupled with the ability to introduce fluorescent reporter proteins that faithfully track and expose the activities of parasite proteins has revolutionized our view of parasite cell biology. The greatest insights have been realized in the erythrocyte stages of *Plasmodium falciparum*. *P. falciparum* invades and remodels the human erythrocyte: it feeds on haemoglobin, grows and divides, and subverts the physiology of its hapless host. Fluorescent proteins have been employed to track and dissect each of these processes and have revealed details and exposed new paradigms.

Life in an erythrocyte – a cell biology challenge

The malaria parasite *Plasmodium falciparum* spends part of its life cycle in the unusual niche of a terminally differentiated mature human red blood cell (RBC). Development of the parasite within this normally quiescent environment results in remarkable modifications to the RBC that support the growth and multiplication of the parasite. The growing parasite expands its core complement of organelles needed for metabolism (i.e. nucleus, endomembrane system, mitochondria and apicoplast) and also develops novel organelles, including a modified lysosome (referred to as the digestive vacuole) and a series of apical organelles (rhoptries, dense granules and micronemes) [1]. Moreover, the parasite manages to export some of its proteins beyond the confines of its own plasma membrane (PM) and the parasitophorous vacuole (PV) membrane out to the surrounding RBC, which results in extensive modifications of the host-cell cytoplasm and PM (for reviews, see Refs [2–4]). Eventually, the RBC membrane becomes distorted with knobby protrusions that are created by the deposition of parasite proteins such as the knob-associated histidine-rich protein (KAHRP) and the adhesion protein *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is inserted into the RBC membrane. To bring about these dramatic changes in the structure and function of the RBC, the parasite has developed a complex system for trafficking proteins to destinations outside its own cell [3,4].

The novel subcellular compartments and unusual trafficking pathways of *P. falciparum* are of substantial cell biological interest. Unfortunately, obtaining morphological information about the organization of the structures within parasitized RBCs has been hampered by poor preservation of samples during preparation for electron microscopy and immunofluorescence. A revolution in the knowledge regarding the generation and organization of different organelles has occurred because of recent transfection experiments that employ fluorescent protein chimeras of parasite components. Fluorescent fusion proteins provide a molecular paint box that can be used to label different compartments in living *P. falciparum* and provide unprecedented images of the cellular landscape. They have enabled cell biologists to follow trafficking to different compartments in real time and to unravel signals that direct proteins to particular destinations.

Generating *P. falciparum* transfectants that express fluorescent protein chimeras

The first successful attempt at transfecting *P. falciparum* was reported over ten years ago [5]. Since then, several hundred different gene constructs have been successfully introduced into malaria parasites [6,7]. The introduction of constructs that express fluorescently tagged proteins, particularly green fluorescent protein (GFP), has been key to understanding cellular organization and dynamics. A detailed description of vectors and transfection methodology is provided by Crabb *et al.* [8]. Additional information and references are provided in the supplementary material online.

This article reviews recent uses of fluorescent proteins to study parasite development and division and presents a series of images that depict organelle growth and trafficking events. Some properties of fluorescent proteins that make them suitable for cell biology applications are given in Box 1. Some useful fluorescence microscopy imaging formats are listed in Box 2. This article concentrates on the intraerythrocytic phase of *P. falciparum* but fluorescent proteins have also been used to visualize other life-cycle stages, other malaria species [9–13] and related parasites such as *T. gondii* [14–16].

Cellular compartments within the malaria parasite

The endoplasmic reticulum and the Golgi

Transgenic parasites that express GFP flanked by the endoplasmic reticulum (ER) entry and retention signals of *P. falciparum* immunoglobin binding protein (PfBiP)

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Box 1. Fluorescent proteins as a molecular paint box

- GFP, a naturally fluorescent gene product from the jellyfish *Aequorea victoria,* can be expressed with proteins fused to either the N or C terminus.
- Enhanced GFP is monomeric, shows good biological and photostability and is readily imaged with the fluorescein filter sets available on most laboratory microscopes.
- Mutation of residues within or near the chromophore generates proteins with a range of spectral characteristics.
- Additional fluorescent proteins from corals and anemones extends the color palette into the red and far-red range and provides photoactivatable fluorescent proteins.
- The low oxygen environment that is used in malaria cultures does not seem to restrict the rate of maturation of the GFP chromophore, and GFP seems to be relatively nontoxic to malaria parasites.
- Although artifacts due to overexpression or mistargeting of fusion proteins are always possible, transfectants that express fluorescent proteins provide an excellent resource to examine the cellular landscape of parasitized RBCs.

Box 2. Some fluorescence microscopy formats

- Conventional (epi)fluorescence microscopy
  - Readily available, relatively inexpensive, multiple colour imaging available.
  - Can be coupled to sensitive digital cameras for long-term imaging with low light levels and/or to special stages to permit 3D deconvolution.
  - Out-of-focus light compromises image quality.
- Confocal laser scanning microscopy
  - Confocal optics rejects out-of-focus light and enables image formation from a single focal plane. This permits imaging of thin optical sections of samples without physically sectioning cells and is good for live cell imaging.
  - 1.4-fold increase in resolution compared with conventional light microscopy.
  - A 3D reconstruction of a specimen can be generated by stacking 2D optical sections.
  - Photobleaching methods are relatively easy to implement.
- Wide-field imaging with digital deconvolution
  - Uses knowledge of the point spread function to remove out-of-focus light.
  - Maximizes light collection and enables 3D reconstruction.
- Spinning-disk confocal microscopy
  - Uses an array of hundreds of pinholes in a rapidly spinning disk.
  - Simultaneous collection of data through multiple pinholes enables faster imaging (but somewhat lower z resolution) than scanning confocal instruments.
  - Ideal for following and quantitating fast processes in live cells.

have been generated [17] (Figure 1a). The ER is initially visualized as a perinuclear ring with two small protrusions that develop into an extended reticular network as the parasite enlarges [17]. Transfectants that express a GFP fusion of a small GTPase protein (*P. falciparum* secretion-associated Ras-related protein: PfSar1p) associated with the cytoplasmic surface of the ER have also been generated [18]. PfSar1p-GFP undergoes rapid lateral diffusion within the plane of the ER membrane but is concentrated in regions that probably represent ER exit sites. Photobleaching analysis indicated that the ER systems of individual merozoites remain connected until late in schizogony.

Transfectants that express a GFP chimera of the *cis*-Golgi protein *P. falciparum* Golgi reassembly stacking protein (PfGRASP-GFP) (Figure 1b) indicate that the *cis*-Golgi is a single, perinuclear structure in ring-stage parasites. In the trophozoite stage, two 'horns' are formed adjacent to but distinct from the ER protrusions [19]. PfGRASP-GFP occupies a compartment that is separate from the compartment that is marked by the *trans*-Gogli marker PfRab6 [19]. The *cis*-Golgi undergoes division in synchrony with the nucleus, which leads to the presence of multiple organelles in more mature-stage parasites (Figure 1b). Each daughter merozoite is furnished with a single Golgi during division of the schizont [19].

In the absence of any signal sequence information, GFP is synthesized on ribosomes in the cytoplasm and remains in this compartment [20] (Figure 1c).

The mitochondrion and the apicoplast

The mitochondrial lumen has been labelled with *Discosoma* red fluorescent protein (DsRed) or yellow fluorescent protein (YFP) fused to the leader sequences of citrate synthase [17,21] (Figure 1d) or other mitochondrial enzymes [22,23]. During the trophozoite stage, the mitochondrion is a single elongated or branched organelle that has looped regions and forms contacts with the PM. During schizogony, the branching is extended and the mitochondrion divides to provide an organelle for each daughter cell [17] (Figure 1d).

GFP or DsRed fused to the leader peptide of the apicoplast protein acyl carrier protein (ACP) (Figure 1e) is directed to the apicoplast [16,17,22]. In the ring stage, apicoplasts are observed as rounded puncta. During early schizogony, the apicoplast elongates and eventually begins to branch. Late in schizogony, the apicoplast undergoes a process of division [17] (Figure 1e). Trafficking of proteins to the apicoplast proceeds through the ER and requires an ER entry signal sequence followed by a plastid transit peptide [24], which might be processed by stromal-processing peptidase then degraded by falcilysin [25]. It is thought that some plastid targeted proteins might be routed through the PM [26]; however, other studies in *P. falciparum* and in *Toxoplasma gondii* argue against obligate transit through the Golgi [27,28].

The mitochondrion and the apicoplast are in close apposition in the merozoite and ring stages. As the two organelles undergo elongation and branching, they retain points of close association. The apicoplast divides before the mitochondrion and the two organelles segregate as a pair into daughter merozoites [17].

The digestive vacuole

The intraerythrocytic parasite feeds using a cytostome (mouth) to ingest small packets of haemoglobin from the host cytoplasm. The haemoglobin-containing vesicles are transported to an acidic digestive vacuole (DV) where the haemoglobin is degraded by the action of a series of proteases [29]. Trafficking of proteases to the digestive vacuole involves a circuitous route. For example, plasmepsin-GFP is directed into the ER and transferred to the cytostome for delivery to the DV [30]. In ring stage parasites, plasmepsin-GFP is visible in the ER and in cytostomal vesicles en route to the DV. In trophozoite stage

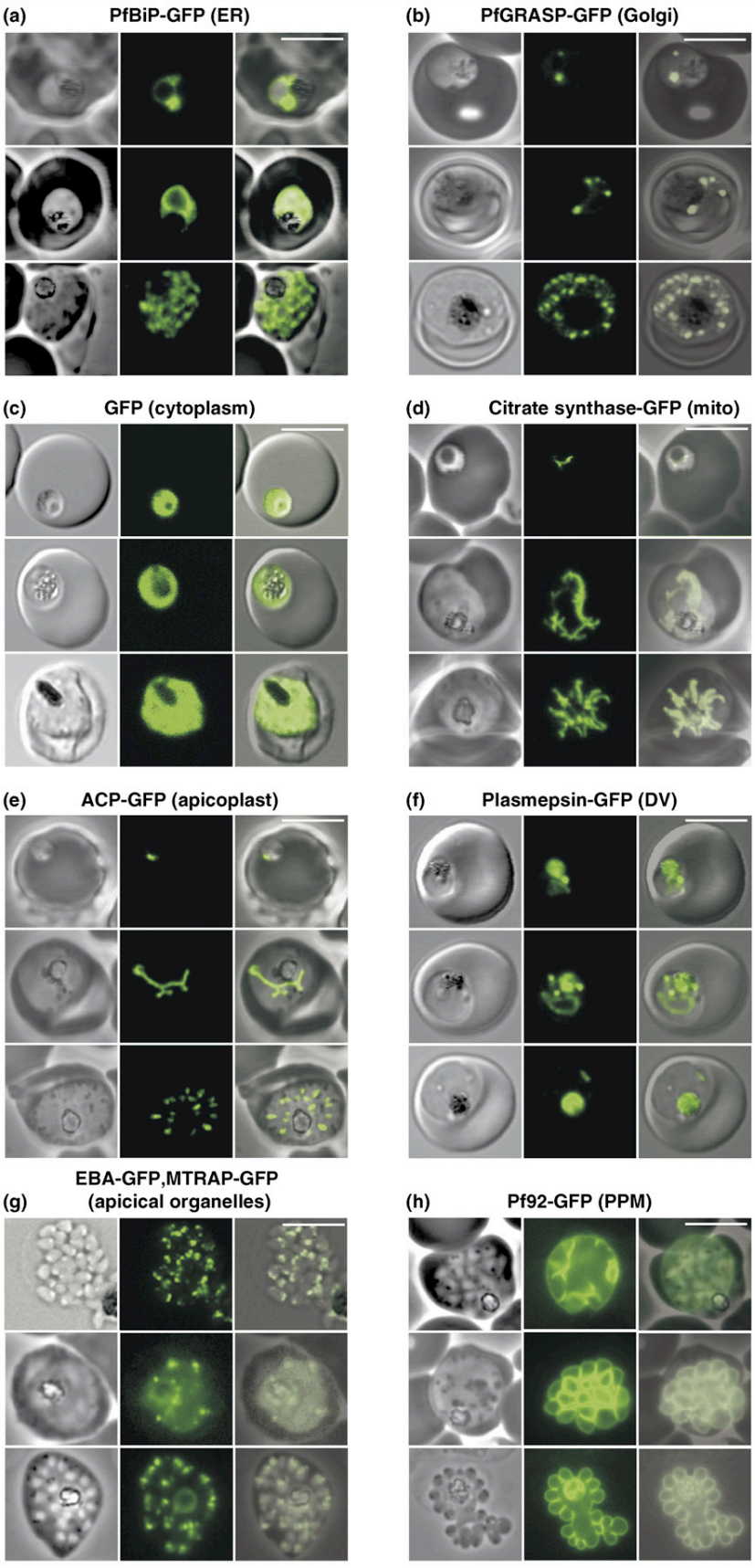

Figure 1. Transfected *Plasmodium falciparum*-infected RBCs expressing GFP chimeras directed to compartments within the parasite. The images represent a differential interference contrast (DIC) or phase image, the fluorescent protein signal and an overlay of these images. Where possible, early-, mid- and late-stage parasites are shown. **(a)** PfBip-GFP transfectant (ER). Ring and early trophozoite stage parasites show a perinuclear ring of fluorescence in the parasite cytoplasm. Schizont stage parasites show

parasites, it accumulates in the DV [30] (Figure 1f). Another DV protease, dipeptide aminopeptidase 1 (DPAP1), seems to accumulate in the PV before uptake into the cytostome and delivery to the DV [31]. The sequence motifs that direct proteases to the DV have not yet been identified; however, the N-terminal region of plasmepsin IV is sufficient to deliver a pH-sensitive GFP variant, pHluorin, to the DV [32]. This construct has been used to estimate the pH of the DV [32].

The apical organelles

Apicomplexan parasites have three sets of regulated secretory organelles at the apical end of their invasive forms: the rhoptries, the micronemes and the dense granules. These organelles have crucial roles in the invasion of RBCs by merozoites and in modifying the host cell after invasion [7,33].

Erythrocyte binding antigen 175 (EBA-175) is a micronemal protein that is transferred onto the surface of *P. falciparum* merozoites and then shed at or around the time of invasion [34]. The ectodomain of EBA-175 binds to glycophorin A and provides one of the invasion pathways for *P. falciparum* [35]. Transfected parasites that express GFP chimeras of EBA-175 have been generated [36] (Figure 1g, top row). Correct trafficking of EBA-175 requires an ER entry signal and a cysteine-rich region in the ectodomain, in addition to appropriate timing of expression [36,37]. The merozoite thrombospondin-related adhesive protein (TRAP) homologue is another micronemal protein for which a transfectant that expresses a GFP-fusion protein has been generated [38] (Figure 1g, bottom rows).

The ring-infected erythrocyte surface antigen (RESA) is a dense granule protein that is secreted into the PV then transferred to the RBC cytoplasm just after reinvasion [39]. Correct trafficking of a RESA-GFP chimera is also dependant on correct timing of expression [39]. It seems probable that proteins synthesized in late schizogony rely on a 'just-in-time' sorting strategy. That is, they might need to be synthesized at the precise moment when the secretory organelles for which they are destined are being formed or when appropriate escorters are available, as described for *T. gondii* [40].

A GFP-fusion protein that is directed to the rhoptries has not yet been generated in *P. falciparum* but precise timing of expression of apical membrane antigen 1 (AMA1) has been shown to be crucial for its correct trafficking to this compartment [41,42]. Trafficking to the apical organelles has been studied extensively in *T. gondii* [14,15,43].

The parasite PM

The parasite PM harbours a series of multi-transmembrane domain transporters that are involved in uptake of nutrients and ion homeostasis [2,44,45]. GFP-tagged versions of PM transporters are not available but a haemagluttinin-tagged phosphate transporter has been generated [44].

As the parasite matures, glycosylphosphatidylinositol (GPI)-anchored proteins form a coat on the surface of the developing merozoites [46,47]. Stable expression of GFP-GPI seems to be toxic to *P. falciparum*; however, an inducible system has been used to express GFP fused to a GPI-anchored Cys_6 protein referred to as Pf92 [47,48] (Figure 1h).

Recent work has shown that components of the unique submembranous motor complex of apicomplexa are present in *P. falciparum* merozoites and seem to function in RBC invasion [38]. A chimera of the glideosome-associated protein 45 concentrates at the merozoite periphery late in asexual development, consistent with the formation of the inner membrane complex [38].

Cellular compartments beyond the parasite PM

The PV lumen

P. falciparum proteins with an ER entry signal but no additional signalling information follow the default secretory pathway to the PV. GFP fusions with the classical signal sequences of the ACP exported protein 1 (Exp1) and histidine rich protein 2 and the noncanonical signal sequence of KAHRP are all released into the PV [16,49–51]. The Exp1 signal sequence linked to GFP displays a 'necklace of beads' pattern in the PV lumen, with occasional looped extensions that presumably represent the tubulovesicular network (TVN) (Figure 2a). Photobleaching analysis shows limited connectivity between subcompartments, indicating close apposition of the PV membrane and the parasite PM in some regions [49] (Figure 3 a,b). A wagon wheel pattern forms in schizont stages as the protein accumulates around individual merozoites (Figure 2a).

Crossing the PV membrane

Some proteins remain within the PV, whereas others are exported beyond the PV membrane to sites in the RBC cytoplasm or RBC membrane. The export process seems to involve a selective transporter in the PV membrane [3,52]. Recent studies that employed fluorescent protein transfectants [53,54] have identified a pentameric host targeting or protein export element (HT–PEXEL) motif that forms the core of the signal that mediates export of proteins across the PV membrane. Additional information in the surrounding amino acids is also likely to contribute to the signal [49]. PfEMP1 represents a second class of exported proteins. It lacks an N-terminal hydrophobic signal sequence and its export is thought to be determined by

rings of fluorescence around individual nuclei [17]. **(b)** PfGRASP-GFP transfectant (*cis*-Golgi). PfGRASP is located in a perinuclear compartment in ring stages that expands to form two ER-associated compartments in trophozoites and undergoes division to form multiple Golgi bodies in schizont-stage parasites [19]. **(c)** GFP alone (parasite cytoplasm). In the absence of any signal information GFP is expressed in the parasite cytoplasm. It is excluded from the DV but gains access to the nucleus [20]. **(d)** Citrate synthase (cit synth)-GFP transfectant [mitochondrion (mito)]. The mitochondrion is initially a single elongated or branched organelle. During schizogony, the branches extend and divide to provide a mitochondrion for each daughter cell. **(e)** ACP-GFP transfectant (apicoplast). In early stage parasites, the apicoplast is a punctate structure that elongates and branches as the parasite matures, then divides late in schizogony. **(f)** Plasmepsin-GFP transfectant (DV). Plasmepsin-GFP is visible in the ER and in cytostome-derived vesicles in ring stage parasites but accumulates in the DV as the parasite matures [30]. **(g)** EBA-GFP and MTRAP-GFP transfectants (micronemes). EBA175-GFP (top row) and MTRAP-GFP (bottom rows) are expressed under the control of a schizont stage promoter. The chimeras are restricted to the apical pole within each merozoite in schizonts and in free parasites [36,38]. **(h)** Pf92-GFP transfectant (parasite PM). Pf92-GFP is also expressed under the control of a schizont stage promoter. It is located at the parasite surface which invaginates around individual merozoites as the schiozont divides. Scale bar = 5 μm. Note: the intensities of the images were adjusted to optimise the fluorescence signal at each parasite stage.

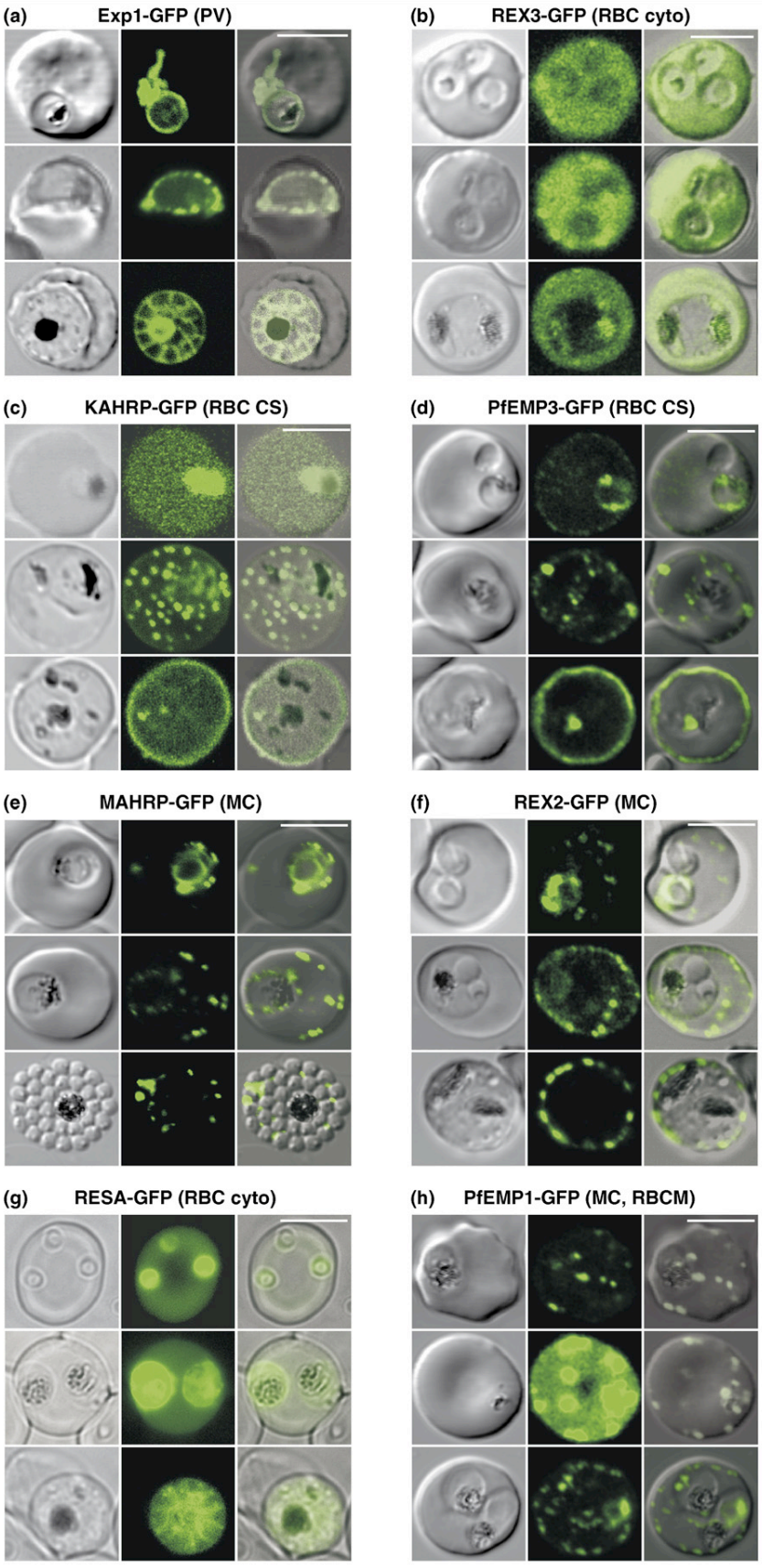

Figure 2. Transfected *Plasmodium falciparum*-infected RBCs expressing GFP chimeras directed to compartments within the host cytoplasm. The images represent a DIC image, the GFP fluorescence signal and an overlay of these images. **(a)** $Exp1_{1-35}$-GFP transfectant (PV). Some ring and trophozoite stage parasites show a 'necklace of beads' pattern, whereas some parasites show distortions and evaginations of the PV to form a TVN. Schizonts show a segmented pattern with a highly fluorescent central

PEXEL-related sequences in the N-terminal region of the protein [53,54].

The RBC cytoplasm

For some exported parasite proteins, such as the ring exported protein 3 (REX3), the bulk compartment of the host-cell cytoplasm seems to be the final destination [55] (Figure 2b). REX3 is one of a relatively small number of proteins expressed in early ring stages and might have a role in initial remodelling of the host-cell compartment. Photobleaching analysis indicates that REX3 could be present as part of a complex [55].

An intriguing question is how integral membrane proteins such as PfEMP1, the STEVORs (subtelomeric variable open reading frame proteins) and Rifins (repetitive interspersed family proteins) are trafficked across the RBC cytoplasm. Given that RBCs completely lack an endogenous vesicle-mediated transport system, the pathway for trafficking across the host-cell cytoplasm clearly involves an unusual mechanism. Early studies using antibodies raised against plasmodial vesicle machinery components led to the hypothesis that the malaria parasite exports trafficking machinery outside the boundaries of its own PM [56–58]. However, recent studies using GFP fusions do not support this proposal [18]. This indicates that the parasite sets up an entirely novel trafficking system that involves components without obvious homologues in other eukaryotes. GFP transfectants have been used to visualize these novel structures and to unravel the unusual transport mechanism.

The Maurer's clefts

Maurer's clefts are disc-like structures that appear at the RBC periphery as the parasite matures. They are thought to be a way station for proteins en route to the RBC membrane. Soluble proteins such as KAHRP and PfEMP3 are transiently associated with the cytoplasmic surface of the Maurer's clefts before redistribution to the cytoplasmic face of the RBC membrane [51,59] (Figure 2c,d, middle rows). Regions of basic amino acid sequence in KAHRP and PfEMP3 seem to be responsible for Maurer's cleft binding [50,51,60].

Several integral membrane proteins are Maurer's clefts residents [61]. These include the membrane-associated histidine-rich protein 1 (MAHRP1) [62], the skeleton binding protein 1 (SBP1) [63–65], the Maurer's cleft two transmembrane proteins (MC-2TM) [66], the STEVORs [67,68], REX1 and REX2 [55,69]. Knockout of SBP1 prevents export of PfEMP1 to the surface of infected RBCs, which indicates the potential importance of the Maurer's cleft-resident proteins [63,65].

The STEVOR, Rifin and MC-2TM families have a hydrophobic ER entry sequence and an HT–PEXEL motif [54,70,71] and presumably transit through the PV membrane by interacting with the same transporter that passages soluble cargo. The transmembrane domain of STEVOR-GFP is also needed for final sorting to the Maurer's clefts [68]. Rifin tagged with GFP in the region between the two transmembrane domains is exported to Maurer's clefts, whereas C-terminally GFP-tagged full-length chimeras are retained in the ER [3,70].

By contrast, MAHRP1, SBP1, REX1 and REX2 have no obvious PEXEL motifs [20,55,63,65]. MAHRP1-GFP accumulates in foci that emanate from the PV membrane before appearing at the Maurer's clefts (Figure 2e). A series of transfectants that express GFP chimeras of MAHRP1 fragments indicated that information within the second half of the N-terminal domain directs MAHRP1 to its final destination, whereas the transmembrane domain is required for entry into the ER [20]. Photobleaching analysis indicates that Maurer's clefts are not directly connected to each other or to the PV membrane or the RBC membrane (Figure 3e,f). REX2-GFP transfectants have also been generated and show similar behaviour to MAHRP1-GFP transfectants [55] (Figure 2f). Thus, a small vesicle-mediated system might not be necessary to transport resident proteins to the Maurer's clefts: they could be inserted into these compartments as they form.

The RBC membrane

Several exported proteins interact with the RBC membrane. For example, KAHRP is the main structural component of the so-called 'knobs', which provide a platform for the presentation of the cytoadherence protein PfEMP1. Analysis of transfectants that express GFP chimeras of KAHRP fragments indicates that KAHRP is released into the RBC cytoplasm in early stage parasites and transits the RBC cytoplasm as a large complex [51] (Figure 2c). KAHRP-GFP briefly associates with the cytoplasmic surface of Maurer's clefts before it is transferred to the RBC membrane [51] (Figure 2c). Photobleach analysis shows that the N-terminal region of KAHRP is sufficient for moderate binding to the RBC cytoskeleton (Figure 3c,d); however, the C-terminal repeat region is crucial for tighter binding and the formation of functional knobs [72,73].

PfEMP3 is another exported protein that associates with the RBC membrane [74]. PfEMP3 is not essential for knob formation but might have a role in PfEMP1

remnant body [49]. **(b)** REX3-GFP transfectant (infected RBC cyto). At each stage, REX3-GFP shows a homogeneous fluorescence pattern in the host-cell cytoplasm [55]. **(c)** $KAHRP_{1–123}$-GFP transfectant [RBC cytoskeleton (CS)]. In early ring-stage parasites, $KAHRP_{1–123}$-GFP is present in the parasite ER and PV. A weak fluorescence signal is observed in the infected RBC cytoplasm. The chimera is briefly associated with the Maurer's clefts before transfer to the cytoplasmic side of the RBC membrane [51]. **(d)** $PfEMP3_{1–500}$-GFP transfectant (RBC CS). In early ring-stage parasites, $PfEMP3_{1–500}$-GFP is present in the parasite endomembrane system with a weak signal in the infected RBC cytoplasm. The chimera is briefly associated with the Maurer's clefts before transfer to the cytoplasmic side of the RBC membrane. **(e)** MAHRP1-GFP transfectants [Maurer's clefts (MC)]. In early-stage parasites, puncta of fluorescence are observed emanating from the PV. These foci might represent nascent Maurer's clefts. In later-stage parasites (≥15 hours), peripheral Maurer's clefts are observed. In a burst schizont, the remnant Maurer's clefts remain associated with the lysed host cell membrane. **(f)** REX2-GFP transfectants (MC). Punctate fluorescence is observed at the PV membrane and in the cytoplasm in early stage parasites. REX2-GFP is concentrated in peripheral Maurer's clefts in mature-stage parasites. **(g)** $RESA_{1–117}$-GFP transfectants (dense granules and RBC cyto). $RESA_{1–117}$-GFP (expressed under control of its own promoter) is present within the parasite and in the RBC cytosol in ring and trophozoite stage parasites. Schizonts show merozoite labelling with some brighter puncta [38]. **(h)** $KAHRP_{119}$-PfEMP1-TM-ATS-GFP transfectants [RBC membrane (RBCM)]. A chimera with the ER signal and PEXEL from KAHRP appended to the transmembrane and C-terminal domains of PfEMP1 is trafficked to Maurer's clefts. A population is also evident in the infected RBC cytoplasm in over-exposed images (middle panel). Some of the chimera is redistributed to the RBC surface [60]. Scale bar = 5 μm. Note: the intensities of the images were adjusted to optimise the fluorescence signal at each parasite stage.

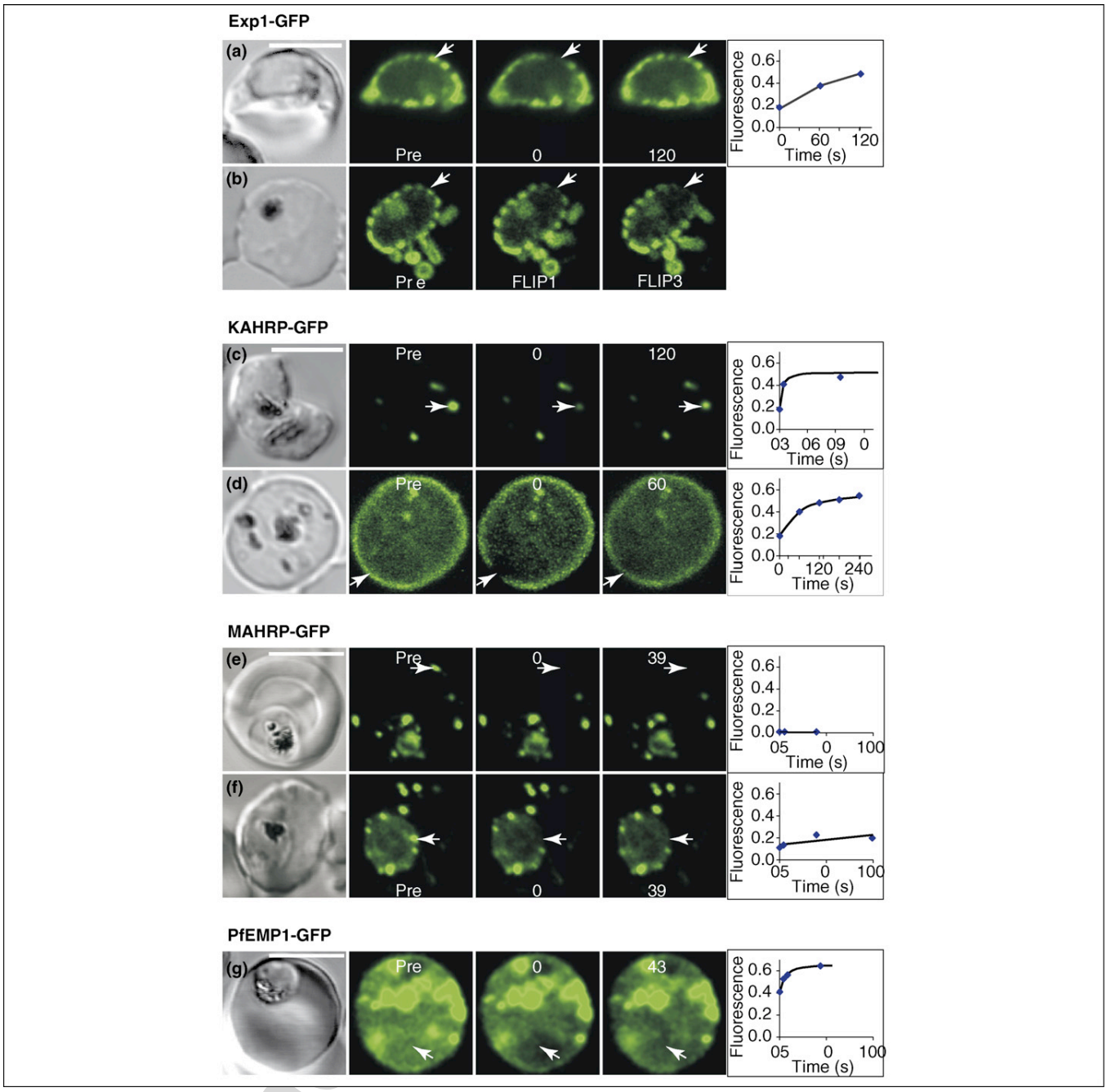

Figure 3. Analysis of the molecular organization of GFP chimeras in transfected *Plasmodium falciparum*-infected RBCs using fluorescence photobleaching. In each case, the first panel shows the DIC image. The fluorescence images (green) comprise prebleach (pre) and postbleach images at the times (seconds) indicated following the bleach pulse or the fluorescence loss in photobleaching (FLIP) images after repeated bleaching. The position of the bleach pulse is indicated by white arrows. The graphs show the recovery of fluorescence intensity over time in the bleached region relative to the prebleach level. **(a)** Trophozoite-stage $Exp1_{1–35}$-GFP transfectant showing a 'necklace of beads' fluorescence pattern. A high intensity laser pulse was applied. Partial recovery into this region was observed over two minutes indicating partial connectivity to surrounding regions. **(b)** $Exp1_{1–35}$-GFP transfectant subject to FLIP analysis. The region indicated by the arrow was repeatedly subjected to an intense laser pulse. Loss of fluorescence was restricted to regions near the bleach spot, which indicated connectivity between adjacent 'beads' but no connectivity to distal compartments. **(c)** $KAHRP_{1–123}$-GFP transfectant. The fluorescence associated with a Maurer's cleft was ablated. **(d)** $KAHRP_{1–123}$-GFP transfectant. Fluorescence associated with a region of the RBC membrane was ablated. Some recovery of the fluorescence was observed because of exchange between cytoplasmic and bound pools of the chimera. **(e)** Bleaching of MAHRP1-GFP in a tethered peripheral Maurer's cleft. **(f)** Bleaching of MAHRP1-GFP in a PV membrane-associated focal region (nascent Maurer's cleft). There is no recovery of the signal, which indicates that the Maurer's clefts are physically separate structures. **(g)** A $KAHRP_{119}$-PfEMP1-TM-C-term-GFP transfectant was imaged at high photomultiplier gain to visualize the faint fluorescence in the RBC cytoplasm. A region of the cytoplasm was subjected to a bleach pulse and recovery was observed over several seconds. The data are consistent with the presence of large complexes of the chimera in the RBC cytoplasm. Scale bar = 5 μm.

trafficking to the RBC surface [59,75]. PfEMP3-GFP is transported into the RBC as a protein complex and undergoes a brief association with the Maurer's clefts before it relocates to the cytoplasmic surface of the RBC membrane [60] (Figure 2d).

The N-terminal regions (~100 amino acids) of exported proteins such as KAHRP, PfEMP3 and RESA contain information for transit across the PV membrane but lack the information for binding to the host cytoskeleton [39,51,60]. GFP-chimeras of these protein fragments

display a homogeneous distribution in the RBC cytoplasm (e.g. $RESA_{1–117}$-GFP) (Figure 2g).

PfEMP1 is a major virulence protein of *P. falciparum*. There is currently some debate regarding the mode of trafficking of PfEMP1 to the RBC membrane. Recent studies showed that a chimera that comprised an ER entry signal, the KAHRP HT–PEXEL and the PfEMP1 transmembrane and C-terminal domains contains sufficient information for trafficking to the RBC membrane and presentation of the N-terminal region at the external surface [76] (Figure 2h). A substantial proportion of the K119-PfEMP1-GFP population remains associated with the Maurer's clefts (Figure 2h, top row) as is the case for endogenous PfEMP1 [77]. Transfectants that are imaged at high photomultiplier gain (Figure 2h, middle row) indicate a population of the chimera in the RBC cytoplasm. Photobleaching and electron microscopy analyses of these transfectants indicate that the GFP chimera is present in the RBC cytoplasm as a large protein complex rather than as a membrane-embedded protein [76] (Figure 3f). Thus, PfEMP1 might transit the host-cell cytoplasm as a chaperoned complex and only insert into a bilayer environment at the Maurer's clefts [78]. However, given the unusual nature of the chimera that is used in the K119-PfEMP1-GFP transfectant, additional studies that employ constructs that more closely resemble endogenous PfEMP1 are needed to confirm these initial findings.

Concluding remarks

A diagrammatic representation of putative trafficking pathways in *P. falciparum* is presented in Figure 4. Analysis of fluorescent protein transfectants has dramatically enhanced the view of cellular organization and the complex trafficking pathways of the intraerythrocytic parasite; however, much remains to be elucidated. The availability of multiple-colour fluorescent proteins proffers the opportunity of viewing interactions between different proteins and different compartments in live cells. The advent of photoactivatable fluorescent proteins provides the possibility of following the reorganization of compartments in real time

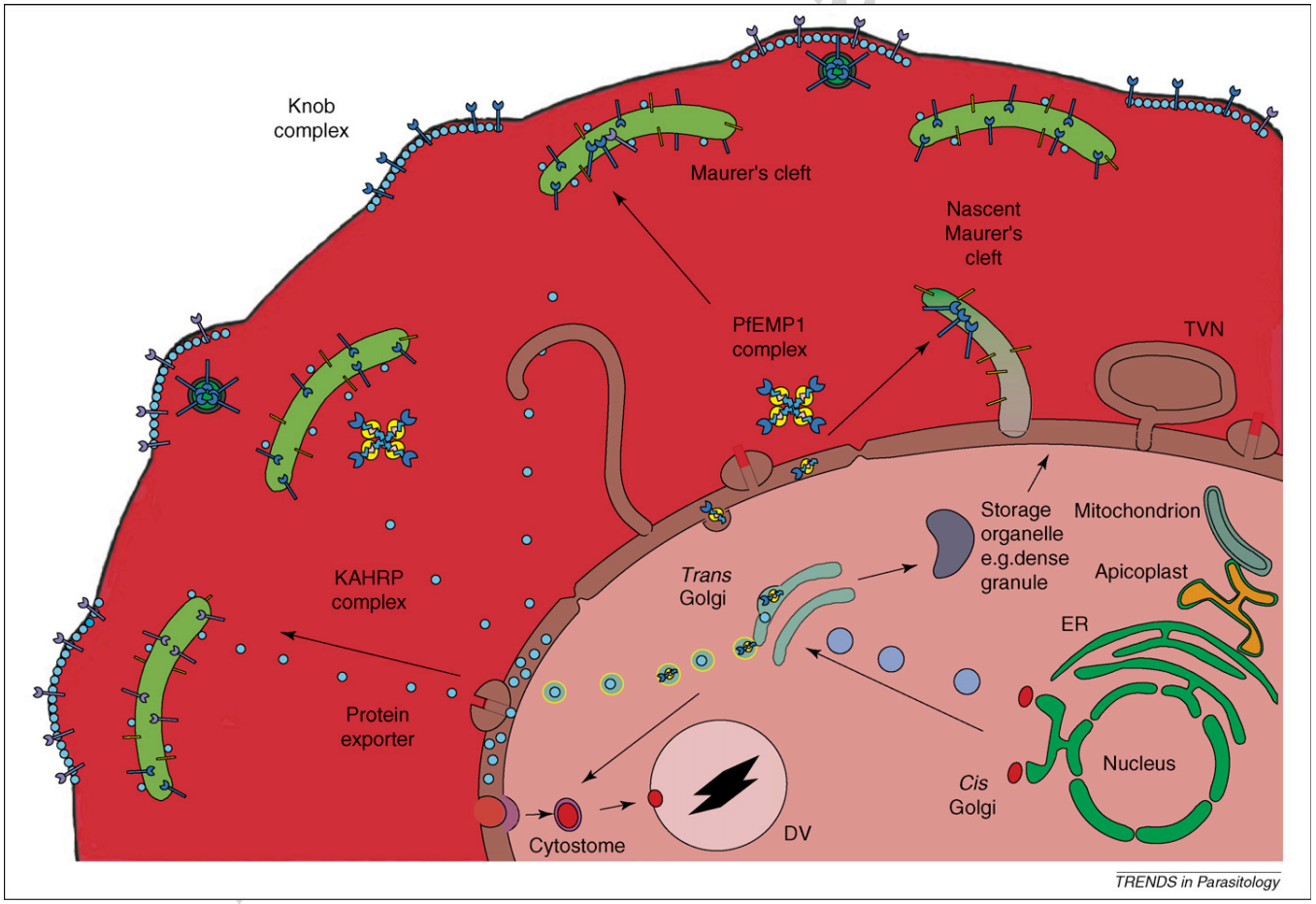

Figure 4. Diagrammatic representation of some putative trafficking pathways in a *Plasmodium falciparum*-infected RBC delineated using GFP transfectants. Soluble proteins destined for export are directed into the ER and pass through exit sites to a closely apposed *cis*-Golgi, then to a more distal *trans*-Golgi en route to the PV. Some proteins are retrieved from the PM or diverted from the ER or Golgi to intracellular organelles such as the DV and the apicoplast or (in the schizont stage) to regulated secretory compartments such as the dense granules, rhoptries and micronemes. Some proteins destined for locations beyond the PV membrane are first released into the PV. Recognition of a PEXEL motif enables translocation of proteins across the PV membrane. Exported soluble proteins can form complexes as they diffuse across the RBC cytosol and might interact with the cytoplasmic surface of the Maurer's clefts before redistribution to the RBC membrane skeleton. Some exported integral membrane proteins, such as PfEMP1, seem to be trafficked through the same PEXEL recognition machinery. The Maurer's clefts contain resident proteins, such as MAHRP1, that accumulate in these structures as they form. PfEMP1 might cross the RBC cytoplasm as a protein complex or might be inserted into nascent Maurer's clefts. The Maurer's clefts seem to bud from the PV membrane and form separate structures tethered to the RBC membrane. Some exported proteins lack a recognizable PEXEL motif and could use alternative pathways.

and the availability of novel fluorescent proteins that sense pH, calcium and oxidative stress provide new and exciting opportunities for probing particular compartments. There is available a molecular tool box that enables researchers to continue to ask crucial questions about the cell biology of this important pathogen.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pt.2007.04.001.

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