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# Molecular & Biochemical Parasitology



# A GFP-Actin reporter line to explore microfilament dynamics across the malaria parasite lifecycle

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# ABSTRACT

Malaria parasite motility relies on an internal parasite actomyosin motor that, when linked to the host cell substrate, propels motile zoites forward. Despite their key role in this process, attempts to visualize actin microfilaments (F-actin) during motility and under native microscopy conditions have not to date been successful. Towards facilitating their visualization we present here a *Plasmodium berghei* transgenic line in which a green fluorescent protein (GFP)-actin fusion is constitutively expressed through the lifecycle. Focused investigation of the largest motile form, the insect stage ookinete, demonstrates a large cytosolic pool of actin with no obvious F-actin structures. However, following treatment with the actin filament-stabilizing drug Jasplakinolide, we show evidence for concentration of F-actin dynamics in the parasite pellicle and at polar apices. These observations support current models for gliding motility and establish a cellular tool for further exploration of the diverse roles actin is thought to play throughout parasite development.

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MOLECULAR & BIOCHEMICAL

Apicomplexa are unicellular obligate intracellular parasites that infect a range of animal hosts and are the cause of significant human diseases including malaria and toxoplasmosis. Despite their specific adaptations to various host and target tissues apicomplexan parasites share a common cell architecture and mode of locomotion. This process, called gliding motility, underlies both cell motility and host cell invasion and relies on a conserved molecular machinery that is unique to the apicomplexan phylum in which secreted adhesins are linked with an internal actomyosin motor [1,2]. The motor is anchored within the supra-alveolar space [3], a compartment that lies between the outer plasma membrane and underlying inner membrane complex (IMC) of the motile parasite (or zoite). The current model for gliding motility has an actin microfilament (F-actin) forming the key rigid element upon which myosin bears to create the necessary rearward traction force for motility. However, the presence of F-actin in the supra-alveolar space has never been definitively described in any apicomplexan cell [4–6]. The most compelling evidence for a critical role of supra-alveolar F-actin comes from drug studies where treatment of

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parasites with actin-specific inhibitors that sequester free globular (G)-actin [7,8] or stabilize forming filaments [8–12] each prevent cell movement and host cell invasion. Electron microscopy images have shown microfilament-like structures, presumed to be composed of actin in *Toxoplasma gondii* tachyzoites following treatment with the actin stabilizing drug Jasplakinolide, JAS. These structures could be seen concentrated in patches at the parasite poles [10,11], associated with the parasite plasma membrane [11] and are consistent with a supra-alveolar localization of F-actin and its role in motility. However, no study to date has been able to visualize actin dynamics under native conditions. The difficulties associated with identifying microfilaments in their physiological state are likely explained by the predicted inherent instability of formed apicomplexan actin filaments [13], their short length and rapid turnover [14–16].

Towards exploring the spatiotemporal dynamics and localization of actin in motile malaria parasite lifecycle stages we generated a constitutively active fluorescently tagged actin in the mouse malaria parasite *Plasmodium berghei*. The N-terminus of *P. berghei* actin I (PBANKA\_145930) was tagged with green fluorescent protein (GFP) in the wild type *P. berghei* ANKA strain to preserve actin function. This strategy has been extensively used to visualize dynamics of the actin pool in a diversity of eukaryotic cells including *T. gondii* [11]. Constitutive expression of GFP-PbActin was achieved using the *P. berghei* elongation factor 1 alpha (*Pb*EF1α) promoter,



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**Fig. 1.** Expression of GFP-PbActin across the malaria parasite life cycle (A) Expression vector schematic of GFP-PbActin under the *Pbef1a* promoter for constitutive expression [17]. Green fluorescent protein (GFP) was excised from the PbGFP<sub>CON</sub> by digesting plasmids with BamHI. In its place a stitched PCR product joining GFP to the N terminus of PbActin was created (using primers GFPfwd 5'-GATCggatccATGAGTAAAGGAGAAGAACTTTCACTGGGAGA-3; GFPrev [PbAct overhang]5-TGAACTTCTGTCACCCATactagtTTTGATAGTTCATCCATGCGACTGTGT-3; PbActfwd [GFP overhang]5-GCATGGATGAACTATACAAAactagtATGGGGGAGAAGAGTTCAAGGCTTTAGTT-3; PbActTow 5-GATCggatccctaggTTAGAAGCATTTCTGTGGGACAATACTGG-3) that, when digested with *BamHI*, was re-ligated into the parent vector to create GFP-PbACT<sub>CON</sub>. This final vector was linearized at the unique *Apal* site to integrate into the genome by single crossover recombination (as per [17]). *P. berghei* parasite transfection, mouse (BALB/C) and mosquito infections, and sample preparation followed standard protocols [17] with all experiments approved by the Walter and Eliza Hall Institute Animal Ethics Committee (AEC) Project 2009-023. (B) Immunoblot of wild type and GFP-PbACT<sub>CON</sub> parasite saponin-treated schizont lysates demonstrating tagged (~69 kDa) and untagged (~42 kDa) proteins probed with antisera raised against either anti-Actin monoclonal (clone C4, Millipore), and visualized via enhanced chemiluminescence (ECL, Amersham Biosciences). Asterisks mark non-specific labelling products. (C) Live and IFA imaging of GFP-PbACT<sub>CON</sub> parasites show actin expression throughout motile (and non-motile) lifecycle stages. Scale bar = 5  $\mu$ m. Imaging by live fluorescence or immunofluoresence assay was as described [1] with anti-GFP (1:50, Roche Applied Biosciences) and DAPI (1:1250, Roche Molecular Biochemicals). Images were acquired at 100× using a Zeiss AxioVert 200M live cell imaging inverted microscope equipped with an AxioCam MRm camera. Images were captured and processed using Axiovision Software (Carl Zeiss) and p



**Fig. 2.** Localization of GFP-PbActin to the ookinete pellicle (A–D) Still images and time-lapse imaging series of GFP-PbACT<sub>CON</sub> ookinetes either untreated (A and C) or in the presence of 1 µM JAS (B and D). Arrows indicate areas of actin concentration at the flexing edge of the parasite in still images from time-lapse series. See also supplemental Movies S1–6. *In vitro* conversion to ookinetes and ookinete motility assays followed Moon et al. [20].

which is active throughout the lifecycle [17] (Fig. 1A), creating the line GFP-PbACT<sub>CON</sub>. Whilst the expression profile of EF1 $\alpha$  is not identical to that of actin, both are expressed strongly, coming on early during asexual development, and show broad expression across parasite lifecycle stages. As expected GFP-PbActin could be detected (as determined by live or immunofluorescence assay (IFA) microscopy) in every stage of parasite development (Fig. 1B and C), and was found to be broadly cytoplasmic. Sporozoites suggested a pellicular concentration of actin (Fig. 1C) whereas ookinetes and merozoites showed more uniform fluorescence (Fig. 1C and data not shown) consistent with the largely monomeric pool of actin in free motile parasite cells [4,18]. A concentration of actin fluorescence in the nucleus was seen in several stages (Fig. 1C), which may suggest that actin functions in nuclear architecture or gene regulation, as is seen in other eukaryotes [19]. No evidence for stable actin filament structures, such as stress fibres was seen, in line with the absence of a stable actin cytoskeleton in apicomplexan cells [6,7].

Ookinetes are the largest motile cell in the malaria parasite lifecycle, and can be readily generated *in vitro* [20]. We focussed on this stage to explore whether filament dynamics could be visualized utilising an *in vitro* motility assay combined with time-lapse imaging of the GFP-PbACT<sub>CON</sub> parasite line [20]. As a tool to explore F-actin turnover we incorporated JAS to stabilize otherwise dynamic actin filament populations [10,11]. The inhibitory effects of this drug treatment in our motility assay were consistent with previous studies [8] (Fig. S1).

No obvious dynamics of parasite actin could be detected in gliding or turning ookinetes in the absence of drug (Fig. 2A, Movie S1–2). Given the short, unstable and rapid turnover of parasite actin filaments [13–16] this inability to differentiate monomer from filament pools using GFP-PbActin is not surprising. However, treatment of parasites with 1  $\mu$ MJAS, which grossly retarded movement (Fig. 2B, S1, Movie S3–6), demonstrated localized centres of actin concentration. These centres likely correlate with sites of high actin turnover [11], with JAS-treated ookinetes demonstrating strongest concentration of GFP-PbActin at apical and basal positions as well as a clear concentration along the cell periphery (Fig. 2B, Movie S3–6). This pattern is similar to that seen in JAS-treated YFP-TgActin tachyzoites [11] and fixed *P. berghei* ookinetes labelled with generic anti-Actin [8]. In several instances, a notable concentration of actin was seen at the flexing edge of ookinetes, where traction with the substrate surface would be predicted to be high (Fig. 2B arrow, Movie S3–6). These data are, we believe, the first such exploration of live actin filament dynamics in a motile malaria parasite lifecycle stage. Furthermore, the concentration of fluorescence at the periphery is entirely consistent with a concentrated population of F-actin in the supra-alveolar space as predicted by current models for gliding motility [1,2].

Combination of this constitutive expressing GFP-Actin line with advanced live molecular level imaging of dynamic events during invasion and gliding will likely improve our understanding of the mechanics of malaria parasite gliding motility. More broadly, increased efforts towards understanding the regulation of actin's spatiotemporal localization, filament turnover and association with the pellicular membranes may reveal new pathways that could prove amenable to drug targeting.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2011.11.008.

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