

Generation, regulation and function of morphology in *Leishmania* and *Trypanosoma*

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“Biologists got obsessed with genes and stopped even noticing there was a question in terms of shape.”

— James Sharpe, EMBL/CRG Systems Biology Unit

Abstract

Little is known about the generation of *Leishmania* morphology and the function of morphology in trypanosomatids, despite every species having characteristic cell shapes and undergoing changes in morphology between life cycle stages. To address this I analysed morphogenesis of the cell body and flagellum through the cell cycle of the *Leishmania* insect (promastigote) life cycle stage using a novel method for determining cell cycle stage from cell size and DNA content. This showed cell body morphology is generated by growth and then remodelling of cell shape around mitosis and cytokinesis. Mathematical modelling of flagellum growth indicated flagellum length continues to increase over multiple cell cycles and does not reach a defined length. I also observed a little link between the cell cycle and flagellum length regulation during differentiation to the mammalian macrophage-inhabiting (amastigote) life cycle stage. Analysis of motility showed the diverse flagellar lengths of promastigote *Leishmania* cells bestow different swimming abilities, and the capacity of *Leishmania* promastigotes for highly directional swimming differs sharply from trypomastigote *Trypanosoma brucei*. This difference did not arise from altered flagellar beating therefore appeared to be linked to morphology. Together these indicate the mechanisms of cell body morphogenesis, flagellum length regulation, life cycle stage differentiation and the swimming abilities of the cells the morphogenetic processes generate differ significantly between *Leishmania* and *T. brucei*. These insights motivated the creation of automated micrograph analysis tools based on a new DNA staining method to support similar future morphometric analyses. This is the first comprehensive comparison of morphogenesis and function of morphology in a promastigote and a trypomastigote and, by considering these new insights in the context of existing molecular biology and the morphological diversity across many trypanosomatid species, give insight into basic *Leishmania* biology, the shared molecular mechanisms underlying morphogenesis and the potential functions of the diverse morphologies which are seen in different trypanosomatid species and life cycle stages.

Declarations

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Excluding references, the text of this thesis consists of 49,995 words.

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Abbreviations

PFR	Paraflagellar rod
FAZ	Flagellum attachment zone
TAC	Tripartite attachment complex
MTOC	Microtubule organising centre
BMDM	Bone marrow-derived macrophages
IFT	Intraflagellar transport
MOI	Multiplicity of infection
SMP	Small myristoylated protein
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
MGB	Minor groove binding
BPI	Base pair intercalating
LPG	Lipophosphoglycan
9v	9 variable
NA	Numerical aperture
DAPI	4',6-diamidino-2-phenylindole
PI	Propidium iodide
BrdU	5-bromo-2'-deoxyuridine
MAT	Medial axis transform

Preface

This thesis is arranged in four results chapters ([Section 2, 3, 4 & 5](#)) along with general introduction ([Section 1](#)), synthesis and conclusion ([Section 6](#)) and methods ([Section 7](#)) sections. Due to the breadth of topics covered and techniques used, each results chapter also includes its own (short) introduction, discussion and methods development and mathematics sections.

To aid clarity, cross-references are made, where possible, between the sections and chapters. They are shown in red with the section heading number, for example ([Section 3.4.1](#)), and in the digital copy of this thesis the section numbers can be followed as hyperlinks.

References to figures are also shown in red, for example ([Figure 3](#)). Some figures incorporate tables so, for simplicity, all tables are listed as figures. In the digital copy of this thesis the figure numbers can be followed as hyperlinks.

References to digital supplementary material which cannot be included in the thesis (computer scripts, videos, etc.) are too shown in red, for example ([Supplementary: Video 1](#)). These files are listed in [Section 9.1](#) and are available on the enclosed DVD.

1 Introduction

Leishmania parasites are fascinating organisms with a history of research which stretches back centuries (Gibson, 1983; Cox, 2002). The modern first written accounts of leishmaniasis come from the Spanish conquistadors, who described skin lesions consistent with cutaneous leishmaniasis (ul Bari, 2006), and reports from Africa and India in the mid-18th century of black fever, or “kala-azar” (Annesley and Pettigrew, 1828; Rogers, 1898), later shown to be visceral leishmaniasis. The first rigorous analyses of this disease came from its namesakes, William Leishman and Charles Donovan, who, in 1903, identified Leishman-Donovan bodies (*Leishmania donovani* amastigotes) in the spleens of kala-azar patients (Donovan, 1903; Leishman, 1903; Ross, 1903). The bodies were tentatively classified as a single celled eukaryotic parasite similar to, but distinct from, African trypanosomes, and these analyses quickly led to a more detailed understanding of the disease.

The early discovery of *Leishmania* means the history of the cell biology of the parasite also stretches back over a century (Gibson, 1983; Cox, 2002). By 1905, *L. donovani* could be grown and differentiated to flagellated forms *in vitro* (Leishman and Statham, 1905), in 1941 transmission was proven to be *via* the bite of the sandfly (Adler and Ber, 1941), in 1976 replication of *Leishmania* in macrophages *in vitro* was demonstrated (Chang and Dwyer, 1976) and in 1994 techniques to allow a complete axenic life cycle of *Leishmania mexicana* were published (Bates, 1994). It is this extensive period of research which has given us the textbook descriptions of the *Leishmania* life cycle, cell biology and interaction with its hosts.

More recently, the sequencing of the *Leishmania major* genome in 2005 (Ivens *et al.*, 2005) (and other species including *L. braziliensis*, *L. infantum* and *L. mexicana* (Peacock *et al.*, 2007; Aslett *et al.*, 2009; Raymond *et al.*, 2011; Rogers *et al.*, 2011)) revolutionised the analysis of the biology of the parasite. The genome sequence itself, development of modern molecular biology, genetic tools for

modification of the *Leishmania* genome (Beverley, 2003; Madeira da Silva *et al.*, 2009; Damasceno *et al.*, 2010) and bioinformatics has allowed researchers to build a well-supported, detailed description of many biochemical processes in these parasites. However, understanding of the cell biology of *Leishmania* has not advanced at the same pace; some of the older data is beginning to show its age and newer more detailed cell biological questions have arisen. Interpretations of experiments concerning *Leishmania* molecular biology therefore sometimes do not have the clarity and certainty that would be desired.

There are some intriguing areas of the ‘textbook’ *Leishmania* infectious life cycle which are not supported thoroughly by existing data, particularly concerning the function of morphology and the flagellum. *Leishmania* has characteristic morphologies at different life cycle stages. The cell cycle and life cycle have to be precisely choreographed to produce these morphologies and they presumably confer advantages at different life cycle stages, although the function of different cell morphologies and precisely how they arise is largely unknown. This thesis therefore begins to address aspects of *how* and *why* these morphologies arise and how they compare to other trypanosomatids to determine why *Leishmania* change morphology with such high fidelity.

1.1 The trypanosomatid cell and cell cycle

Leishmania are members of the order *Trypanosomatida* (trypanosomatids), an exclusively parasitic group of protozoa which includes the human pathogens *Leishmania* (which cause leishmaniasis), *Trypanosoma cruzi* (American trypanosomiasis, Chagas disease) and *Trypanosoma brucei* (African trypanosomiasis, sleeping sickness). All trypanosomatids have a rigid morphology and ultrastructure which are preserved through all cell cycle and life cycle stages (Vickerman, 1985, 1994). Nonetheless large morphological variation is still seen (Figure 1) (Hoare and Wallace, 1966) which may help the parasites to adapt to their current environment (Vickerman, 1985, 1994).

1.1.1 Cell ultrastructure

The cell body of trypanosomatids is typically vermiform and approximately axially symmetric with single copies of several major organelles; including the nucleus, the mitochondrion (and the mitochondrial DNA, the kinetoplast) and the flagellum (subtended by the basal body). A regular array of sub-pellicular microtubules lies under the cell membrane, shown in *T. brucei* to be oriented

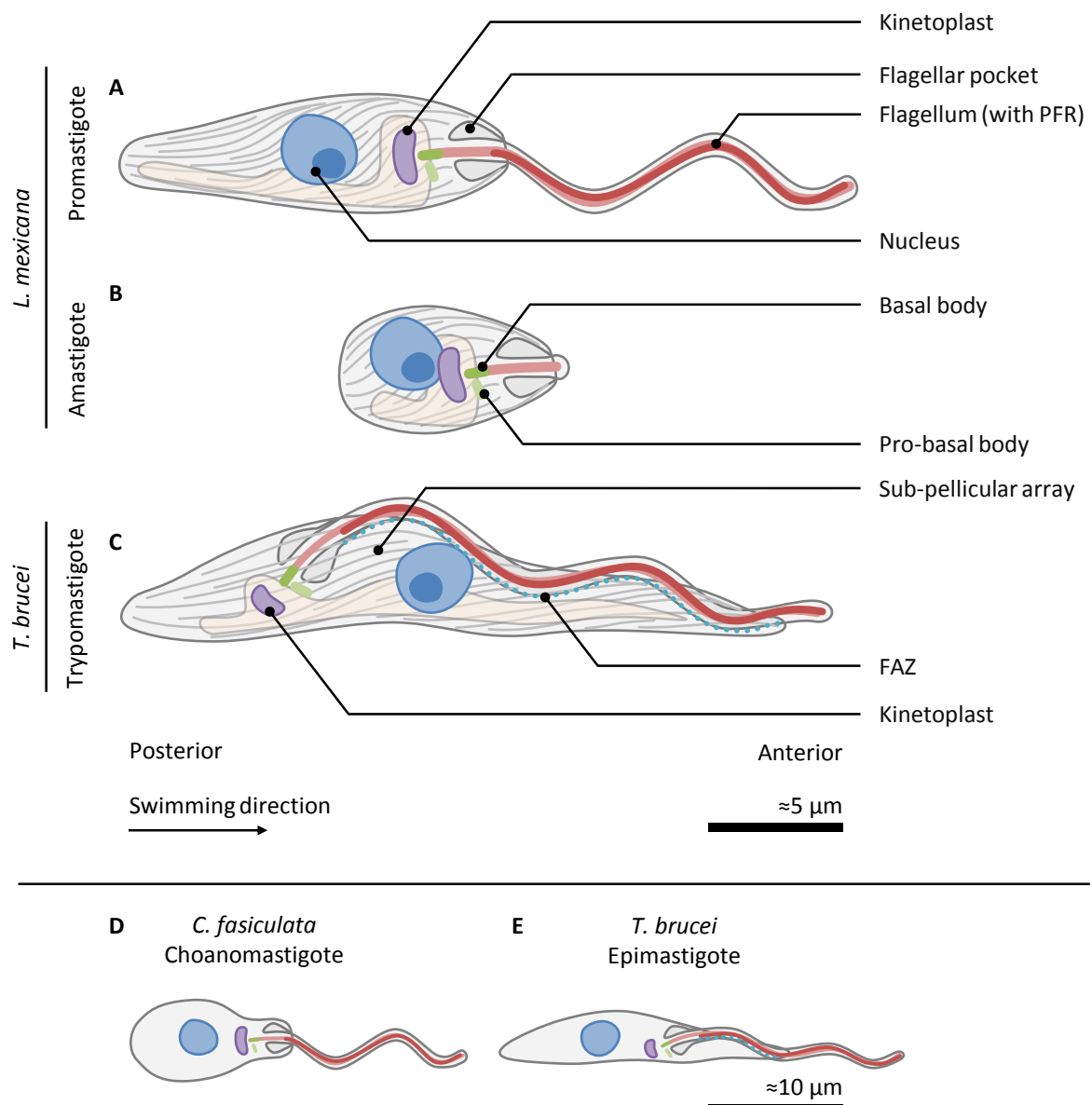


Figure 1. The morphology and ultrastructure of trypanosomatids, focusing on *L. mexicana* and *T. brucei*. A. Promastigote; flagellar pocket at the cell anterior, no extensive lateral connection from the flagellum to the cell body. B. Amastigote; no long external flagellum. C. Trypomastigote; flagellar pocket posterior to the nucleus, extensive lateral connection between the flagellum and the cell body. Two related morphologies are shown in less detail. D. Choanomastigote; similar to the promastigote. E. Epimastigote; similar to the trypomastigote, but with the kinetoplast anterior of the nucleus. (Hoare and Wallace, 1966).

with the + end toward the posterior (Sherwin and Gull, 1989b; Robinson *et al.*, 1995). In *T. brucei* increase or decrease circumference is associated with additional nucleation or termination of sub-pellicular microtubules (Sherwin and Gull, 1989b) and disruption of the regular sub-pellicular microtubule spacing through RNAi knockdown of the calpain-like protein CAP5.5 causes altered morphology and cytokinesis defects generating anucleate daughter cells (Olego-Fernandez *et al.*, 2009). This cytoskeleton precisely defines morphology and different trypanosomatid species, life cycle stages and cell cycle stages can often be identified on this basis (Hoare and Wallace, 1966).

Several major organelles are found associated with the basal body. The kinetoplast is a concatenated network of the circular mitochondrial DNA molecules and is associated with the basal body via the tripartite attachment complex (TAC) which provides a structural linkage through the mitochondrial membranes (Robinson and Gull, 1991; Ogbadoyi *et al.*, 2003). The basal body (with an associated pro-basal body) is found associated with the bottom of an invagination of the cell membrane, the flagellar pocket. The flagellum extends from the basal body, through the flagellar pocket, and out of the flagellar pocket neck to reach the extracellular space. In *T. brucei* electron tomography has been used to describe these structures in detail (Lacomble *et al.*, 2009, 2010). The flagellar pocket and flagellum are the only parts of the plasma membrane not underlain by the sub-pellicular microtubules and it is thought that the flagellar pocket, flagellar and cell membranes are distinct membrane domains with different compositions (Landfear and Ignatushchenko, 2001; Overath and Engstler, 2004; Souza, 2006). All endo- and exocytosis is thought to occur in the flagellar pocket (Overath and Engstler, 2004).

The flagellum structure is typical of eukaryotic flagella/cilia. Motile trypanosomatid flagella have a canonical 9+2 axoneme structure with 9 circumferential microtubule doublets and a central microtubule pair. Trypanosomatid flagella also possess an additional paraxial structure, the paraflagellar rod (PFR), a paracrystalline structure which lies parallel to the axoneme for the portion of the flagellum outside of the flagellar pocket (Section 1.3.1).

There are different morphological classes of trypanosomatid defined by kinetoplast and nucleus location and lateral attachment of the flagellum to the cell body (Hoare and Wallace, 1966). Different life cycle stages often have different morphologies (Figure 2). *Leishmania* are found as a promastigote (Figure 1A) or amastigote (Figure 1B) (Section 1.2), distinguished by flagellar length; the amastigote flagellum is too short to protrude beyond the flagellar pocket. The choanomastigote, similar to the promastigote but with a pronounced collar around the flagellar pocket neck (Figure 1D), is seen in

	No external flagellum	Laterally attached flagellum		Free flagellum	
	Amastigote ^a	Trypomastigote ^a	Epimastigote ^a	Promastigote ^a	Choanomastigote ^a
<i>Trypanosoma brucei</i> ^{bc}		mammal (bloodstream ^{4*}) tsetse fly (midgut ¹ & salivary glands ^{2*}) salivary glands ^{3*})	tsetse fly (salivary glands ²)		
<i>Trypanosoma cruzi</i> ^d	mammal (intracellular ³)	triatomine bug (hindgut ^{2*}) mammal (bloodstream ^{4*})	triatomine bug (midgut ¹)		
<i>Trypanosoma lewisi</i> ^e	flea (stomach epithelial cells ²)	flea (stomach ¹³ , stomach epithelial cells ²) rat (bloodstream ^{6*})	flea (rectum ^{4*}) rat (bloodstream ⁵)		
<i>Trypanosoma avium</i> ^f		bird (bloodstream ^{3*} & bone marrow ³) louse flies (hindgut ^{2*})	louse flies (midgut ¹)		
<i>Trypanosoma cobitis</i> ^g		fish (bloodstream ^{4*}) leech (stomach ¹ & proboscis sheath ^{3*})	leech (stomach ²)		
<i>Leishmania mexicana</i> ^{hi}	mammal (macrophage ^{3*})			sand fly (midgut ¹ & mouthparts ^{2*})	
<i>Phytomonas elmassiani</i> ^j				milkweed bug (gut ¹ & salivary glands ^{2*}) milkweed plant (sap/latex ^{3*})	
<i>Blastocrithidia gerridis</i> ^{kl}	environment ⁴ water strider (rectum ^{3*})		water strider (midgut ¹)	water strider (hindgut ²)	
<i>Leptomonas oncopelti</i> ^j	environment ³ milkweed bug (rectum ^{2*})			milkweed bug (gut ¹)	
<i>Crithidia fasciculata</i> ^m	environment ³ mosquito (rectum ^{2*})				mosquito (gut ¹)

Figure 2. Occurrence of different morphologies through the life cycle of trypanosomatids. Morphologies of seven dioxenous and three monoxenous trypanosomatids at different stages of their life cycles. The host organism and location within the host are listed for each morphology of each parasite species. Superscript numerals indicate the order in which morphologies arise during the life cycle (starting with the first vector stage) and asterisks indicate transmissive stages. Some life cycle stages (e.g. proventricular *T. brucei* trypomastigotes) are omitted for clarity. ^a (Hoare and Wallace, 1966), ^b (Vickerman, 1985), ^c (Matthews, 2005), ^d (Goldenberg and Avila, 2011), ^e (Minchin and Thompson, 1915), ^f (Baker, 1956), ^g (Letch, 1980), ^h (Bates, 2007), ⁱ (Handman and Bullen, 2002), ^j (Jankevicius *et al.*, 1989), ^k (Becker, 1923), ^l (Laird, 1959), ^m (Patton, 1908).

Crithidia. *T. brucei* are often trypomastigote (Figure 1C) distinguished by a posterior kinetoplast position and the lateral attachment of the flagellum, by the flagellum attachment zone (FAZ), along the cell body (Figure 1C). Some *T. brucei* life cycle stages are epimastigote; similar to the trypomastigote but with an anterior kinetoplast and shorter laterally attached flagellum (Figure 1E). Evidence as to any functional importance of these different morphologies is very limited.

1.1.2 Duplicating the cell ultrastructure through division

The complex and high fidelity organisation of trypanosomatids demands a complex and high fidelity division to build the morphology of the daughter cells. Trypanosomatids progress through a canonical eukaryotic cell cycle with nuclear G₁, S and G₂ phases, followed by mitosis and cytokinesis. The timing of these cell cycle stages has been analysed for several trypanosomatid species including *T. brucei* (Sherwin and Gull, 1989a; Woodward and Gull, 1990), *T. cruzi* (Elias *et al.*, 2007), *Leishmania donovani* (Bhattacharya and Ghosh, 1985) and *Crithidia fasciculata* (Cosgrove and Skeen, 1970). The kinetoplast has pre-S, S and post-S-phases followed by division, analogous to the nucleus, the timing of which have also been analysed in these species (Cosgrove and Skeen, 1970; Bhattacharya and Ghosh, 1985; Sherwin and Gull, 1989a; Elias *et al.*, 2007). Flagellum duplication is mediated by duplication of the basal body and flagellum outgrowth, which have been analysed in particular detail for *T. brucei* and also occur at precisely defined points in the cell cycle (Gull *et al.*, 1990; Lacomble *et al.*, 2010). A similar series of events is likely to occur in *Leishmania*.

Trypanosomatids do not have a single microtubule organising centre (MTOC) (Gull *et al.*, 2005). The single centriole-like MTOC pair is always in use as a basal body and associated pro-basal body; no trypanosomatid species have been identified in which the flagellum disassembles for mitosis. Mitosis occurs without breakdown of the nuclear envelope and the spindle MTOCs are morphologically dissimilar to centrioles (Gull *et al.*, 2005), shown particularly clearly for *T. cruzi* (Solari, 1980). Separation of the centriole-like MTOCs (basal bodies) plays a vital role in partition of

kinetoplast DNA via the TAC (Ogbadoyi *et al.*, 2003; Gluenz *et al.*, 2011). RNAi targeting p166 or ectopic overexpression of AEP-1, components of the TAC, cause failure in kinetoplast division resulting in daughter cells with either an abnormally large or an absent kinetoplast (Ochsenreiter *et al.*, 2008; Zhao *et al.*, 2008). The sub-pellicular microtubule array is nucleated from many MTOCs distributed within the array and also does not disassemble at any point during the cell cycle, shown in particular detail for *T. brucei* (Gull *et al.*, 1990). Trypanosomatids do not have a conventional actin cytoskeleton which could drive cytokinesis; filamentous actin structures have not been detected in *T. brucei* or *L. mexicana* (Amar *et al.*, 1988; García-Salcedo *et al.*, 2004; Sahasrabuddhe *et al.*, 2004) and RNAi knockdown of actin in procyclic *T. brucei* only causes morphological defects in the Golgi apparatus (García-Salcedo *et al.*, 2004). Remodelling of the cell shape during cytokinesis and furrow ingression must therefore occur through remodelling of the sub-pellicular microtubules. Organelle positioning and furrow ingression during cytokinesis must be precise; in *T. brucei* detachment of the flagellum through RNAi targeting FAZ1 or FLA1 causes defects in these processes generate abnormal daughter cell morphologies and anucleate cells through missegregation of organelles (LaCount *et al.*, 2002; Vaughan *et al.*, 2008).

1.1.3 Altering the cell ultrastructure

Mechanisms for morphogenesis during division are of interest as they may be adapted to generate altered morphology during life cycle stage differentiation. This has been demonstrated in *T. brucei* where specialised division events generate daughter cells with different morphology, enabling transition from a trypomastigote to epimastigote in the tsetse fly (Sharma *et al.*, 2008). During this process the position of the nucleus and the cleavage furrow are altered relative to normal proliferation (Gull *et al.*, 1990; Sharma *et al.*, 2008). *T. brucei* cell division events therefore appear to fall into two classes; proliferative division, which generates daughter cells identical to the parent, or differentiation division, where the daughter cells have undergone some degree of morphological change (Matthews and Gull, 1994; Tyler *et al.*, 2001; Sharma *et al.*, 2008). The

mechanisms of cell body and flagellum morphogenesis used during the *Leishmania* cell cycle may similarly be adapted to achieve morphological change during life cycle stage differentiation (Figure 3).

1.2 *Leishmania* life cycle

The *Leishmania* life cycle progresses through two hosts, as a promastigote in the sandfly vector (where it replicates in the digestive system) and as an amastigote in mammalian hosts (where it proliferates inside parasitophorous vacuoles in macrophages) (Chang and Dwyer, 1976) (Figure 1 & 4). The parasite undergoes biochemical adaptation to its current microenvironment (described in Section 1.2.2). As *Leishmania* is a single celled parasite all life cycle stage differentiation processes must work within the framework of the cell cycle, and the capacity for morphological change is constrained by the ultrastructure of the cell.

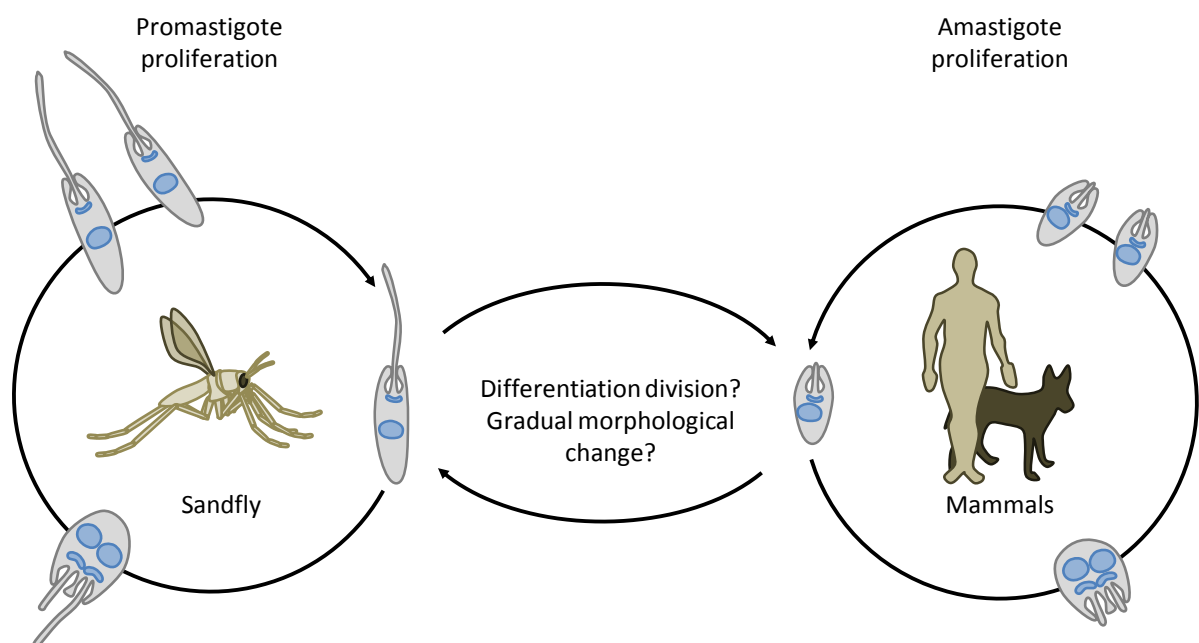


Figure 3. A simplified view of the *Leishmania* lifecycle in the context of the cell cycle. The parasite proliferates in the promastigote forms (Figure 5) in the sandfly vector and the amastigote form inside macrophages in the mammalian host. The parasite undergoes morphological and biochemical changes between the lifecycle stages. The nature and timing of many changes are known but the mechanism of morphological change and the interplay of these changes with the cell cycle is not.

1.2.1 The promastigote in the sandfly

There are many species of sandfly (family *Phlebotominae*, genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World) which transmit *Leishmania* (Lewis, 1971). Different parasite species are only transmitted by a small number of sandfly species which complicates analysis of vector-parasite interactions. The most detailed analyses concern *L. mexicana* and *Lutzomyia longipalpis* (Figure 5). For these species a series of parasite life cycle substages defined by morphology (Figure 6) arise in succession, associated with approximately two doublings of parasite numbers, during progression of an infection (Rogers *et al.*, 2002; Bates, 2007, 2008). Ingested parasites are initially amastigotes. These differentiate into procyclic promastigotes then nectomonad promastigotes in the abdominal midgut, followed by leptomonad promastigotes in the thoracic midgut. Finally the number of metacyclic promastigotes begins increase (in the anterior gut) coupled with a gradual decrease in number of leptomonads. Some nectomonad and leptomonad promastigotes are also present at these later stages of an infection (Rogers *et al.*, 2002). Some promastigotes attach to the gut surfaces

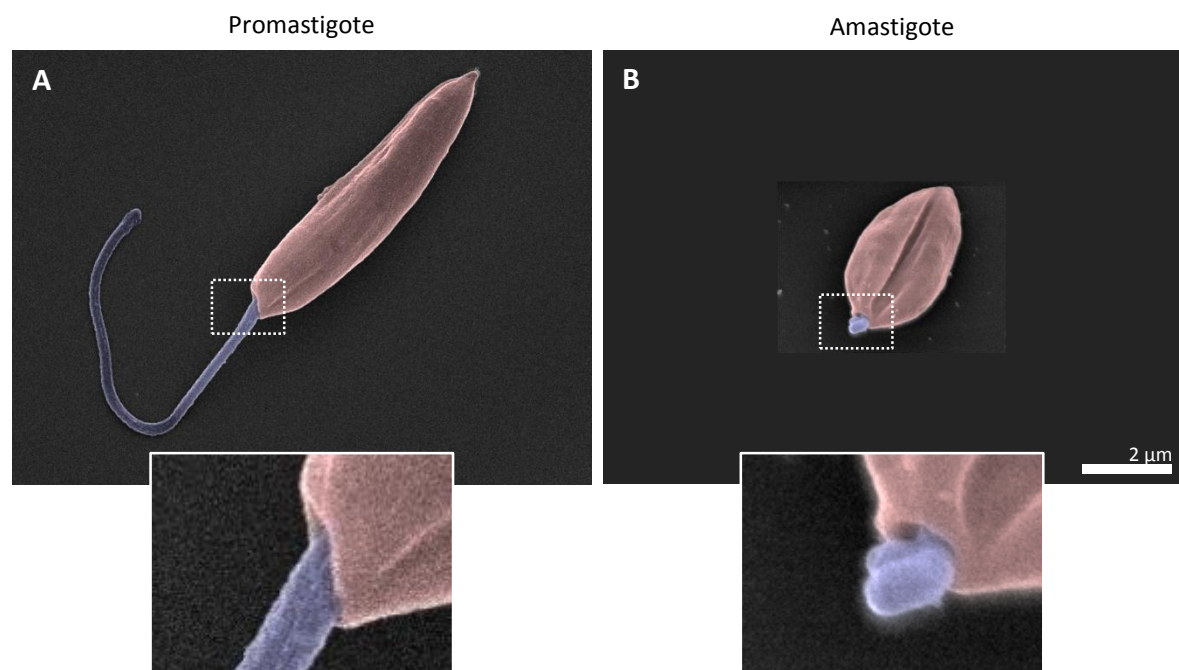


Figure 4. Scanning electron micrographs of the two major lifecycle stage morphologies of *L. mexicana*. A. The promastigote. B. The amastigote. The flagellum is pseudocoloured blue and the cell body is pseudocoloured red.

(haptomonad promastigotes) (Section 1.3.2). Of these morphologies the procyclic and leptomonad promastigote are described as proliferative (Bates, 2007, 2008) (Figure 5).

In a mature sandfly infection *Leishmania* secrete high levels of chitinase (Rogers *et al.*, 2008) which damages the alimentary canal interior (Schlein *et al.*, 1992) and secrete a filamentous proteophosphoglycan (Ilg *et al.*, 1996) which alters feeding behaviour and promotes regurgitation of infected saliva during feeding (Rogers *et al.*, 2002, 2004). The combination of these factors increases the number of parasites egested into the mammalian host during feeding (Bates, 2007). The large number of metacyclic promastigotes present in mature sandfly infections (Rogers *et al.*, 2002) are pre-adapted for mammalian infection (Sacks and Perkins, 1984), have decrease in abundance and a 2

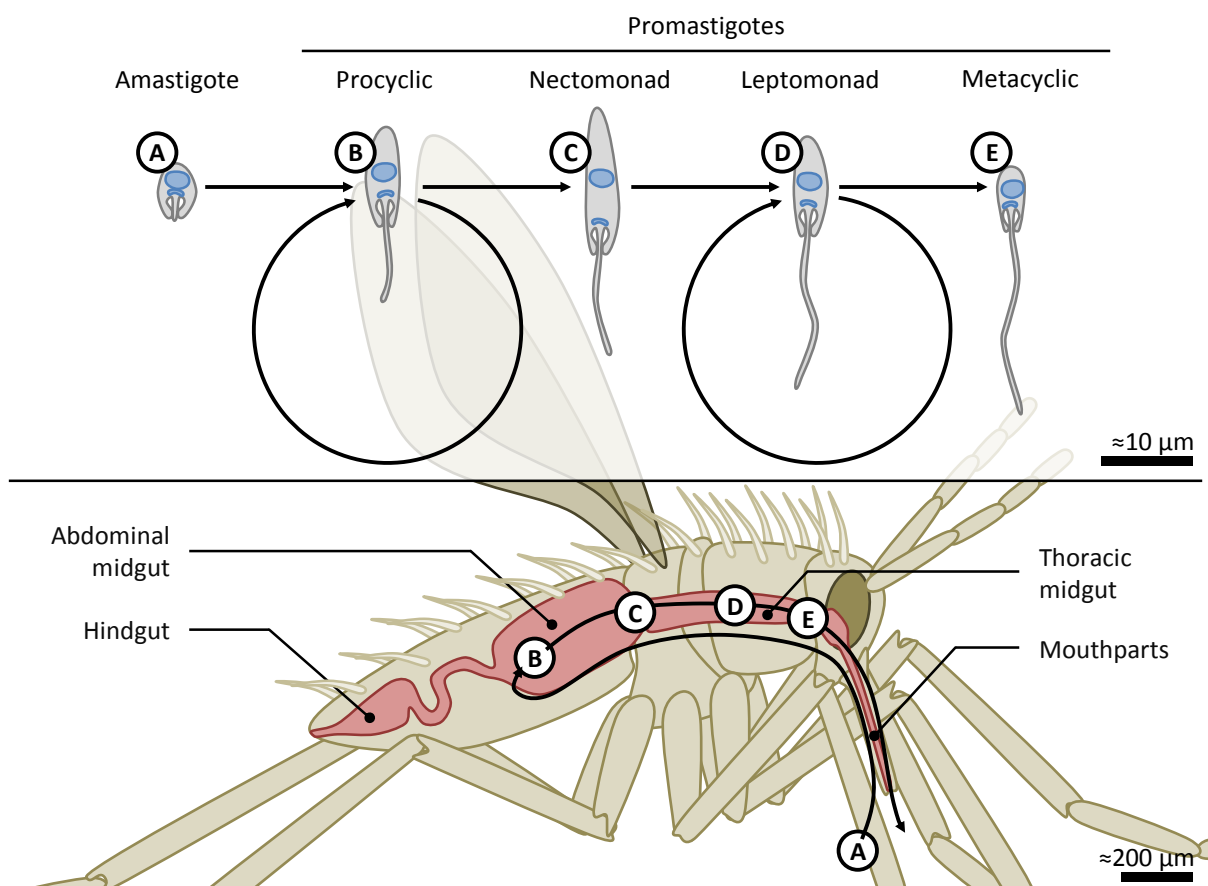


Figure 5. Promastigote *L. mexicana* life cycle substages in *L. longipalpis*. According to (Bates, 2007). A. The amastigote as taken up in a bloodmeal. B. Proliferative procyclic promastigote, formed by differentiation from the amastigote, in the ingested bloodmeal in the abdominal (posterior) midgut. C. Non-proliferative nectomonad promastigote responsible migration to the anterior midgut. D. Proliferative leptomonad promastigote in the thoracic (anterior) midgut. E. Non-proliferative metacyclic promastigotes in the anterior thoracic midgut and posterior mouthpart components. Haptomonad promastigotes (cuticle-attached in the thoracic midgut) are not shown.

to 3 fold increase in glycan size of the lipophosphoglycan (LPG) surface coat (Sacks, 1992) and are resistant to complement-mediated lysis (Mallinson and Coombs, 1989).

1.2.1.1 *In vitro* promastigote culture

Promastigote *Leishmania* is widely grown in axenic semi-defined cell culture media (Kar *et al.*, 1990; Bates, 1994). Formation of metacyclic promastigotes can be triggered *in vitro* by transfer to acidic media (pH 5.5) (Bates and Tetley, 1993; Bates, 1994) or by allowing the culture to acidify during growth to stationary phase (Saraiva *et al.*, 2005). Interpretation of parasite behaviour and biochemistry using the second of these protocols is complicated by the additional effects of starvation in depleted medium. *In vitro* generated metacyclic promastigotes have stalled in the cell cycle in G₁, are more readily taken up by macrophages, and have undergone biochemical changes similar to those seen for sandfly-derived metacyclic promastigotes (Bates and Tetley, 1993) (Section 1.2.1).

Axenic promastigotes are similar morphologically to those found in the sandfly gut and procyclic, nectomonad, leptomonad and metacyclic promastigote-like morphologies (Figure 5 & 6) occur successively in *in vitro* culture during differentiation from amastigotes (Gossage *et al.*, 2003). Whether these morphologies are present in established exponentially growing culture is not clear. A simplified view of axenic promastigote culture is sometimes taken (Saraiva *et al.*, 2005); that

	Amastigote	Procyclic promastigote	Nectomonad promastigote	Leptomonad promastigote	Metacyclic promastigote
Cell body length	ND	> 6 µm < 12 µm	> 12 µm	> 6 µm < 12 µm	< 8 µm
Cell body width	ND	ND	ND	ND	< 1 µm
Flagellum length	0	< Cell body length	ND	> Cell body length	> Cell body length

Figure 6. Morphological definitions of *L. mexicana* life cycle substages. Definitions from cell body and flagellum dimensions according to (Rogers *et al.*, 2002). ND indicates that morphological feature was not used for definition of the life cycle substage morphology.

logarithmically growing *in vitro* cultures are procyclic promastigotes and stationary phase cultures are metacyclic promastigotes. This view may not be accurate. Typical flagellum length range in *in vitro* culture (Erdmann *et al.*, 2006; Tull *et al.*, 2010) indicates promastigote morphological diversity is unlike procyclic promastigotes in sandflies (Rogers *et al.*, 2002) and the metacyclic content of stationary phase cultures varies; stationary phase *L. amazonensis* and *L. donovani* can consist of less than 30% metacyclic promastigotes (Saraiva *et al.*, 2005). More rigorous comparison of the range of *Leishmania* morphologies which arise in *in vitro* culture and how they relate to the *in vivo* developmental processes in the sandfly would therefore be valuable.

1.2.2 The amastigote in the macrophage in the mammal

In the mammalian host *Leishmania* primarily infect macrophages; upon the bite of an infected sandfly, saliva containing metacyclic promastigotes is transferred into the mammal skin where they are taken up by macrophages. Uptake of *Leishmania* is a passive process (from the point of view of the parasite) and uses the normal macrophage phagocytic pathways. Other professional phagocytes (neutrophils and monocytes) are also likely to be involved (Williams, 1988; Gueirard *et al.*, 2008). Infection of other cell types with phagocytic capability is possible *in vitro* (Rittig and Bogdan, 2000) but the relevance of this *in vivo* is probably limited. Several ligands/receptor interactions have been identified including the parasite surface components LPG and gp63 (Mosser *et al.*, 1987; Da Silva *et al.*, 1989; Peters *et al.*, 1995), opsonising factors from the host serum bound to the *Leishmania* (Blackwell, 1985; Brittingham and Mosser, 1996) and phosphatidylserine indicating a role for cooperative apoptosis or mimicry of apoptosis in promoting uptake (Barcinski *et al.*, 2003; Wanderley *et al.*, 2009; Wanderley and Barcinski, 2010). Uptake is more efficient for amastigote and metacyclic promastigote parasites than other promastigote forms (Bates *et al.*, 1992; Bates and Tetley, 1993).

Following uptake, the metacyclic promastigotes undergo morphological and metabolic changes to differentiate into the amastigote life cycle stage, triggered by the increase in temperature from 26

to 28°C, to 32 to 37°C (Shapira *et al.*, 1988; Alcolea *et al.*, 2010). Changes include increased nuclease and proteinase activity (Rainey *et al.*, 1991; Bates *et al.*, 1992; Pral *et al.*, 1993; Bates, 1994), metabolic changes to favour anaerobic catabolism (Castilla *et al.*, 1995; Gupta *et al.*, 1999), lower levels of surface LPG (Saar *et al.*, 1998), decreased membrane microviscosity (Gupta *et al.*, 1996) and upregulation of amastigote-specific membrane markers such as the A2 proteins (Zhang *et al.*, 1996; Barak *et al.*, 2005). The process by which morphological change occurs is not known, but it occurs within the 24 h (Bates, 1994; Barak *et al.*, 2005; Alcolea *et al.*, 2010).

Leishmania perturb the normal endocytic pathway of the macrophage and the phagocytic vacuole develops into a parasitophorous vacuole (Chang and Dwyer, 1976; Antoine *et al.*, 1998). Membrane is recruited to the developing vacuole through fusion with endosomes and lysosomes (Chang, 1983; Barbieri *et al.*, 1985; Alexander and Russell, 1992) and it develops into a mature parasitophorous vacuole over 2-5 days, depending on *Leishmania* species (Antoine *et al.*, 1998). The classification of the mature parasitophorous vacuole (in the context of the normal endocytic pathway) is ambiguous (Russell *et al.*, 1992; Antoine *et al.*, 1998; Ndjamen *et al.*, 2010). It is acidic (pH 5 (Antoine *et al.*, 1990)), similar to late endosomal compartments (Alexander and Vickerman, 1975; Brazil, 1984; Veras *et al.*, 1996), but also has unusual features like recruiting membrane from the endoplasmic reticulum (Ndjamen *et al.*, 2010). *Leishmania* go on to modulate macrophage behaviour; parasitophorous vacuole biogenesis continues to increase vacuole size (for *Leishmania* species proliferating in commensal vacuoles) and allow division of the vacuole (for species in individual vacuoles) (Real *et al.*, 2010; Real and Mortara, 2012). *Leishmania* alter host cell signalling, inhibiting the action of the NF- κ B, AP-1 and CREB transcription factors and the KAK/STAT signalling pathway. This leads to inhibition of production of inflammatory cytokines (IL-1 and TNF- α), decreased nitric oxide production and reduced sensitivity to IFN- γ (Gregory and Olivier, 2005; Olivier *et al.*, 2005). *Leishmania* also alter macrophage metabolism through scavenging of essential nutrients, including purines and haem, from the parasitophorous vacuole (Naderer and McConville, 2008).

1.2.2.1 *In vitro* axenic amastigote culture

The amastigote of many *Leishmania* species cannot readily be grown in axenic culture. Therefore *in vitro* infection of macrophages (macrophage-like cell lines (for example Raw 264.7 (Raschke *et al.*, 1978)), bone marrow-derived macrophages (BMDM) and peripheral blood mononuclear cell-derived macrophages) are often used. High temperature (32 to 37°C depending on species), acidic (pH 5.5), growth conditions can induce *in vitro* axenic differentiation into amastigotes of some species, originally described for *L. mexicana* (Pan, 1984). The similarity of these cells to amastigotes in macrophages is debated (Gupta *et al.*, 2001) but axenic *L. mexicana* and *L. donovani* amastigotes have been well characterised and are similar in many respects. Morphology is similar with a similar cell size, a short flagellum and lack of PFR (Doyle *et al.*, 1991; Bates *et al.*, 1992). Axenic amastigotes are more infective than promastigotes, and are of comparable infectivity to macrophage-derived amastigotes (Bates *et al.*, 1992; Saar *et al.*, 1998). Axenic amastigotes can also revert back to promastigote forms *in vitro* (Pan, 1984; Doyle *et al.*, 1991). All of the biochemical differences between promastigotes and amastigotes described above (Section 1.2.2) are also observed in axenic amastigotes (reviewed by (Gupta *et al.*, 2001)). Axenic and macrophage-derived amastigotes are, however, not identical and the metabolic profile of axenic *L. donovani* (Gupta *et al.*, 1999) and mRNA expression levels in axenic *L. mexicana* (Holzer *et al.*, 2006) share features of both promastigote and amastigotes.

1.3 The multifunctional *Leishmania* flagellum

The *Leishmania* flagellum is present during all cell cycle stages of all life cycle stages and is always found at the anterior of the cell in a flagellar pocket, although the flagellar structure does vary between life cycle stages. It is thought the *Leishmania* flagellum has several roles at different stages of the life cycle, in motility, physical attachment and possibly mediation of communicating with a host macrophage (Gluenz *et al.*, 2010a). This diversity of function is similar to that of the *T. brucei* flagellum which is also required for motility and physical attachment (Bastin *et al.*, 2000).

The basal body, through its roles in division, has emerged as a ‘master organiser’ of cell morphogenesis (Davidge *et al.*, 2006) (Section 1.1.2). In addition the trypanosomatid flagellum also plays a role in directly defining the ultrastructure of the cell. The flagellar pocket region is highly organised with the flagellum forming a major part of the structure (Lacomble *et al.*, 2009). Disruption of the collar at the neck of the pocket in *T. brucei* by RNAi knockdown of the protein BILBO1 causes failure in assembly of the new flagellar pocket with the flagellum instead emerging directly from the surface of the cell body (Bonhivers *et al.*, 2008). This malformation is lethal (Bonhivers *et al.*, 2008) and it is plausible that the *Leishmania* flagellum plays a similar vital role in maintaining the ultrastructure of the cell anterior and flagellar pocket.

1.3.1 Basic structure and function of the flagellum

The flagellum of most trypanosomatids, including the *Leishmania* promastigote, has a structure typical of the motile eukaryotic flagellum/cilium (Fliegeauf *et al.*, 2007). The flagellum is subtended by the basal body with 9 circumferential microtubule triplets. The microtubules of the basal body extend to form the flagellum, initially through the transition zone, characterised by 9 circumferential microtubule doublets, to the basal plate. At this point a central pair of microtubules is nucleated and the axoneme proper, with a 9+2 structure, extends from the basal plate. The 9 outer microtubule doublets connect via the radial spoke structure to the central microtubule pair (which are found at a fixed rotation relative to the doublets (Gadelha *et al.*, 2006)) and via dyneins motor proteins to the neighbouring doublets (Gibbons and Rowe, 1965; Gibbons and Gibbons, 1973). The basal body and transition zone are short in comparison to the length of the flagellum and the basal plate is found near the bottom of the flagellar pocket. The combined action of the dyneins, to generate a sliding force between microtubules in the axoneme, the radial spoke structure, to hold the axoneme in its circular arrangement, and the constraint of the microtubules by the basal body translates motor protein action into axoneme bending and is responsible for generating flagellum movement (Sale and Satir, 1977). The central pair is essential for flagellum motility (with a few

exceptions (Marchese-Ragona and Holwill, 1980), including metazoan nodal cilia (Praetorius and Spring, 2005)) and some immotile trypanosomatid flagella lack them (Gluezn *et al.*, 2010b) (Section 1.3.3).

The precise beat pattern which arises from flagellum bending varies by species (Section 4.1.1) however the beat which gives rise to directional movement in *Leishmania*, *Crithidia*, *T. cruzi* and *T. brucei* is caused by an approximately symmetric tip-to-base flagellum-type beat (Walker, 1961; Gadelha *et al.*, 2007; Rodríguez *et al.*, 2009; Ballesteros-Rodea *et al.*, 2012). Bloodstream form *T. brucei* has been shown to have two modes of motility; swimming (which achieves a directed movement) and tumbling (in which the cell does not translocate) (Uppaluri *et al.*, 2011; Zaburdaev *et al.*, 2011).

In addition to the axoneme most trypanosomatid flagella also contain a PFR. This paracrystalline structure runs parallel to the axoneme, extends from the point at which the flagellum exits the flagellar pocket up to the flagellum tip and lies in a constant orientation relative to the axoneme and axoneme central pair (Gadelha *et al.*, 2006). Assembly of the PFR structure is dependent on PFR1 and 2, first identified in *C. fasciculata* (Russell *et al.*, 1983), and in *Leishmania* PFR assembly fails following ablation of either PFR1 or 2 (Maga *et al.*, 1999). The PFR is essential for motility; in *T. brucei* RNAi targeting PFR2 reduces average swimming speed, flagellum movement frequency and amplitude although some flagellar movement still occurs (Bastin *et al.*, 1998). A similar effect is seen in the *L. mexicana* PFR2 (Santrich *et al.*, 1997) and PFR1 deletion mutants (Maga *et al.*, 1999). A large PFR structure, as seen in *T. brucei*, *L. mexicana* and *C. fasciculata*, is not essential for motility in all trypanosomatids. A reduced 'cryptic' PFR structure in *C. deanei* is sufficient to allow motility (Gadelha *et al.*, 2005, 2007). Formation of a similar reduced PFR in *L. mexicana* through ectopic expression of *C. deanei* PFR1 in the *L. mexicana* PFR1 deletion mutant restores some motility (Gadelha *et al.*, 2005). While a PFR structure is universally found in motile trypanosomatid flagella the immotile *Leishmania* amastigote flagellum lacks a PFR (Bates *et al.*, 1992) in addition to lacking the axoneme central pair (Gluezn *et al.*, 2010b).

The mechanism through which the PFR is essential for motility has not been firmly established. It is a large, multicomponent, structure which may prove to have several important roles. The PFR has been implicated in formation of hemidesmosomes during attachment to the gut (Bastin *et al.*, 1996; Beattie and Gull, 1997; Maga *et al.*, 1999), involvement in cAMP (Oberholzer *et al.*, 2007) or calcium (calmodulin-mediated) (Ridgley *et al.*, 2000) signalling and involvement in energy transduction or recognition of metabolites through metabolic enzymes in the PFR (Portman *et al.*, 2009). The paracrystalline structure may be indicative of a more structural primary role with an involvement in flagellum stiffening (Fuge, 1969), generation of the helical flagellum path along the *T. brucei* cell (Branche *et al.*, 2006) or providing a preferred direction of bending allowing formation of the bihelical flagellum beat (Koyfman *et al.*, 2011; Hughes *et al.*, 2012) proposed to occur in *T. brucei* (Rodríguez *et al.*, 2009) (Section 4.1.1).

1.3.2 The flagellum in the sandfly

Formation of the long promastigote flagellum during differentiation from the amastigote is vital to sustain an infection of a sandfly. In *L. donovani*, overexpression of ADP-ribosylation factor-like protein 3A (LdARL-3A) prevents formation of a long flagellum (Cuvillier *et al.*, 2000) and causes abortive sandfly infection (Cuvillier *et al.*, 2003). The flagellum is used for attachment to the interior of the gut which prevents clearance during defecation and helps confer parasite/vector specificity (Pimenta *et al.*, 1994; Sacks *et al.*, 2000). Interaction with the gut surface occurs through two mechanisms. In the midgut the flagellum interacts with microvilli and, as there are no ultrastructural changes, may reflect a receptor ligand (Bates, 2008) or non-specific interaction. In cuticle-lined gut segments interaction of haptomonad promastigotes with the gut surface is via hemidesmosome-like structures (Killick Kendrick *et al.*, 1974; Wakid and Bates, 2004), similar to *T. congolense* and *T. brucei* epimastigotes during attachment to the tsetse mouthparts or salivary glands respectively (Tetley and Vickerman, 1985; Beattie and Gull, 1997).

All *Leishmania* in the sandfly host are promastigotes and many are found free-swimming in the gut lumen. Different promastigote life cycle substages have been described as having different swimming abilities, notably the metacyclic promastigote is said to swim quickly (Bates, 2007), although quantitative data is lacking. Different swimming abilities of life cycle stages could be important in progression of an infection. There is some evidence for osmotaxis in *Leishmania* (Bray, 1983; Oliveira *et al.*, 2000; Leslie *et al.*, 2002; Barros *et al.*, 2006; Detke and Elsabrouty, 2008) (Section 4.1.2) although in the absence of more precise descriptions of the normal motility of *Leishmania* these data are difficult to interpret. It is proposed that chemotaxis may be involved in the gradual anterior migration of *Leishmania* in the sandfly as the infection progresses (Bates, 2008).

1.3.3 The flagellum in the macrophage

Amastigote *Leishmania* also possesses a single flagellum although it is very short and does not protrude far from the flagellar pocket. It lacks the axoneme central pair and the outer doublets are not found in the fixed nine-fold symmetry, with some doublets invading the central region of the axoneme (Gluenz *et al.*, 2010b). This collapsed 9+0 axoneme structure, a 9 variable (9v) axoneme, cannot be motile (Praetorius and Spring, 2005) and is similar to sensory cilia in mammals (Gerdes *et al.*, 2009). The amastigote flagellum tip can be found in tight contact with the parasitophorous vacuole membrane (Gluenz *et al.*, 2010b). This raises the possibility that the flagellum plays a sensory role, possibly mediating interaction with the macrophage (Gluenz *et al.*, 2010b) although there is not yet any biochemical evidence for communication. The short 9v axoneme structure of the amastigote flagellum is generated during differentiation from the promastigote but the mechanism by which this occurs is not known.

The multifunctional role of the *Leishmania* flagellum makes it important to understand when and how it grows, and when and how it changes function during differentiation between life cycle stages. Many proteins in *Leishmania* are known to be important for normal flagellum length and

morphology (Bengs *et al.*, 2005; Erdmann *et al.*, 2006; Casanova *et al.*, 2009; Tull *et al.*, 2010) (Section 3.1.2) however little is known about when the *Leishmania* flagellum grows and how its growth responds to environmental cues. Flagellum growth is likely to be intimately linked with the cell cycle, as in *T. brucei* (Sherwin and Gull, 1989a; Tyler *et al.*, 2001; Farr and Gull, 2009).

1.4 Objectives and aims

Modern microscope, imaging and computer technologies provide new opportunities for interrogating the *Leishmania* cell which have not been fully explored and have the potential to give insight into the generation, regulation and function of cell morphology. In comparison the technologies available in the 20th century for the original analyses of the *Leishmania* life cycle were limited. I have therefore chosen to revisit three areas of the cell biology of the parasite life cycle related to morphology and morphogenesis making use of these new technologies; firstly morphogenesis through cell division, secondly flagellum length regulation and thirdly the impact of morphology on motility. Where necessary new techniques for analysis of micrographs have also been developed.

1.4.1 Chapter 2 – Cell morphogenesis through the promastigote *Leishmania* cell cycle

Given the complex cellular ultrastructure, rigid morphologies of different life cycle stages and faithful duplication of morphology during division cell it appears morphology plays an important role in *Leishmania* biology. How *Leishmania* morphology arises through division is therefore of interest. Existing descriptions of morphogenesis through the cell and life cycle of *T. brucei* (Section 1.1.2) have revealed that morphogenesis is firmly rooted in the cell cycle (Gull *et al.*, 1990; Robinson *et al.*, 1995) and at least some of the morphological changes through the *T. brucei* life cycle are linked to modified division events (Sharma *et al.*, 2008). I have performed a detailed analysis of the *Leishmania* cell cycle as a foundation for understanding *Leishmania* cell body morphogenesis.

1.4.2 Chapter 3 – *Leishmania* flagellum growth and length change in the cell and life cycles

The *Leishmania* flagellum has diverse functions through the life cycle (Section 1.3) including roles in surface adhesion and motility (Bates, 2008). Therefore the mechanisms by which *Leishmania* flagellum length is modulated are of interest. For example how the flagellum lengths observed in promastigote culture arise may give insight into the function and origin of the different promastigote life cycle stages in the sandfly. The mechanism of generation of the short, immotile, amastigote flagellum is also of interest because of its potential importance in parasite-host cell interaction (Gluenz *et al.*, 2010b) and to determine how the 9v axoneme structure is generated. With reference to a mathematical model based on the balance point model of eukaryotic flagellum length regulation (Marshall *et al.*, 2005) I have determined how promastigote *Leishmania* flagellum length may be regulated and how the amastigote flagellum morphology is attained.

1.4.3 Chapter 4 – Roles of trypanosomatid cell and flagellum morphology in motility

While the flagellum beat of *Leishmania* (Gadelha *et al.*, 2007) and the modes of swimming of *T. brucei* (Uppaluri *et al.*, 2011; Zaburdaev *et al.*, 2011) are well understood (Section 1.3.1) the role of flagellum length in *Leishmania* swimming and the role of trypomastigote and promastigote morphologies on swimming are not known. The effects of different trypanosomatid morphologies on swimming may give insight into the function of these different cell shapes. I therefore analysed the swimming capability of a range of trypanosomatids with different morphologies and have determined a possible functional role of the trypomastigote and epimastigote morphologies in achieving highly directional swimming.

1.4.4 Chapter 5 – New approaches for automating morphometric analysis of trypanosomatids

The value of understanding morphogenesis is not limited to *Leishmania* as all trypanosomatids appear to undergo similarly high fidelity changes in morphology through their life cycles. In addition many mutants and chemical insults perturb these processes giving insight into parasite biology. Tools to assist analysis of trypanosomatid morphology would therefore be powerful. Micrographs contain an enormous quantity of morphological data and I have developed automated micrograph analysis tools which, combined with a novel means of analysing the kinetoplast and nucleus number, position and DNA content, generating high quality morphometric data quickly. Their value extends beyond analysis of morphological function and morphogenesis in *Leishmania* to related analyses of other species.

1.4.5 Chapter 6 – Synthesis and conclusion

In the preceding results chapters morphogenesis and the function of morphology in trypanosomatids is analysed by comparison between my data concerning promastigote *L. mexicana* and literature concerning other species, predominantly *T. brucei*, and the conclusions drawn are critically analysed and summarised in the discussion section of each chapter. However, to move towards a wider perspective on morphological origin and function these conclusions must account for the range of morphologies which occur in other trypanosomatid species. This thesis therefore concludes with a synthesis of the conclusions from the results chapters concerning morphogenesis and function of morphology in the context of cell shape in more diverse trypanosomatid species.

2 Cell morphogenesis through the promastigote *Leishmania* cell cycle

The work presented in this chapter was published in 2011; Wheeler RJ, Gluenz E and Gull K. (2011) “The cell cycle of *Leishmania*: morphogenetic events and their implications for parasite biology”. *Mol. Microbiol.* 79 (3): 647-62. All work included in this chapter is my own except the TEM data presented in Figure 16 (Section 2.2.5).

2.1 Introduction

It is likely generation of *Leishmania* cell morphology is linked with the processes of division, and, like *T. brucei*, that division is a highly choreographed process (Section 1.1.2). A detailed, quantitative, and comprehensive description of the *Leishmania* cell cycle is therefore vital for understanding morphogenesis. *L. mexicana* was selected as the model species for cell cycle analysis as the whole life cycle can be simulated in axenic conditions (Bates, 1994) and the morphological changes which occur in the sandfly life cycle stage are well understood (Gossage *et al.*, 2003).

2.1.1 Previous analyses of the *Leishmania* cell cycle

Pre-existing descriptions of the *Leishmania* cell cycle show it is typical of trypanosomatids, where division of the nucleus and kinetoplast and duplication of the flagellum occur at precise points during the cell cycle (Section 1.1.2). *Leishmania* kinetoplast and nuclear S-phase are approximately synchronous and mitosis and kinetoplast division occur late in the cell cycle (Chakraborty and Das Gupta, 1962; Bhattacharya and Ghosh, 1985). The length and timing of kinetoplast and nucleus S-phases is dependent on temperature (Bhattacharya and Ghosh, 1985). More recent, qualitative, description of *L. major* division (Ambit *et al.*, 2008) suggest the cell cycle is similar to *L. donovani*, with mitosis occurring near the end of the cell cycle and kinetoplast division occurring shortly after

onset of mitosis. Onset of new flagellum growth is said to occur near the end of kinetoplast and nucleus S-phase and cytokinesis and mitosis are illustrated with altered morphology. This description of the cell cycle is not, however, supported by data.

These pre-existing descriptions of the *Leishmania* cell cycle are limited. Rigorous quantitative data exists only for the timing of kinetoplast and nuclear S-phase in *L. donovani* (Bhattacharya and Ghosh, 1985), and these were obtained under growth conditions which allowed only slow growth in comparison to even slightly more modern growth medium (Kar *et al.*, 1990). More comprehensive description of the cell cycle is not supported by data (Ambit *et al.*, 2008). Quantitative analysis of flagellum growth and cell body change, processes central to morphogenesis, do not exist.

2.1.2 Mechanisms for trypanosomatid cell morphogenesis

The morphology of the cell body and the flagellum are the primary contributors to the overall shape of a *Leishmania* cell. While it is not known how the cell body and flagellum grow in *Leishmania* these processes have been analysed in detail in *T. brucei* which gives insight into the mechanisms which may be available to *Leishmania* for generation of daughter cell morphology through division.

In *T. brucei* the sub-pellicular cytoskeleton progresses through a series of morphological changes through the cell cycle (Gull *et al.*, 1990). Tyrosinated α -tubulin can be used as a marker of newly assembled microtubules in *T. brucei* as α -tubulin is gradually detyrosinated following assembly into the sub-pellicular array and the axoneme (Sasse and Gull, 1988). Immunogold TEM and immunofluorescence using the anti-tyrosinated α -tubulin antibody YL 1/2 revealed that *T. brucei* uses two routes for altering the sub-pellicular array; elongation of the posterior of the array by extension of existing microtubules and increasing the circumference of the array by insertion of new microtubules between existing members of the array (Sherwin and Gull, 1989b; Robinson *et al.*, 1995). These processes are cell cycle regulated; the cell normally extends by 6 μm through the cell cycle (Robinson *et al.*, 1995) and disrupting normal progression from G_1 to S-phase by RNAi

degradation of Cdc2-related kinases CRK1 and 2 (Tu and Wang, 2005) or PHO80 cyclin related CycE1/CYC2 (Li and Wang, 2003) disrupts this morphogenesis and allows abnormal extension of the cell. *Leishmania* may use related mechanisms for remodelling the sub-pellicular array. *L. mexicana* α -tubulin has a C-terminal tyrosine and the YL 1/2 epitope, a C-terminal tyrosine or phenylalanine preceded by a negatively charged amino acid (Wehland *et al.*, 1984). YL 1/2 may be a useful tool for analysing the dynamics of the sub-pellicular array of *Leishmania*.

Cytokinesis is the final step in generating the daughter cell body morphology during division and occurs through remodelling of the sub-pellicular microtubule array. Cytokinesis must accurately segregate the daughter nuclei, kinetoplasts and flagellar pocket complex which lie at different positions along the long axis of the cell. In *T. brucei* this is achieved by furrow ingression from the anterior to posterior along an approximately longitudinal axis. Division is not bilaterally symmetric and the furrow follows a helical path starting near the anterior and finishing near the posterior without bisecting either (Sherwin and Gull, 1989a). The correct plane of cytokinesis is critical for morphogenesis. In *T. brucei* and RNAi knockdown of FLA1 or FAZ1, involved in attachment of the flagellum to the cell body, alter the plane of cytokinesis and result in abnormal daughter cells (LaCount *et al.*, 2002; Vaughan *et al.*, 2008) (Section 1.1.2). As the flagellum is not attached to the cell body the plane of cytokinesis in *Leishmania* must be defined by a different mechanism.

Cytokinesis has not been analysed in detail for *Leishmania* but qualitative descriptions of *L. major* suggests it may be more bilaterally symmetric (Ambit *et al.*, 2008) than *T. brucei*. Analysis of the positioning of the nuclei and kinetoplasts and the path along which the cleavage furrow ingresses will give insights into the mechanisms used to generate the promastigote (rather than trypomastigote) daughter cell morphology of *Leishmania* and provide a foundation for determining if asymmetric division may be a tool for achieving morphological change during life cycle stage differentiation.

The process of basal body duplication, maturation and flagellum growth has not previously been analysed in detail for a *Leishmania* species, although new flagellum growth has been qualitatively described as initiating late in the cell cycle (Ambit *et al.*, 2008). These processes have been analysed in more detail in *T. brucei*. A G₁ cell is found with a basal body (subtending the single flagellum) and an associated pro-basal body. During early kinetoplast S-phase the pro-basal body matures and begins to elongate to form the new flagellum which protrudes into the pre-existing flagellar pocket of the old flagellum. This is closely followed with formation of two new pro-basal bodies next to the basal body of both the old and new flagellum (Sherwin and Gull, 1989a; Woodward and Gull, 1990). A complex process of reorientation and separation of the basal bodies then occurs (Lacomble *et al.*, 2010). This is associated with the division of the flagellar pocket (Lacomble *et al.*, 2010), and division of the kinetoplast (Gluezn *et al.*, 2011) and positions the new flagellum toward the posterior of the cell. In *Leishmania*, understanding the timing of basal body maturation and onset of flagellum growth will be important for understanding normal flagellum growth and may be important for determining at which point of the cell cycle commitment to formation of an amastigote or promastigote flagellum occurs.

The newly formed *T. brucei* flagellum continues growth from its initial formation at a constant rate (Tyler *et al.*, 2001) through the remainder of the cell cycle and reaches its near-final length (>90%) at the end of cytokinesis (Sherwin and Gull, 1989a; Davidge *et al.*, 2006; Absalon *et al.*, 2008). By the next cell cycle the flagellum length has stabilised to its final length; dividing (biflagellate) *T. brucei* cells have a narrow range of old flagellum lengths (Tyler *et al.*, 2001). Some limited flagellum growth or turnover of flagellar tip material may occur up to 1.5 cell cycles following initial flagellum formation (Farr and Gull, 2009). *Leishmania* does not appear to have a similarly consistent flagellum length, either in the sandfly (Rogers *et al.*, 2002) and *in vitro* (Erdmann *et al.*, 2006; Tull *et al.*, 2010). Determining when and how the *Leishmania* flagellum grows to generate these more diverse morphologies is an important aspect of morphogenesis.

2.1.3 The cell cycle in interpreting mutant behaviour

In *T. brucei*, where detailed descriptions of the cell cycle preceded availability of genetic tools, the impact of mutation on cell cycle progression is routinely analysed, sometimes revealing unexpected functions. For example flagellum attachment and correct FAZ structure were demonstrated, through RNAi targeting of FLA1 (LaCount *et al.*, 2002) and FAZ1 (Vaughan *et al.*, 2008), to be involved in morphogenesis through defining the plane of cytokinesis. In the absence of supporting cell cycle data this function may not have been detected. The *L. mexicana* genome has been sequenced (Rogers *et al.*, 2011) and genetic tools for modification of this species are diverse and powerful (Beverley, 2003; Madeira da Silva *et al.*, 2009; Damasceno *et al.*, 2010) although *L. mexicana* does lack RNAi machinery (Peacock *et al.*, 2007). A quantitative understanding of the cell cycle would support future analysis mutants created using these genetic tools.

Here I have used cytometry from micrographs, supported by flow cytometry, time lapse videomicroscopy and electron microscopy, to generate a comprehensive quantitative description of the DNA content and morphological changes through the promastigote cell cycle. These confirmed that nucleus and kinetoplast S-phase are long and synchronous, showed that the cell undergoes rapid dramatic morphological change (decreasing in length and increasing in width) for cytokinesis, yielding insights into the mechanisms used for cell body morphogenesis, and demonstrated that flagellum length does not have a simple correlation with cell cycle stage. Onset of new flagellum growth is preceded by basal body maturation and duplication similar to *T. brucei* and mitosis and cytokinesis are subtly asymmetric. As anticipated these give insight into the capacity for morphological change in the life cycle.

2.2 Results

Promastigote *Leishmania* grown *in vitro* progress through the cell cycle asynchronously therefore exponentially growing cultures contain cells from all stages of the cell cycle. In order to analyse the

cell cycle culture growth must either be synchronised or other techniques to analyse the cell cycle must be developed. Techniques for synchronising trypanosomatids fall into two broad classes; those which use chemical treatments to stall cells at a certain stage of the cell cycle, including hydroxyurea to stall cells in S-phase (Chowdhury *et al.*, 2008) (through inhibition of ribonucleotide reductase (Yarbro, 1992)), and those which extract only cells from a certain cell cycle stage from culture, for example fluorescence activated cell sorting of cells in G₂ using a vital DNA stain (Kabani *et al.*, 2010) or size based sorting using elutriation (Archer *et al.*, 2011). Cells recovered from either technique unfortunately lose synchrony over the first few cell cycles (Chowdhury *et al.*, 2008; Kabani *et al.*, 2010). The unusual environment, particularly when using chemical treatments, also may have complex side effects; it takes 6 h for *T. brucei* to recover from hydroxyurea treatment (Chowdhury *et al.*, 2008). Cell cycle synchronisation techniques were therefore not used for this analysis.

2.2.1 Onset of new flagellum growth, kinetoplast division and mitosis occur late in the cell cycle

In exponentially growing *L. mexicana* cultures cell body length, width and flagellum length vary over an approximately twofold range. As exponentially growing cultures include only proliferating cells, the range of individual cell morphologies observed represent the different cell cycle stages. The single flagellum (F), kinetoplast (K) and nucleus (N) can be used as discrete morphological markers as each replicates once during the cell cycle for the daughter cells to inherit. Using fluorescence and phase contrast microscopy the kinetoplast, nucleus and flagellum number and configuration in a population of growing cells were analysed. Flagella were identified by PFR immunofluorescence which allowed identification only of flagella which protruded from the flagellar pocket. Morphologies with 1K1N1F (1 kinetoplast, 1 nucleus and 1 flagellum), 1K1N2F, 1K2N2F and 2K2N2F were observed (Figure 7A–F). As the flagellum, nucleus and kinetoplast each duplicate once during the cell cycle 1K1N1F morphologies (Figure 7A–C) must precede 1K1N2F

morphologies (Figure 7D), 1K1N2F must precede 1K2N2F morphologies (Figure 7E) and 1K2N2F must precede 2K2N2F morphologies (Figure 7F). Therefore during the cell cycle onset of new flagellum growth occurs first (Figure 7D), followed by mitosis (Figure 7E). Kinetoplast division occurs after onset of mitosis, when mitosis is in late anaphase or telophase (Figure 7F).

Utility of tyrosinated α -tubulin as a marker for newly formed microtubules in *L. mexicana* using the antibody YL 1/2 (Sasse and Gull, 1988) was tested. Many *L. mexicana* cells did not contain high levels of tyrosinated α -tubulin (Figure 7A–C), only biflagellate cells contained high levels of tyrosinated α -tubulin throughout their sub-pellicular microtubule array (Figure 7D–F). Tyrosinated α -tubulin in the axoneme was concentrated toward the proximal portion (Figure 7D–F). This staining pattern was unlike *T. brucei* in which all cell cycle stages feature a posterior region of

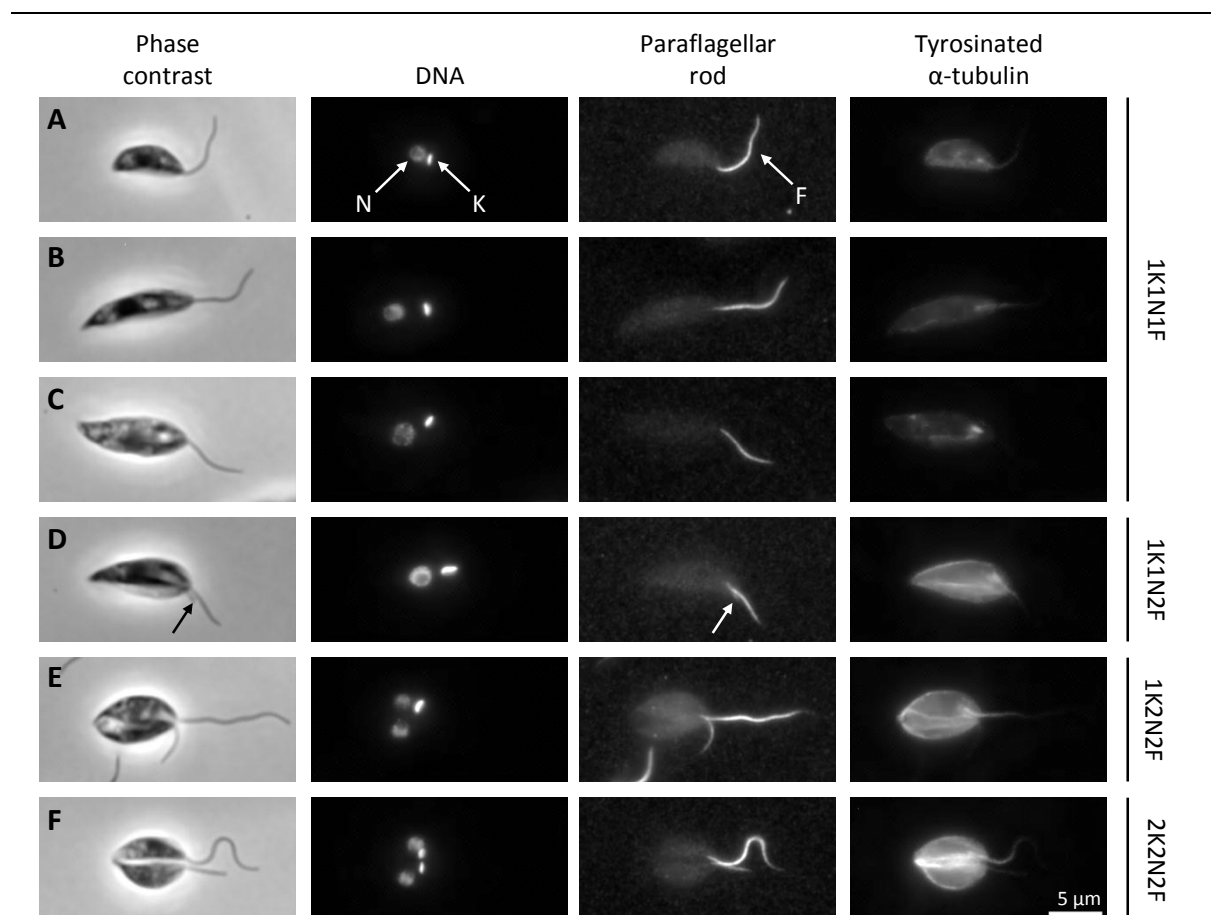


Figure 7. Stages of the *L. mexicana* cell cycle by light microscopy. DNA was labelled with DAPI, the PFR was labelled using the monoclonal antibody L8C4 and tyrosinated α -tubulin was labelled using the monoclonal antibody YL1/2. Images are a maximum projection of a z-stack. Cells were arranged in order based on kinetoplast (K), nucleus (N) and flagellum (F) number. The kinetoplast (K), nucleus (N) and flagellum (F) are indicated in A, the short new flagellum is arrowed in D.

tyrosinated α -tubulin corresponding to sub-pellicular microtubule extension. During cytokinesis tyrosinated α -tubulin staining also increases, particularly along the cleavage furrow (Sasse and Gull, 1988). Unlike *L. Mexicana*, tyrosinated α -tubulin content of the *T. brucei* axoneme is concentrated at the distal tip, particularly on growing flagella (Sasse and Gull, 1988). It therefore seems the dynamics of α -tubulin detyrosination in *L. mexicana* prevent the use of YL 1/2 as a marker for newly assembled microtubules.

The time at which onset of flagellum growth, mitosis and kinetoplast division occur within the cell cycle can be calculated from the proportion of cells observed with each K/N/F morphology during exponential growth and the doubling time (Williams, 1971). The growth properties of *L. mexicana* *in vitro* were therefore analysed to determine the densities between which growth occurred logarithmically. Between 10^5 and 10^7 cells/ml (densities under 10^5 cells/ml were not analysed) growth was exponential (Figure 8A). With repeated subculture to 10^6 cells/ml to maintain culture density below 10^7 cells/ml the population remained in logarithmic growth with a doubling time of 7.1 h (Figure 8B). From a sample of a population in logarithmic growth, light microscopy was used to classify 980 cells (from three independent sample preparations) by K/N/F configuration (Figure 8C). Using these data the timings of cell cycle events were calculated, both in hours since the start of the 7.1 h long cell cycle and as the percentage progress through the unit cell cycle. Onset of new flagellum growth, mitosis and kinetoplast division all occur late in the cell cycle at 5.2 h (83% cell cycle progress), 6.3 h (88%) and 6.6 h (93%) respectively.

2.2.2 Delayed abscission is common

In exponentially growing cultures a sub-population of cells was attached posterior-to-posterior. These cells appeared to have completed division except for abscission leaving a connection, which may be analogous to the midbody in mammalian cells during late cytokinesis. These cells were termed ‘doublets’ and they are distinct from previously described ‘rosettes’ which comprise a larger number of cells positioned side-to-side with their flagellum toward the centre of the cluster (Dwyer

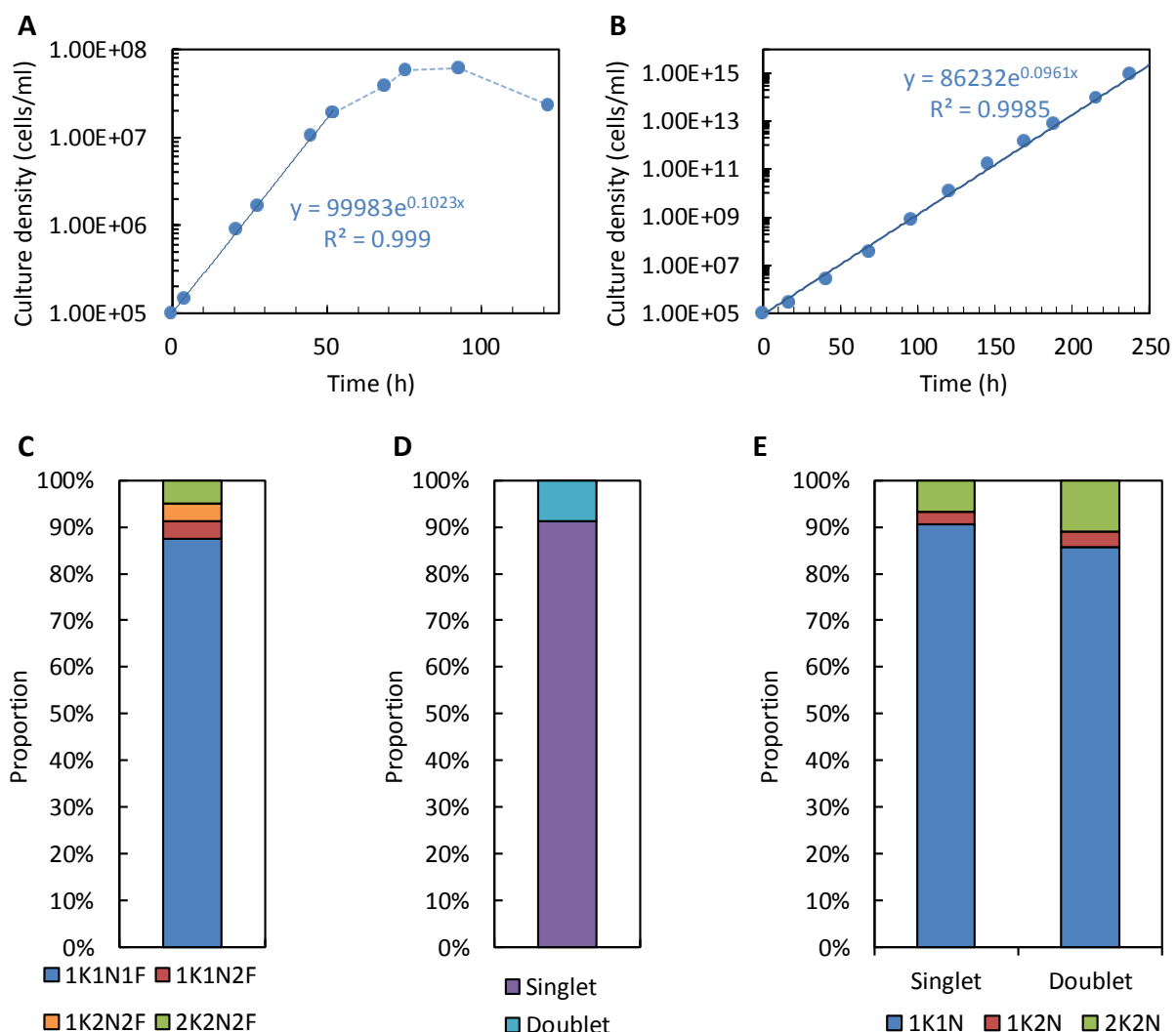


Figure 8. Properties of logarithmic growth of *L. mexicana*. **A.** Growth of *L. mexicana* promastigotes from 10^5 cells/ml at 28°C in M199 with 10% FCS at pH7.4. The first six data points are shown with an exponential fitted curve. **B.** Calculated growth of *L. mexicana* promastigotes maintained in logarithmic phase growth by repeated 9 successive subcultures to 10^6 cells/ml, shown with an exponential fitted curve. **C.** Proportion of cells with different kinetoplast (K), nucleus (N) and flagellum (F) numbers, $n = 980$. **D.** Proportion of cells found as doublets and singlets, $n = 1114$. **E.** Proportion of singlet ($n = 942$) and doublet ($n = 182$) cells with different K/N numbers. All samples taken from an exponentially growing population.

et al., 1974). A similar doublet arrangement has also been observed for *T. brucei* (Vaughan and Wheeler, Unpublished).

Using a centrifugation-free microscopy sample preparation protocol, to maximise preservation of this structure (Section 2.4.1) exponentially growing *L. mexicana* were observed by fluorescence and scanning electron microscopy (SEM). Light microscopy showed that approximately 10% of cells were as doublets (counting doublets as two cells) (Figure 8D). Staining of with DAPI indicated the proportion of 1K1N, 1K2N and 2K2N doublet cells was not significantly different to normal 'singlet' cells ($p > 0.25$, Pearson's χ^2 test) (Figure 8E) indicating the cell cycle of the doublets is not abnormal. Doublets were therefore included as two separate cells in any further analyses. The two cells in a doublet were universally found with the same number of kinetoplasts and nuclei showing their cell cycles are approximately synchronous. This may indicate a cytoplasmic link between the two cells through which cell cycle regulating factors could travel. SEM confirmed that the stages of the cell cycle of singlet cells (Figure 9A–D) all have doublet counterparts (Figure 9E–H), based on external morphology. The morphology of late cytokinesis singlet cells (Figure 9D) and early cell cycle doublets (Figure 9E) are indistinguishable, supporting the proposed origin of doublet cells as cells which have delayed abscission. The pleated appearance of the cells was consistently observed and was seen when cells were fixed for 2 h with either 2.5% or 4% glutaraldehyde or 4% paraformaldehyde. This appearance is therefore not thought to be a fixing artefact. The seam-like structure on cells during late cytokinesis along the position of the cleavage furrow (Figure 9D–E) was also consistently observed.

These analyses revealed the order and timing of key morphogenetic events in the *Leishmania* cell cycle; onset of new flagellum growth, mitosis and kinetoplast division. All three of these events occur in the last 20% of the cell cycle leaving a large proportion of the cell cycle with no clear morphological markers.

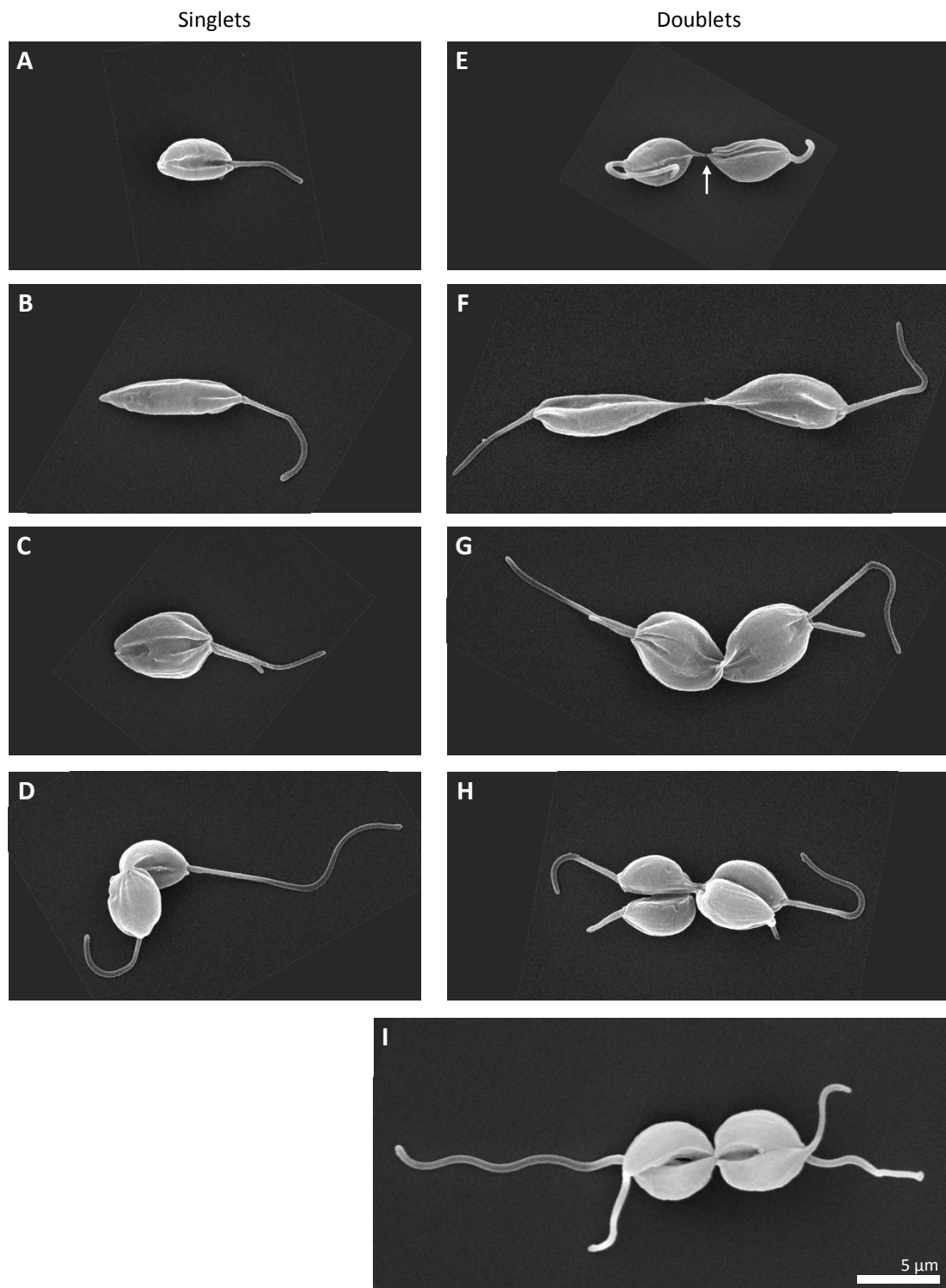


Figure 9. Stages of the *L. mexicana* cell cycle by SEM. A–D. Micrographs of the major morphologies of normal ‘singlet’ cells arranged in increasing cell cycle progress from A to D based on morphologies observed by light microscopy (Figure 7). E–H. Micrographs of morphologies of ‘doublet’ cells corresponding to the cell cycle stages shown in A–D. The thin cytoplasmic bridge which connects the two cells is arrowed in E. I. An example quadriflagellate doublet cell with a clear view of four flagella of different length.

2.2.3 Morphological and DNA content changes through the cell cycle

To analyse the events of the first 80% of the cell cycle, particularly the cell growth and morphogenetic processes which give rise to the wide range of morphologies, other cellular properties must be measured. Therefore analysis moved away from the discrete morphological markers of kinetoplast, nucleus and flagellum number to continuous quantitative measures, for example the quantity of nucleus DNA and cell length and width. Morphological measurements were collected from cells during logarithmic growth at three culture densities, 3.0×10^6 , 5.0×10^6 and 1.3×10^7 cells/ml, each grown from logarithmically growing cells subcultured to a density of 1.0×10^6 cells/ml, to analyse the cell cycle and detect any changes in morphology with density. For each density, cell body length, cell body width, flagellum (or flagella) length, kinetoplast DNA content and nuclear DNA content were measured (Figure 10A). Cell body length showed a broad distribution, 6 to 12 μm , with no significant variation between culture densities (Figure 10B). Total DNA content varied over an approximately twofold range, also with no significant variation between culture densities (Figure 10C). Flagellum length showed a broad distribution, 3 to 16 μm , with a significant increase with culture density (Figure 10D). Cell body width showed a tighter distribution, 2 to 5 μm , with a significant decrease with culture density (Figure 10E).

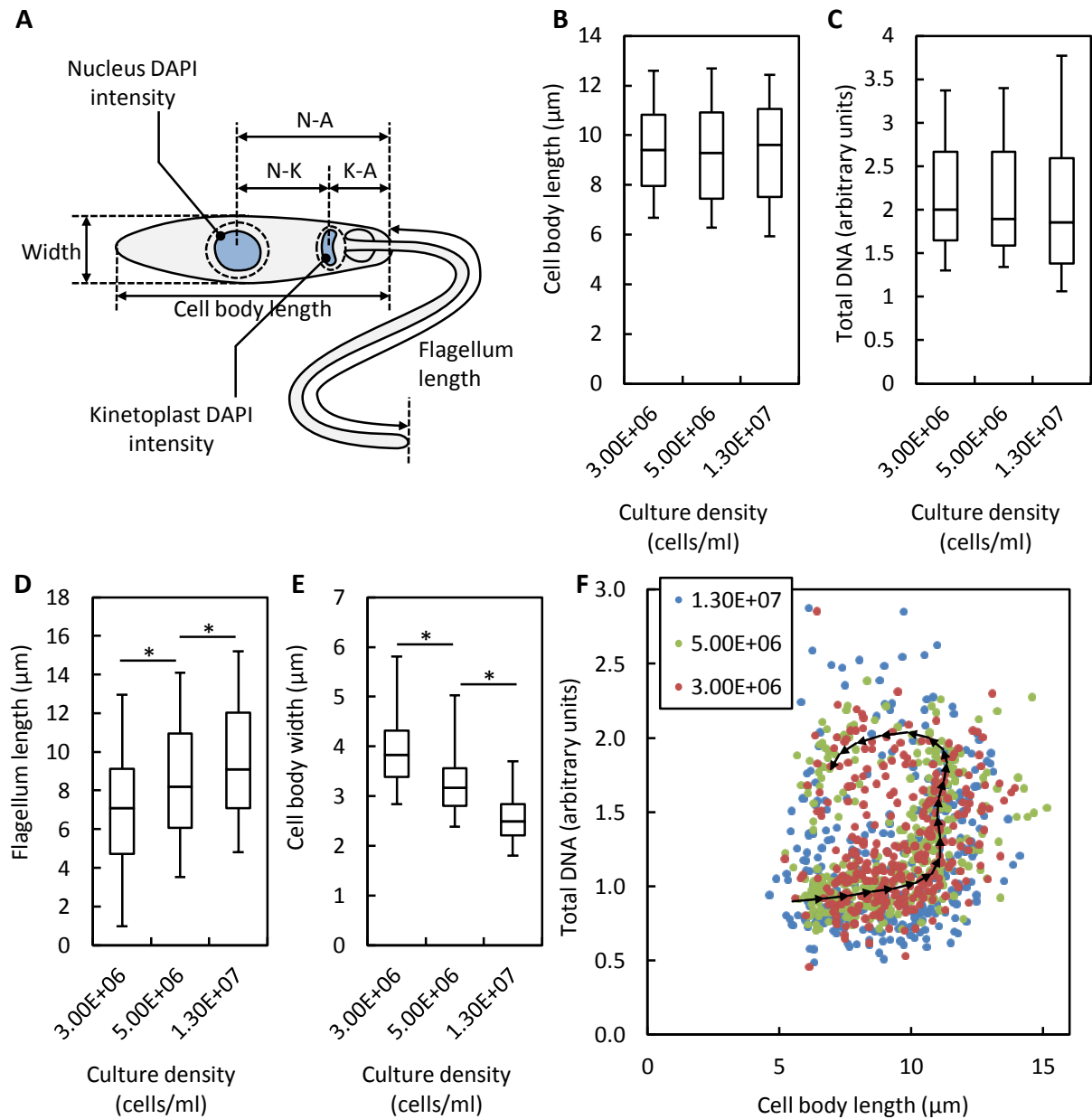


Figure 10. Morphometric analysis of *L. mexicana*. **A.** Cartoon illustrating the cellular features measured from light micrographs; cell body length and width, flagellum length, nucleus DNA and kinetoplast DNA content (from DAPI intensity), kinetoplast-anterior distance (K-A), nucleus-anterior distance (N-A) and nucleus-kinetoplast distance (N-K). If organelle duplication had occurred then both daughters were measured. **B-E.** Box-whisker plots of cell body length, total DNA, longest flagellum length and cell body width for three logarithmic culture densities. Boxes and whiskers indicate the median, upper and lower quartiles and 5th/95th percentiles. All significant differences ($p < 0.01$, Student's t-test) are indicated with an asterisk (*). **F.** Scatter plot of cell body length against total DNA content for the three logarithmic culture densities. The best-fit curve (black arrows) is shown. $n = 320$ (3.0×10^6 cells/ml), $n = 341$ (5.0×10^6 cells/ml), $n = 321$ (1.7×10^6 cells/ml).

2.2.3.1 Determining cell cycle progress from cell size and DNA content

To translate these morphological measurements to an analysis of the cell cycle, a method to derive cell cycle progress from continuous changes in morphology must be determined. The ability to calculate cell cycle progress from morphology would allow the precise determination of cell cycle stage of cells in an asynchronous culture on a cell by cell basis. In any stable proliferative cell cycle a cell must achieve two targets; the cell volume increase to that of two daughter cells (typically a doubling) and the DNA content must be duplicated. Measurement of cellular DNA content is well established method for determining the position of a cell within the cell cycle through flow cytometry (Chowdhury *et al.*, 2008; Archer *et al.*, 2011) or quantitative fluorescence microscopy (Siegel *et al.*, 2008). Measurement of *L. mexicana* morphology indicated there was an approximately twofold range of cell lengths (Figure 10B) and a narrow range of cell widths (Figure 10E), therefore cell length is approximately proportional to cell volume. I predicted that the combination of both cell length and cellular DNA content would allow the calculation of cell cycle progress. A scatter plot of total DNA against cell body length (Figure 10F) revealed a distinctive correlation which was used as the basis for determining the position of each cell, based solely on its morphometric measurements, in the cell cycle.

At the start of each cell cycle a *Leishmania* cell is expected to have a single copy of cellular DNA and a small volume, corresponding to a short cell length. I therefore analysed the scatter plot of total DNA against cell body length (Figure 10F) using a stepwise path walking algorithm (Section 2.4.3) to draw a best-fit curve through the plot, starting from a short cell length and single unit of DNA. This curve fitting method follows the regions of highest point density in the plot and, if accurate, depicts the changes in cell body length and DNA content of an ideal cell as it progresses through the cell cycle.

If a cell starts with a short cell body and a single copy of DNA, the best-fit curve indicates it initially grows in length maintaining a single copy of DNA (G_1 phase). This is consistent with 1K1N1F cell body sizes observed by light microscopy (Figure 7A–C). The cell body length then stabilises and instead DNA content increases (S-phase). Finally, while maintaining a constant DNA content, the cell body decreases in length (G_2 , mitosis and cytokinesis). Again this is consistent with the decrease in cell body length and increase in width during progression from 1K1N2F to 1K2N2F then 2K2N2F as observed by light microscopy (Figure 7D–F).

The density of points around the best-fit curve varied depending on the stage of the cell cycle, the density was highest around G_1 and S-phase and least around G_2 , mitosis and cytokinesis (Figure 10F). The density of points is proportional to the time spent during that stage of the cell cycle; high point densities correspond to slow processes and low point densities correspond to fast processes. On this basis the timing of changes in morphology could be calculated (Section 2.4.3). This calculated cell cycle progress value (derived from cell length and total DNA content) was a powerful tool as it allowed correlation of morphometric data with the precise position of each cell within the cell cycle. This was used to analyse change in kinetoplast and nucleus DNA content, cell body length and width, old and new flagellum length and kinetoplast and nucleus location through the cell cycle.

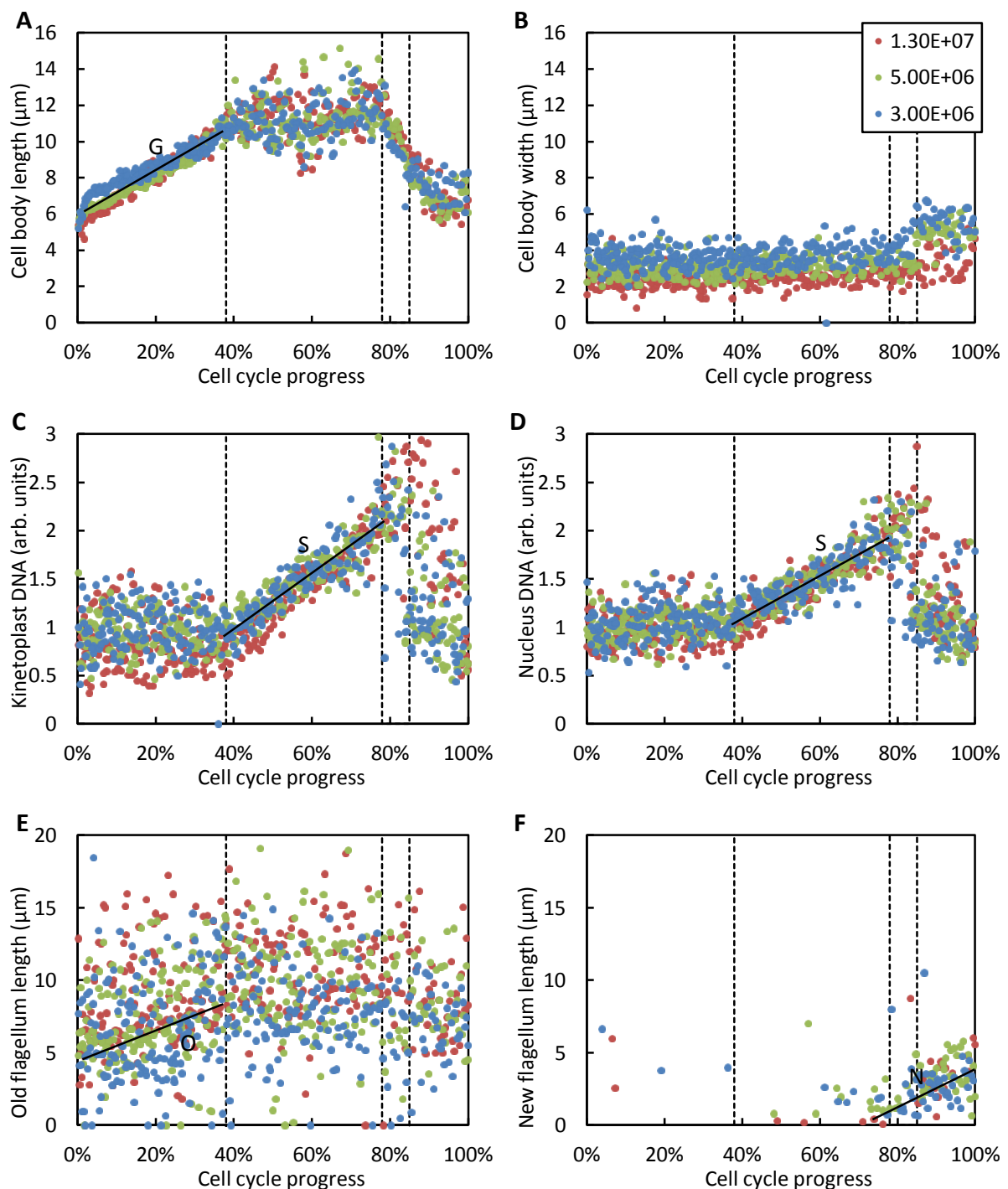


Figure 11. Changes in morphology and DNA content through the *L. mexicana* cell cycle. A-B. Correlation of cell body length and width with cell cycle progress calculated from cell body length and total DNA content of the cell (Figure 10F). C-D. Correlation of kinetoplast and nucleus DNA content with calculated cell cycle progress. DNA content of individual kinetoplasts and nuclei (i.e. not the total cellular kinetoplast/nuclear DNA content) is shown. E-F. Correlation of the old (long, present at the start of the cell cycle) and new (short, starting growth partway through the cell cycle) flagellum length with calculated cell cycle progress. Data from three logarithmic culture densities are shown, $n = 320$ (3.0×10^6 cells/ml), $n = 341$ (5.0×10^6 cells/ml), $n = 321$ (1.7×10^6 cells/ml).

2.2.3.2 *Leishmania* undergo large morphological changes through the cell cycle

Figure 11A–F show the correlation of morphometric measurements with calculated cell cycle progress (along the x -axis) which approximately corresponds to a straightened version of the fit line in Figure 10F. These plots allow the direct identification of the timings and rates of morphological changes. Cell body length and width correlated with cell cycle progress (Figure 11A–B). The cell body entered the cell cycle with a length of 6 μm and width (dependent on culture density) of approximately 3 μm (see also Figure 10E) then grew in length to 11 μm by 2.7 h (38%) at a rate of approximately 1.7 $\mu\text{m}/\text{h}$. Cell length and width remained constant from 2.7 h (38%) to 5.6 h (78%). This was followed by a rapid decrease in length back to 6 μm between 5.6 h (78%) to 6.4 h (90%) which occurred at the same time as an increase in width to 5 μm . The approximate volume and surface area of the cell was calculated by treating it as a scalene spheroid. This indicated the increase in width and decrease in length preserved cell volume (approximately 400 μm^3) and surface area (approximately 320 μm^2) as the cell dimensions changed from 11 $\times$ 3 $\times$ 3 to 6 $\times$ 3 $\times$ 5 μm . This rapid morphological change is during mitosis and cytokinesis and occurred at the same stage of the cell cycle as increased tyrosinated α -tubulin content of the sub-pellicular microtubules (Figure 7).

2.2.3.3 Kinetoplast and nucleus S-phases are long and synchronous

The quantity of kinetoplast and nuclear DNA also showed distinctive correlations with cell cycle progress (Figure 11C–D). DNA content of both organelles remained constant from the start of the cell cycle to 2.7 h (38%), the period in which cell body length increased. Between 2.7 h (38%) and 5.6 h (78%) the DNA content of both organelles increased linearly and synchronously, indicating the S-phases of the kinetoplast and nucleus are synchronous. On reaching duplicated DNA content (around 5.6 h (78%)) the kinetoplast and nucleus divided, returning the organelles to a single copy of DNA. This occurred immediately after the end of S-phase (there is no prolonged G_2 phase). Mitosis occurred between 5.6 h (78%) and 6.4 h (90%), the same time as the cell underwent its rapid decrease in length. Kinetoplast division occurred slightly later, typically later than 6.4 h (90%), when the

nucleus was late anaphase (see [Figure 7E–F](#) in which sister chromatids have separated prior to kinetoplast division). The timings of these events are consistent with the proportion of cells observed with different K/N configurations ([Figure 8C](#)) ([Section 2.2.4](#)).

2.2.3.4 Flagellum length does not have a simple correlation with the cell cycle

Unlike cell body shape and DNA content, flagellum length did not show a clear correlation with cell cycle progress ([Figure 11E–F](#)). The length of the flagellum present on cells entering the cell cycle (the ‘old flagellum’) ([Figure 11E](#)) was very varied, typically between 5 and 15 μm . There was a weak positive correlation of flagellum length with cell cycle progress up to 2.7 h (38%) suggesting the flagella may be growing at a rate of approximately 1 $\mu\text{m}/\text{h}$, although this is a tenuous conclusion because of the weak correlation. The onset of new flagellum growth was clearer and occurred between 5.0 h (70%) and 5.7 h (80%), near the end of S-phase, and grew at a rate of approximately 3 $\mu\text{m}/\text{h}$ up to completion of cytokinesis at which point it had reached 4 to 5 μm . Number of flagella present on the cell was not used in calculating cell cycle progress, yet 90% of cells with two flagella were calculated as being in the last 2.9 h (40%) of the cell cycle. This is consistent with the proportion of cells observed with different K/N/F configurations ([Figure 8C](#)) and corroborates my method of using cell body length and total DNA content for calculating where a cell is in the cell cycle.

Timing and magnitude of cell body length change ([Figure 11A](#)) and timing of kinetoplast and nuclear DNA synthesis and division ([Figure 11C–D](#)) were not significantly different for cells undergoing logarithmic growth at three culture densities, 3.0×10^6 , 5.0×10^6 and 1.3×10^7 cells/ml (each grown from logarithmically growing cells subcultured to a density of 1.0×10^6 cells/ml). However cell body width and flagellum length both showed significant correlation with culture density ([Figure 10D–E](#)), although both followed similar trends through the cell cycle (increase in cell width during mitosis and cytokinesis and an increase in flagellum length during G_1) at all densities ([Figure 11B & E–F](#)). It seemed plausible that the variation of cell body width and flagellum length

width may be linked, however there was no significant correlation of cell body length with flagellum length (Figure 12A) so these culture density dependent phenomena are unlikely to be directly related.

Kinetoplast and nucleus position were measured in addition to cell length and width (Figure 10A). The kinetoplast was positioned at an approximately constant distance from the anterior end of the cell at a distance of around 2.5 μm (Figure 12B). The nucleus position showed a positive correlation with cell length, its position can be expressed as:

$$n \approx 2.5 + 0.2l$$

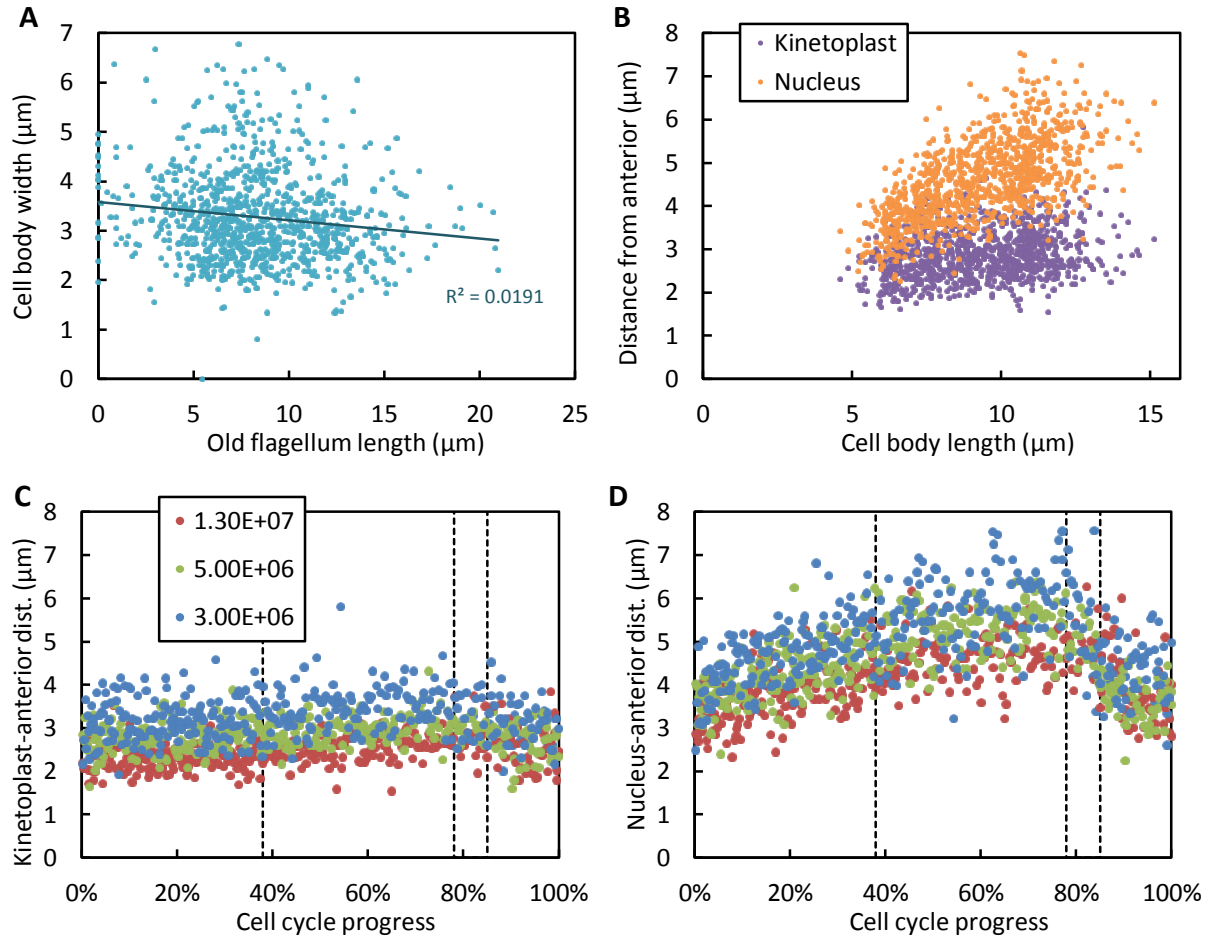


Figure 12. Additional morphological features of *L. mexicana* and their relationship with the cell cycle. A. Correlation of cell body length with old flagellum length (pooled data from three logarithmic culture densities). B. Correlation of kinetoplast–anterior and nucleus–anterior distances with cell body length (pooled data from three logarithmic culture densities). C–D. Correlation of kinetoplast–anterior and nucleus–anterior distance with calculated cell cycle stage. $n = 320$ (3.0×10^6 cells/ml), $n = 341$ (5.0×10^6 cells/ml), $n = 321$ (1.7×10^6 cells/ml), $n = 982$ (pooled data).

where n is the nucleus-anterior distance and l is the cell body length in micrometres. Kinetoplast position did not show a strong correlation with cell cycle progress (Figure 12C) while nucleus position (Figure 12D) followed a similar trend to cell body length (Figure 11A). This shows nucleus position depends on cell length and indicates that in short cells, particularly in early G_1 , the kinetoplast and nucleus lay very close to each other.

2.2.4 Validation of cell cycle events

The method of using cell length and total DNA content of a cell to calculate its cell cycle progress (Figure 10A) yields results (Figure 11) consistent with the morphologies of cells observed in culture (Figure 7), but is a new technique. The measurement of DNA content, and therefore timing of S-

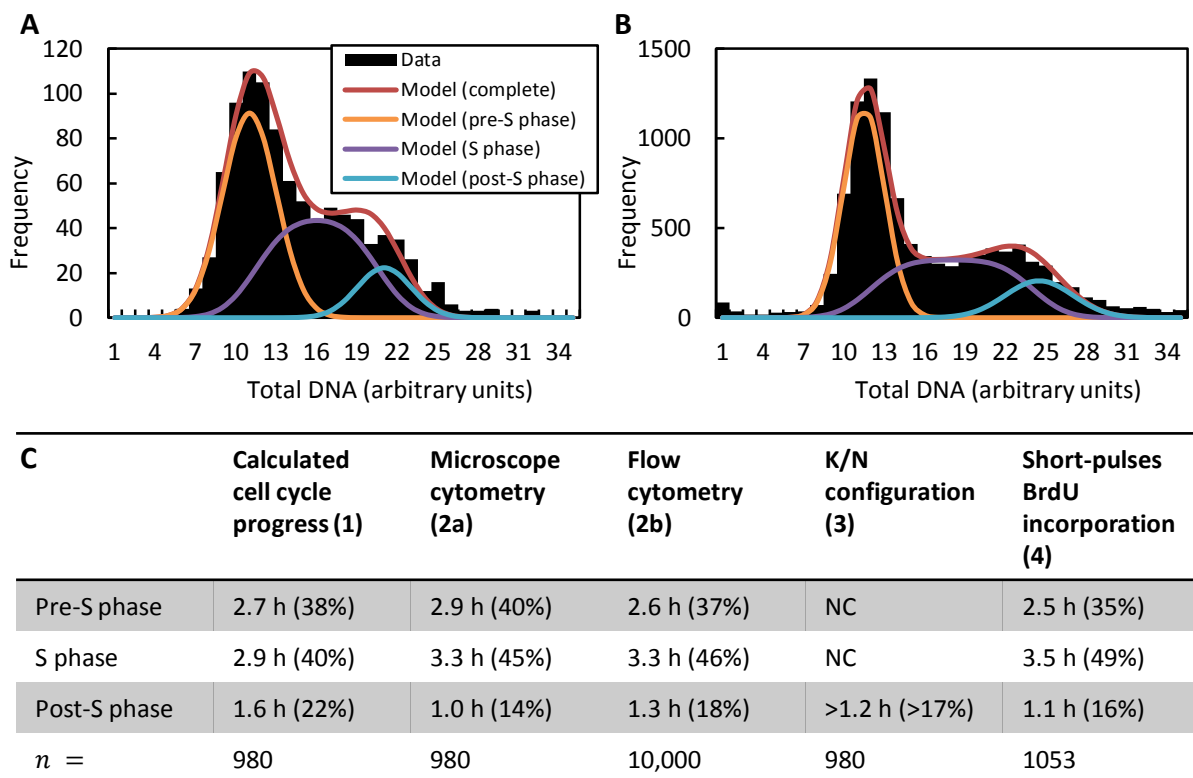


Figure 13. The length and timing of *L. mexicana* S-phase. A-B. Calculation of the length of S-phase from microscope and flow cytometry. Histograms of total DNA content of cells fitted to a model of the cell cycle (Section 2.4.4) to determine the number of cells in pre-S-phase, S-phase and post-S-phase. A. Microscope cytometry data, DNA content measured from DAPI staining intensity, $n = 980$. B. Flow cytometry data, DNA content measured from PI staining intensity, $n = 10,000$. C. The length of pre-S-phase, S-phase and post-S-phase of *L. mexicana* determined by four methods. 1. As determined from correlation of kinetoplast and nucleus DNA with calculated cell cycle progress (Figure 11C-D). 2. From curve fitting to a histogram of total cellular DNA content measured by either a, microscope cytometry (Figure 13A) or b, flow cytometry (Figure 13B). 3. From the proportion of cells which have entered mitosis (Figure 8C) therefore can no longer be in S-phase. 4. From incorporation of BrdU into synthesising DNA. NC indicates the timing for that cell cycle stage is not calculable with that analysis method.

phase, is sensitive to the accuracy of measurement of DNA content from fluorescence micrographs. It also yielded a surprisingly long kinetoplast and nucleus S-phase in comparison to *T. brucei* (Woodward and Gull, 1990), although it is consistent with previous analysis of *L. donovani* (Bhattacharya and Ghosh, 1985). The changes in cell length and width during mitosis and cytokinesis were also unexpectedly large and fast in comparison to *T. brucei* (Gull *et al.*, 1990). These results were therefore validated with different methods.

2.2.4.1 Validation of S-phase length and timing

The length of S-phase was validated by three methods: 1, histogram analysis of the DNA content of cells in the population; 2, pulse-labelling of replicating DNA with BrdU and 3, constraining the length of S-phase based on the proportion of cells with different K/N configurations observed. The first method was based on histograms of the total DNA content of cells in an exponentially growing population. This histogram is made up of cells in pre-S-phase (single DNA content), post-S-phase (doubled DNA content) and S-phase (intermediate DNA content) and therefore can be fitted to a curve to give a measurement of the number of cells in each of these cell cycle stages (Section 2.4.4). This method was applied to both DNA content measured by intensity of DAPI staining by microscopy (data shown in Figure 10C, and shown fitted to the model in Figure 13A) and DNA content measured

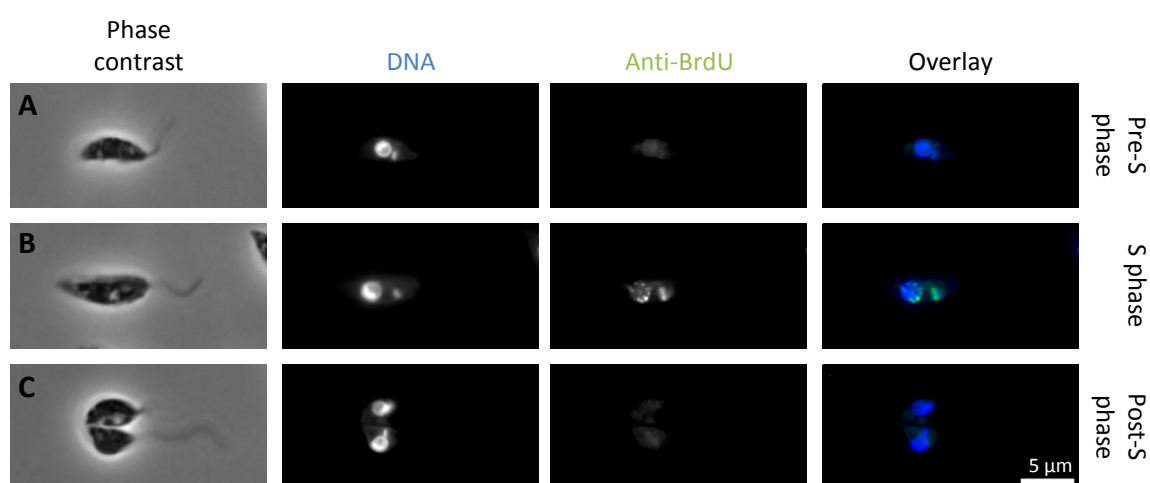


Figure 14. BrdU incorporation in promastigote *L. mexicana*. Examples of anti-BrdU immunofluorescence following 15 min incubation of logarithmically growing promastigotes with BrdU, DNA was labelled with DAPI. **A.** Pre-S-phase cell. **B.** S-phase cell with BrdU incorporation in the nucleus and kinetoplast. **C.** Post-S-phase (cytokinetic) cell.

by flow cytometry of propidium iodide (PI) stained cells (Figure 13B). The resulting measurements of cell cycle stage lengths are summarised in Figure 13C.

The second method used a short (15 min) incubation of exponentially growing cells with BrdU which is incorporated into newly synthesised DNA. These cells were then fixed and analysed by anti-BrdU immunofluorescence, BrdU incorporation was seen at multiple point-like foci in the nucleus and the kinetoplast in some 1K1N cells (Figure 14). All cells analysed ($n = 1053$) were either positive for BrdU incorporation into both the kinetoplast and nucleus or neither organelle, confirming that the S-phases of these two organelles are synchronous. The proportion of cells in each K/N configuration which were BrdU positive was analysed and from these data, assuming a short G_2 phase prior to mitosis, I calculated the length of S-phase (Figure 13C).

The third method was based on the simple assumption that S-phase must be complete before the onset of mitosis, therefore the minimal length of post-S-phase can be calculated from the proportion of 1K2N and 2K2N cells in the population (data shown in Figure 8C, result shown in Figure 13C). The duration of S-phase determined by my calculated cell cycle progress analysis Figure 11C-D gave pre-S-phase, S-phase and post-S-phase durations of 2.7 h (38%), 2.9 h (40%) and 1.6 h (22%) respectively. The data from all three validation methods (Figure 13C) were in agreement, supported these measurements and together indicated the S-phase of *L. mexicana* lasts 40% of the cell cycle.

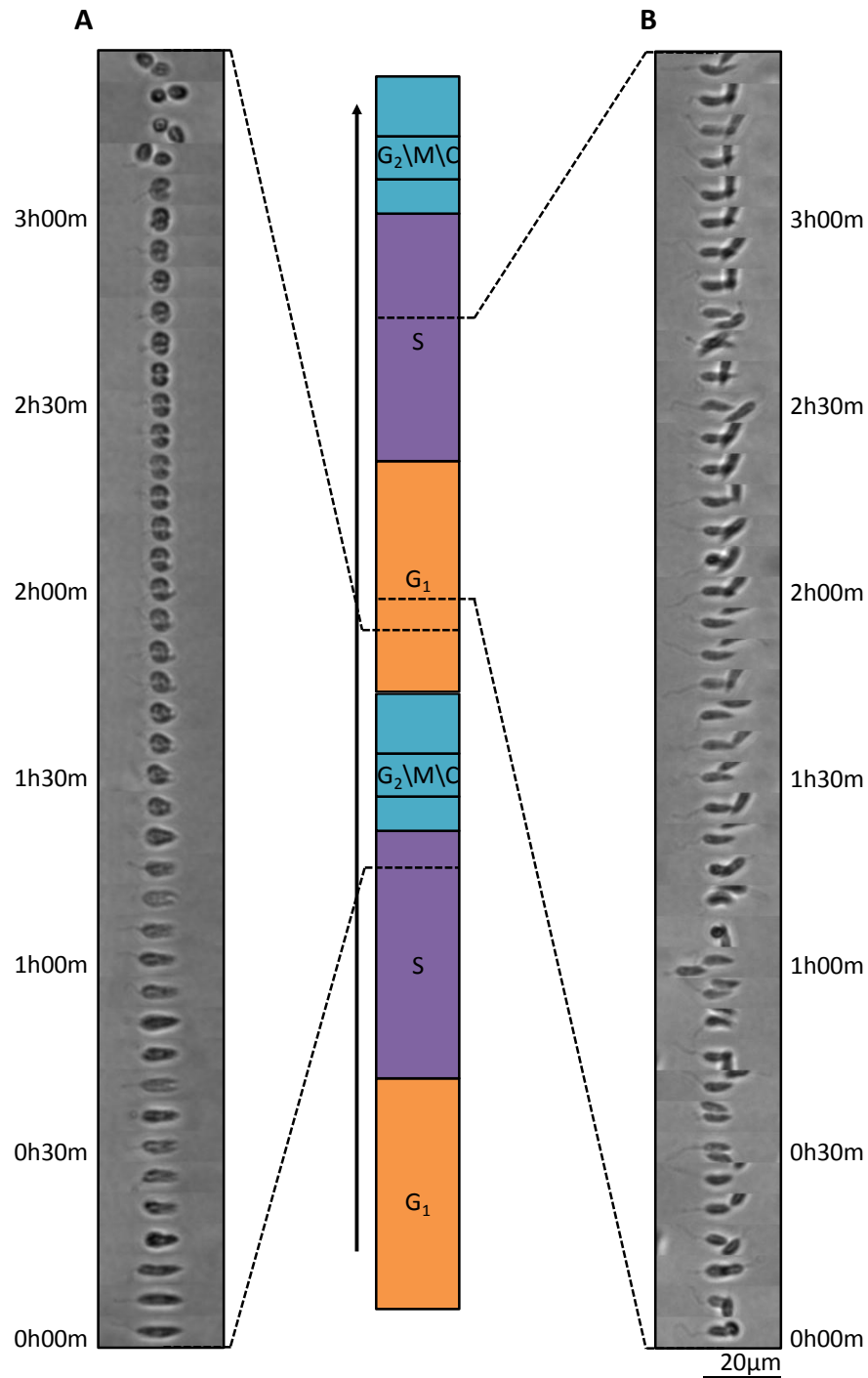


Figure 15. Time-lapse of individual *L. mexicana* cells progressing through the cell cycle. Montages of video frames from time-lapse observation of *L. mexicana* cells immobilised in small fluid pockets in agarose. **A.** A cell leaving S-phase, undergoing morphological changes for mitosis and generating two daughter cells which remain trapped in the same liquid pocket following division. **B.** Two cells (likely to be sister cells) trapped in the same liquid pocket growing in length. Both cells are growing in length at the same rate. The approximate position of the cell in the cell cycle for each video is indicated in the centre.

2.2.4.2 Validation of morphological change with time lapse microscopy

The magnitude and speed of change in cell body shape observed (Figure 11A–B) was surprising given the structure of the sub-pellicular microtubule array. Morphological changes were therefore validated by time-lapse microscopy. Time-lapse observations of *Leishmania* are challenging as promastigotes are fast swimmers, capable of moving one body length in under a second (Gadelha *et al.*, 2007). A method to trap promastigotes in agarose was therefore developed (Section 2.4.5). This immobilisation allowed the cells to move and tumble within a small liquid pocket, but not travel any significant distance. Videos with one z-stack captured every minute for 12 h were recorded and the slice from the z-stack in best focus at each time point was selected. Flagellum movement was seen to slow after the 5.0 h therefore only the first 3.5 h, where cell behaviour is likely to be minimally perturbed by the unusual cellular environment, was used for analysis. Many (>50) cells at a variety of stages of the cell cycle, including those undergoing cytokinesis (Figure 15A) and those undergoing a growth in cell body length (Figure 15B), were observed (Supplementary: Video 1). These direct observations of changes in morphology are consistent with the morphometric data shown in Figure 11A–B, and based on this data the time-lapse videos can be mapped to particular parts of the cell cycle (Figure 15).

2.2.5 Flagellum growth, duplication and reorientation of basal bodies

Analysis of flagellum lengths by light microscopy were performed on only the external portion of the flagellum. The portion in the flagellar pocket could not be visualised. The newly growing flagellum emerged from the pocket near the end of S-phase (Figure 11C– D & F). Transmission electron microscopy (TEM) of thin serial sections was used to analyse the timing of initiation of new flagellum growth and formation and re-orientation of the basal bodies (Figure 16). The flagellum of all 1K1N1F cells was subtended by a basal body, adjacent to which was a pro-basal body ($n = 7$) (Figure 16A–C & J). In 1K1N2F cells in which the new flagellum was still contained fully within the flagellar pocket both basal bodies had an associated pro-basal body, oriented orthogonally to its partner basal body ($n = 9$) (Figure 16D–F & K). In mitotic cells with a dividing kinetoplast and two flagella the pro-basal bodies had reoriented to lie parallel to their partner basal body ($n = 9$)

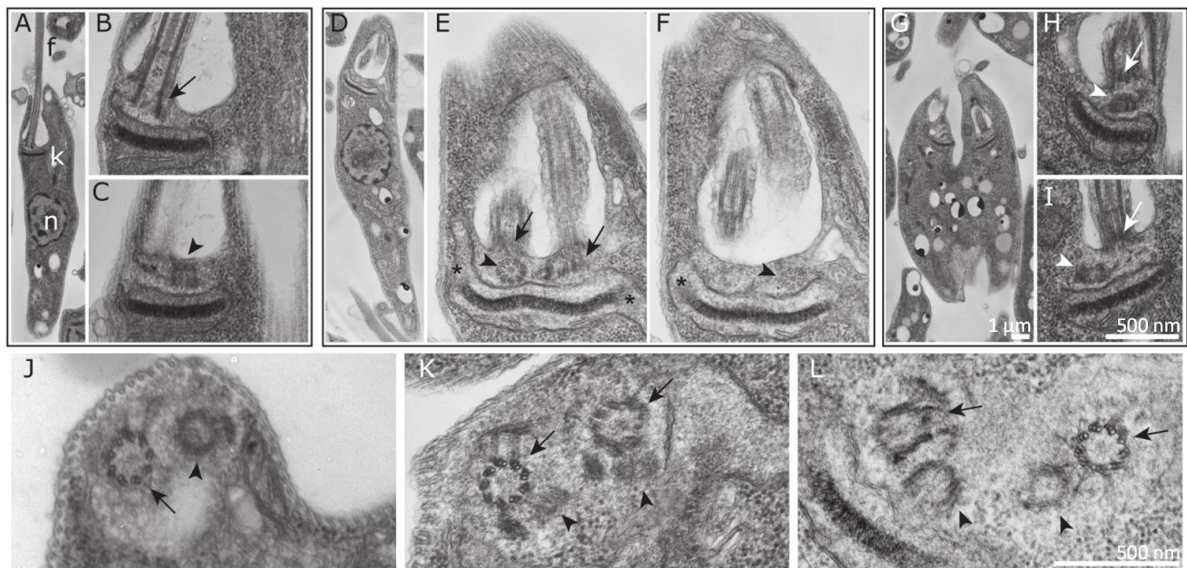


Figure 16. The basal body duplication cycle in *L. mexicana*. Electron microscopy of serial thin sections of stages of basal body duplication and onset of new flagellum growth, from (Wheeler *et al.*, 2011). **A–C.** 1K1N cell, longitudinal sections. One basal body (at the base of the flagellum, arrow in **B**) and one, parallel, pro-basal body (arrowhead in **C**) are associated with the kinetoplast. **D–E.** 1K1N cell, longitudinal sections. The kinetoplast is elongated and has polar filamentous regions (asterisk in **E–F**), therefore it is in S-phase. Two basal bodies (arrows in **E–F**) are associated with the kinetoplast and subtend two flagella, one of which is short and does not protrude from the flagellar pocket. Two pro-basal bodies lie orthogonal to the two basal bodies (arrowheads in **E–F**). **G–I.** 2K2N cell, longitudinal sections. Two basal bodies (arrows in **H–I**) are each associated with a kinetoplast and subtend a flagellum in a separate flagellar pocket. Two pro-basal bodies (arrowheads in **H–I**) lie parallel to the two basal bodies. **J–L.** Example transverse sections through basal body pro-basal body pairs. The following sets of images are at the same magnification: **A, D & G; B, C, E, F, H & I; J, K & L.**

(Figure 16L). Each daughter cell inherited one basal body/pro-basal body pair ($n = 5$) (Figure 16G–I). This sequence of onset of new flagellum growth and basal body duplication, basal body reorientation and basal body/pro-basal body inheritance is similar to seen in both *T. cruzi* (Elias *et al.*, 2007) and *T. brucei* (Sherwin and Gull, 1989a).

TEM indicated that the earliest new flagellum growth occurred during kinetoplast S-phase (Figure 16D–F) while the growing flagellum became visible outside of the flagellar pocket near the end of S-phase (Figure 11C–D & F). By this stage the new flagellum has extended through the flagellar pocket and has reached an estimated external length of 1 to 2 μm to become clearly visible next to the old flagellum. New flagellum growth rate is approximately 3 to 4 $\mu\text{m}/\text{h}$ (Figure 11F) and the flagellar pocket is approximately 2.5 μm deep, therefore basal body maturation and initial onset of new flagellum growth occurred approximately 1 h prior to the end of S-phase.

2.2.6 *Leishmania* division is subtly asymmetric

Leishmania division is not wholly symmetric and the two daughter cells are not identical. Light microscopy revealed that the plane of cytokinesis in *Leishmania* is approximately longitudinally symmetric but the positioning of the kinetoplast and nucleus prior to onset of mitosis is not. This leads to asymmetries in organelle positioning during mitosis and kinetoplast division (Figure 17A–F). 1K1N cells enter mitosis with the kinetoplast and nucleus positioned on the opposite side of the cell to the newly growing (shorter) flagellum (Figure 17A). As mitosis occurs one daughter nucleus is pushed toward the far side of the cell at a more posterior position (Figure 17B–C). Kinetoplast division occurs in a similar fashion with the kinetoplast associated with the new flagellum moving to join its associated nucleus (Figure 17D–E). The cytokinesis furrow then ingresses from the anterior along the longitudinal cell axis (Figure 17F); progress of furrow ingression from the anterior to posterior is clearly visible by SEM (Figure 18).

The asymmetry in nucleus and kinetoplast position during division is small but sufficient to accurately determine which is the old flagellum and which is the new one from the kinetoplast and nucleus location. In 1K1N2F cells the new flagellum is positioned on the opposite side of the cell to the kinetoplast and nucleus (Figure 17A), in 1K2N2F and 2K2N2F cells the new flagellum is positioned on the same side of the cell as the more posterior nucleus (Figure 17C–D). The cell with the new flagellum is harder to identify at late stages of cytokinesis (Figure 17F).

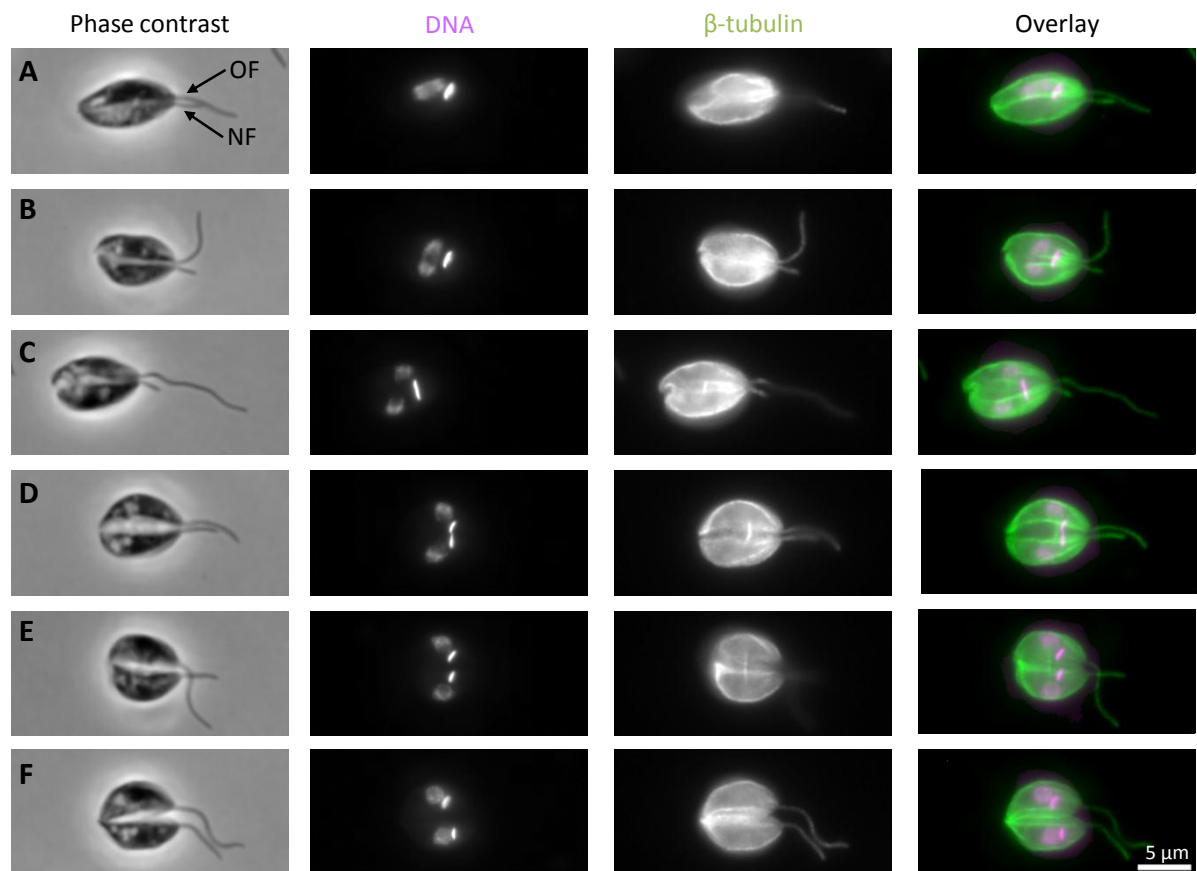


Figure 17. Asymmetries in mitosis and cytokinesis of *L. mexicana*. A–F. Light micrographs of biflagellate cells arranged in order of cell cycle progress. DNA was labelled with DAPI, β -tubulin was labelled using the monoclonal antibody KMX-1. The cell in each panel is orientated with the longer old flagellum (OF) on the upper side of the image and the shorter new flagellum (NF) on the lower side. Phase contrast and DNA images are a maximum projection of a z-stack, β -tubulin images are a single focal slice through the plane of the nuclei.

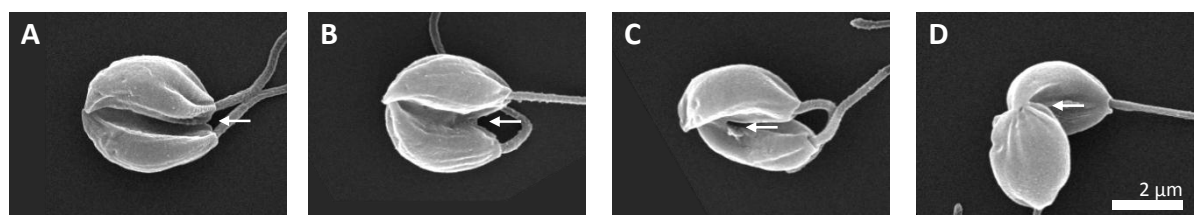


Figure 18. Furrow ingression in *L. mexicana*. A–D. Scanning electron micrographs of cytokinetic cells arranged in order of increasing furrow ingression. Arrows indicate the leading edge of furrow ingression.

To further analyse the asymmetric position of nuclei during mitosis, an anti- β -tubulin monoclonal antibody (KMX) was used to determine the orientation and positioning of the mitotic spindle relative to the separating chromosomes and surrounding sub-pellicular microtubule array (Figure 17A–F). Unfortunately the anaphase diamond-shaped spindle (expected to be visible at the earliest stages of mitosis, such as Figure 17B) was not visible above the staining of the sub-pellicular array (Figure 17A–B) so analysis of early spindle orientation was not possible. It was, however, notable how long the telophase spindle persisted; it was often observed in cells with clearly separated daughter kinetoplasts and a division furrow visible in the sub-pellicular microtubules (Figure 17D–E).

The old and new flagella at the end of the cell cycle are often not the same length (Figure 7F, Figure 9D, Figure 11E & Figure 17). One daughter cell inherits the shorter new flagellum and one inherits the longer old flagellum. To gather more comprehensive data on this phenomenon the flagella lengths on 296 cells growing a new flagellum were measured. The pairs of flagella length measurements were plotted in order of increasing new flagellum length (Figure 19), equivalent to increasing cell cycle progress; analysis of new flagellum length correlation with cell cycle progress indicated the new flagellum grows at an approximately constant rate (Figure 11E). This method was used to analyse new flagellum growth in *T. brucei* (Tyler *et al.*, 2001). The new flagellum, assessed by

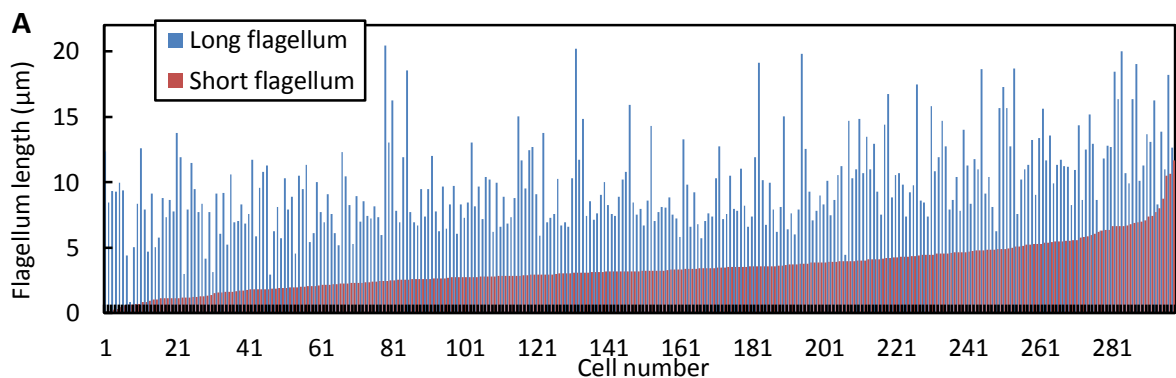


Figure 19. Asymmetries in flagellum length during *L. mexicana* division. Bar chart indicating lengths of the long old flagellum and short new flagellum for 296 biflagellate cells. Data for the cells is plotted in order of increasing new flagellum length with the old flagellum length plotted next to the corresponding new flagellum length.

flagellum length and kinetoplast and nucleus positioning, was always shorter than the old flagellum, indicating that at division the flagellum lengths on the daughter cells will always differ. The distribution of new flagellum lengths was approximately linear indicating a constant growth rate and a flagellum length at division of 5 μm . The rate of flagellum growth could therefore also be estimated at 4 $\mu\text{m}/\text{h}$, similar to that derived from Figure 11F. While the new flagellum appeared to reach a consistent length by division the old flagellum length was very variable. This indicated the flagellum is not maintained at a fixed length through the cell cycle giving rise to variable old flagellum length at the time a cell re-enters mitosis/cytokinesis.

2.3 Discussion

These data provide the first comprehensive, quantitative description of how a *Leishmania* promastigote progresses through the cell cycle. I have determined the timing of the major events in the cell cycle, the timing of DNA replication in the kinetoplast and nucleus and the periods of cell body growth and morphology change, summarised in Figure 20. These data are primarily based on a new method for calculating cell cycle progress of individual cells from their length and DNA content (Figure 10F), the validity of which was confirmed through multiple techniques; flow cytometry and BrdU incorporation for analysis of DNA content and S-phase timing and time lapse videomicroscopy for cell body morphological change. These results are supported by light and electron microscopy to analyse cytokinesis and basal body duplication in detail. *In vitro* cultures of promastigote *Leishmania* contain cells with a diverse range of morphologies and these data show that these morphologies can all be explained by asynchronous progression of cells through the cell cycle.

In *T. brucei* YL 1/2 is a marker for newly assembled microtubules, however in *L. mexicana* the staining pattern with YL 1/2 was inconsistent with this anticipated specificity. 1K1N1F *Leishmania* (undergoing cell body extension) (Figure 7A–C) and the distal portion of growing new flagella (Figure 7D–F) were YL 1/2 negative. The dynamics of α -tubulin detyrosination in *L. mexicana*

therefore differ from *T. brucei* and prevent use of YL 1/2 to identify growing regions of the cytoskeleton. Mitotic and cytokinetic cells possessed more tyrosinated α -tubulin (Figure 7D–F), possibly indicating increased incorporation of new tubulin and decreased α -tubulin detyrosination at the late cell cycle stages.

2.3.1 The cell cycle of *L. mexicana* in comparison to other *Leishmania* species

This description of the cell cycle is consistent with the older descriptions of the cell cycle of *Leishmania donovani* which indicated mitosis and cytokinesis occur late in the cell cycle (Chakraborty *et al.*, 1962; Bhattacharya and Ghosh, 1985) and that S-phase can take up a large proportion of the cell cycle (Bhattacharya and Ghosh, 1985). Shortly after this description of the *L. mexicana* cell cycle

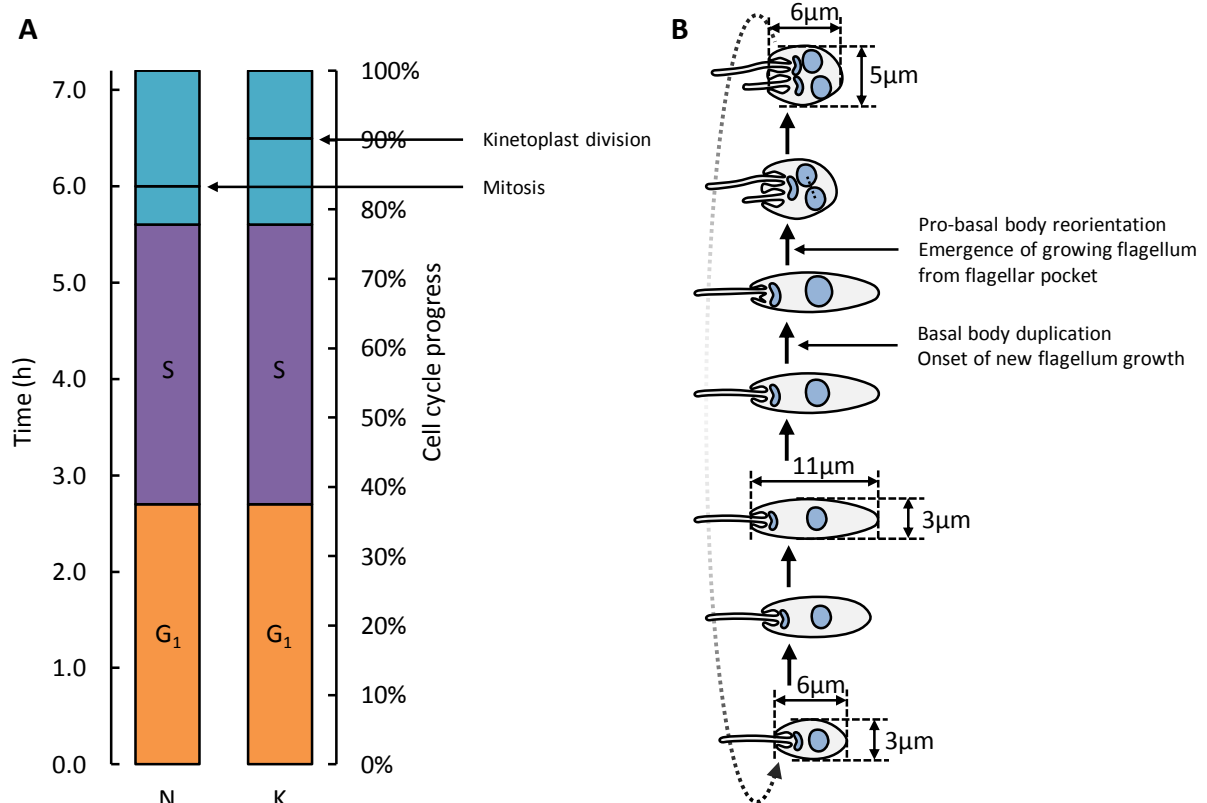


Figure 20. The cell cycle of promastigote *L. mexicana*. **A.** The timings of G₁ (orange), S (purple) and post-S-phase (cyan) for the nucleus (N) and kinetoplast (K). The time at which mitosis and kinetoplast division occur is indicated. **B.** Cartoon of promastigote cell morphologies, drawn on the same vertical time scale as **A.** Approximate cell body dimensions for each morphology are indicated. Approximate time of basal body duplication, onset of new flagellum growth, pro-basal body reorientation and emergence of the new flagellum from the flagellar pocket are also indicated.

was published (Wheeler *et al.*, 2011), a study outlining *Leishmania major* division was also published (Ambit *et al.*, 2011). The cell cycle of *L. major* takes longer than *L. mexicana* (10.2 h (Ambit *et al.*, 2011) in comparison to 7.1 h (Figure 7A)) however the order and timing of onset of new flagellum growth, kinetoplast division and mitosis are very similar. Morphological changes during G₁ and mitosis are also similar. Unfortunately (Ambit *et al.*, 2011) did not analyse the length of S-phase for *L. major* but the proportion of the cell cycle pre- and post-mitosis is also similar to that of *L. mexicana*. Flagellum length in *L. major* and its changes through the cell cycle were also not analysed by this study.

The primary difference between *L. mexicana* and *L. major* revealed by (Ambit *et al.*, 2011) was the behaviour of the anti- β -tubulin monoclonal antibody KMX-1. In *L. mexicana* KMX-1 labelled all microtubules present in the cell at all cell cycle stages, including the mitotic spindle, sub-pellicular microtubule array and the flagellar axoneme approximately equally (Figure 17A–F). (Ambit *et al.*, 2011) reported that the spindle in mitotic cells, the cleavage furrow in cytokinetic cells and a sub-population of 1K1N cells were labelled by KMX-1 more intensely, proposed to be due to an unknown, cell cycle stage dependent, post-translational modification of β -tubulin. While the modulation of recognition of β -tubulin by KMX-1 by post-translational modification in a trypanosomatid is unprecedented it is plausible and KMX-1 may identify newly assembled regions of microtubules in this species.

2.3.2 The cell cycle of *Leishmania* in comparison to other trypanosomatids

The cell cycle of *Leishmania* shares canonical features of the trypanosomatid cell cycle with other related trypanosomatids such as *C. fasciculata* (Cosgrove and Skeen, 1970), *T. brucei* (Sherwin and Gull, 1989a; Gull *et al.*, 1990) and *T. cruzi* (Elias *et al.*, 2007). The order of cell cycle events differs between species; in *C. fasciculata*, *T. cruzi* and *T. brucei* duplicated kinetoplast DNA segregates prior to the onset of mitosis (Cosgrove and Skeen, 1970; Woodward and Gull, 1990) while in *L. mexicana* (and

L. major (Ambit *et al.*, 2011)) a nuclear spindle has formed and the nucleus is in anaphase prior to kinetoplast division (Figure 17).

L. mexicana nuclear S-phase is long (40% of the cell cycle (Figure 11C–D & Figure 13C)) in comparison to *T. brucei*, *C. fasciculata* and *T. cruzi* (Figure 21). The nuclear DNA content of *L. mexicana* (approximately 64 Mbase) is similar to that of *T. brucei* and *C. fasciculata* and is approximately half that of *T. cruzi*. This suggests *L. mexicana* achieves only half the nuclear DNA synthesis rate of *T. brucei*, *C. fasciculata* and *T. cruzi* during S-phase (Figure 21). It is not clear if this difference arises from processivity of DNA replication, differences in chromosome number and genome architecture (Van der Ploeg *et al.*, 1984; Berriman *et al.*, 2005; El-Sayed *et al.*, 2005; Ivens *et al.*, 2005) or some other effect.

		<i>T. brucei</i> (procyclic)	<i>C. fasciculata</i>	<i>T. cruzi</i> (epimastigote)	<i>L. mexicana</i>
Nucleus S-phase	Cell cycle proportion	18% ^a	22% ^b	10% ^c	40%
	Time (h)	1.51 ^a	1.35 ^b	2.4 ^c	2.9
	DNA content (pg)	0.097 ^{de}	0.095 ^e	0.156 ^{ef}	≈ 0.090 ^g
	Synthesis rate (pg/h)	<i>0.064</i>	<i>0.070</i>	<i>0.065</i>	≈ 0.310
Kinetoplast S-phase	Cell cycle proportion	12% ^a	16% ^b	10% ^c	40%
	Time (h)	0.99 ^a	0.98 ^b	2.4 ^c	2.9
	DNA content (pg)	0.004 ^h	0.032 ^e	0.047 ⁱ	≈ 0.050 ^j
	Synthesis rate (pg/h)	<i>0.004</i>	<i>0.033</i>	<i>0.020</i>	≈ 0.017
Generation time (h)		8.50 ^a	6.23 ^b	24.0 ^c	7.10

Figure 21. The length of S-phases and rates of DNA synthesis for kinetoplasts and nuclei in different trypanosomatid species. Normal font indicates directly measured values, *italic* font indicates calculated or approximate values and **bold** font indicates new data for *L. mexicana* from this study. ^a (Woodward and Gull, 1990), ^b (Cosgrove and Skeen, 1970), ^c (Elias *et al.*, 2007), ^d Calculated from the total nuclear genome size of 70 Mbase (Berriman *et al.*, 2005), ^e (Borst *et al.*, 1982), ^f Calculated from the total nuclear genome size of 120 Mbase (El-Sayed *et al.*, 2005), ^g Calculated from the total nuclear genome size of 64 Mbase (Rogers *et al.*, 2011), ^h (Borst *et al.*, 1982), ⁱ (Henriksson *et al.*, 1996), ^j Calculated from minicircle and maxicircle size and approximate number (Rogers and Wirth, 1988).

The quantity of *L. mexicana* kinetoplast DNA has not been directly measured but can be calculated from minicircle and maxicircle size and number (Rogers and Wirth, 1988) and is similar to *T. cruzi* and greater than *C. fasciculata* (Figure 21). The length of kinetoplast S-phase indicates kinetoplast DNA synthesis rate in *L. mexicana* is similar to *T. cruzi* and *C. fasciculata*. In comparison *T. brucei* has particularly slow kinetoplast DNA synthesis (Figure 21). *T. brucei* minicircles have a different pattern of accumulation on the kinetoplast during kinetoplast DNA replication. In most species (*C. fasciculata*, *Leishmania tarentolae*, *L. donovani*, *T. cruzi* and *Phytomonas serpens*) newly synthesised DNA at the antipodal sites becomes redistributed around the circumference while in *T. brucei* it remains concentrated at the kinetoplast poles (Guilbride and Englund, 1998). *T. brucei* minicircles also have a high sequence diversity, unlike the homogenous sequence observed in other species (Steinert and Van Assel, 1980). One of these factors may be the reason for slower kinetoplast DNA replication.

2.3.3 *Leishmania* flagellum growth and length regulation

The earliest events during onset of new flagellum growth in *L. mexicana* occur in a similar fashion to in *T. brucei* (Sherwin and Gull, 1989a; Woodward and Gull, 1990; Lacomble *et al.*, 2010); first the pro-basal body matures and begins to elongate to form the new flagellum, closely followed by formation of new pro-basal bodies (Figure 16). These events occur near the end of kinetoplast S-phase, again similar to *T. brucei* where kinetoplast S-phase is coincident with the complex series of events (including onset of new flagellar growth) which result in pocket duplication (Lacomble *et al.*, 2010; Gluenz *et al.*, 2011) culminating in kinetoplast division driven by basal body separation (Robinson and Gull, 1991).

The link between onset of new flagellum growth and kinetoplast S-phase defines the time for which a flagellum can grow prior to division. In *T. brucei* and *T. cruzi* the period between the end of kinetoplast S-phase and completion of cytokinesis is long (Woodward and Gull, 1990; Elias *et al.*,

2007). In both these species the new flagellum has also reached a long length at the time of division. In contrast the end of the *L. mexicana* kinetoplast S-phase is near the end of the cell cycle leaving little time for new flagellum growth and the new flagellum length at the time of division is short. On this basis *C. fasciculata*, in which kinetoplast S-phase ends near the end of the cell cycle (Cosgrove and Skeen, 1970), would be expected to have a short new flagellum at division. Qualitative analysis suggests this is correct (data not shown). The structure of the cell cycle therefore seems to directly impact flagellum morphogenesis.

Leishmania promastigotes which inherit the new flagellum from a dividing parent cell possess a flagellum that is often much shorter (by up to approximately 10 μm) than their sister cell (Figure 17G). Furthermore flagellum lengths among promastigotes are highly variable and do not have a clear link to cell cycle stage (Figure 11E & Figure 17G). To generate this diverse range of flagellum lengths the *Leishmania* flagellum must continue to grow for some time following division, possibly over several cell cycles. There is weak evidence this additional growth occurs during G_1 (Figure 11E). This situation differs from *T. brucei* where, at the point of division, old and new flagellum length are within a narrow range (Tyler *et al.*, 2001) and growth of a flagellum following its initial formation is limited to a small amount in the following G_1 phase (Farr and Gull, 2009). The rate of growth of the new *Leishmania* flagellum is comparable to *T. brucei*; new flagellum growth rate is 8 $\mu\text{m}/\text{h}$ in *T. brucei* (Tyler *et al.*, 2001) and 3 to 4 $\mu\text{m}/\text{h}$ in *L. mexicana* (Figure 11E & Figure 17G). To understand how the flagellum grows during the cell cycle and to understand the impact this has for flagellum morphogenesis in the life cycle further analysis of the regulation of promastigote flagellum length and growth is required.

2.3.4 Cell body morphogenesis through *Leishmania* division

The cell body of *Leishmania* is elongate with the flagellar pocket at the anterior. Through the G_1 the cell body grows in length, approximately doubling in length. This growth presumably occurs

through elongation of the microtubules of the sub-pellicular array as observed in *T. brucei* (Sherwin and Gull, 1989b; Robinson *et al.*, 1995). Little change in cell body occurs though S-phase followed by a rapid decrease in length and increase in width during mitosis which conserves cell surface area and volume indicating a conservation of cytoplasm, sub-pellicular microtubules and membrane. *T. brucei* does not undergo a similar degree of rapid morphological change during the cell cycle indicating the *L. mexicana* sub-pellicular array is, in comparison, plastic. To achieve this morphological change any microtubules which run the entire length of the cell must halve in length and the number of microtubules lying in parallel around the circumference must approximately double. The mechanism by which this occurs is not clear but the cell cycle-dependent recognition of β -tubulin by KMX-1 in *L. major* (Ambit *et al.*, 2011) may provide a method for interrogating sub-pellicular array dynamics, like using YL 1/2 with *T. brucei*.

While *L. mexicana* mitosis and cytokinesis are superficially bilaterally symmetrical there are subtle similarities with the non-bilaterally symmetric division of *T. brucei* in which the daughter nuclei and kinetoplasts separate longitudinally. (Sherwin and Gull, 1989a). The orientation of the *L. mexicana* mitotic spindle is not orthogonal to the anterior/posterior axis of the cell and one daughter nucleus (which will be inherited by the cell which inherits the new flagellum), is initially found at a more posterior position than its sister (Figure 17). This behaviour has now also been observed in *L. major* (Ambit *et al.*, 2011).

2.3.5 Linking the cell cycle to life cycle stage differentiation

This new description of cell body morphogenesis through the proliferative promastigote cell cycle can be used to speculate how altered *Leishmania* morphology could be generated through the life cycle. Metacyclic promastigotes and amastigotes are both characterised by short, small cell bodies. The rapid decrease in cell length for promastigote division and the small (5×3 μ m) cell body of young, G₁, daughter cells (Figure 11A–B) suggests the generation of the metacyclic and amastigote

morphologies may be via a division process. Alternatively the ability of the sub-pellicular array to undergo a rapid decrease in length may be applied, outside the cell cycle, coupled with autophagy to reduce cell volume to reach the small metacyclic and amastigote cell body morphologies. The reverse differentiation, from an amastigote to a promastigote, may occur through extension of the cell posterior, similar to cell body growth during G₁. These hypotheses could be tested by analysis of either *in vitro* or *in vivo* differentiation, although greater understanding of flagellum growth will be required to analyse the differentiation process and determine how the characteristic long and very short flagella of metacyclic promastigotes and amastigotes arise. Morphological changes also occur during promastigote proliferation in the sandfly (Section 1.2.1) (Rogers *et al.*, 2002). Some of these life cycle substages match the cell body dimensions of different cell cycle stages. There may therefore be a link between promastigote morphogenesis in the sandfly and the cell cycle.

This quantitative description of *Leishmania* morphogenesis during division presents many interesting possibilities for greater understanding of morphogenesis through the *Leishmania* life cycle and morphogenesis across different trypanosomatid species. The major, and somewhat surprising, limitation of this analysis was in understanding growth of the *Leishmania* flagellum. Flagellum length does not have a simple correlation with cell cycle stage (Figure 11E) so an alternative analysis approach is required to determine how it grows and how its length is regulated.

2.4 Methods development and mathematics

This section covers only mathematics and method development specific to this results chapter (for standard materials and protocols see Section 7).

2.4.1 Centrifugation-free microscopy sample preparation

For light microscopy of doublet cells, 5×10^6 to 1×10^7 cells trypanosomatid cells during the logarithmic phase of growth were transferred, in culture medium, to a 1 cm² square on a silanised slide outlined using a fat pen (DakoCytomation S2002). Cells were allowed to settle for 15 min.

Culture medium was then aspirated and replaced with 100 μ l 2% v/v paraformaldehyde in PBS and incubated at room temperature for 15 min then washed three times by immersion in PBS. Normal staining protocols were then used (Section 7.4.3).

2.4.2 Calculation of timings of cell cycle events

Cell division generates two daughter cells both of which are at the start of a new cell cycle, therefore at any instant in an exponentially growing population there are twice as many cells at the start of the cell cycle as at the end of the cell cycle. To account for this bias the time through the cell cycle at which the event occurs, x , is calculated with (Williams, 1971):

$$x = t \frac{\ln\left(1 - \frac{1}{2}y\right)}{-\ln(2)}$$

where y is the cumulative proportion of cells up to the cell cycle event for which the timing is being calculated and t is the population doubling time. In the case $t = 1$, x gives the fractional progress through the unit cell cycle on a scale of zero to one, presented here as percentages.

2.4.3 Calculation of cell cycle progress from cell length and width

A best fit curve was fitted to the plot of cell body length and total cellular DNA content (Figure 11A–F) by a stepwise path walking algorithm. This used the set of n data points (a_i, b_i) , normalised with the variance of measurement error in the values of a , v_a , and b , v_b , $\mathbf{p}_i = \left(\frac{a_i}{v_a}, \frac{b_i}{v_b}\right)$, to draw the fit curve, with coordinates $\mathbf{q}_j = (c_j, d_j)$. The algorithm proceeded in a stepwise manner, calculating the next fit curve point from the preceding two fit curve points and the data. It therefore used two starting positions, $\mathbf{q}_0 = (c_0, d_0)$, set to (5, 1) (a cell body length of 5 μ m and a single copy of DNA), and $\mathbf{q}_1 = (c_1, d_1)$, set to (6, 1). For each step the following calculation was made:

$$\mathbf{q}_{j+1} = (c_{j+1}, d_{j+1}) = \left(\frac{1}{n} \sum_{i=0}^n w a_i, \frac{1}{n} \sum_{i=0}^n w b_i \right)$$

$$w = \begin{cases} e^{-|p_i - q_j|^2}, & -\sqrt{2}/2 < q_j \cdot (q_j - q_{j-1}) < \sqrt{2}/2 \\ 0, & \text{otherwise} \end{cases}$$

This is a Gaussian weighted average of all points within 90° of the current direction of travel to give the next point in the line. v_a and v_b , were estimated from a Gaussian curve fitted to histograms of cell body length and total DNA content; $v_a = 1.25 \mu\text{m}$ and $v_b = 0.21$ units.

Cell cycle progress of cells was calculated from their cell body length and total cellular DNA by reference to this fitted curve. Each normalised data point, $p_i = \left(\frac{a_i}{v_a}, \frac{b_i}{v_b}\right)$, was assigned to the nearest point on the curve and all data points were ranked in ascending order of distance along the curve. The position of each cell through the cell cycle was calculated incorporating the observation bias towards early stages of the cell cycle (Section 2.4.2); $y = r/n$, where r = rank of the current data point, was used to calculate the cell cycle progress, x , of each cell. Calculations were performed for each culture density separately.

2.4.4 Calculation of cell cycle phase lengths from histograms of DNA content

A histogram of DNA content of cells during logarithmic growth is made up of cells in three stages of the cell cycle; pre-S-phase (single), post-S-phase (duplicated) and S-phase (intermediate DNA content). Assuming a constant rate of DNA synthesis during S-phase, the theoretical distribution of DNA content, $D(x)$, is described by four parameters; n_a , n_b and n_c , the number of cells in pre-S-phase, S-phase and post-S-phase, and d the quantity of DNA in a pre-S-phase cell:

$$D(x) = \begin{cases} n_a, & x = d \\ \frac{n_b}{d}, & d < x < 2d \\ n_c, & x = 2d \end{cases}$$

A measured distribution of DNA content of logarithmically growing cells deviates from $D(x)$ due to two primary experimental errors; d_0 , the systematic zero error in measured DNA quantity and

v_d , the variance of measurement error. The measured distribution of DNA content, $D'(x)$, can therefore be modelled as convolution of the ideal distribution of DNA with a Gaussian measurement error, offset by the zero error:

$$D'(x) = (D * G)(x) + d_0$$

$$G(x) = e^{-\frac{x^2}{2v_d}}$$

This distribution was fitted to experimentally measured histograms of total cellular DNA content to determine n_a , n_b and n_c . It is too complex to fit analytically so a genetic algorithm was used. Starting values for the six parameters were selected manually and 10,000 mutants were generated by adding 1% random variation to each parameter. The best fitting mutant (smallest R^2) was selected and further mutated. This process was repeated for 10,000 generations.

2.4.5 Immobilisation of live *Leishmania* and time-lapse imaging

Live *Leishmania* were immobilised using low melt temperature agarose (Flowgen Bioscience, H15619) with a melting and gelling point of approximately 60°C and 20°C respectively. Molten 1% w/v low-melt agarose in M199 was prepared by mixing molten, sterile 2% w/v low-melt agarose in water (at 60°C) with double-concentration M199 (at room temperature). An equal volume of molten 1% low-melt agarose in M199 and promastigote culture were then mixed and placed in a 0.5 mm deep well on an FCS3 heated chamber (Biopetechs). The agarose was allowed to solidify by cooling to approximately 20°C. Finally the solidified agarose, containing immobilised cells, was surrounded with M199 via the perfusion channels and observed while maintained at a temperature of 28°C.

A z-series of 10 images was captured every 5 min with a spacing of 2 µm using a 10× numerical aperture (NA) 0.40 HC plan apochromat (Leica Microsystems, 11506285) objective. Each frame was analysed using ImageJ (Collins, 2007) and the most in-focus z slice for each cell was manually selected and rotated to give the cell a constant orientation (Figure 15).

3 *Leishmania* flagellum growth and length change in the cell and life cycles

Parts of the work presented in this chapter (the data presented in [Figures 22 to 25](#)) were published in 2011; [Wheeler RJ](#), Gluenz E and Gull K. (2011) “The cell cycle of *Leishmania*: morphogenetic events and their implications for parasite biology”. *Mol. Microbiol.* 79 (3): 647-62. All work included in this chapter is my own except for production of the SMP::eGFP::TY *L. mexicana* cell line ([Section 3.2.4.1](#)).

3.1 Introduction

Analysis of the *Leishmania* cell cycle indicated that the length of the flagellum does not have a straightforward correlation with cell cycle stage and may continue to grow over several cell cycles ([Section 2](#)). The mechanism of flagellum length regulation of *Leishmania* is not known, and the difference in comparison to *T. brucei* ([Section 2.1.2](#)), is of interest as the flagellum undergoes a dramatic change in length, coupled with a change in ultrastructure from the 9+2 to 9v axoneme, between the promastigote and amastigote life cycle stages (Gluenz *et al.*, 2010a). More subtle flagellum length changes also occur between the different promastigote life cycle substages found in the sandfly (Rogers *et al.*, 2002). Understanding of how the *Leishmania* flagellum grows, how its length is regulated, and how this links to the progression of the cell cycle will therefore provide a foundation for understanding how different life cycle stages achieve their distinctive morphologies.

3.1.1 Eukaryotic flagellum growth and length regulation

Assembly of a *Leishmania* flagellum begins with the cell cycle-linked maturation of a basal body which then begins to extend through the flagellar pocket to form a new flagellum. This process is

linked with formation of new pro-basal bodies, division of the flagellar pocket and division of the kinetoplast which occur in a similar fashion to *T. brucei* (Section 2) (Wheeler *et al.*, 2011). The elongation of a growing trypanosomatid flagellum has been shown, in *T. brucei*, to occur through intraflagellar transport (IFT) of flagellum components to the growing tip, as in other eukaryotes (Scholey, 2008), through a comprehensive analysis of 9 conventional and 5 novel IFT proteins (Absalon *et al.*, 2008). IFT particles (more recently termed ‘trains’ because of their variable length (Pigino *et al.*, 2009)) are found as one of two complexes, comprising 6 proteins (complex A) and 11 proteins (complex B), of which 11 proteins have detectable homologues in *L. major* (Gouveia *et al.*, 2007). Together these suggest that, like *T. brucei*, canonical IFT operates in *Leishmania*. IFT uses two microtubule motor complexes with a trimeric kinesin II responsible for anterograde IFT (toward the flagellum tip) (Kozminski *et al.*, 1995; Cole *et al.*, 1998; Snow *et al.*, 2004) and a dynein complex responsible for retrograde IFT (toward the flagellum base) (Pazour *et al.*, 1999; Porter *et al.*, 1999; Signor *et al.*, 1999). In *L. mexicana* the dynein *LmxDHC2.2* is required for normal flagellum growth and may play a role in retrograde IFT (Adhiambo *et al.*, 2005).

IFT assembly of flagella is proposed to confer a mechanism of flagellum length regulation termed the balance point model, originally described for *Chlamydomonas reinhardtii* (Marshall, 2002; Marshall *et al.*, 2005). This model states that the rate of flagellum elongation is limited by the rate at which flagellum components can be transported to the flagellum tip by IFT and growth is balanced by random disassembly of the flagellum tip independent of IFT. IFT-mediated growth is limited by availability of IFT machinery, originally modelled as a limited number of IFT particles (Marshall *et al.*, 2005). The model has been challenged by discovery of a correlation of flagellum length and number of IFT particles per flagellum (Dentler, 2005) and IFT trains of various length (in contrast to particles of a single size) (Pigino *et al.*, 2009). Nonetheless the balance point model, with the minor adaptation of considering total available IFT machinery rather than number of IFT trains, has proven resilient (Engel *et al.*, 2009). Turnover of material at the flagellum tip is required for the

balance point model (Marshall, 2002), a process which has been observed in *T. brucei* (Farr and Gull, 2009). Therefore it seems likely that IFT-mediated flagellum growth in *Leishmania* is modulated by the balance point model. The balance point model alone cannot explain all the changes in flagellum length through the *Leishmania* life cycle as it only considers flagellum lengths attained during steady state growth (Marshall *et al.*, 2005). The decrease in flagellum length during promastigote to amastigote differentiation must also incorporate additional regulatory effects.

3.1.2 *Leishmania* flagellum growth and length regulation

Genetic modification of *Leishmania* has revealed many proteins which have a function in either flagellum growth or flagellum length regulation which are not canonical flagellum components or involved in canonical assembly pathways. These proteins include two which may alter microtubule stability; overexpression of a kinesin 13 LmjKIN13-2 with microtubule disassembly activity (Blaineau *et al.*, 2007) and a katanin-like protein KAT60b (Casanova *et al.*, 2009) both give rise to reduced flagellum lengths. Two proteins with a potential actin-mediated function have also been identified, a putative ADF/cofilin homologue (Tammana *et al.*, 2008; Kumar *et al.*, 2012) and myosin XXI (Katta *et al.*, 2010), are required for long flagella. SMP1, a myristoylated and palmitoylated membrane protein which is proposed to have a role in stabilising sterol- and sphingolipid-rich flagellar membrane domains, is also required for long flagella (Tull *et al.*, 2010). Finally MAPK-like kinases appear to modulate flagellum length; overexpression and deletion of different members of a set of MAPK-like protein kinases (*LmxMKK*, *LmxMPK3* and *LmxMPK9*) variously cause an increase or decrease in flagellum lengths (Wiese *et al.*, 2003; Bengs *et al.*, 2005; Erdmann *et al.*, 2006).

Despite this range of mutants they have provided limited insight into how the *Leishmania* flagellum actually grows during the cell cycle. These proteins have been identified and analysed through deletion and constitutive overexpression mutants. Inducible mutants have not been used. Analysis of the phenotypes is therefore limited to the steady state effect of the change rather than

the response to an increase or decrease of protein levels. This limits the value of these mutants in moving toward a more mechanistic understanding of the action of any of these proteins and, in turn, flagellum length regulation in *Leishmania*. For example it is not possible to unequivocally determine if shorter flagella arising from a constitutive mutation have occurred from a change in flagellum length regulation or a failure in flagellum growth. Understanding precisely how flagellum growth in *Leishmania* occurs may assist in extracting this additional mechanistic detail from these pre-existing mutant cell lines.

3.1.3 Flagellum length change during the *Leishmania* life cycle

The change in flagellum length from the promastigote to amastigote and *vice versa* is the most dramatic in the life cycle and is likely, based on analysis of *T. brucei* differentiation, to be linked with the normal proliferative cell cycle (Section 1.1.3). The change in flagellum morphology is more than a change in length, the axoneme structures in the promastigote and amastigote flagella differ (Gluenz *et al.*, 2010b). It is known that promastigote to amastigote morphological change can occur in under 24 h for *L. mexicana* (Bates, 1994) and, for *L. donovani*, occurs following a 6 hour lag phase during which cells accumulate in G₁ (Barak *et al.*, 2005). The mechanisms involved are not known, but several hypotheses can be made. Firstly flagellum length may decrease through modulation of normal flagellum length regulation and undergo a gradual disassembly. Secondly the flagellum may disassemble rapidly through specialised pathways, for example making use of a microtubule destabilising protein like KAT60b (Casanova *et al.*, 2009). Thirdly the flagellum may be severed and shed rather than being disassembled and resorbed. Fourthly more complex mechanisms involving an asymmetric differentiation division, in which the old flagellum on one daughter cell retains a promastigote length and ultrastructure while the new flagellum on the second daughter cell is constructed with the amastigote length and ultrastructure, may be responsible.

The more subtle flagellum length changes which occur between different promastigote life cycle substages in the sandfly (Rogers *et al.*, 2002) have not been linked with a change in the function or the ultrastructure of the flagellum (with the exception of the gut surface-adherent haptomonad promastigote in which the PFR may have an altered structure (Beattie and Gull, 1997)). Analysis of flagellum growth *in vitro* may give insight into how these promastigote morphologies in the sandfly could arise.

The PFR is a major component of the promastigote flagellum but whether it plays a role in life cycle stage differentiation is unknown. It represents a significant proportion of the protein content of the flagellum, filling a volume similar to the axoneme, and is a complex multi-component structure which, including metabolic enzymes and proteins potentially associated with calcium and cAMP signalling, making a role in environment sensing or signal transduction plausible (Portman *et al.*, 2009). The PFR may therefore have a role in differentiation signal perception. Alternatively the PFR may present a challenge for flagellum disassembly due to its bulk. In either case the kinetics and capacity of the flagellum to undergo flagellum length change in the absence of PFR will give insights into its role in differentiation.

To analyse flagellum growth and length regulation the growth of flagella in promastigote culture and the disassembly of flagella during differentiation from the promastigote to the amastigote axenically and in macrophages were analysed. Axenic differentiation of the PFR1 deletion mutant (Maga *et al.*, 1999) was also analysed. These were analysed with the support of a quantitative mathematical model of flagellum growth based on the balance point model, extended to incorporate the hypothetical contribution of synthesis and degradation of flagellum components. Fitting this model to the flagellum lengths observed in an exponentially growing promastigote culture indicated flagellum growth on promastigotes can be partially accounted for by the balance point model and that flagellum growth continues (predominantly in G₁ and post-S-phase) over multiple cell cycles. Flagellum length change during promastigote to amastigote differentiation was similar

to predicted by the balance point model suggesting flagellum length change occurs through modulation of normal flagellum length regulation. This thorough description of the cell biology underlying flagellum length regulation in *Leishmania* adds value to the existing biochemical, molecular biological and ultrastructural understanding of the flagellum, improves understanding of how the parasite progresses through its life cycle and sets the stage for use of *Leishmania* as model species for understanding conserved mechanisms of flagellum length regulation in eukaryotes.

3.2 Results

As previously described (Section 2) (Wheeler *et al.*, 2011) the length of the externally visible flagellum (protruding from the flagellar pocket) in promastigote *L. mexicana* did not follow a simple correlation with the cycle. Replotting this morphometric data to show the correlation of old flagellum length with total cellular DNA content, cell length and calculated cell cycle progress did not reveal strong correlation with any of these measures (Figure 22A–C). Therefore an alternative approach was taken. A computer model of flagellum growth was built to simulate flagellum length data for comparison to the flagellum length data. This model was designed to be of the minimum complexity required, therefore only concerned flagellum length change arising from IFT, to avoid overfitting. This provided a starting point for understanding the dynamics of the flagellum and how it grows.

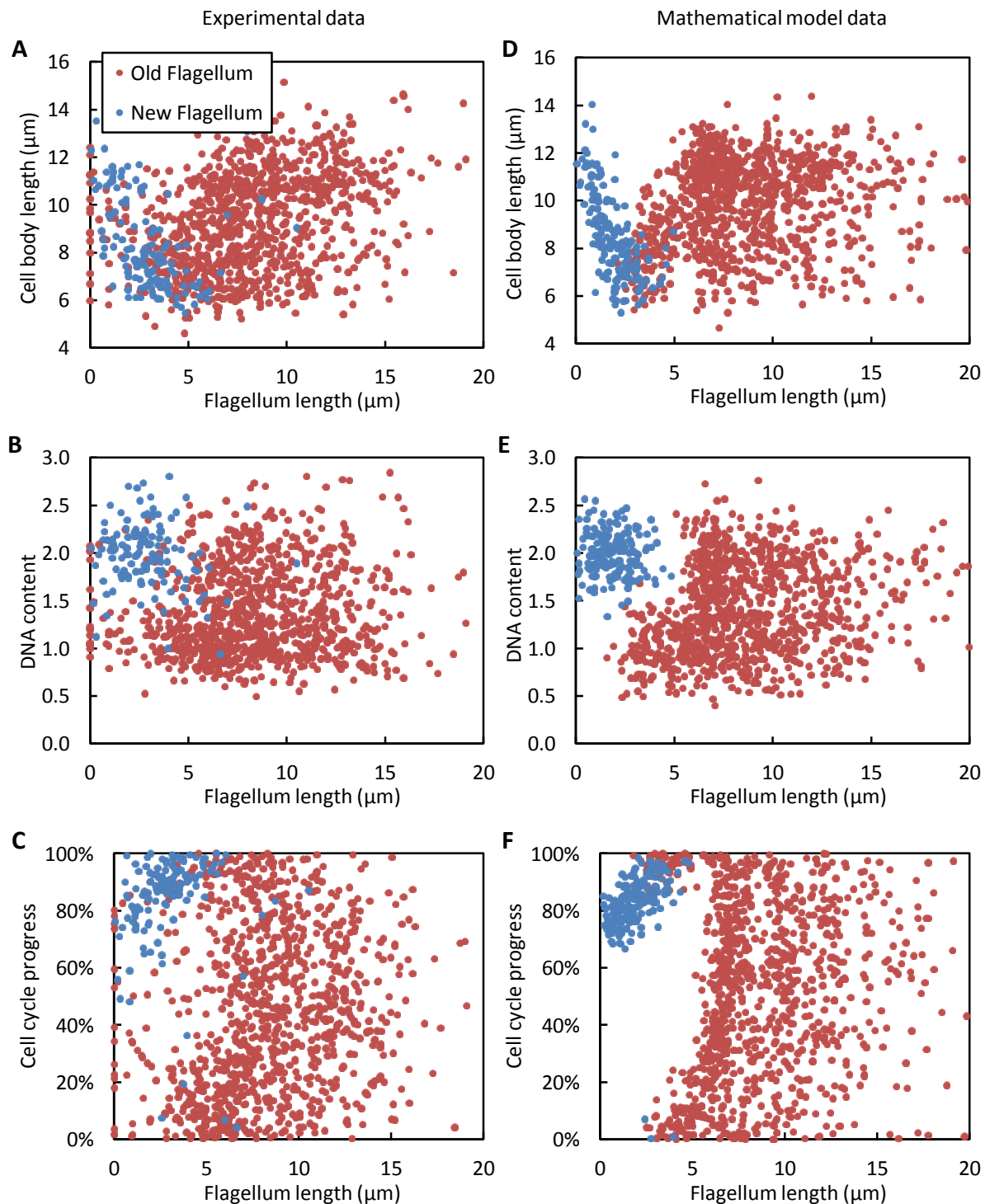


Figure 22. Experimentally determined correlations of cell morphological features through the cell cycle can be replicated through modelling of flagellum growth. A–C. Experimentally determined correlation of cell body length, cell DNA content and cell cycle progress with flagellum length, data from Section 2.2.3. D–F. Simulated correlation data for cells allowed to undergo unconstrained logarithmic growth. $n = 980$ for experimental data.

3.2.1 Mathematical modelling of promastigote flagellum growth

In the model of flagellum growth a pool of cytoplasmic components is generated by synthesis of flagellum components and modulated by flagellum component degradation. Flagellum growth then occurs through a balance of length-dependent flagellum assembly and length-independent disassembly as described by the balance point model (Marshall *et al.*, 2005). Flagellum assembly rate is further modulated by the concentration of flagellum components in the cell; depletion of the cytoplasmic pool of flagellum components also decreases flagellum growth rate. Change in flagellum length therefore arises from the four rate constants (Figure 23). The balance point model alone could not account for the correlation of cell cycle stage and flagellum length observed (Figure 22C); additional modulation of flagellum growth through the times of the cell cycle at which flagellum components were synthesised was also required for the model to match the experimental observations (Figure 24). The final model was fitted to the experimental external flagellum length data (Section 3.4.1.1).

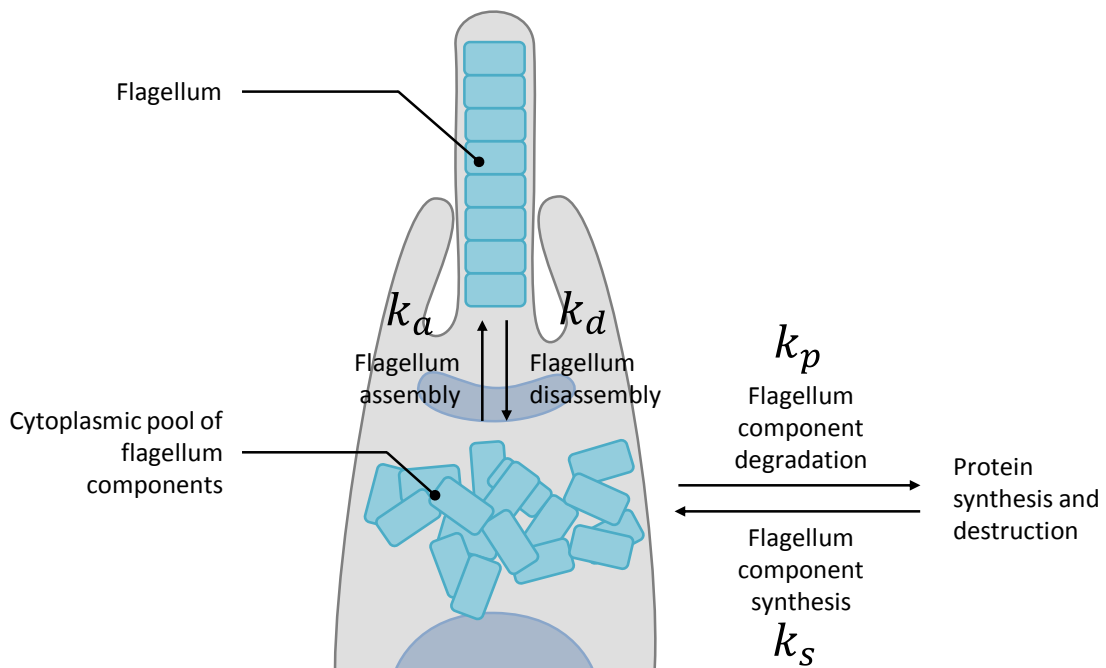


Figure 23. The model of *Leishmania* flagellum growth. Flagellum length arises from the interplay of two processes, flagellum assembly/disassembly and flagellum component synthesis/degradation from the intermediate pool of flagellum components.

To confirm the fitting process was accurate, external flagellum length of an exponentially growing population of cells was simulated. Cells were allowed to progress through 20 cell cycles in unconstrained growth. For cell cycles 16 to 20 the flagellum length distribution in the population had stabilised, indicating the equilibrium state had been reached. The simulated correlation of equilibrium state flagellum length with total cellular DNA content, cell body length and cell cycle progress (Figure 22D-E) was similar to the experimental data (Figure 22A-C). This showed this simple flagellum growth model can explain the range of flagellum lengths observed in culture,

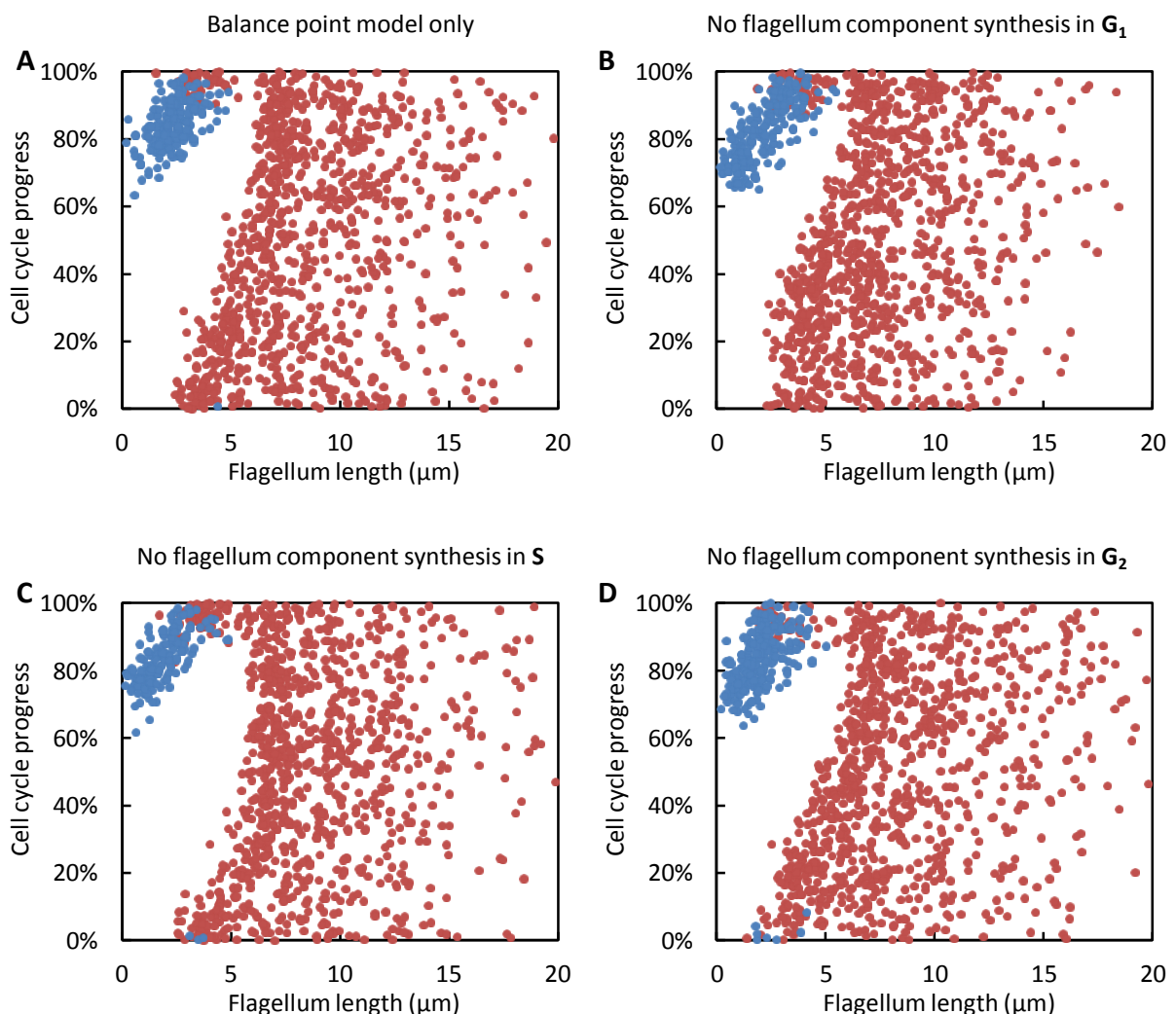


Figure 24. The balance point model alone cannot accurately predict flagellum growth. Simulated correlation of old and new flagellum length with cell cycle stage for different cell cycle-dependent modulations of flagellum component synthesis (confer Figure 8C). **A.** The balance point model alone with no change in flagellum component synthesis through the cell cycle. **B.** With no synthesis of flagellum components in G_1 . **C.** With no synthesis of flagellum components in S-phase. **D.** With no synthesis of flagellum components in G_2 , during mitosis or cytokinesis. Total quantity of flagellum components synthesised per cell cycle are equal for each simulation.

although may not be the only model which can do so. If correct the model indicated the flagellum grew during all stages of the cell cycle except S-phase and the flagellum did not have a rigidly defined target length, instead it continued to grow over multiple cell cycles, gradually slowing as IFT became unable to sustain further increase in length. Overall flagellum growth was slow relative to the length of the cell cycle.

This model of flagellum growth makes testable predictions about flagellum length in logarithmically growing culture. The fitted model indicated that flagellum component synthesis does not occur during S-phase, resulting in limited flagellum growth during this stage of the cell cycle. A histogram of external flagellum lengths for cells in S-phase was therefore expected to be multimodal, with flagella which were one, two, three, etc. cell cycles old (flagellum generation number) being found in different peaks (Figure 25A). A histogram of experimentally determined S-phase flagellum length data (Figure 25B) had a similar multimodal distribution. The validity of this prediction supports the accuracy of the model.

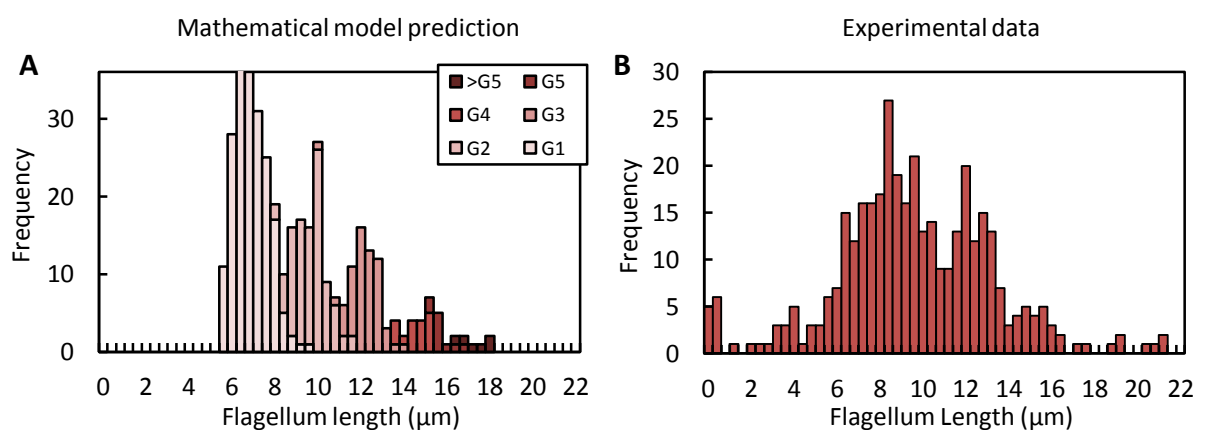


Figure 25. Predicted flagellum length behaviour in S-phase and corresponding experimental data. A and B. Modelled (A) and experimentally determined (B) histograms of flagellum length of cells during S-phase from a logarithmically growing culture. Simulated data is sub-categorised by age of the flagellum in generations. $n = 365$ for experimental data.

3.2.2 Mathematical modelling of flagellum length change during differentiation

The model of flagellum growth successfully simulated flagellum length for promastigotes undergoing exponential growth; it was therefore tested whether it could make predictions for the flagellum length change during life cycle stage differentiation assuming flagellum length change involves modulation of normal length regulation (Section 3.1.3, hypothesis one). Promastigote to amastigote differentiation and *vice versa* are likely to involve cessation and initiation of flagellum component synthesis respectively. During differentiation to the amastigote autophagy is upregulated (Rainey *et al.*, 1991; Bates *et al.*, 1992; Pral *et al.*, 1993; Bates, 1994; Besteiro *et al.*, 2007) therefore it is likely that flagellum components are degraded during this process. The degradation rate of flagellum components would be likely to be around 4 μm /cell cycle as a typical promastigote flagellum (80% have a length of 12 μm or less (Section 2) (Wheeler *et al.*, 2011)) disassembles within 24 h (Bates, 1994; Barak *et al.*, 2005; Alcolea *et al.*, 2010); approximately 3 promastigote cell cycles. Remodelling of flagellum ultrastructure is omitted from this model.

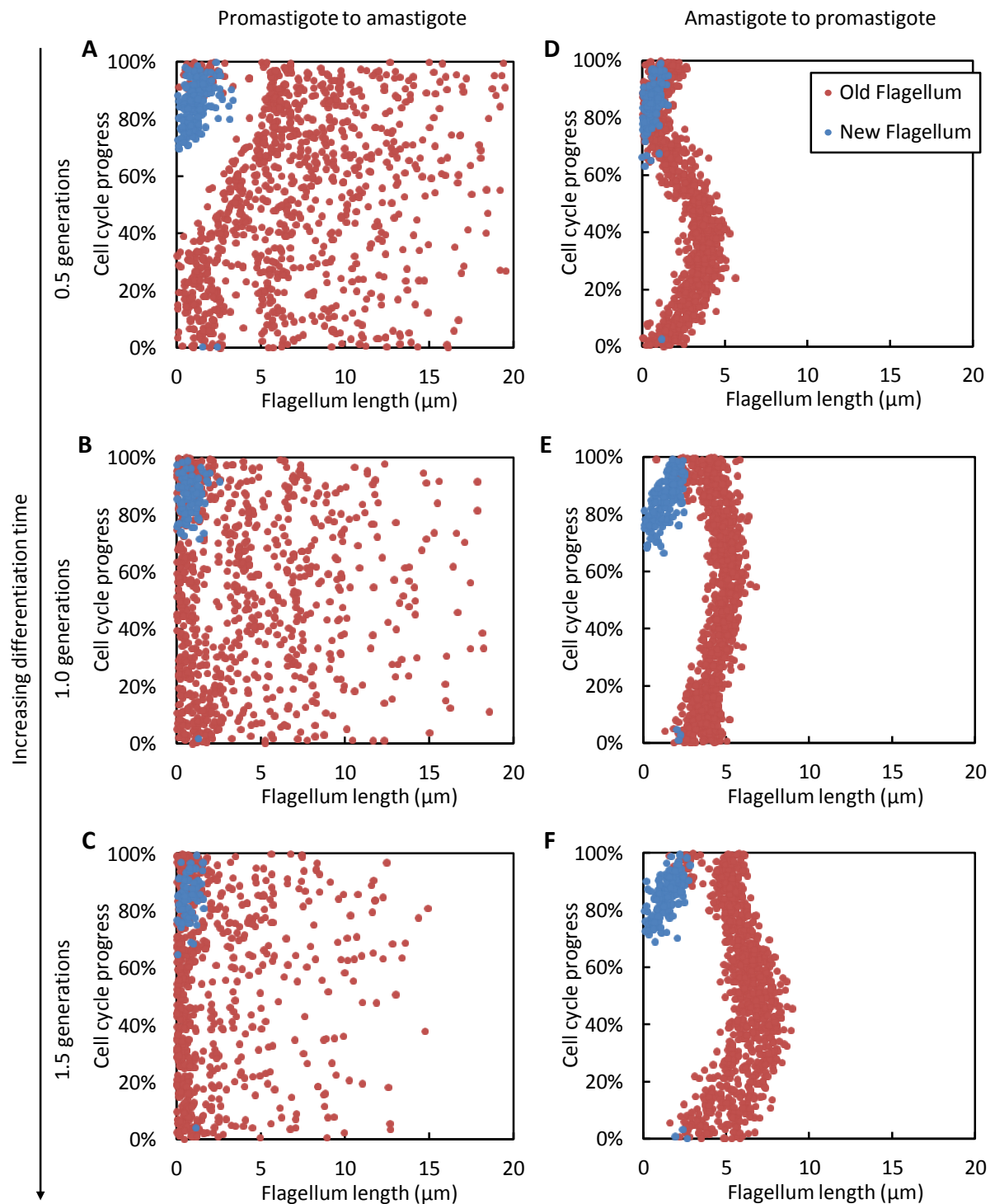


Figure 26. Predicted flagellum length changes during lifecycle stage differentiation from promastigotes to amastigotes and vice versa. A–C. Old and new external flagellum lengths in a simulated promastigote population 0.5 (A), 1.0 (B) and 1.5 cell cycles (C) following cessation of flagellum component synthesis. D–F. Old and new external flagellum lengths in a simulated amastigote population 0.5 (D), 1.0 (E) and 1.5 (F) following initiation of flagellum component synthesis.

To simulate promastigote to amastigote differentiation a population of cells (in exponential growth) was subjected to a new flagellum component synthesis rate of zero and a rate of degradation of flagellum components of 4 $\mu\text{m}/\text{cell cycle}$. The cells were then simulated through a further 1.5 cell cycles (approximately 12 h at promastigote doubling times) (Figure 26A–C). Cessation of synthesis of flagellar components resulted in a rapid decrease of flagella growth rates, both for dividing cells and for cells during G_1 . 0.5 cell cycles following the start of differentiation the new flagellum on biflagellate cells no longer grew at a sufficient rate to extend further than 2 μm from the flagellar pocket prior to division and cells during the early cell cycle (0 to 40% progress) showed no increase in flagellum length relative to those completing cytokinesis (90 to 100% progress) (Figure 26A). These effects were sustained through later differentiation time points (Figure 26A–C). The flagella on cells with a long ($>2 \mu\text{m}$) flagellum gradually disassembled manifesting as a decrease in typical flagellum length (Figure 26A–C).

This simulation of promastigote to amastigote differentiation (Figure 26A–C) made testable predictions about the changes in flagellum length during axenic differentiation. The earliest detectable effect would be the cessation of growth of the new flagellum beyond the flagellar pocket on dividing cells yielding 2K2N1F cells morphologies in place of 2K2N2F cell morphologies expected from the promastigote cell cycle (Section 2) (Wheeler *et al.*, 2011). Division of these cells would give rise to an increasing population of 1K1N cells with no long flagellum. Finally, flagellum length on 1K1N cells with a long flagellum would decrease through flagellum disassembly.

To simulate amastigote to promastigote differentiation, a population of cells was set to a starting cytoplasmic flagellum component pool and external flagellum length of zero (a flagellar pocket-contained 3 μm flagellum length), then simulated through 1.5 cell cycles (Figure 26D–F). Initiation of flagellum component synthesis resulted in immediate onset (within 0.5 cell cycles) of flagellum growth of cells in G_1 (Figure 26D). This was followed (within 1.0 cell cycles) by onset growth of the new flagellum on dividing cells (Figure 26E). As differentiation progressed beyond 1.0 cell cycles

average flagellum length increased and became more equal between different cell cycle stages (Figure 26E-F). Over further cell cycles the distribution of flagellum lengths gradually approached the logarithmic promastigote external flagellum length distribution shown in Figure 22F.

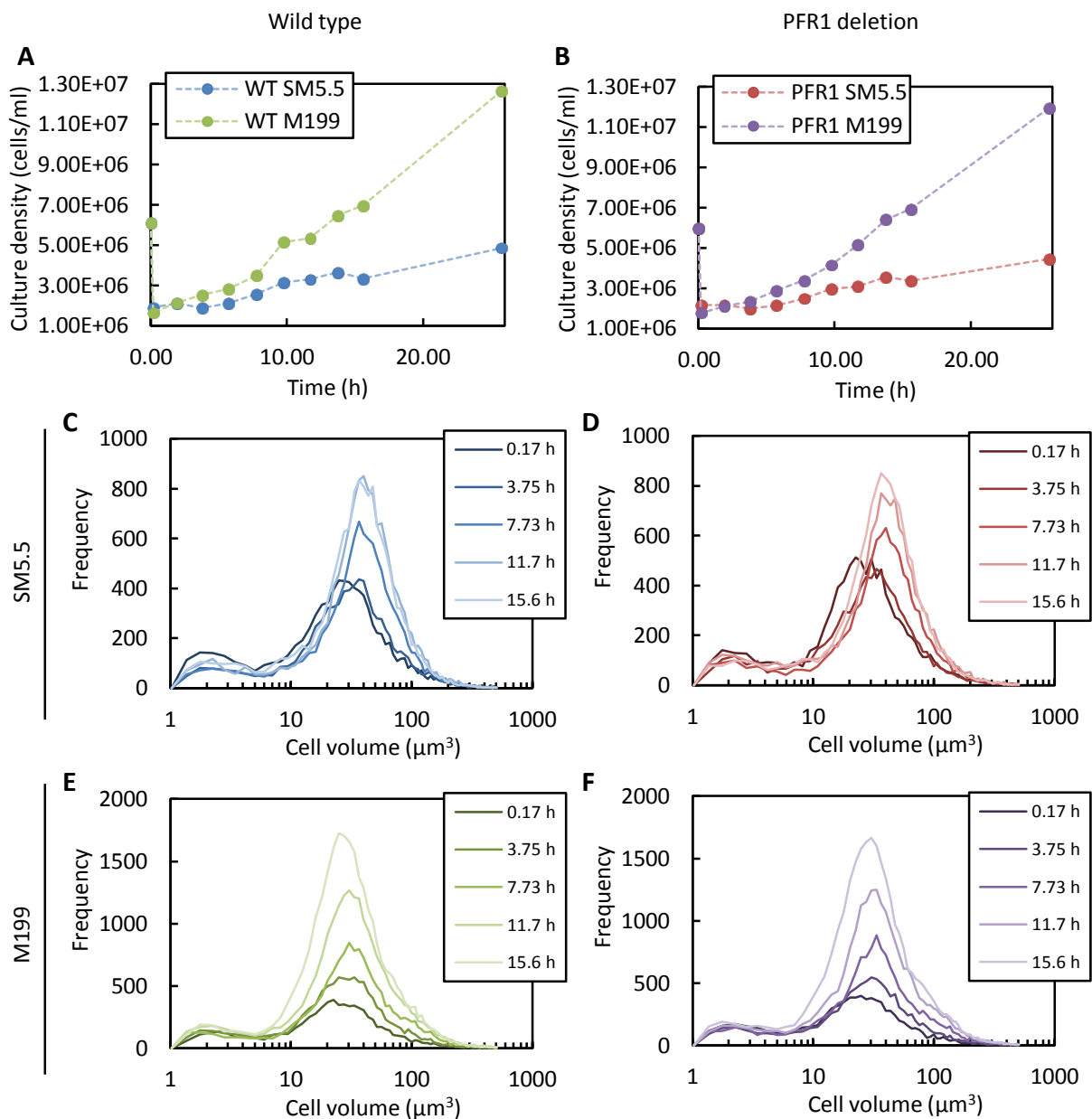


Figure 27. Promastigote growth and cell volume change during axenic differentiation. A-B. Growth of *L. mexicana* wild type (A) or PFR1 deletion (B) promastigotes following subculture into either M199 pH 7.4 28°C (promastigote conditions) or SM pH 5.5 34°C (amastigote conditions). C-F. Change in distribution of *L. mexicana* wild type (C-E) or PFR1 knockout (D-F) promastigotes cell volume following subculture into either SM pH 5.5 34°C (C-D) or M199 pH 7.4 28°C (E-F). $n > 20,000$ for each cell volume distribution.

3.2.3 Flagellum length change in axenic promastigote to amastigote differentiation

I have outlined four hypotheses as to how the amastigote flagellum could arise during differentiation from the promastigote (Section 3.1.3), and simulated how flagellum lengths would change if the first hypothesis was correct. Axenic promastigote to amastigote differentiation of *L. mexicana* was therefore analysed to test these hypotheses. In addition the axenic differentiation of the *L. mexicana* PFR1 deletion mutant (Maga *et al.*, 1999) was also analysed to assess the dependence of flagellum morphology change on promastigote motility and the presence of the PFR.

Axenic differentiation was induced by temperature increase and pH decrease (Bates *et al.*, 1992) by subculture of promastigotes to 2×10^6 cells/ml into either promastigote (M199 pH 7.4 28°C; M199) or axenic amastigote (SM5.5 pH 5.5 34°C; SM5.5) culture conditions. Culture density (Figure 27A–B) and cell volume distributions (Figure 27C–F) were measured every two hours. Following subculture into M199, cells continued exponential growth with little change in cell volume (Figure 27A–B, E–F). Following subculture into SM5.5 growth immediately ceased and there was a small decrease in cell number over the first 4 h (Figure 27A–B). Following this initial lag, growth resumed at a reduced rate with a doubling time of approximately 19 h. Over the first 4 h, cell volume increased slightly,

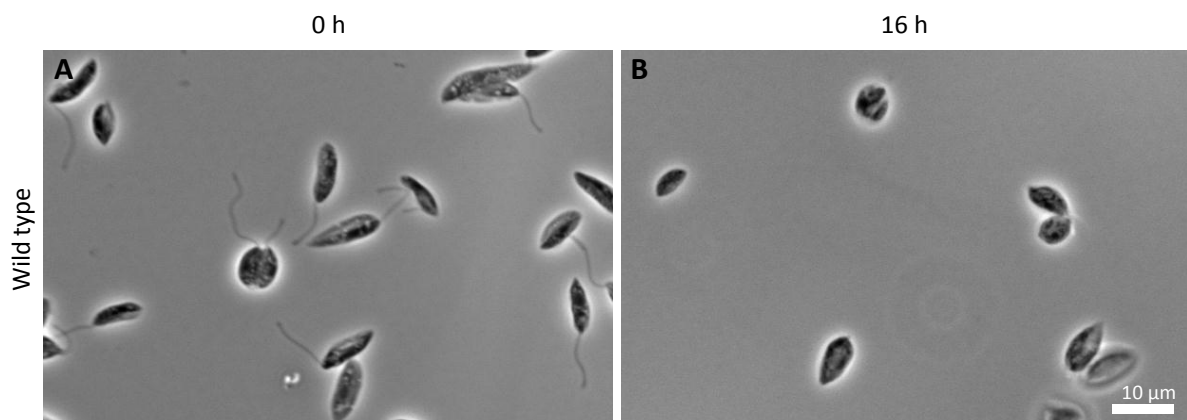


Figure 28. *L. mexicana* morphology changes during axenic differentiation. A. Phase contrast micrograph of wild type *L. mexicana* promastigotes during exponential growth. B. Phase contrast micrograph 16 h following transfer to axenic amastigote growth conditions.

resumption of growth was associated with no further increase in volume (Figure 27C–D). This may be indicative of growth in cell volume while division had stalled. Light microscopy confirmed that following 16 h >95% of cells had no long external flagellum (consistent with the original description of axenic differentiation (Bates *et al.*, 1992)) (Figure 28). No detached flagellar fragments were present, indicating the long flagellum is not lost by shedding (Section 3.1.3, hypothesis 3).

To analyse flagellum disassembly, external flagellum length was measured from phase contrast micrographs at 2 h intervals following subculture of PFR1 deletion and wild type *L. mexicana* promastigotes into SM5.5. Only 1K1N cells were analysed as a large majority of these (97% based on the promastigote cell cycle (Section 2.2.1) (Wheeler *et al.*, 2011)) are expected to possess only a single flagellum and none are in the process of division which would complicate the analysis. The proportion of cells with a very short or no external flagellum (< 2 µm external length) increased from 5.1% to 77.5% (wild type) or 2.4% to 64.2% (PFR1 deletion mutant) over 16 h (Figure 29A–B). This change predominantly occurred following the initial 4 h lag period; 95.7% and 85.8% of the increase in cells with a very short or no external flagellum occurred between 4 h and 16 h for the wild type and PFR1 deletion mutant respectively. Histograms of external flagellum length (Figure 29C–D) showed that typical flagellum length on cells with a long (>2 µm) external flagellum also decreased from 11.12 to 8.7 µm 10.8 to 5.2 µm from 0 h to 16 h for the wild type and PFR1 deletion mutant respectively.

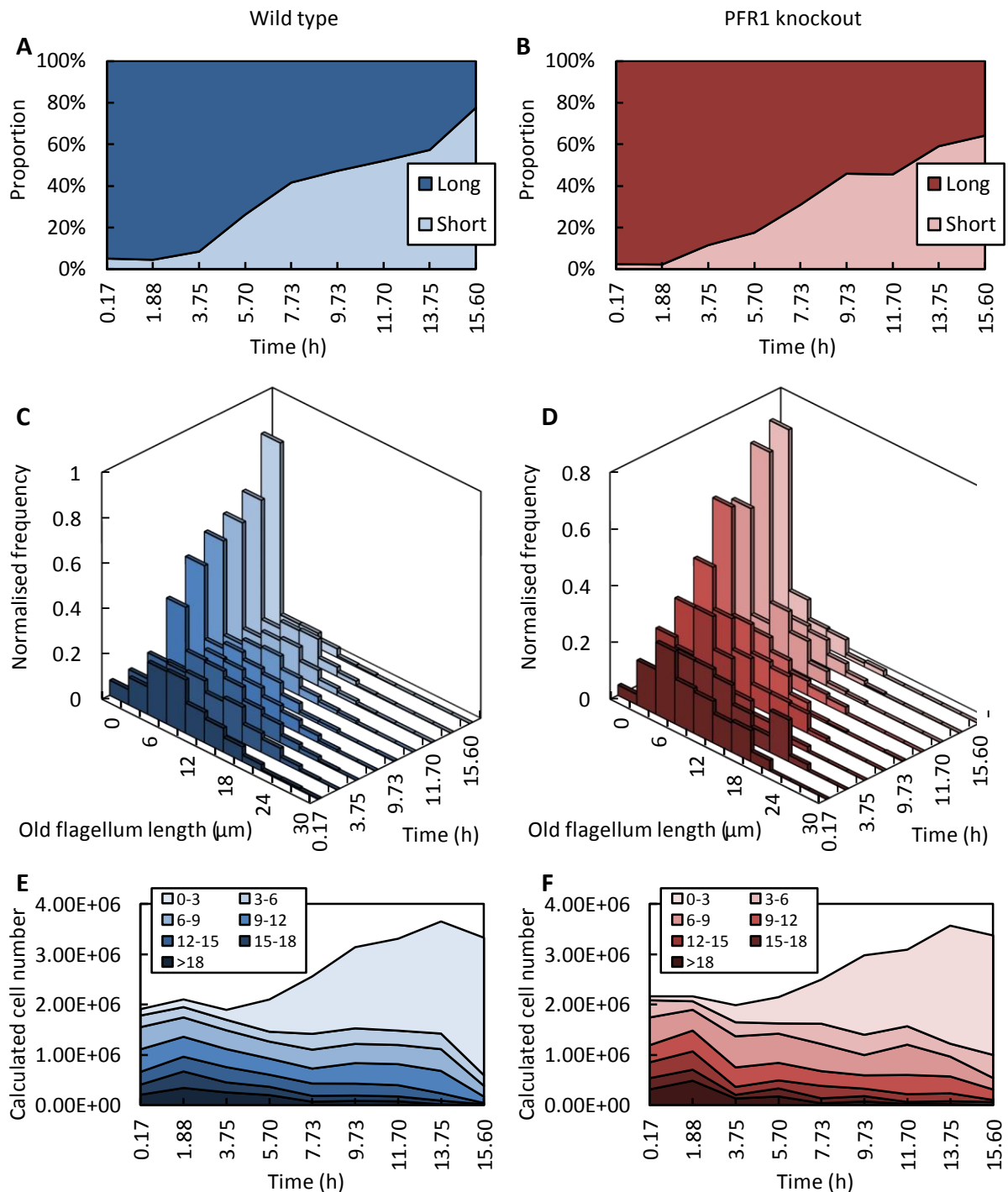


Figure 29. Promastigote flagellum length change during axenic differentiation. A-B. Change in proportion of *L. mexicana* wild type (A) or PFR1 knockout (B) promastigotes with a long or short (<2 μm) flagellum following subculture into SM pH 5.5 34°C. C-D. Histograms of *L. mexicana* wild type (C) or PFR1 knockout (D) promastigote flagellum length following subculture into SM pH 5.5 34°C. $n > 550$ for each time point for wild type and $n > 90$ for PFR1. E-F. Calculated number of *L. mexicana* wild type (E) or PFR1 knockout (F) cells with different flagellar lengths following subculture into SM pH 5.5 34°C, derived from C and D and the growth curves in Figure 27A-B.

Flagellum length data (Figure 29C–D) were combined with culture growth data (Figure 27A–B) to extrapolate the total number of cells present in the culture with different flagellum lengths for each time point (Figure 29E–F). The number of cells with a long external flagellum decreased over time while the number of cells with no long external flagellum increased. No cell death was observed; therefore the decrease in cells with a long external flagellum occurred through disassembly of the flagellum. Rate of increase of cells with no long external flagellum was faster than population growth. Some of these cells were generated by flagellum however the remainder must be generated by cell division. These data therefore show that two processes are occurring: division of cells to generate one or both daughter cells with no long external flagellum and disassembly of the external portion of existing flagella (Section 3.1.3, hypothesis 1 & 2). The proportion of cell divisions which generate daughter cells with two, one or no long external flagella cannot be determined from these data.

Analysis of 1K1N cells during differentiation suggested that if a *Leishmania* cell with a long external flagellum divides it generates one daughter cell with amastigote-like morphology (no long external flagellum) and the second with a gradually disassembling long flagellum. To test this hypothesis and determine the role of division in axenic promastigote to amastigote differentiation (Section 3.1.3, hypothesis 4) external flagellum lengths on dividing wild type cells were analysed. Dividing promastigote cells with two nuclei are expected to have two visible external flagella (Section 2.2.1) (Wheeler *et al.*, 2011), therefore flagellum lengths on all 1K2N and 2K2N cells were measured. If one or both flagella were not visible then they were recorded with an external flagellum length of zero. After 2 h the number of dividing cells with two long flagella dropped sharply (Figure 30A–B) reaching 15.4% by 6 h. The majority of dividing cells had one long flagellum, although cells with no long flagellum became gradually more prevalent reaching 12.5% by 8 h. Histograms of the new (shorter) flagellum length (Figure 30C) showed a sharp switch from lengths typical of promastigote division (2 to 6 μm) to a flagellum length of $<2 \mu\text{m}$ at the 4 h time point.

Histograms of the old (longer) flagellum length (Figure 30D) were similar to the distributions seen for 1K1N cells (Figure 29C), including a small decrease in typical flagellum length by 8 h. These data confirm the hypothesis that growth of the new flagellum on dividing promastigotes in SM5.5 is limited, generating one daughter cell with an amastigote-like morphology and a second with a long external flagellum.

Taken together the cessation of growth of new flagella on dividing cells and the gradual decrease in average flagellum length of cells with a long flagellum during axenic differentiation were

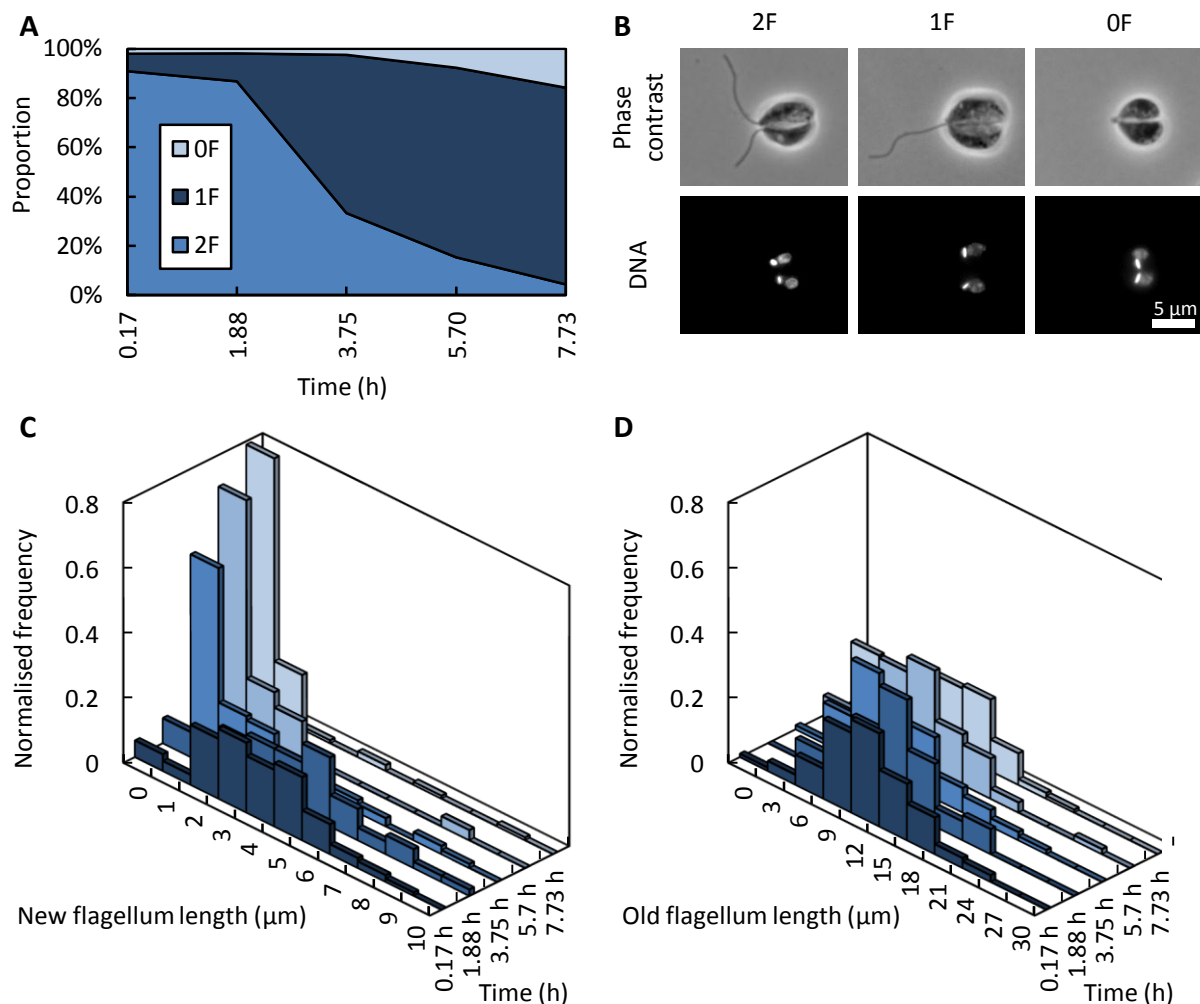


Figure 30. Flagellum length on dividing cells during axenic differentiation. **A.** Change in proportion of dividing *L. mexicana* cells with two nuclei and two (>2 μm) flagella (2F), one (1F) or zero long flagella (0F) following subculture into SM pH 5.5 34°C. **B.** Example immunofluorescence micrographs of dividing *Leishmania* with two, one or zero long flagella illustrating these morphologies. DNA was stained with DAPI. **C–D.** Short (new, **C**) and long (old, **D**) flagellum length on *L. mexicana* wild type cells with two nuclei following subculture into SM pH 5.5 34°C. $n > 50$ for each time point.

consistent with the promastigote to amastigote differentiation behaviours predicted from my flagellum growth model (Figure 26A–C). This was surprising given the simple assumptions the promastigote to amastigote simulation was based upon, but nonetheless further supports the validity of the flagellum growth model. This also suggests the hypothesis that flagellum length change through promastigote to amastigote differentiation by modulation of normal flagellum length control may be correct (Section 3.1.3, hypothesis 1).

3.2.4 Flagellum length change in promastigote to amastigote differentiation in macrophages

To validate these results under conditions more similar to a mammalian infection the promastigote to amastigote differentiation was analysed using *in vitro* infection of macrophages, with the primary aim of determining flagellum disassembly kinetics.

3.2.4.1 SMP1::eGFP::TY is a luminal flagellar membrane marker

To visualise the promastigote flagellum inside a macrophage, an *L. mexicana* cell line expressing eGFP-tagged SMP1 (Gluenz, Unpublished) was used. SMP1 is both myristoylated and palmitoylated and localises to the flagellum in *L. major* and cell fractionation analysis indicated it is associated with the membrane fraction of the parasite (Tull *et al.*, 2004). SMP1 in *L. mexicana* was tagged with eGFP at the endogenous locus, to generate the SMP1::eGFP::TY cell line, and the tagged gene is flanked by the endogenous 5' UTR and the *C. fasciculata* phosphoglycerate kinase A 3' UTR (Gluenz, Unpublished). The N-terminal position of the eGFP::TY tag should not alter myristoylation or palmitoylation; N-terminal HA tagging of SMP-1 has previously been performed in *L. major* without disruption of normal localisation, myristoylation or palmitoylation (Tull *et al.*, 2010).

SMP1::eGFP::TY localised to the flagellum in *L. mexicana* promastigotes, axenic amastigotes and amastigotes in BMDMs (Figure 31A–C). This was unlike in *L. major* where SMP1 could not be detected by antibody in amastigotes (Tull *et al.*, 2004). The exogenous UTR downstream of SMP1::eGFP::TY (*C. fasciculata* phosphoglycerate kinase A UTR) may be responsible for this disruption of normal life cycle stage-specific expression (Campbell *et al.*, 2003). In both life cycle stages SMP1::eGFP::TY was

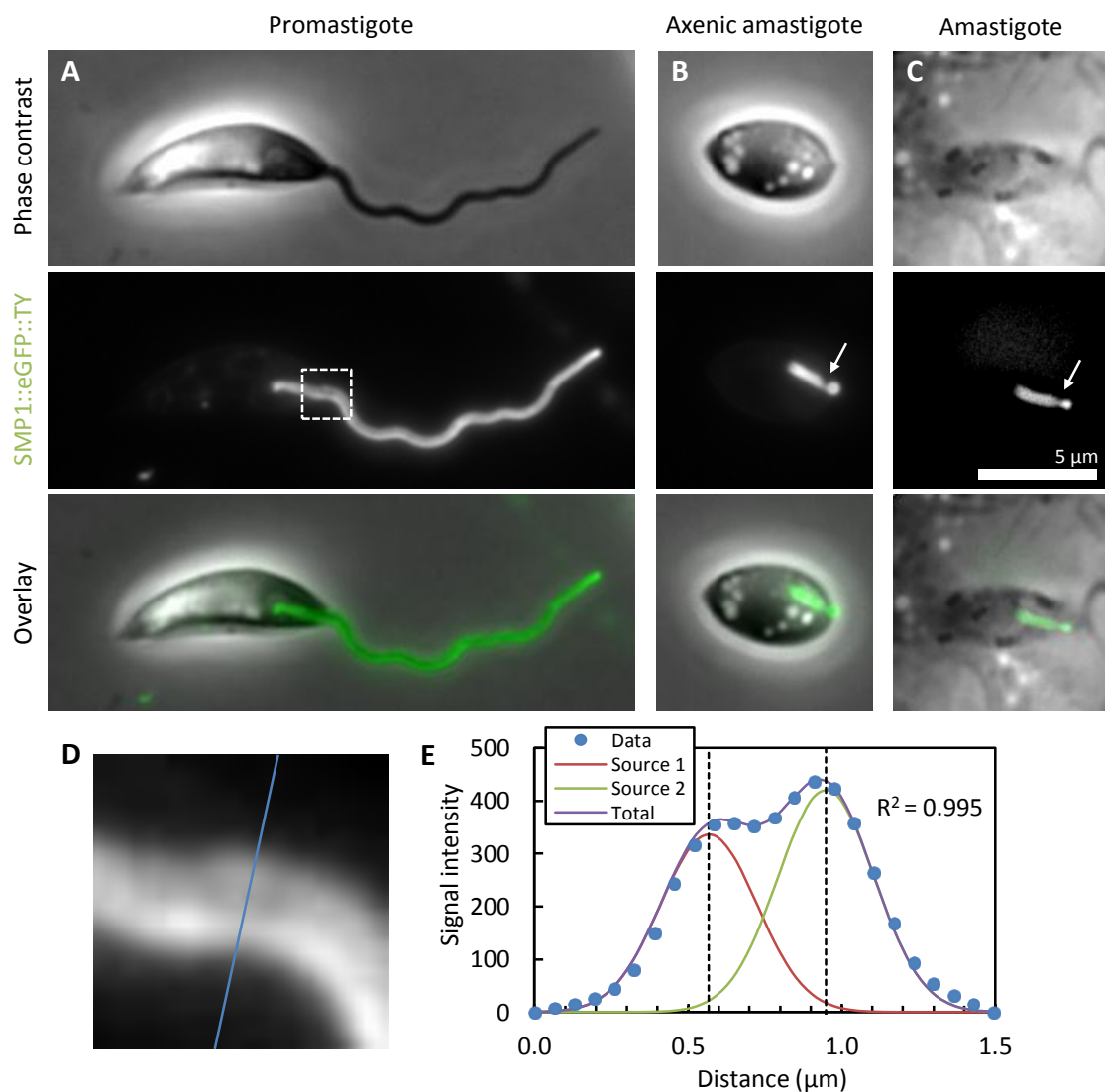


Figure 31. SMP1::eGFP::TY localises to the flagellum membrane. **A.** Phase contrast and fluorescence micrographs of live promastigote *L. mexicana* expressing SMP1::eGFP::TY adhered to a slide. **B.** Phase contrast and fluorescence micrographs 5 days following transfer of promastigotes to SM pH 5.5 34°C. The arrow in **B** indicates a constriction in SMP1::eGFP::TY staining on the amastigote flagellum at the flagellum pocket neck. **C.** Phase contrast and fluorescence micrographs of live *L. mexicana* inside a parasitophorous vacuole in a BMDM, 5 days following infection of the macrophage with promastigotes. The arrow indicates the flagellar pocket neck as in **B**. **D.** Detail of a portion of the promastigote flagellum (indicated in **A**) lying precisely in the focal plane. **E.** eGFP fluorescence intensity profile along the blue line indicated in **D**. The distribution of fluorescence intensity is consistent with the total fluorescence signal (purple curve) resulting from two point sources (red and green curves). The vertical black dashed lines indicate the location of the two point sources.

present on the entirety of the *L. mexicana* flagellum, including the 2 to 3 μm portion within the flagellar pocket which can be seen in the overlay of the phase contrast and SMP1::eGFP::TY fluorescence images (Figure 31A–C). Portions of the flagellum which lay precisely in the focal plane showed two parallel lines of SMP1::eGFP::TY fluorescence (Figure 31D). The fluorescence signal intensity profile of these regions featured a near-bimodal distribution and the distribution of fluorescence signal was very similar to the combined emissions of two closely spaced point sources (Figure 31E), in this example at a separation of 0.38 μm (Section 3.4.2). This was consistent with a flagellar membrane localisation. The amastigote flagellum featured a distinctive constriction in width near the exit point from the flagellar pocket leaving a spheroid (radius $\approx$ 0.2 μm) portion of flagellum membrane in contact with the extracellular environment (Figure 31B–C). This was consistent with the amastigote flagellum structure seen by electron microscopy (Gluenz *et al.*, 2010b).

To determine whether SMP1 was localised on the extracellular or luminal face of the flagellar membrane accessibility of the eGFP epitope to anti-GFP antibody was tested. Detergent treatment of promastigotes expressing SMP1::eGFP::TY altered accessibility of eGFP to anti-GFP antibody (Figure 32). Anti-GFP antibody could not recognise the GFP epitope on live cells (Figure 32C) or 2% PFA fixed cells (Figure 32A) however treatment of live cells with 0.01% NP40 detergent (Figure 32D) or fixed cells with 0.1% NP40 (Figure 32B) caused an increase in anti-GFP immunofluorescence signal which colocalised with the native SMP1::eGFP::TY fluorescence. Treatment of live cells with 0.1% NP40 resulted in extraction of the cytoplasm and membrane, similar to seen for treatment of *T. brucei* with 1% NP40 (Sherwin and Gull, 1989a). This treatment removed a large proportion of SMP1::eGFP::TY as observed by both native SMP1::eGFP::TY fluorescence and anti-GFP immunofluorescence (Figure 32E) consistent with near-complete extraction of the flagellar membrane, although may also be due to release of proteases and protein degradation. The change in accessibility of the GFP epitope on detergent treatment of cells indicated that SMP1::eGFP::TY is on

the interior face of the flagellar membrane. Presence of SMP::eGFP::TY at this location is unlikely to alter action of the host macrophage on the parasite as the exterior-exposed flagellum membrane face is not altered.

3.2.4.2 Flagellum length in *in vivo* promastigote to amastigote differentiation

To analyse disassembly of the promastigote flagellum upon infection of a macrophage, BMDMs were infected with SMP1::eGFP::TY *L. mexicana* at a multiplicity of infection (MOI) of 3 then the interaction was observed by time lapse videomicroscopy. BMDMs were treated with rhodamine-dextran prior to infection to pre-load the endocytic system with a fluorescent marker (Section 3.4.4). Once taken up by a BMDM a promastigote became immobilised (excepting the slower

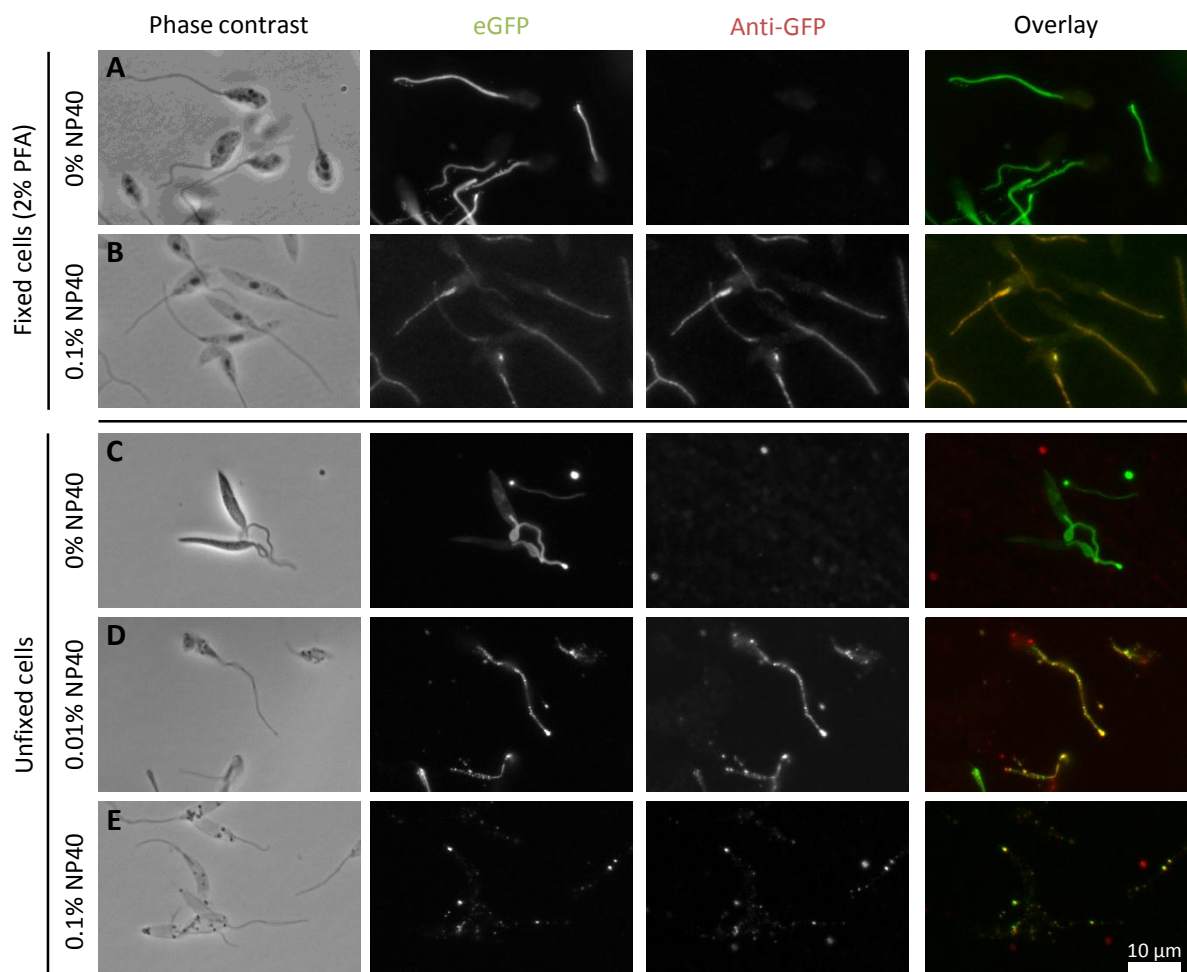


Figure 32. Antibodies can only recognise SMP1::eGFP::TY following disruption of the flagellum membrane by detergent. Micrographs of native eGFP fluorescence and anti-GFP immunofluorescence. A–B. Promastigote *L. mexicana* fixed with 2% PFA then either permeabilised with NP40 detergent or left untreated. C–E. Promastigote *L. mexicana* adhered live to a glass slide then permeabilised with NP40 or left untreated.

movement of the BMDM) (Figure 33A), unlike during axenic differentiation where they were free to continue swimming. This allowed the analysis of flagellum length change on individual parasites over time unlike analysis of axenic differentiation where analysis was limited to ‘snapshots’ at different time points (Section 3.2.3).

The uptake of *L. mexicana* SMP1::eGFP::TY by a BMDM was followed by one of two behaviours. In 12.7% ($n = 102$) of cases the *Leishmania* parasite was killed and degraded over the course of approximately 4 to 8 h (Figure 33B) (Supplementary: Video 2). Death was visible through three effects; rapid decrease in SMP1::eGFP::TY signal (Figure 33B, 0.33 to 0.44 h), accumulation of dextran in cytoplasm (indicative loss of *Leishmania* membrane integrity) (Figure 33B, 0.44 to 0.88 h) and disruption of *Leishmania* shape and decrease in size (Figure 33B, 1.55 to 2.67 h). In the remaining 87.3% of cases the *Leishmania* parasite migrated toward the centre of the BMDM (towards the nucleus) and the flagellum length gradually decreased (Figure 33C) (Supplementary: Video 2).

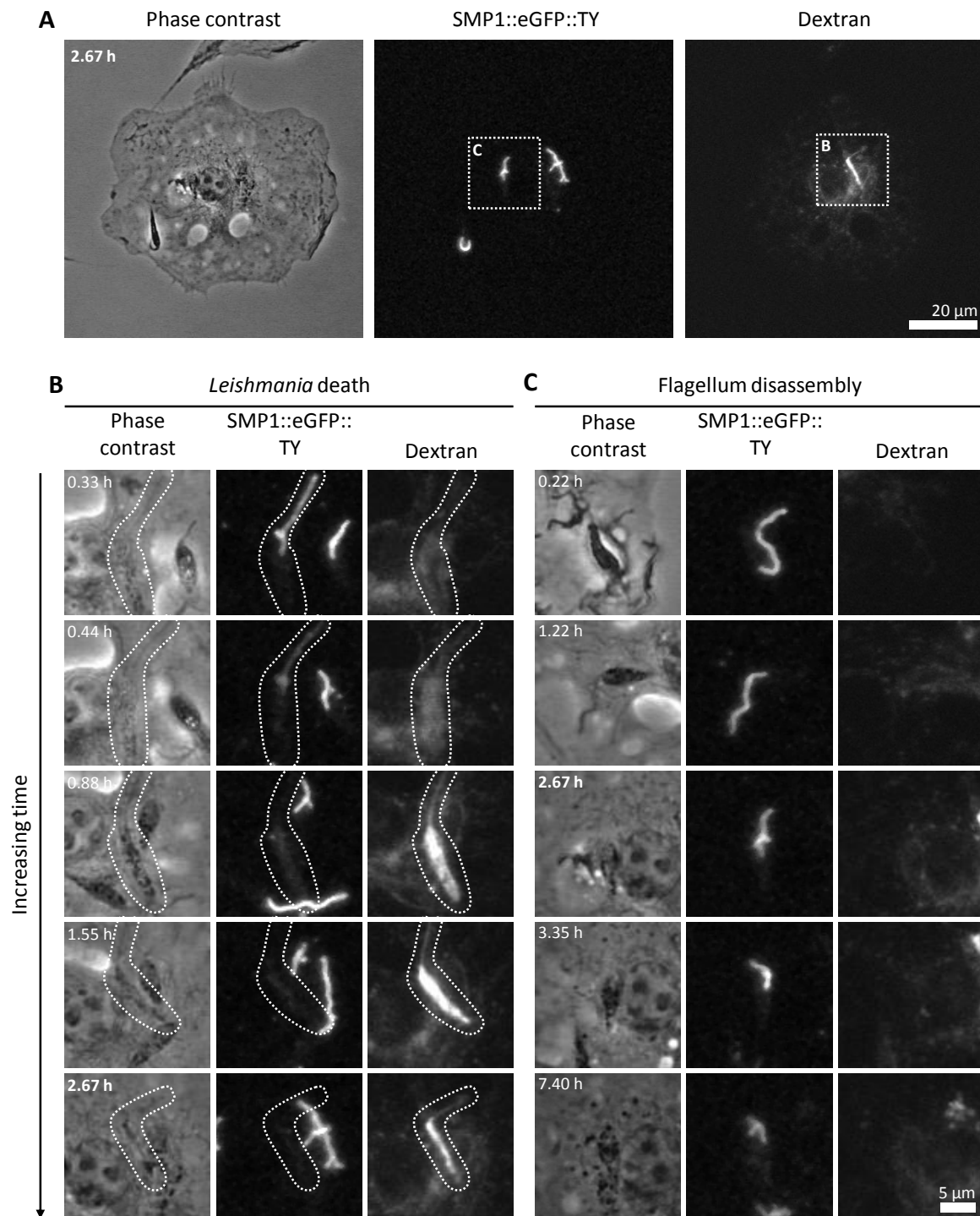


Figure 33. Dynamics of flagellum disassembly in macrophages visualised with SMP1::eGFP::TY. Micrographs of native eGFP and rhodamine-dextran fluorescence from a time-lapse videomicrograph of a single BMDM infected with promastigote *L. mexicana* SMP1::eGFP::TY. Times indicate time since addition of promastigotes to the BMDMs. **A.** Single frame showing the example BMDM. Development of the region indicated in the dextran image (B) over time is shown in **B**, development of the region indicated in the SMP1::eGFP::TY image (C) over time is shown in **C**. **B.** Series of frames showing a single *Leishmania* parasite within the BMDM in **A** which lost eGFP signal and membrane integrity (inferred from increased rhodamine-dextran signal). The position of the cell is indicated with a dotted outline. **C.** Series of frames showing another single *Leishmania* parasite within the BMDM in **A** which retained eGFP signal and membrane integrity and underwent a decrease in flagellum length.

To determine the flagellum disassembly kinetics, the length of the flagellum on individual *Leishmania* cells ($n = 38$) was measured semi-automatically (using a skeleton-based method, see [Section 5.2.6](#)) for the first 24 h of interaction with macrophages which revealed flagella disassemble to total length of approximately 3 μm over a time period of 3 to 16 h. Disassembly occurred at a wide range of rates ([Figure 34A & 35](#)). No dividing cells with two flagella were analysed. The flagellum length data (up to the first time at which it reached less than 3 μm) for each cell was fitted to both a linear and exponential curve ([Figure 34B](#)) which yielded two measures of disassembly rate; the decay constant and the decay rate respectively. The mean R^2 of these fits were similar, 0.724 ± 0.202 and 0.767 ± 0.202 (mean $\pm$ standard deviation) for the linear fit and exponential fit respectively, although the exponential fit was better in 67% of cases. Both measures of disassembly rate had broad distributions ([Figure 34C–D](#)) and neither correlated with the maximum (i.e. initial) flagellum length observed ([Figure 34E–F](#)). The mean decay rate (from the linear fit) and the mean half-life (from the exponential fit) of flagellum length were $0.97 \pm 0.66 \mu\text{m}/\text{h}$ and $2.03^{+10.49}_{-0.92} \text{ h}$ respectively (mean $\pm$ standard deviation).

To compare differentiation in BMDMs to differentiation under axenic conditions the two data sets must be represented in the same way. The differentiation in BMDMs data can be represented as histograms of flagellum lengths at different time points ([Figure 36B](#)), as used to analyse axenic differentiation ([Figure 29C](#)). Both sets of histograms show a similar broad starting distribution of flagellum lengths and a similar increase in cells with a very short flagellum, although this increase began approximately 2 h later in axenic differentiation. Both sets of histograms also show a gradual decrease in flagellum length for cells with a long flagellum. Overall flagellum length change is therefore similar for the two different analyses, suggesting the flagellum disassembly kinetics underlying axenic differentiation are similar to those observed during differentiation in BMDMs.

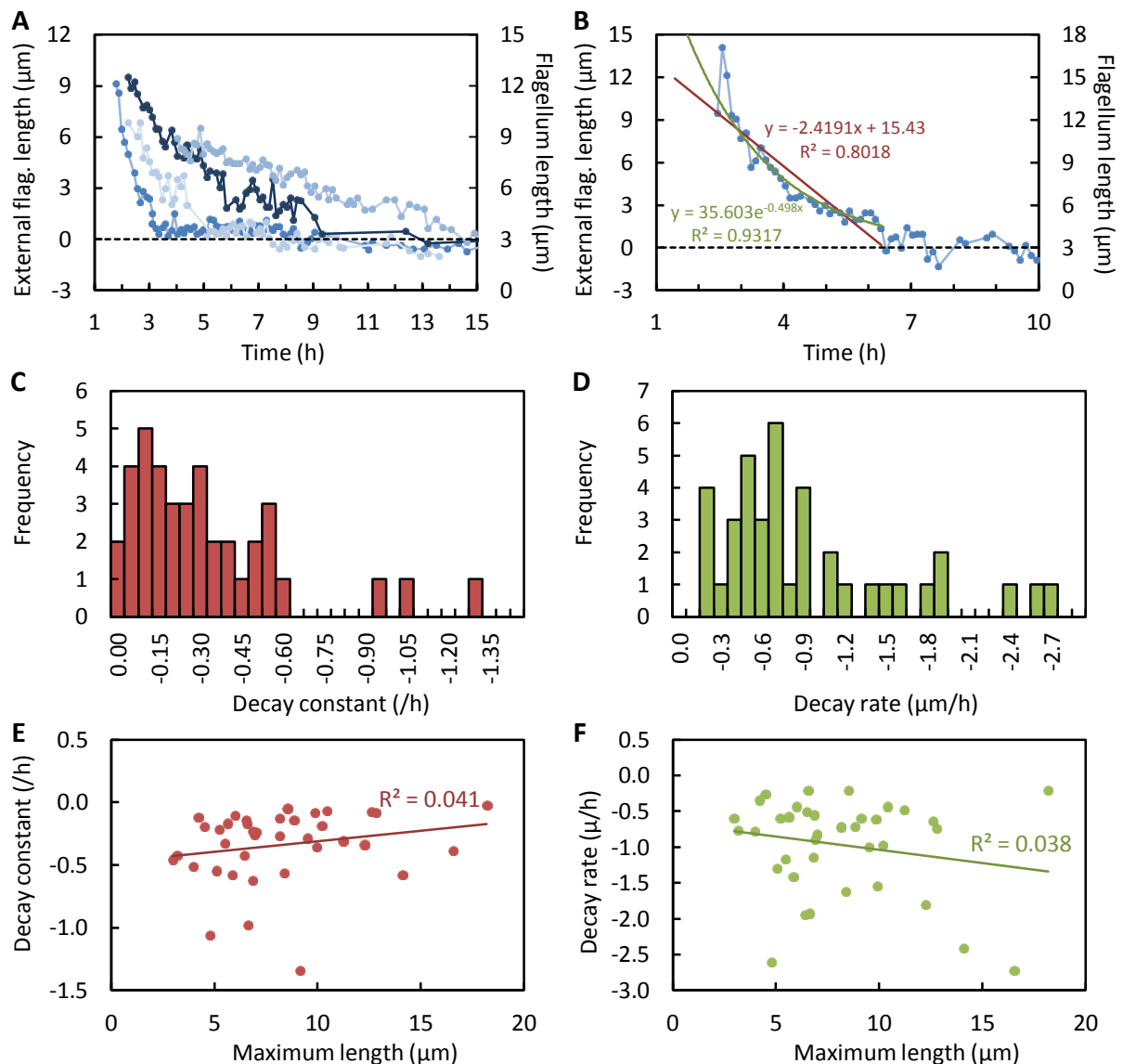


Figure 34. Diversity of flagellum disassembly rates following uptake of *L. mexicana* by BMDMs. **A.** Change in flagellum length of four representative *L. mexicana* cells following uptake by a BMDM. The approximate depth of the flagellar pocket (3 μm) is indicated by the dashed line. Flagellar length measurements began at the time of uptake by a BMDM. **B.** Example of curve fitting to flagellum length of one *L. mexicana* cell. Flagellum lengths up to the first time point where flagellum length fell below 3 μm was fitted by linear (red) and exponential (green) regression. **C.** Histogram of the decay constants determined from the exponential fit ($n = 38$). **D.** Histogram of the decay rates determined from the linear fit ($n = 38$). **E-F.** Correlation of maximum (initial) flagellum length with decay constant and decay rate respectively, linear correlation indicated the gradient was not significantly non-zero in either case ($n = 38$).

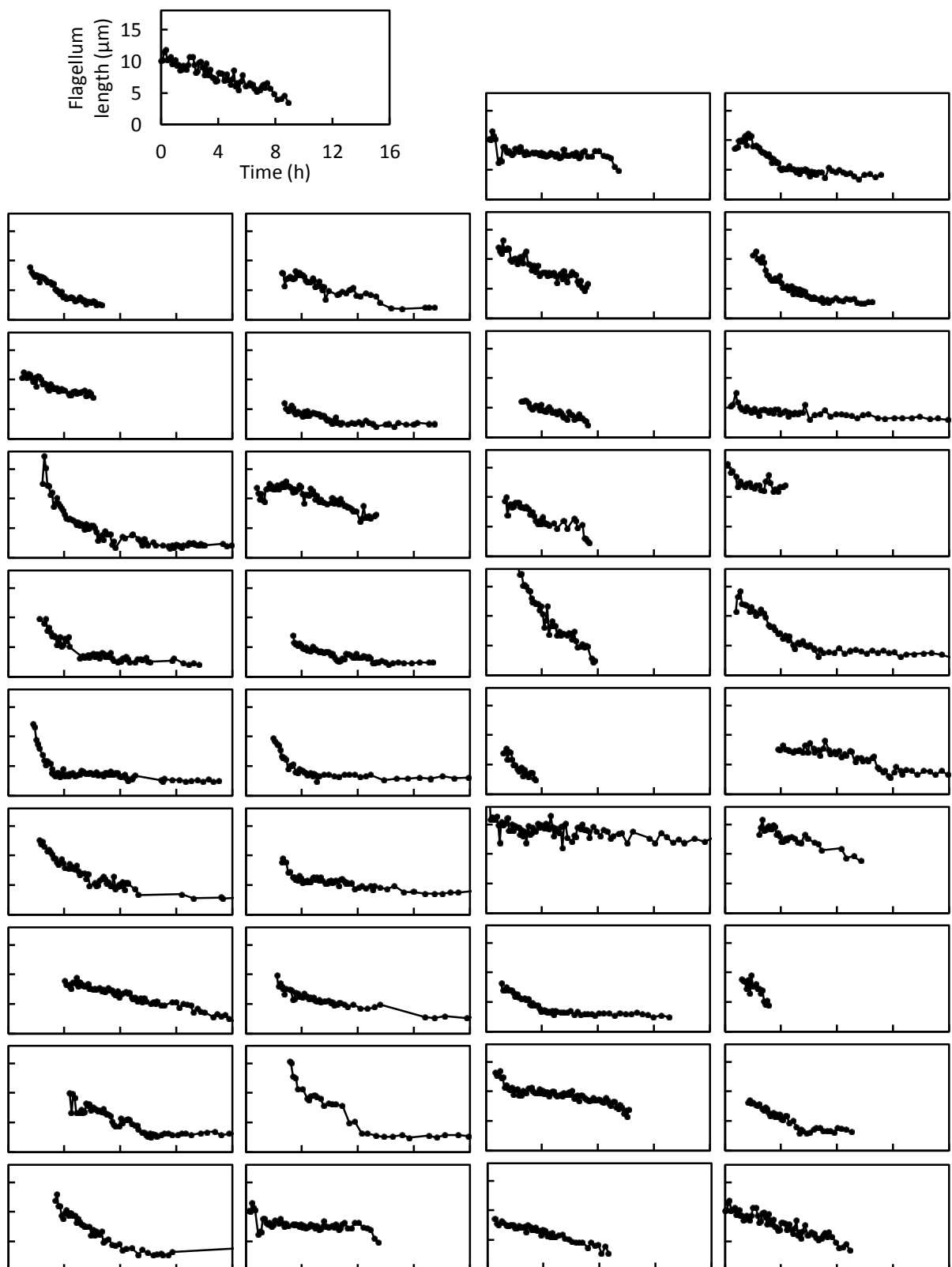


Figure 35. Complete set of flagellum disassembly curves following uptake of *L. mexicana* by BMDMs. Change in flagellum length of all 38 *L. mexicana* cells analysed following uptake by a BMDM. All graphs are to the same scale, shown in the top left.

3.3 Discussion

These analyses of the growth of the *Leishmania* promastigote flagellum have indicated that its growth and length regulation differ from that previously observed in *T. brucei*. A mathematical model of flagellum growth based on the balance point model (Figure 23) could replicate the complex correlation of flagellum length with cell cycle progress, cell body length and cellular DNA content (Figure 22) although could only do so when an additional cell cycle-dependent regulation of flagellum component synthesis was included in addition to the effects of the balance point model. The fitting of the model to the data was poorly constrained due to noise in the experimental data; nonetheless it could qualitatively predict the distribution of flagellum lengths on promastigotes in S-phase arising from growth over multiple cell cycles (Figure 25). It may be possible to use flagellum lengths on doublet cells (Section 2.2.2) (Wheeler *et al.*, 2011) (which are sister cells at the same stage of the cell cycle) to confirm growth occurs over multiple cell cycles unequivocally. Using this model, flagellum length change during differentiation to and from the amastigote was simulated (Figure 26).

The process of promastigote to amastigote differentiation axenically and in macrophages (using SMP1::eGFP::TY as a flagellum marker (Figure 31)) was analysed and compared to the prediction of

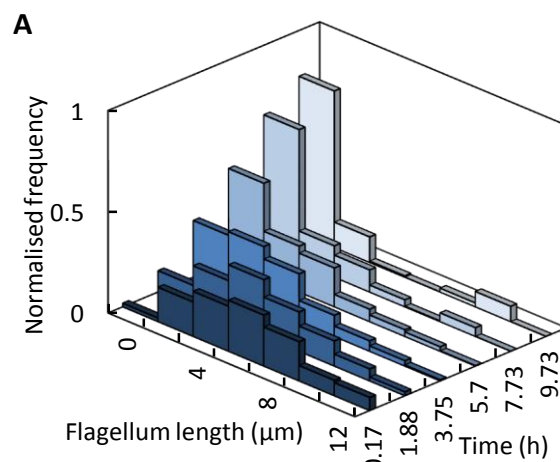


Figure 36. Population flagellum lengths during promastigote to amastigote differentiation in BMDMs. A. Normalised histograms of flagellum lengths over the first 10 h of differentiation in BMDMs, data from Figure 29C. These histograms were derived from 2463 flagellum length measurements over 38 cells.

the quantitative model of *Leishmania* flagellum growth. No evidence was observed for shedding of flagella (Figure 28). In-macrophage differentiation (Figure 33) and axenic differentiation (Figure 29) indicated (directly and indirectly respectively) that long flagella gradually decreased in length over the first 16 h in an amastigote environment. Flagellum length change occurred following a 4 h lag in population growth (Figure 27), similar to the 6 h lag previously observed in axenic *L. donovani* differentiation (Barak *et al.*, 2005). In addition axenic differentiation indicated cell division could occur during flagellum disassembly generating one daughter cell with a short amastigote-like flagellum and one with a long promastigote-like flagellum, still undergoing disassembly (Figure 30). This process was similar to the predictions of the model of *Leishmania* flagellum growth (Figure 26). In a small majority of cases the kinetics of flagellum disassembly better matched an exponential decrease in flagellum length than the linear decrease, predicted by the balance point model, and were very diverse with decay rates varying by a factor of 20 (Figure 34).

3.3.1 Promastigote cell cycle and flagellum length

The *Leishmania* flagellum does not grow to a rigid target length; instead it undergoes multiple rounds of growth in successive cell cycles. This has the effect that older flagella are longer

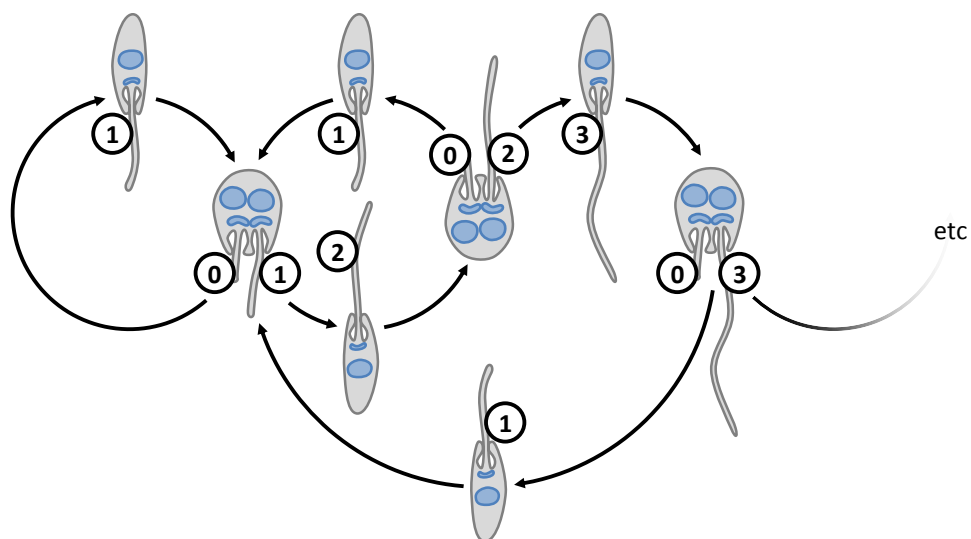


Figure 37. Promastigote flagellum growth over multiple cell cycles. Numbers indicate the age of each flagellum in generations and older flagella are longer.

(Figure 37). Previous studies have asserted, without reference to data, that the *Leishmania* flagellum is found at a constant length following its initial growth. For example (Adhiambo *et al.*, 2005) stated “*Leishmania* flagella are stable and do not shorten or elongate during the cell cycle”. It will be vital for future analyses of flagellar length to incorporate this new understanding of promastigote flagellum growth into interpretation of the data.

The pattern of flagellum growth of *L. mexicana* is distinctly unlike the other trypanosomatid for which the process of flagellar growth has been analysed in detail; *T. brucei*. In procyclic *T. brucei* flagellum growth is almost completely confined to the new flagellum of 2F cells (Tyler *et al.*, 2001). Following completion of cytokinesis the flagellum on the cell which inherited the new flagellum undergoes a small extension while the flagellum on the cell which inherited the old flagellum does not grow (Farr and Gull, 2009). In contrast flagellum growth in *L. mexicana* appeared to be dominated by the effects of the balance point model and was comparatively unconstrained.

The ongoing growth of the *L. mexicana* flagellum over multiple cell cycles has direct impacts for the interpretation of the sandfly portion of the *Leishmania* life cycle. The morphologies of sandfly life cycle substages (as defined by cell body and flagellum length (Rogers *et al.*, 2002)) follow the trend that longer flagellar lengths occur on parasites found at later stages of an infection. The simplest explanation for the origin of these morphologies is therefore that the flagella are older and have had more time to grow. In particular the non-replicating metacyclic promastigotes may undergo ongoing flagellum growth to generate their distinctive long flagellum.

3.3.2 Promastigote to amastigote differentiation

The generation of the short amastigote flagellum during differentiation from the promastigote occurred through two pathways; the disassembly of existing long external flagella (Figure 29 & 36) and the limited growth of the new flagellum on dividing cells (Figure 30), the latter of which was only observed during axenic differentiation. The mechanism involved therefore appears to be a

modulation of normal flagellum length regulation which resulted in disassembly of flagella in a cell cycle-independent manner. The diversity of flagellum disassembly rates and the nonlinear disassembly of many flagella cannot, however, be explained by the balance point model. The wild type and PFR1 deletion mutant behaved identically during differentiation (Figure 29) indicated the PFR is not important for signal perception or flagellum disassembly.

Flagellum disassembly is an efficient method for generating the amastigote morphology as every cell in the population is able to undergo differentiation and every cell which divides during the differentiation process also generates one amastigote-shaped daughter cell and one which can continue flagellum disassembly. Cells which underwent division during differentiation generated asymmetric daughter cells, one with a long external flagellum and one without. Unlike the procyclic promastigote to epimastigote differentiation process used by *T. brucei* (Sharma *et al.*, 2008) this was not the dominant mechanism to achieve cell body morphological change and a reduced flagellum length.

The amastigote life cycle stage has upregulated proteolysis and autophagy pathways in comparison to the promastigote (Rainey *et al.*, 1991; Bates *et al.*, 1992; Pral *et al.*, 1993; Bates, 1994). The smaller cell body of the amastigote suggest that autophagy may be a major pathway in generating its morphology. As the promastigote flagellum is disassembled (rather than shed) autophagy may also be required for breaking down the unwanted flagellum components, although disassembly of the flagellum of the PFR1 deletion mutant (which lacks the considerable bulk of the PFR) was no faster than the wild type (Figure 29) suggesting autophagy, if involved, is not the limiting factor. Nonetheless it will be interesting to determine if disruption of proteolysis or autophagy also disrupts flagellum disassembly, for example through use of lactacystin to block ubiquitin-mediated proteolysis by the proteasome (Robertson, 1999).

While I have shown the process by which the short amastigote flagellum length is attained when and how the 9v axoneme structure characteristic of the amastigote (Gluezn *et al.*, 2010b) is formed was not analysed. The short flagella formed by disassembly of long promastigote flagella over 24h in a macrophage (Figure 33) did not have an identical morphology to *Leishmania* amastigotes which had been in either a parasitophorous vacuole or SM5.5 for 5 days (Figure 31). These had developed a constriction in the flagellar membrane near the flagellar pocket neck which may be indicative of a 9v axoneme structure at that point. If the amastigote 9v axoneme structure proves to be important for function of the amastigote flagellum then the mechanism by which it is generated will be of interest. It may be the case that more than 24 h is required for flagellum disassembly to generate the 9v axoneme structure. Alternatively construction of a new flagellum in a dividing cell may be required to form a 9v axoneme and a 9+2 promastigote flagellum cannot simply disassembly to form the collapsed axoneme structure. These two processes could be differentiated using serial section TEM of the anterior portion of dividing cells to determine axoneme ultrastructure during the first 24 h of differentiation.

3.3.3 From model to molecular mechanisms

The mathematical model of flagellum growth in *Leishmania*, based on the balance point model, explained the origin of diverse flagellum length in an exponential promastigote culture (Figure 25) and accurately predicted the changes in flagellum length during promastigote to amastigote differentiation (Figure 26). The model was designed to be simple to prevent over fitting of the data, its ability to replicate and predict experimental observations was impressive considering its simplicity. The model was not, however, able to capture all subtleties of *Leishmania* flagellum growth. Specifically the model predicts the same distribution of flagellum lengths for all exponentially growing cultures while a modest increase in average flagellum length occurred in logarithmic promastigote cultures at higher densities (Section 2.2.3) (Wheeler *et al.*, 2011). A small

increase in the length of the cell cycle may account for this effect by leaving additional time for flagellar growth.

The simplicity of the flagellum growth model also makes it abstract, while it was designed with specific biological behaviours in mind there are alternative mechanisms which would give rise to a similar model, similar kinetics and similar behaviour. The model was based on the concept of a cytoplasmic pool of flagellum components driving flagellum growth and cell and life cycle dependent production of these components. This was based on the observations that, in *T. brucei*, abundance of transcripts for flagellar and paraflagellar proteins vary through the cell cycle (Archer *et al.*, 2011). Temperature-sensitive mRNA stability is also a mechanism for regulation of gene expression between the *Leishmania* promastigote and amastigote, as used by HSP83 (Larreta *et al.*, 2004), and amastigote *Leishmania* possess decreased levels of major flagellar components, including β -tubulin and PFR1 (Bates *et al.*, 1992), relative to the promastigote. While regulation of synthesis of flagellum components is a plausible means by which flagellum growth could be modulated an alternative, but functionally similar mechanism, could be cell and life cycle dependent regulation of IFT.

Signalling-mediated regulation of IFT is particularly interesting as overexpression and deletion of some mitogen-activated protein kinase-related proteins (LmxMKK (Wiese *et al.*, 2003), LmxMPK9 (Bengs *et al.*, 2005) and LmxMPK3 (Erdmann *et al.*, 2006)) alter flagellar length. The case for involvement of LmxMPK9 in regulating flagellum length (rather than being directly involved in flagellum growth) is particularly convincing as LmxMPK9 null mutants have an *increased* flagellum length (Bengs *et al.*, 2005). The upstream effectors and downstream targets of these kinases are not yet known but a role for LmxMPK3, LmxMKK and LmxMPK9 in regulating flagellum length change during differentiation to the amastigote has been proposed based on the change in expression level between life cycle stages as all three are present at higher levels in the promastigote (Bengs *et al.*, 2005; Erdmann *et al.*, 2006). An alternative view is that the regulatory function of the kinases only

occurs in the promastigote where they are present at high levels and therefore could form part of signalling cascades to regulate flagellum growth. In either case these are strong candidates for molecular basis of modulation of flagellum growth in the cell and life cycles.

These analyses have revealed how the promastigote and amastigote flagellum morphologies are attained through the cell cycle and through promastigote to amastigote differentiation respectively. Flagellum growth and disassembly in *L. mexicana* occurred largely independently of the progression of the cell cycle with behaviours similar to predicted by the balance point model, although additional modulation of growth in a cell cycle and differentiation stage-dependent manner was required to precisely match the observed behaviour. This behaviour was unlike that previously described for *T. brucei* and yields the first insights into the mechanisms *Leishmania* utilises to achieve morphological change to the flagellum through the life cycle.

3.4 Methods development and mathematics

This section covers only mathematics and method development specific to this results chapter (for standard materials and protocols see [Section 7](#)).

3.4.1 Flagellum length modelling

Flagellum length simulation was based on the balance point model (Marshall *et al.*, 2005). Changes in flagellum length and cytoplasmic flagellum components were calculated for 100 time steps per cell cycle, change in flagellum length, δ_f , and flagellum component pool, δ_c , per time step were given by:

$$\delta_f = \frac{k_g[c]}{f} - k_d$$

$$\delta_c = k_s - k_p - \delta_f$$

where f is flagellum length, c is the cytoplasmic pool of components, k_g is the flagellum growth rate constant and k_d , k_s and k_p are the flagellum disassembly, flagellum component synthesis and flagellum component degradation rates respectively (Figure 23). The net production of flagellar components is given by $k_s - k_p$. For simplicity, cell volume was assumed always to equal one, therefore $[c] = c$, and c used units of μm (μm of flagellum the components could assemble). Onset of new external flagellum growth (elongation beyond the flagellum pocket) started at 80% cell cycle progress (Section 2.2.3.4) (Wheeler *et al.*, 2011) with an initial length, f , of 3 μm , equal to the depth of the flagellar pocket.

Timing of cell cycle phases and changes in cell body length and DNA content were determined from existing cell cycle data (Section 2.2.3.2 & 2.2.3.3) and were simulated as follows: S-phase occurred between 40 and 80% cell cycle progress. Mitosis occurred at 90% cell cycle progress. Cell length grew at a linear rate from 7.2 to 11.3 μm during G_1 and decreasing at a linear rate back to 7.2 μm between the end of S-phase and mitosis. DNA increased at a linear rate from 1 to 2 during S-phase.

3.4.1.1 Model fitting

The model of flagellum growth was fitted to available flagellum length data by adjusting four variables; (1) k_a , (2) k_d , (3) $k_s - k_p$ and (4) the phases of the cell cycle during which flagellum synthesis occurred. Variables 1 to 3 were adjusted based on the flagellum lengths of cells in exponentially growing promastigote *L. mexicana* (Section 2.2.3). Net flagellum component synthesis must occur at a rate of approximately one flagellum's-worth per cell cycle (approximately 10 μm). The ratio of flagellum growth and disassembly rates must give rise to a maximum flagellum length of around 20 μm . Flagellum growth rate must be slow in comparison to the cell cycle; few cells attain a flagellum at the equilibrium point of maximum length. Given these constraints the value of variables 1 to 3 were adjusted by comparison of simulated flagellum length data to experimental data (Section 2.2.3). The final fitted values were (1) $k_a = 70$ /cell cycle, (2) $k_d = 13 \mu\text{m}/\text{cell cycle}$, (3)

$k_s - k_p = 6.4 \mu\text{m}/\text{cell cycle}$. Finally synthesis of flagellum components during different stages of the cell cycle (variable 4) was tested; flagellum component synthesis was allowed to occur during pre-S-phase, S-phase, post-S-phase and combinations thereof (Figure 24).

3.4.2 Analysis of SMP1::eGFP::TY signal distribution

The SMP1::eGFP::TY fluorescence intensity profile across a transverse section of the flagellum was fitted to a model treating the two portions of membrane lying orthogonal to the focal plane as two point sources. The diffraction-limited images of these point sources were simplified to Gaussian distributions, as previously described (Zhang *et al.*, 2007):

$$I(x) \approx I_0 e^{-x^2/w^2}$$

where I is the resultant signal intensity, x is the distance from the point source, I_0 is the total intensity and w is the distribution width.

$$w = 0.84\lambda N$$

where λ is the wavelength of the emitted light (509 nm, the eGFP emission maxima (Patterson *et al.*, 1997)) and N is the numerical aperture of the objective lens (1.4). The position and intensity (I_0) of the two point sources were adjusted manually to minimise the R^2 value to achieve the best fit.

3.4.3 Live *Leishmania* immunofluorescence

5×10^6 to 1×10^7 *Leishmania* cells were harvested from culture by centrifugation at 3000 g for 3 min and washed twice by resuspension in 0.5 ml PBS and centrifugation at 3000 g for 3 min followed by resuspension in PBS to a final volume of 100 μl . The suspension was applied to a 1 cm^2 square on a silanized slide outlined using a fat pen (DakoCytomation S2002) and allowed to settle for 10 min. 0.00%, 0.02% or 0.20% NP40 (Sigma Aldrich) in PBS was then added and the slides were incubated for 5 min at room temperature, then washed twice by addition and aspiration of 200 μl PBS. A mixture of 1:200 anti-GFP (rabbit IgG) (Life Technologies (Molecular Probes), A11122) and 1:100 rhodamine

isothiocyanate goat anti-mouse IgG (Jackson ImmunoResearch 115-025-166) in PBS was then added and the slide was incubated at room temperature for 10 min, then washed thrice by addition and aspiration of 200 µl PBS and observed immediately.

3.4.4 Macrophage time-lapse microscopy

BMDMs (Section 7.3.2) were incubated in 35 mm glass-bottom plates in growth medium supplemented with 250 µg/ml rhodamine-conjugated dextran (average molecular weight 10,000; D1824 Life Technologies) for 18 h. The medium was then removed by aspiration and the cells were washed with 2 ml normal growth medium three times. Stationary phase (Section 7.3.3) *L. mexicana* SMP1::eGFP::TY were added to an MOI of 3 and the culture was immediately observed on a LSM 510 inverted confocal microscope with a 40× NA 0.8 objective lens (Carl Zeiss). Samples were maintained at 34°C with 5% CO₂ using an environmental chamber.

Infected macrophages were imaged by phase contrast and fluorescence microscopy. Fluorescence micrographs were captured with minimum confocality and minimum laser power to minimise phototoxicity. Images were captured from 5 positions evenly spaced around the sample and a 3×2 tiled array of images with 5% image overlap was captured at each position allowing reconstruction of five 630×440 µm fields of view. One set of micrographs was captured every 5 min at each tiled array location at each position for 24 h.

Customised phase contrast-based autofocus was used to account for long time scale sample movement. A z-series of 20 images at 0.5 µm spacing were captured at each time point from an additional reference position in the centre of the sample. The highest contrast image was selected and z-position recorded. A rolling average of the z-position of the highest contrast image from the previous 10 time points was used for image capture for the next time point.

4 Roles of trypanosomatid cell and flagellum morphology in motility

All work included in this chapter is my own except for production of the GM6 RNAi *T. brucei* cell line (Section 4.2.4) and the TEM data presented in Figure 49 (Section 4.2.5.3).

4.1 Introduction

The long external flagellum on the *Leishmania* promastigote is a multifunctional organelle with roles in both motility and attachment to the vector gut wall (Pimenta *et al.*, 1994; Sacks *et al.*, 2000; Bates, 2008) (Section 1.3). A long flagellum is essential to prevent clearance of *L. amazonensis* from *Lutzomyia longipalpis*; overexpression of LdARL-3A-Q70L prevents formation of a long flagellum and, while parasites grow normally, they are cleared from the vector gut (Cuvillier *et al.*, 2003). A long external flagellum therefore appears important in the sandfly. More recently an *L. braziliensis* isolate was identified which does not form a long flagellum in promastigote growth conditions and can infect sandflies (Zauli *et al.*, 2012) which suggests a flagellum may not be vital for sandfly infection in all species. This isolate has not yet been shown to be capable of establishing a transmissible infection. While further work is required to determine whether a long external flagellum is universally vital for infection of a sandfly it appears to play an important role.

Evidence for a role for the promastigote flagellum in attachment to the gut surface (Pimenta *et al.*, 1994; Sacks *et al.*, 2000; Bates, 2008) is better developed than for the function of motility in the sandfly where quantitative data concerning parasite swimming is limited. It has not been shown whether motility is vital for sustaining and transmitting an infection. Based on qualitative observations it is hypothesised that the nectomonad promastigote is highly motile and responsible for expansion of infection to the anterior midgut and that the metacyclic promastigote is also highly

motile (Bates, 2007). One can therefore speculate there may be a link between promastigote flagellum and cell body morphology and swimming involved in progression through life cycle substages in a sandfly.

All human infective trypanosomatids (*Leishmania*, *T. brucei* and *T. cruzi*) progress through a digenetic life cycle with insect vector and human life cycle stages. Each of these species has motile insect life cycle stages suggesting motility has a functional purpose, although it has not been rigorously shown whether motility is vital for sustaining and transmitting an infection of any species. Nonetheless many parasites are motile in the insect host. Unlike *Leishmania* the morphology of motile *T. brucei* and *T. cruzi* life cycle stages when in the insect are trypomastigote or epimastigote rather than promastigote. The function of these different morphologies and their potential role in motility is, like the function of the diverse promastigote morphologies in *Leishmania*, not known.

4.1.1 Generation of cell movement

Trypanosomatid swimming arises from the motion of the single flagellum. Based on analysis of *C. fasciculata*, *Crithidia deanei* and *L. major* (Gadelha *et al.*, 2007), directional swimming of *Leishmania* is driven by a flagellum-type beat similar to *Crithidia* where a planar, sinusoid-like beat propagates from the flagellum tip to the base (Gadelha *et al.*, 2007). In *T. brucei* the flagellum beat which gives rise to directional swimming also progresses from the tip to base (Walker, 1961) but has been described as having a three dimensional bihelical shape (Rodríguez *et al.*, 2009), although this is contested (Heddergott *et al.*, 2011). The flagellum of *Leishmania* is also capable of undergoing a reversed asymmetric ciliary-type beat which causes rapid cell reorientation. *Leishmania* and *Crithidia* flagella can switch between ciliary and flagellar-type beats separated by a pause in motion (Sugrue *et al.*, 1988; Gadelha *et al.*, 2007). The *T. brucei* flagellum can also undergo a reversed base to tip beat which causes a reversed motion and reorientation (Heddergott *et al.*, 2011).

Swimming behaviours have been analysed in detail for bloodstream form *T. brucei* (a trypomastigote). Parasites undergo one of two behaviours, directional swimming or tumbling in which the flagellum continues to move but the cell does not translocate rapidly (Uppaluri *et al.*, 2011; Zaburdaev *et al.*, 2011; Weiße *et al.*, 2012). *T. cruzi* epimastigotes also display similar swimming and tumbling behaviours (Ballesteros-Rodea *et al.*, 2012). While the swimming behaviour of *Leishmania* has not previously been analysed in detail, the ability of the cells to undergo rapid switches between flagellar and ciliary-type beats suggests they may also undergo a similar swim and tumble behaviour (Gadelha *et al.*, 2007). Direct comparison of the swimming of different species has not been performed but would allow analysis of these behaviours arise and whether it is dependent on morphology.

4.1.2 Functions of motility in trypanosomatids

Motility of trypanosomatids has multiple proposed roles and understanding how trypanosomatids swim assists interpretation of several areas of their biology. Trypanosomatids may undergo chemotaxis or osmotaxis to achieve directed locomotion. This is of interest as there are similarities between the swim and tumble behaviours in *T. brucei* (Uppaluri *et al.*, 2011; Zaburdaev *et al.*, 2011; Weiße *et al.*, 2012), the alternation between flagellar and ciliary-type beats in *L. major* (Gadelha *et al.*, 2007) and the swim and tumble behaviour of *Escherichia coli* (Berg, 2004) which may be conducive to chemotaxis. This may be of use in navigation through the insect host. Chemotaxis has not been detected in *T. brucei* (Hill, 2003) or *T. cruzi* (although an optical tweezers-based experimental approach has been proposed (Thomaz *et al.*, 2009)) but chemotaxis and osmotaxis have been reported in *Leishmania*.

Directed movement of *Leishmania* in response to sugar concentration gradients has been reported (Bray, 1983; Oliveira *et al.*, 2000) although was later reinterpreted as a general osmotactic response (Leslie *et al.*, 2002). More recently ATP, acting through the mitochondrial adenine nucleotide

transporter, has been shown to have a chemorepellent effect (Detke and Elsabrouty, 2008). These studies contradicted each other regarding suitable methodologies for detecting chemotaxis; one measured alteration of mean time of straight line movement between tumbles (Barros *et al.*, 2006) while the other could not detect distinct swim and tumbling behaviours (Detke and Elsabrouty, 2008). Therefore while there is evidence for chemotaxis and osmotaxis in *Leishmania* (which may have a role in anterior migration during a sandfly infection (Barros *et al.*, 2006)) these analyses are limited by our understanding of the basic behaviour of *Leishmania* while swimming.

Motility may have a role in completion of cytokinesis in *T. brucei*. Cells immobilised by RNAi targeting the radial spoke protein *rsp3* are described as forming clusters of posterior-to-posterior connected cells. This is proposed to arise through failure of abscission, suggesting a mechanical effect of the flagellar beat of two antiparallel cells in completing cytokinesis (Ralston *et al.*, 2006). This mechanism is of interest as *Leishmania* cultures include approximately 10% posterior-to-posterior connected doublet cells which also appear to have delayed abscission generating 'doublets' (Section 2.2.2) (Wheeler *et al.*, 2011). Any differences in motility between *T. brucei* and *L. mexicana* may give insight into why these doublets arise.

Locomotion is vital for bloodstream form *T. brucei* (Broadhead *et al.*, 2006) and one of its roles is thought to be in assisting recycling of large surface proteins and clearance of surface-bound immunoglobulins. This is proposed to occur by hydrodynamic forces generated while swimming driving the movement of larger molecules toward the posterior of the cell where the flagellar pocket (the site of all endo- and exocytosis) is situated (Dean and Matthews, 2007; Engstler *et al.*, 2007). In some trypomastigotes the flagellar pocket can be positioned at large distances from the posterior of the cell (such as procyclic *T. brucei* where the posterior to basal body distance is 2.5 μm (Robinson *et al.*, 1995)) in which cases this mechanism may also be of diminished relevance. Nonetheless it is one of few rigorously investigated mechanisms linking motility to morphology. An

inverse process, hydrodynamic-assisted enrichment of *small* surface proteins at the promastigote flagellar pocket, may occur in *Leishmania*.

4.1.3 Function of morphology in motility

Major morphological features of the trypanosomatid cell involve the flagellum, the degree of lateral attachment of the flagellum to the cell body and flagellum length, and are therefore likely to affect motility. Previous analysis of the flagellar beat of *L. major*, *C. fasciculata* and *C. deanei* revealed a correlation of flagellum length with flagellum beat wavelength and amplitude (combining the data from all three species) (Gadelha *et al.*, 2007) however the impact this had on the resulting cell motility was not analysed. One correlate between morphology and motility has previously been described for *T. brucei*; swimming bloodstream form *T. brucei* are more elongated and less contorted than those undergoing tumbling motion. The explanation for this effect was increased rigidity of the cell increasing the straightness of the cell, thus a mechanism by which morphology alters swimming behaviour (Uppaluri *et al.*, 2011). However this data could also be interpreted as the type of flagellum movement which occurs during tumbling behaviour contorting cell body shape.

To address the impact of trypanosome morphology on motility, the swimming behaviours of four species with promastigote or promastigote-like morphology (*L. mexicana*, *L. major*, *C. fasciculata* and *Phytomonas davidii*), one species with trypomastigote morphology (*T. brucei*) and a *T. brucei* mutant with epimastigote-like morphology were analysed using identical experimental methods. In addition the contribution of the morphology (cell body and flagellum length) to swimming speed of individual promastigote cells was assessed, taking advantage of the range of cell morphologies and flagellum lengths which occur in exponential promastigote culture (Section 2) (Wheeler *et al.*, 2011). These gave insight into the different capacities of species and morphological variants for swimming and the contribution of both the promastigote and trypomastigote morphologies to motility. On this

basis a theory of spin-stabilisation to allow highly directional swimming of microorganisms was developed.

4.2 Results

Analysis of trypanosomatid swimming typically uses cell track data; the paths followed by cells as they swim. These must be captured microscopically therefore previously described methods typically use shallow chambers (<50 μm depth) to attempt to restrict cell movement to a two dimensional space and hold cells in the focal plane (Gadelha *et al.*, 2007; Uppaluri *et al.*, 2011; Weiße *et al.*, 2012). This allows the generation of long tracks; however it also places the parasite in an artificially constrained environment where they interact with the chamber floor and ceiling as well as the surrounding fluid. An alternative approach in which cells are free to move in three dimensions and their behaviour is not perturbed by their enclosure was therefore developed.

4.2.1 Cell swimming can be analysed by focus-limited cell tracking

Dark-field videomicroscopy of a 250 μm deep chamber, much larger than the dimensions of *T. brucei* and *Leishmania*, containing normal growth medium was used for motility analysis and only the central portion of the volume was imaged. To generate large data sets, cells in the videomicrographs were identified and tracked automatically using custom image analysis programs (Section 4.4.1.3).

To assess the performance of cell identification, the quantity of defocus the cell tracking algorithm could tolerate before a cell could not be identified accurately (i.e. determine the coordinates of a single point per cell near the centre of the cell) was determined. Using a sample of *L. mexicana* adhered to the slide at the base of the observation chamber. The algorithm was able to identify in-focus cells accurately in 99.1% ($n = 867$) of cases. The number of cells accurately identified decreased as defocus increased and was quantified for defocus distances from 0 to 40 μm

(Figure 38A). The ability to identify cells was reduced twofold with a defocus of 10 μm . Errors in identification were rare (the location of cells were still accurately determined) however sensitivity (the ability to detect any given cell) was reduced. The identification of cells for generating cell tracks was therefore limited to a depth range of around 25 μm , comparable to previous studies using physical confinement (Gadelha *et al.*, 2007; Uppaluri *et al.*, 2011; Weiße *et al.*, 2012).

The performance of the cell tracking was determined from a sample of logarithmically growing *L. mexicana* from a single dark-field videomicrograph (405 s; 1024 frames captured at 5 Hz). The tracking algorithm yielded 3142 cell tracks, of which 522 were over 15 s (75 frames). The distribution of track length followed an exponential decay with a half-life of approximately 30 s (Figure 38B) with tracking primarily limited by cells moving out of the plane of focus. This method gave a large and unbiased collection of cell movement tracks over 15 s long which were confined to a narrow (i.e. near planar) depth range through defocus suitable for numerical analysis.

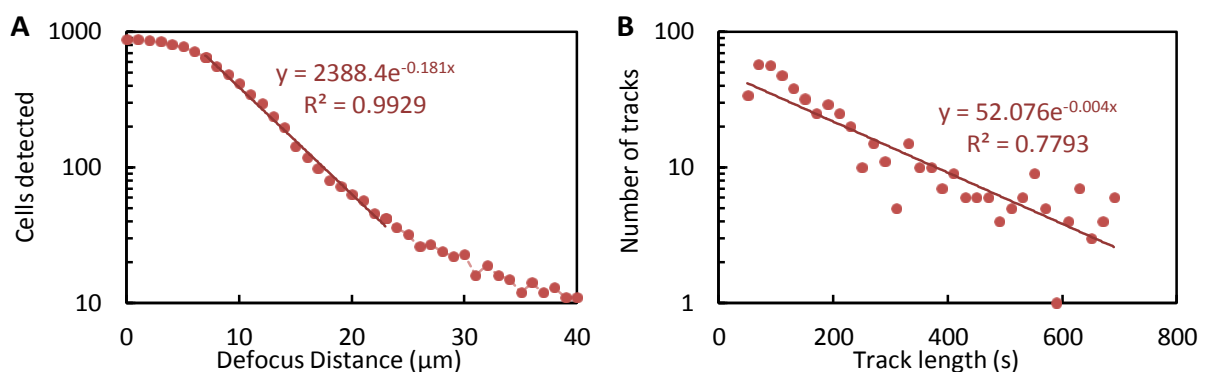


Figure 38. Performance of cell detection for focus-limited cell tracking. **A.** Correlation of the number of cells detected and defocus distance for live *L. mexicana* cells adhered to a slide using dark-field microscopy and the particle detection algorithm. The solid line is an exponential fit line for defocus distances between 8 and 22 μm . **B.** Distribution of cell track lengths (over 50 s in length) achieved by the automated particle tracking algorithm analysing *L. mexicana* ($n = 522$). The solid line is an exponential fit.

4.2.2 Promastigote *L. mexicana* and trypomastigote *T. brucei* have different patterns of motility

The motility of exponentially growing procyclic (trypomastigote) *T. brucei* and procyclic (promastigote) *L. mexicana* were analysed in their culture media (SDM79 and M199 respectively). The shape of cell tracks (Figure 39A–B) differed greatly. Procyclic *T. brucei* either underwent directional movement in near-straight path or did not undergo directional movement ('tumbled') (Figure 39A),

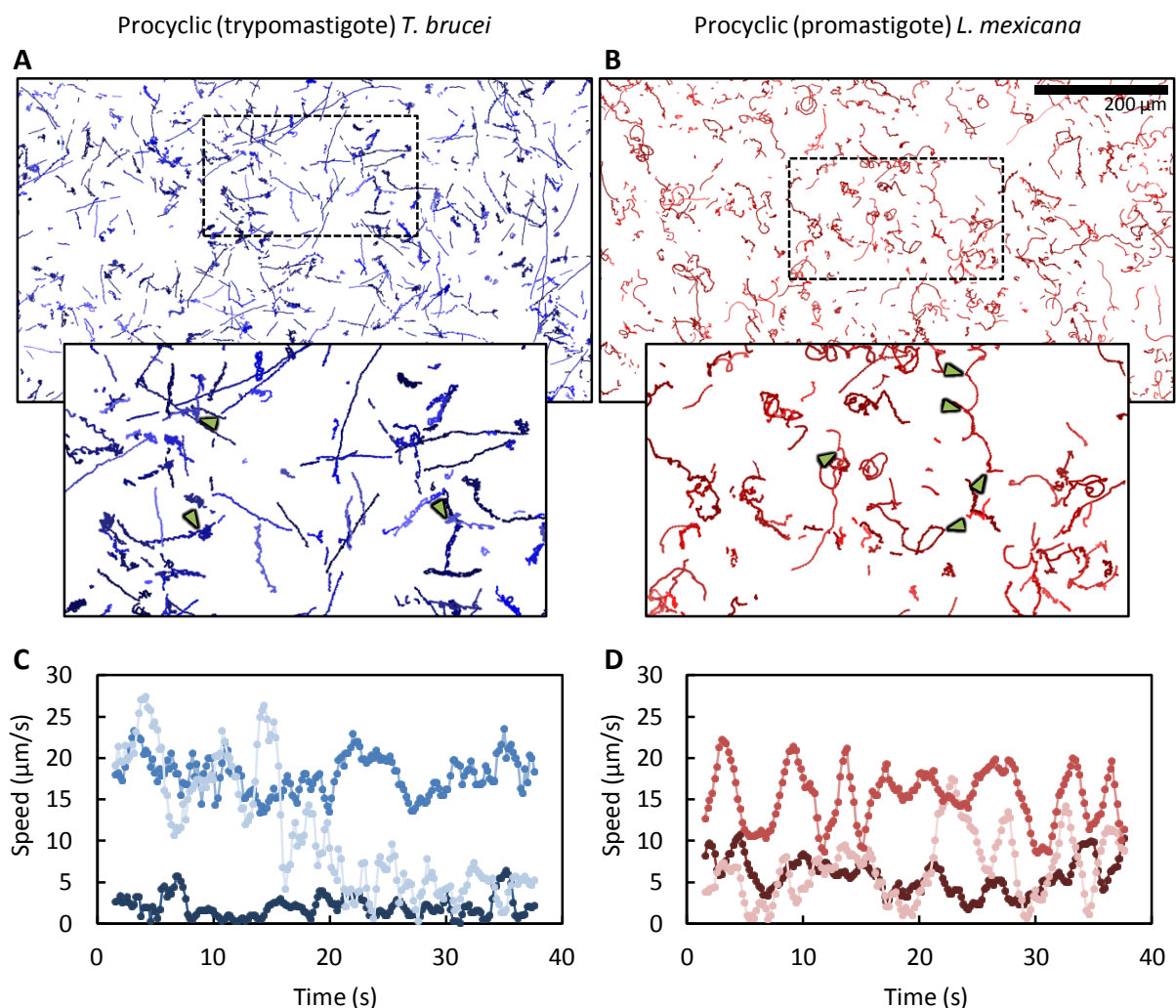


Figure 39. Procyclic trypomastigote *T. brucei* and promastigote *L. mexicana* have different patterns in motility. **A.** Paths followed by *T. brucei* during unconstrained swimming ($n = 1481$). Green arrowheads indicate switches from swimming to tumbling motion and vice versa. **B.** Paths followed by *L. mexicana* during unconstrained swimming ($n = 1130$). Green arrowheads indicate rapid changes in direction of cells. **C.** Changes in speed over time of three example *T. brucei* cells representative of swimming behaviours observed; one tumbling (dark blue), one swimming (mid blue) and one switching from swimming to tumbling (light blue). **D.** Changes in speed over time of three example *L. mexicana* cells representative of the diversity in swimming speed. *L. mexicana* cells do not have distinct swimming/tumbling motions.

similar to described for bloodstream (trypomastigote) form (Uppaluri *et al.*, 2011). Promastigote *L. mexicana* did not display distinct swimming/tumbling behaviours and instead swam along undulating or spiral paths punctuated by rapid changes in direction (Figure 39B), consistent with the description of flagellar and ciliary beat patterns in *L. major* (Gadelha *et al.*, 2007). The speed of individual cells over time illustrated the difference between these two behaviours (Figure 39C–D); procyclic *T. brucei* cells could be classified as either swimmers or tumblers with some cells which transition between these behaviours (Figure 39C). *L. mexicana* cells underwent large fluctuations in swimming speed and could not be classified into distinct swimming or tumbling behaviours (Figure 39D).

To analyse these behaviours, quantitative measures of cell speed, average velocity and directionality of swimming were required. To calculate these statistics accurately the appropriate time step over which to perform calculations must first be selected; short time scale motion is dominated by cell reorientation and undulation due to the flagellar beat while at longer time scales this high frequency motion will average to zero and cell motion will be dominated by swimming. The appropriate time step for calculations was determined using the average angular persistence of a large sample of cells, as previously described for bloodstream form *T. brucei* (Zaburdaev *et al.*, 2011). Angular persistence is a mathematical descriptor of path directionality which quantifies how much the direction of motion of a particle (cell) changes over a time step on a scale of one (continues in the same direction) through zero (no correlation in direction) to minus one (reverses direction). As larger time steps are used the average persistence over a large number of cells within the sample will decay towards zero as the direction of cell motion has had more time to become randomised. This assumes no overall directionality to motion arising from a chemoattractant or bulk flow of the medium. The time scale at which this decay (relaxation) occurs is characteristic of different types of motion.

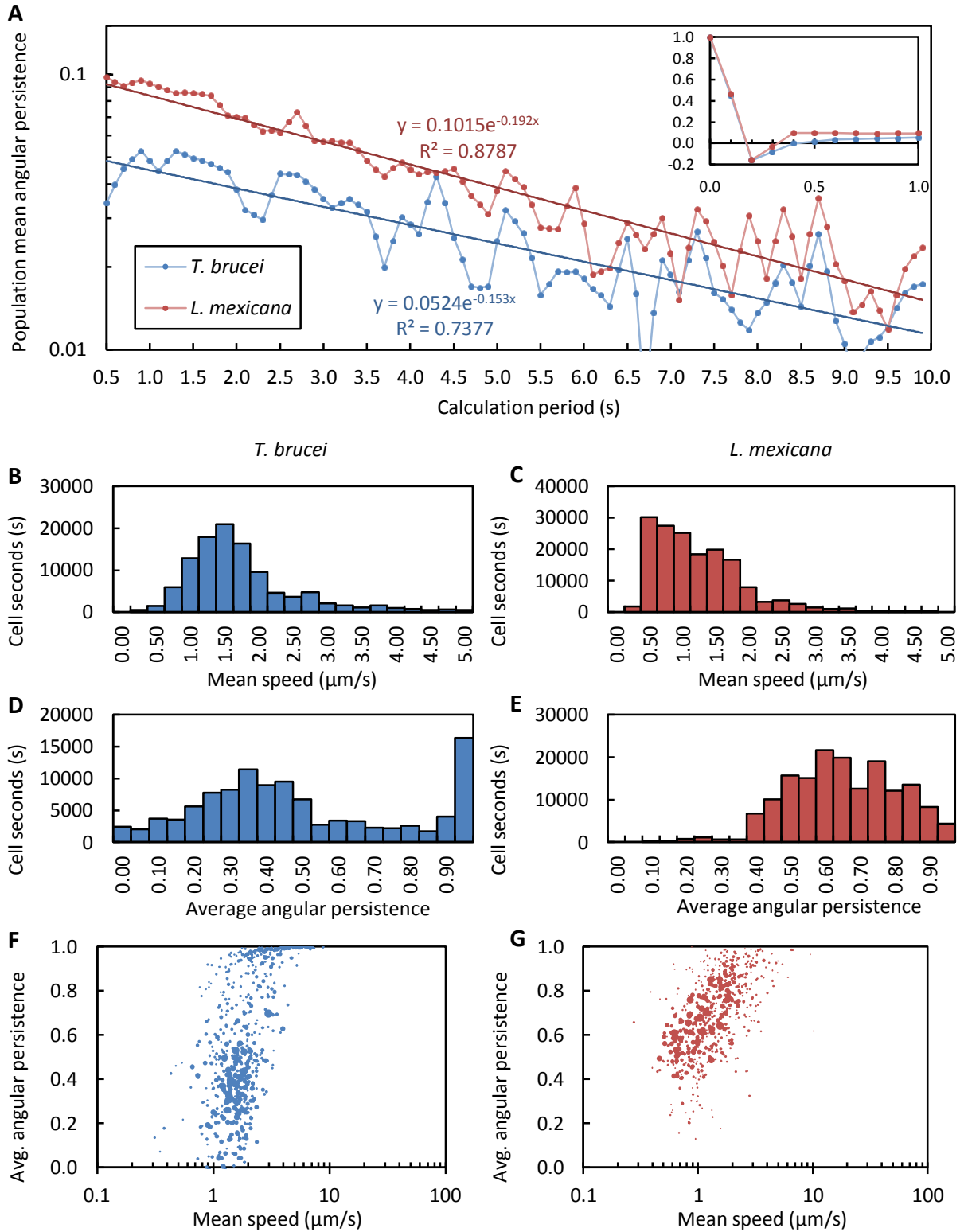


Figure 40. *L. mexicana* and *T. brucei* have similar swimming speeds but distinctive directional persistence. **A.** Self-correlation of angular persistence with time, averaged over the population. The solid lines are exponential fits. Self-correlation of angular persistence with time for 0 to 1 s is shown on a different scale on the inset graph. **B–C.** Histograms of mean swimming speed for tracks at least 15 s long from *T. brucei* ($n = 641$) and *L. mexicana* ($n = 568$) respectively. **D–E.** Histograms of mean angular persistence of swimming in tracks at least 15 s long from *T. brucei* and *L. mexicana* respectively. Negative persistence values were rare (<2.5%) and so are not shown. **F–G.** Correlation of mean speed with mean angular persistence for tracks from *T. brucei* and *L. mexicana* respectively. Point area is proportional to the time for which that cell was observed (Section 4.4.1.4).

This method was applied to procyclic *T. brucei* and *L. mexicana* to determine the time scale at which flagellar beat-driven cell oscillation and cell swimming occur. Using calculation intervals between 0 and 10 s the population average of angular persistence of cell motion was calculated (Figure 40A). The average angular persistence of both species showed a very rapid relaxation over the first 0.5 s (Figure 40A, inset) including a brief drop to negative average angular persistence indicative of reciprocating motion. This was due to the cell oscillation or undulation as a direct result of the flagellar beat (Zaburdaev *et al.*, 2011). Following this initial relaxation both species showed a second, slower, relaxation time corresponding to the properties of cell swimming. The half-lives were 4.53 s and 3.61 s for procyclic *T. brucei* and *L. mexicana* respectively, similar to bloodstream form *T. brucei* with a half-life of approximately 5 s (Zaburdaev *et al.*, 2011). A calculation interval of 1 s was therefore used for analysis of the properties of the swimming behaviours of these cells; at this time scale the cell oscillation due to the flagellar beat will average to zero.

The cell swimming speed (averaged over each cell track) followed broad distribution for procyclic *T. brucei* and *L. mexicana* and was similar for both species (Figure 40B–C). These broad distributions arose from the switch between swimming and tumbling behaviour of procyclic *T. brucei* and the large range and fluctuations in swimming speed of *L. mexicana*. In contrast the average angular persistence (averaged over each cell track) followed very different distributions for the two species. The distribution for procyclic *T. brucei* featured two populations of cells, one with low (<0.5) and one with very high (>0.9) persistence (Figure 40D) while the distribution for *L. mexicana* featured one population of cells with intermediate (>0.4 and <0.9) persistence (Figure 40E). Correlations of average speed and persistence (Figure 40F–G) highlight these different modes of motility. For both procyclic *T. brucei* and *L. mexicana* average cell speed and angular persistence followed a loose positive correlation, again similar to bloodstream form *T. brucei* (Zaburdaev *et al.*, 2011).

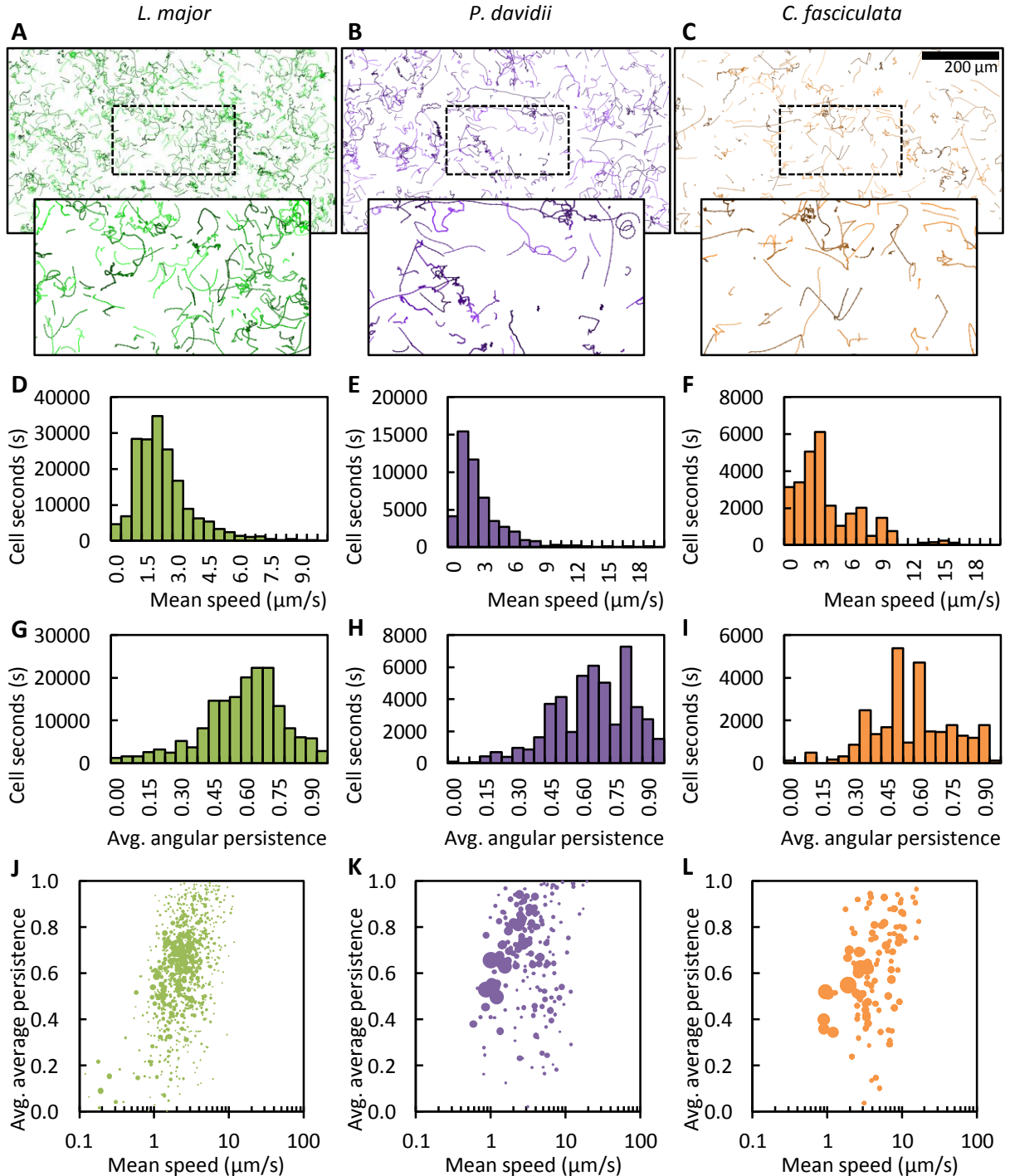


Figure 41. Swimming directional persistence is consistent among promastigote-like morphologies. A–C. Paths followed by *L. major* ($n = 2115$), *P. davidii* ($n = 945$) and *C. fasciculata* ($n = 1378$) respectively during unconstrained swimming. D–F. Histograms of mean swimming speed for tracks at least 10 s long from *L. major* ($n = 894$), *P. davidii* ($n = 195$) and *C. fasciculata* ($n = 186$) respectively. G–I. Histograms of mean angular persistence for tracks at least 10 s long from *L. major*, *P. davidii* and *C. fasciculata* respectively. J–L. Correlation of mean speed with mean angular persistence for tracks from *L. major*, *P. davidii* and *C. fasciculata* respectively. Point area is proportional to the time for which that cell was observed (Section 4.4.1.4).

Taken together these data indicated that while *T. brucei* and *L. mexicana* swim at similar average speeds their behaviour while doing so differs. *L. mexicana* swimming behaviour was homogenous; all cells swam with similar undulating paths (with occasional reorientation events) albeit at different average swimming speeds (Figure 39B & D). The monomodal distribution of average angular persistence (Figure 40G) supported this observation. In contrast *T. brucei* underwent distinctive tumbling or swimming behaviour with occasional switches between these swimming modes (Figure 39A & C). This was reflected by the distribution of average angular persistence of *T. brucei* which indicated motion may either be very high or low in directionality (Figure 40F). Additionally *T. brucei* was capable of undergoing swimming with very high directionality (average angular persistence >0.95), a behaviour which was rare in *L. mexicana* (Figure 40F–G).

The primary morphological difference between the trypomastigote and promastigote is the lateral attachment of the flagellum to the cell body in the trypomastigote in contrast to the free flagellum of the promastigote. This raises the possibility that this morphological difference is the cause of the different swimming behaviours (Figure 39 & 40). Many other trypanosomatid species have motile life cycle stages with promastigote-like morphology, therefore three were analysed to determine if this correlation with swimming behaviour was consistent; promastigote *L. major*, promastigote *Phytomonas davidii* and choanomastigote *C. fasciculata*. Track shape in all three species was qualitatively similar to *L. mexicana* with curved and undulating paths (Figure 41A–C). Swimming speed in the three species varied significantly from *L. mexicana* (KS test, $p < 0.05$), for example modal swimming speed of *C. fasciculata* was six times that of *L. mexicana*. The higher swimming speeds of *P. davidii* and *C. fasciculata* reduced typical cell track length as cells moved out of focus more rapidly; number of cell tracks over 10 s long analysed for these species was correspondingly lower. The distribution of average swimming speed in all species was similar (Figure 41D–F). Distribution of average angular persistence was also similar to *L. mexicana* with a monomodal distribution and few cells with very high persistence (Figure 41G–I). Correlation of average

swimming speed and persistence (Figure 41J–L) confirmed a similar mode of swimming to *L. mexicana* and not *T. brucei* (Figure 40F–G).

4.2.3 Promastigote *L. mexicana* swimming speed depends on flagellum length

L. mexicana swimming speed and persistence was highly varied, and I have previously shown that flagellum length on exponentially growing *L. mexicana* has a similarly large range (Section 2.2.3)

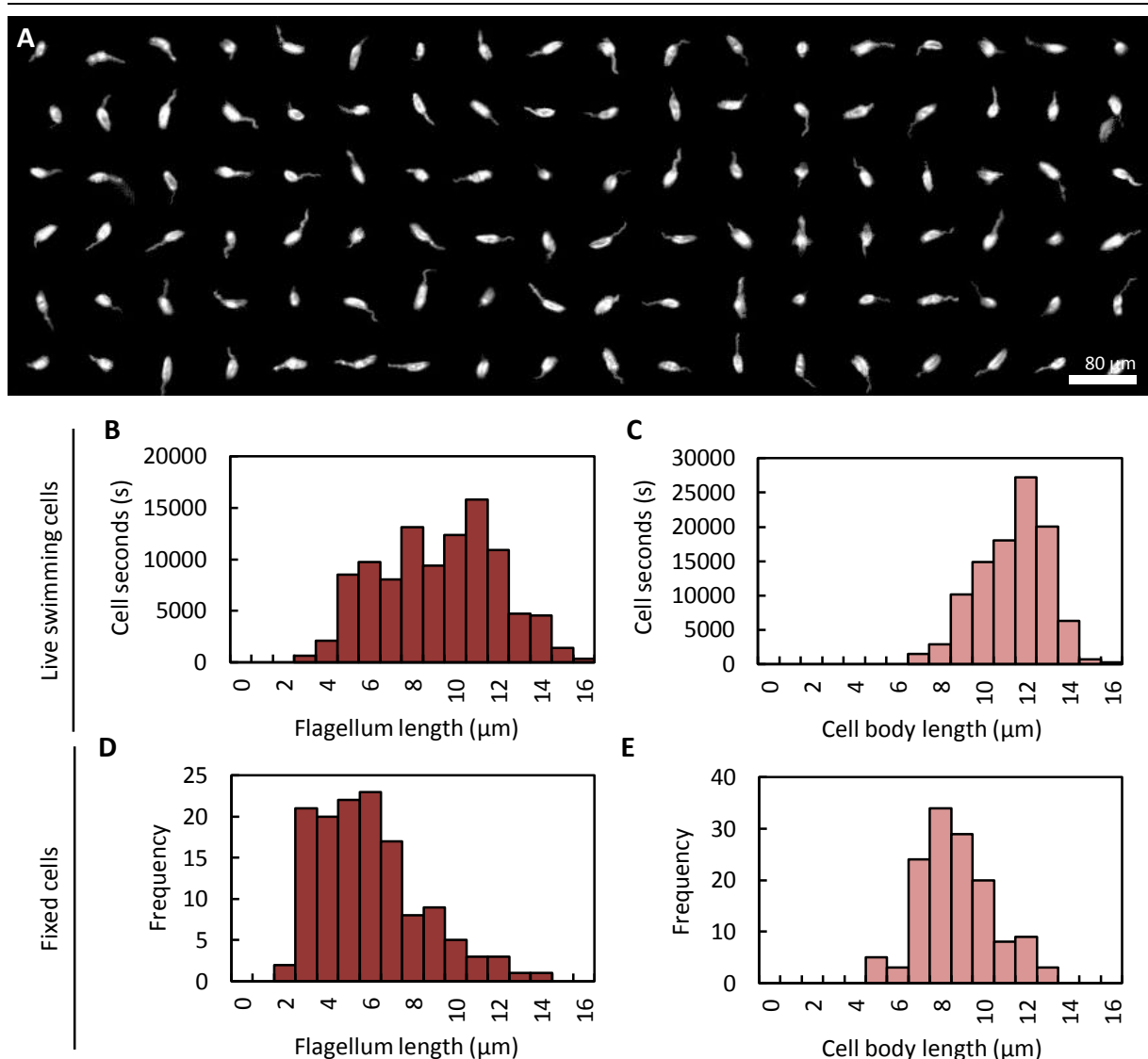


Figure 42. Measurement of morphology from motility analysis micrographs. A. 108 example still images of *L. mexicana* (from the motility analysis dark-field videomicrograph) from which cell and flagellum length were measured. B–C. Histograms of flagellum and cell length measured from the motility analysis dark-field videomicrograph ($n = 308$). D–E. Histograms of cell and flagellum length as measured from a fixed sample from the same population of cells ($n = 135$).

(Wheeler *et al.*, 2011). Whether flagellum length diversity is one of the contributing factors to this diversity in swimming was therefore analysed. The dark-field videomicrographs were captured with sufficiently short (2 ms) exposure times to freeze flagellar motion of *T. brucei*, *Leishmania* and *Crithidia* which have typical beat frequencies of under 50 Hz (20 ms period) (Gadelha *et al.*, 2007; Rodríguez *et al.*, 2009). The videomicrograph therefore consists of hundreds of images of the cell which provided many images where the cell was completely in focus from which measurements of cell body and flagellum length were made (Figure 42A). Only non-dividing cells (cells with one flagellum) were included in the analysis. To validate this method of measuring cell morphology the distribution of flagellum and cell body length measured from videomicrographs was compared to those measured from phase contrast images of a sample of fixed cells taken from the same population (Figure 42B–E). The range of cell and flagellum lengths were similar, and similar to previous measurements (Section 2.2.3) (Wheeler *et al.*, 2011). There was a bias towards longer flagellum and cell body lengths in the live sample. This may indicate a selection bias in which more cells with a long flagellum are found swimming in the centre of the sample chamber rather than interacting with the chamber ceiling or floor.

Flagellum length and cell body length both have a weak correlation with swimming speed (Figure 43B–C) however flagellum length and cell body also have a weak positive correlation (Figure 43A). Correlation of flagellum and cell body length is consistent with my previous data; while the flagellum can grow over multiple cell cycles (Section 3) and flagellum and cell body length do not have a strong correlation (Section 3.2.1) (Wheeler *et al.*, 2011) both predominantly increase in length through the cell cycle giving rise to a weak positive correlation. As a result if swimming speed arose from either cell body or flagellum length it would correlate with both morphological measures. To attribute the contribution of these features to swimming speed, principal component analysis was used to determine the two orthogonal (non-correlated) morphometric measures and isolate the contributions of cell and flagellum length; component 1 (dominated by the contribution

of cell length) and component 2 (dominated by flagellum length) (Figure 44A). Average cell speed showed a significant correlation with component 2 and not with component 1 (Figure 44B and C). Therefore flagellum length is the main morphological feature of promastigotes which contributes to the speed of swimming.

The bias previously observed, where average flagellum length of fixed cells was shorter than that of live cells imaged for the motility analysis (Figure 42B and D), may therefore have arisen from sedimentation of slower cells with short flagella to the bottom of the chamber and out of the observed volume. This is consistent with qualitative observation of *C. fasciculata* and *P. davidii* where average swimming speeds are faster than *L. mexicana* and less sedimentation of cells was observed.

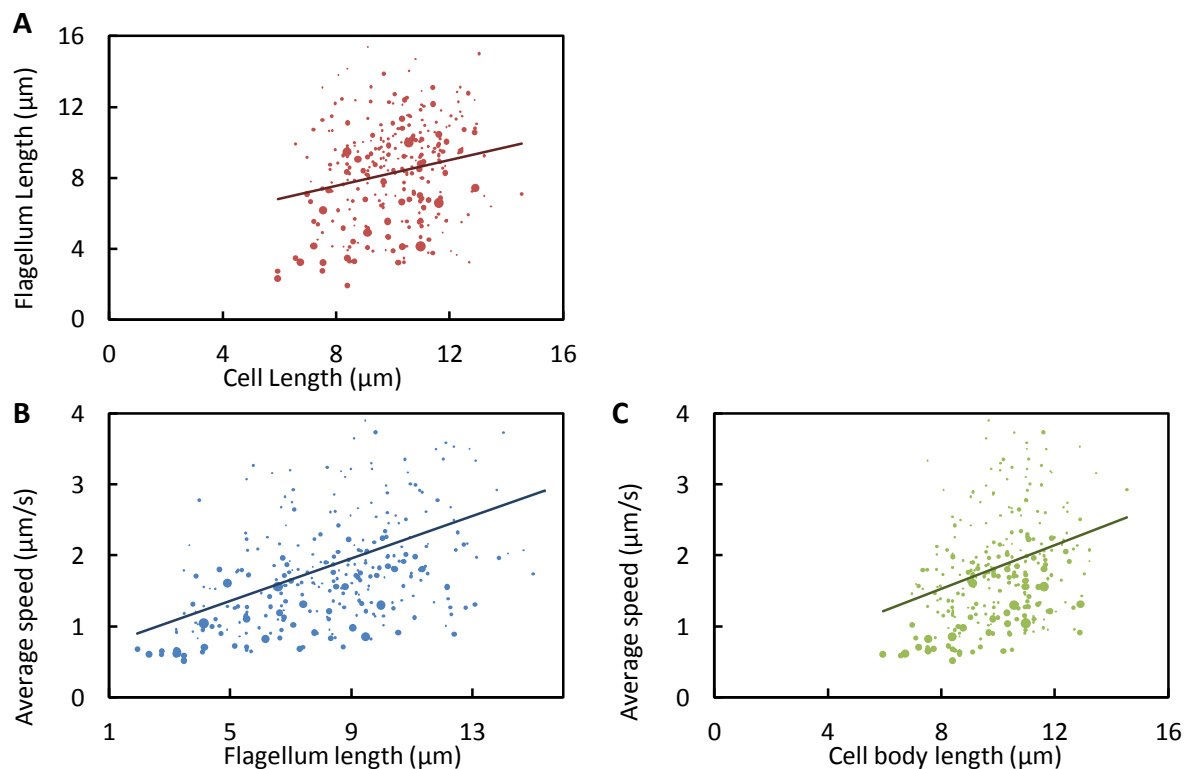


Figure 43. *L. mexicana* swimming speed correlates with flagellum length and cell body length. **A.** Scatter plot of cell length against flagellum length. The gradient of the linear correlation is significantly non-zero ($p < 0.0003$). **B.** Correlation of swimming speed with flagellum length. The gradient of the linear correlation is significantly non-zero ($p < 0.0001$). **C.** Correlation of speed with cell length. The gradient is significantly non-zero ($p < 0.0001$). Point area is proportional to the time for which that cell was observed (Section 4.4.1.4). In all plots $n = 308$.

4.2.4 Trypomastigote and epimastigote-like *T. brucei* have similar motility

The morphological difference of a laterally attached or free flagellum may be the cause of the different swimming behaviours of procyclic *T. brucei* and *L. mexicana*. GM6 is a high molecular weight protein which localises to the FAZ of *T. brucei* and includes calpain-like domains and many (at least 60) 68 amino acid-long GM6 repeats (Varga *et al.*, Unpublished; Müller *et al.*, 1992; Olego-Fernandez, 2010). Induction of RNAi targeting this protein causes an increase in proportion of cells with an epimastigote-like morphology with a long portion of the flagellum free from lateral attachment to the cell body and overhanging the anterior of the cell. These epimastigote-like cells have no detectable growth defect and, beyond 48 h induction, the population morphology. Following

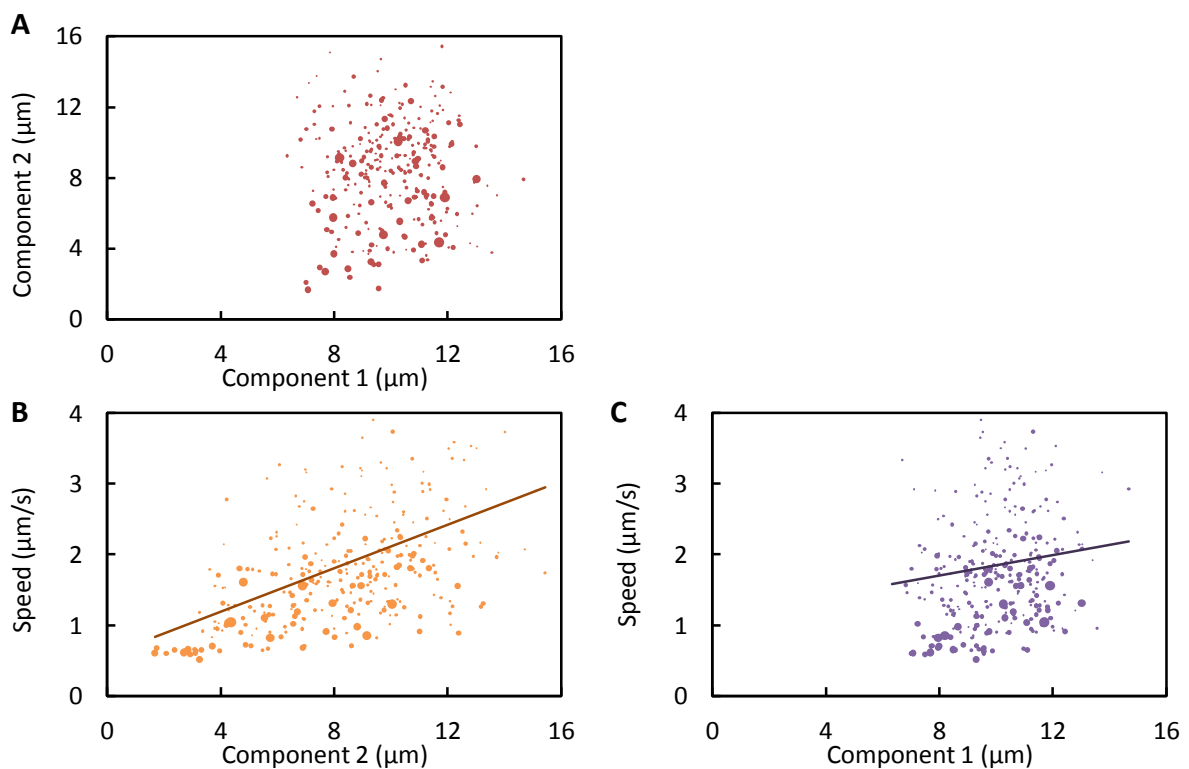


Figure 44. Variation in *L. mexicana* swimming speed arises from flagellum length and not cell body length. **A.** Scatter plot of the data in Figure 44A following use of principal component analysis to split the data into uncorrelated orthogonal components. **B.** Correlation of swimming speed with principal component 2 (dominated by flagellum length). The gradient of the linear correlation is significantly non-zero ($p < 0.0001$) and has a high Spearman's rank correlation coefficient (0.58). **C.** Correlation of speed with principal component 1 (dominated by cell length). The gradient is not significantly non-zero ($p > 0.05$) and has a low Spearman's rank correlation coefficient (0.19). Point area is proportional to the time for which that cell was observed (Section 4.4.1.4). In all plots $n = 308$.

induction for 72 h 90% of cells had an epimastigote-like ($>5\text{ }\mu\text{m}$ flagellum overhang at the anterior) morphology and the portion of the flagellum overhanging the anterior of 1K1N cells increased from $2.7\pm 0.6\text{ }\mu\text{m}$ (uninduced, mean $\pm$ standard deviation, $n = 28$) to $12.2\pm 2.8\text{ }\mu\text{m}$ (epimastigote-like cells, 72 h induced, $n = 27$) while total flagellum length was not significantly altered; $18.0\pm 1.6\text{ }\mu\text{m}$ (uninduced, $n = 27$) in comparison to $19.1\pm 1.5\text{ }\mu\text{m}$ (72 h induced, $n = 27$) (Varga *et al.*, Unpublished). This mutant therefore provides a means to analyse the contribution of the trypomastigote morphology to the swimming behaviours of procyclic *T. brucei*.

The swimming paths followed by both uninduced (trypomastigote) and 72 h induced (epimastigote-like) GM6 RNAi (Figure 45A–B) are similar to wild type *T. brucei* (Figure 39A) and featured cells undergoing either directional swimming or tumbling. The half-life of the population average of angular persistence of cell motion was also similar for the uninduced (6.24 s), induced (2.73 s) (Figure 45C) and wild type (4.53 s) (Figure 40A) which indicated the overall ability of the cells to achieve directional swimming is similar. Correlation of average swimming speed and persistence revealed no significant change in mode of motility following induction of RNAi for 72 h (Figure 45D–E). The proportion of observation time of cells with very high (>0.9) persistence was similar for uninduced, induced and wild-type *T. brucei* (16.15%, 15.14% and 18.82% respectively), and higher than *L. mexicana* (7.77%), *L. major* (4.93%), *P. davidii* (8.67%) and *C. fasciculata* (6.86%). The uninduced sample featured more cells with intermediate swimming speed and angular persistence than the wild type (Figure 40F & 45D) however both induced and uninduced GM6 RNAi mutant had patterns of motility more similar to wild type *T. brucei* than *L. mexicana*.

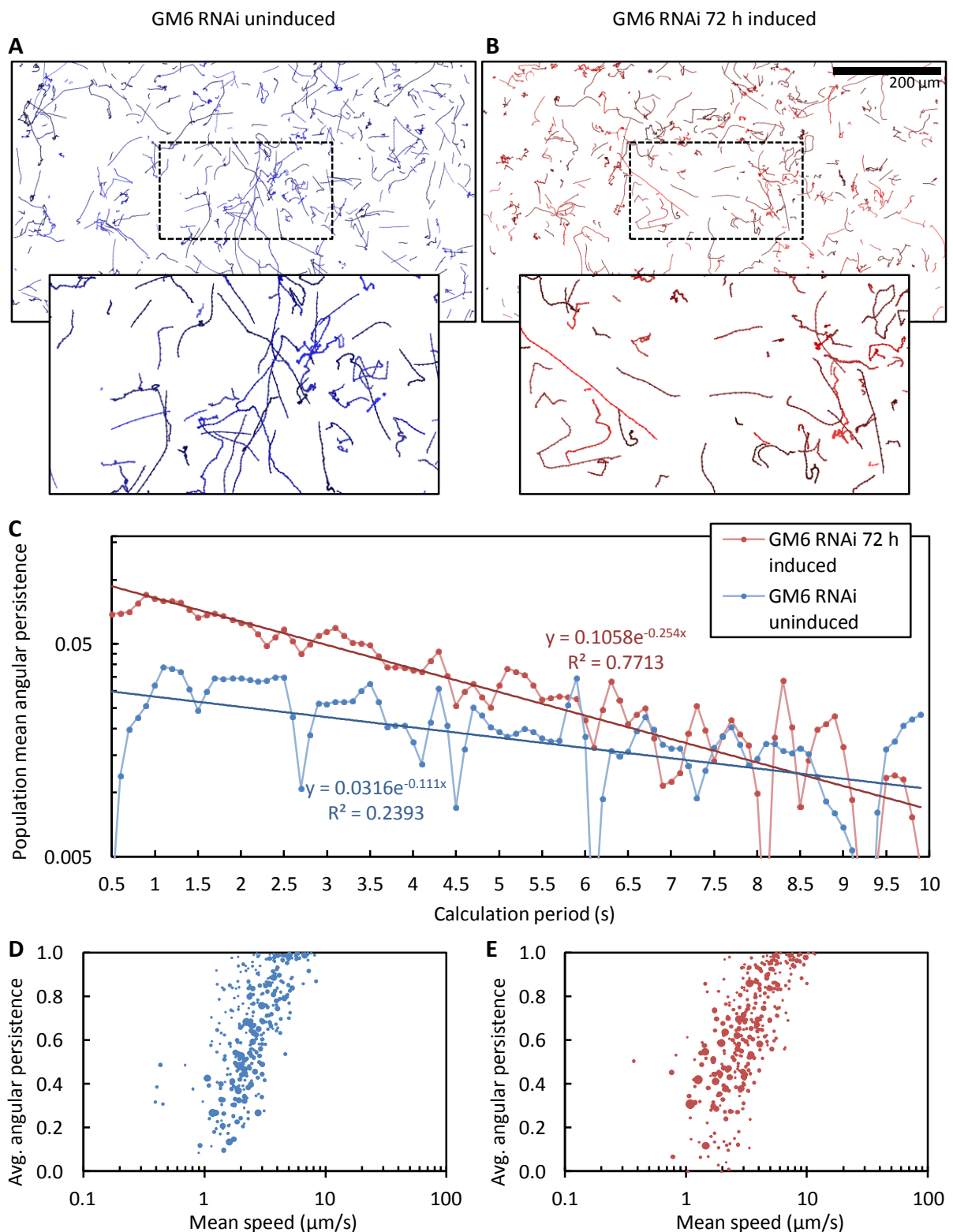


Figure 45. Procytic *T. brucei* swimming is not affected by change to an epimastigote-like morphology. **A.** Paths followed by the uninduced GM6 RNAi mutant during unconstrained swimming ($n = 1011$). **B.** Paths followed by GM6 RNAi mutant during unconstrained swimming following 72 h induction of RNAi ($n = 1002$). **C.** Self-correlation of angular persistence with time, averaged over the population. The solid lines are exponential fits. **D–E.** Correlation of mean speed with mean angular persistence for tracks at least 15 s long from uninduced and 72 h induced GM6 RNAi mutant respectively ($n = 345$ and $n = 353$ respectively). Point area is proportional to the time for which that cell was observed (Section 4.4.1.4).

4.2.5 Biomechanical origin of motility differences between promastigotes and trypomastigotes

Procyclic *T. brucei* and *L. mexicana* have overall similar swimming speed, however *T. brucei* demonstrated highly directional swimming (with occasional switches to and from tumbling behaviour) while *L. mexicana* underwent intermediate directionality swimming, typically along undulating or spiral paths (Figure 39A–B & Figure 40D–E). The primary morphological difference between these two cell types is whether the flagellum is attached laterally to the cell. Large morphological changes to procyclic *T. brucei*, altering it from a trypomastigote to epimastigote-like morphology, which featured a long free portion of flagellum increasing its similarity to the promastigote, did not alter *T. brucei* swimming to be more like *L. mexicana* (Figure 45).

From these data I formed two hypotheses as to the biomechanical origin of the different swimming behaviours:

Firstly the beat structure of the flagellum may determine swimming behaviour. It has been proposed that the flagellum in *T. brucei* has a bihelical (Rodríguez *et al.*, 2009; Koyfman *et al.*, 2011; Hughes *et al.*, 2012) rather than a canonical planar flagellar beat found in *L. major*, *C. fasciculata* and *C. deanei* (Gadelha *et al.*, 2007). The bihelical beat structure, if accurate (Heddergott *et al.*, 2011), may be responsible for the *T. brucei* pattern of swimming behaviour.

Secondly *T. brucei* may experience greater spin-stabilisation while swimming. It has previously been reported that bloodstream form *T. brucei* rotates rapidly (approximately once every 6 flagellar beats) along its long axis as it swims (Heddergott *et al.*, 2011). I hypothesise that attachment of the flagellum to one side of the cell, even for a short distance (as in the epimastigote), causes this rotation which allows a ‘spin-stabilisation’. This effect may balance uneven swimming or drag forces for procyclic *T. brucei* but not *L. mexicana*.

4.2.5.1 There is no evidence for a *T. brucei* bihelical beat

To assess if a bihelical *T. brucei* beat structure is responsible for its highly directional swimming evidence for a bihelical beat was critically analysed. The bihelical beat model has remained controversial as simple, falsifiable, predictions about the appearance of a swimming cell undergoing a bihelical flagellum beat have not been tested. There is at least one simple to observe, qualitative, difference between a planar and a bihelical beat. With a planar beat there are angles of observation where the amplitude of flagellum movement appears to be zero. This situation arises when the plane in which the flagellum movement occurs lies parallel to the angle of observation, i.e. the observer is looking at the plane of the flagellum beat ‘side-on’. This situation can never arise with a helical or bihelical beat; in these cases the three dimensional beat structure means that for every observation angle there will always be some component of flagellum movement which creates movement orthogonal to the angle of observation.

A mathematical description of planar and bihelical flagellar beats was produced (Section 4.4.3) to confirm this qualitative distinguishing feature and predict the appearance of the cell body shape

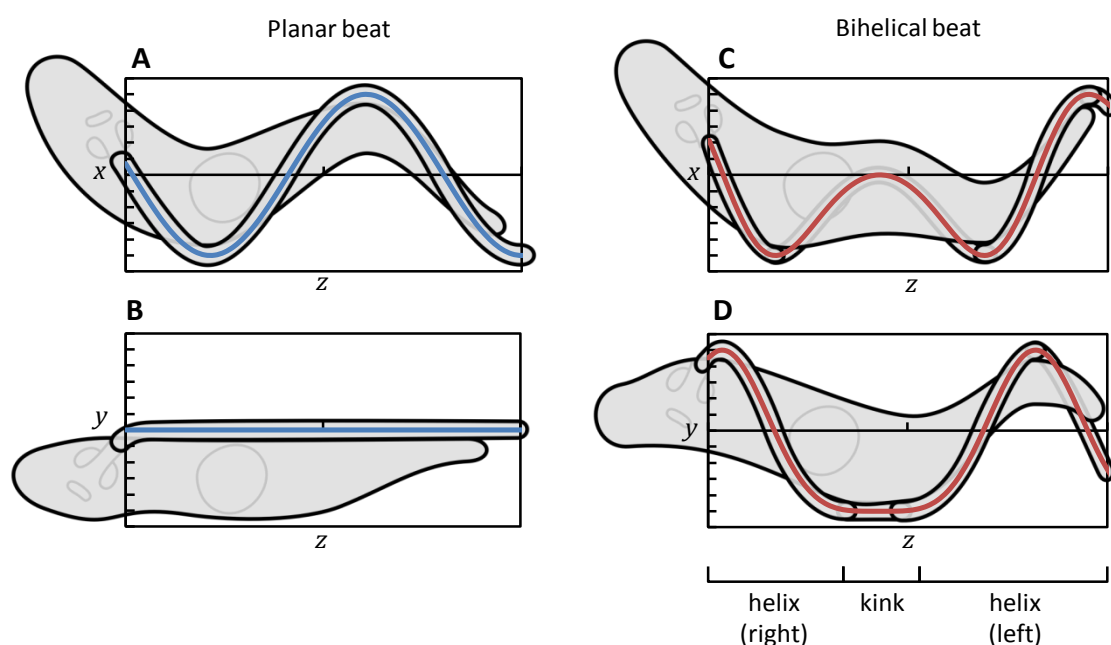


Figure 46. Mathematical models of planar and bihelical beat structures. Graphs of the mathematical model of planar (A–B) and bihelical (C–D) flagellar beats at a single time point overlaid with cartoons of trypanosomatid cell morphology. The helical and kinked regions of the bihelical beat are indicated.

from different orientations (Figure 46). There are orientations where both the planar and bihelical beat descriptions can give qualitatively similar flagellar movement (Figure 46A & C). When viewed from a second orientation it is immediately apparent if the flagellar beat is planar or bihelical (Figure 46B & D). This effect is independent of precise beat shape; it is a fundamental result of whether or not the flagellum beat causes two or three dimensional motion. These predictions allow the unbiased analysis of high frame-rate videomicrographs of swimming *T. brucei* to determine which beat structure the videomicrograph data most closely matches.

High frame-rate videomicrographs of procyclic *T. brucei* swimming were captured and cell motion was digitally subtracted to produce videos of cells ‘swimming on the spot’ (Gadelha *et al.*, 2007). Qualitative analysis showed that two types of movement dominate the swimming of procyclic *T. brucei*; a high frequency flagellum beat and a lower frequency oscillation or rotation of the cell along the direction of travel ($n = 6$) (Figure 47A). The latter was apparent from the regular flipping in orientation of the posterior tip of the cell which, as the flagellum is not attached to the cell at this point, could not be directly due to the flagellar beat. The periodic oscillation or rotation of the cell along its long axis occurred every 4 to 6 flagellar beats and allowed the observation of the flagellar beat from different angles by analysing different time points in the video. Videomicrographs consistently included cell orientations where the apparent amplitude of cell oscillation was approximately zero (Figure 47A). These orientations occurred at regular intervals through the videos and were coincident with changes in cell rotation along its long axis. It was not possible to resolve the flagellum next to the cell body in these videomicrographs.

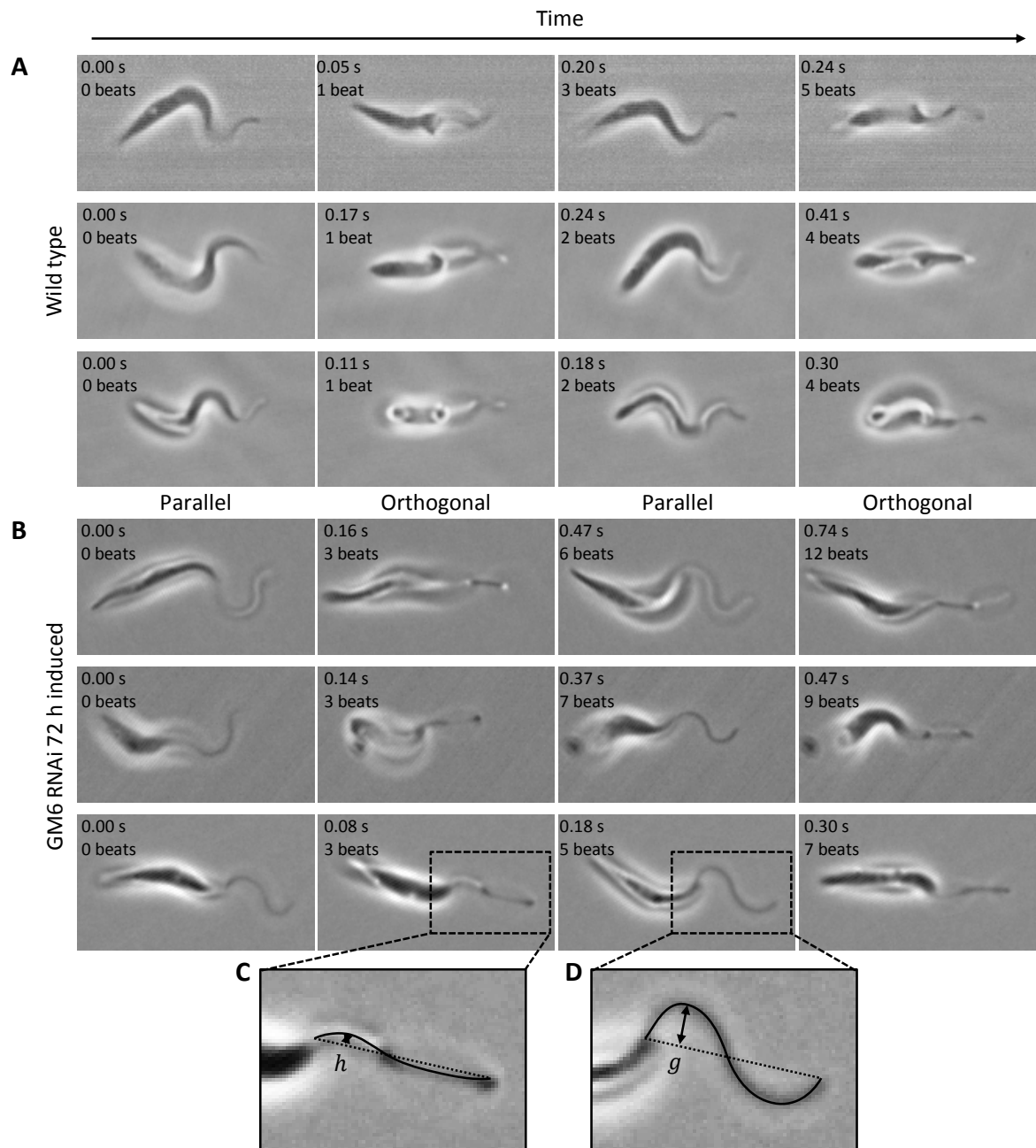


Figure 47. The flagellum beat structure of *T. brucei*. Individual frames from three high frame-rate videomicrographs of procyclic *T. brucei* swimming. Frames were selected to show periodic variation in the apparent amplitude of flagellum beat over time, from near zero (columns 2 and 4) to a large amplitude (columns 1 and 3). Elapsed time and elapsed number of flagellar beats are indicated for each image. **A.** Wild type. All cells progress through alternating periods of high and low apparent amplitude movement over the course of 1 to 2 flagellar beats. **B.** Epimastigote-like *T. brucei* (72 h induced GM6 RNAi cell line). All cells progress through alternating periods of high and low apparent amplitude movement over the course of 2 to 4 flagellar beats. **C–D.** Detail of the detached portion of the flagellum on an epimastigote-like cell during minimum apparent amplitude, h , (**C**) and maximum apparent amplitude, g , (**D**) of flagellum beating.

To analyse movement of the flagellum unfettered by the influence of the cell body the 72 h induced GM6 RNAi cell line, where the epimastigote-like morphology allowed more precise analysis of flagellum beat structure, was analysed. Swimming motion was, as for the wild type, dominated by two movements; the fast flagellar beat and a slower rotation or oscillation around the long axis of the cell ($n = 8$) (Figure 47B). Rotation was slower than the wild type and occurred once every 6 to 14 flagellar beats. These videomicrographs also consistently included frames where the apparent amplitude of flagellum movement was approximately zero, coinciding with rotation of the cell (Figure 47B). However, unlike in the wild type, it was possible to resolve the flagellum and the flagellum shape closely matched the predicted amplitudes of the planar beat model (Figure 46A–B).

The planar beat model predicts that the side-on view of the plane of flagellum beat should give a maximum flagellum displacement of zero, i.e. the flagellum should appear to follow a perfectly straight line. Any deviation from this may indicate some small bihelicity of beat. The limited frame rate of the videomicrographs means a perfectly side-on view of the beat is unattainable, however the images did allow the calculation of an upper bound of deviation from a planar beat. The maximum possible degree of bihelicity can be calculated from the ratio of maximum (g) to minimum (h) apparent amplitude of the beat (indicated in Figure 47C–D) (Section 4.4.3). This was measured for 55 instances over 8 cells giving 0.0607 ± 0.0053 , yielding an r value of 8.23 ± 0.72 which is very large, showing a near planar beat.

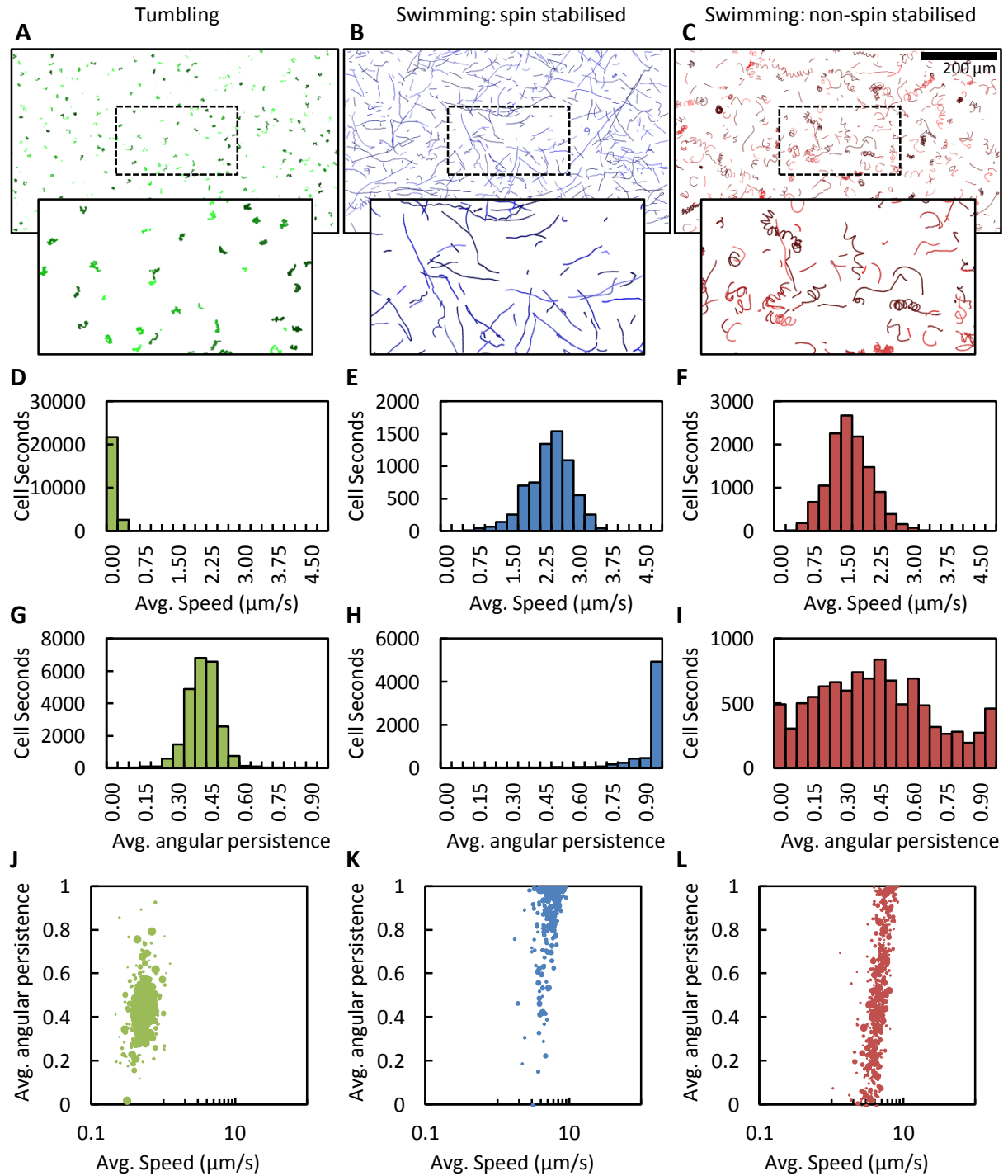


Figure 48. Simulated behaviour of spin stabilised and non-spin stabilised parasite swimming. **A.** Paths followed parasites undergoing only Brownian motion ($n = 444$). **B.** Paths followed spin stabilised parasites ($n = 1000$). **C.** Paths followed by non-spin stabilised parasites ($n = 1000$). **D–F.** Histograms of mean swimming speed for tracks at least 15 s long from Brownian motion only ($n = 444$), spin-stabilised ($n = 601$) and non-spin stabilised ($n = 761$) parasites respectively. **G–I.** Histograms of mean angular persistence of swimming in tracks at least 10 s long from Brownian motion only, spin-stabilised and non-spin stabilised parasites respectively. **J–L.** Correlation of mean speed with mean angular persistence for tracks from Brownian motion only, spin-stabilised and non-spin stabilised parasites respectively. Point area is proportional to the time for which that simulated cell was observed (Section 4.4.1.4).

4.2.5.2 Simulation of spin-stabilisation can model differences in promastigote and trypomastigote swimming

A swimming cell will not necessarily travel in a straight line; asymmetries in the cell structure may cause asymmetric hydrodynamic drag or propulsion leading to a curved path. I hypothesised that trypanosomatid cells may use rotation around the direction of travel to balance these uneven forces and achieve more directional motion. Bloodstream form *T. brucei* (Heddergott *et al.*, 2011), wild type procyclic *T. brucei* (Figure 47A) and epimastigote-like 72 h induced GM6 RNAi cell line (Figure 47B) rotate around their direction of travel on a similar time scale as flagellar beat (typically one rotation in around 0.4 s (Figure 47)). In contrast *Leishmania* and *Crithidia* remain in a similar orientation for long periods (longer than 10 s) (Gadelha *et al.*, 2007) but have a tendency to swim in undulating, curved and spiral paths (Figure 39B).

To test this hypothesis I developed a model of cell swimming (Section 4.4.4) which incorporated the motile force arising from flagellar beating, Brownian motion (translational and rotational) and a hypothetical rotational moment which causes the direction of swimming to change over time (inferred from curved swimming paths followed by *L. mexicana* (Figure 39B) and species with a similar morphology (Figure 41)). Three populations of cells were simulated; one which was not swimming and underwent only Brownian motion (tumbling cells), one which swam while rotating along its long axis at a rate of 15.71 radian/s (spin-stabilised, one rotation every 0.4 s) and one which swam while rotating along its long axis at a rate of 0.63 radian/s (non-spin-stabilised, one rotation every 10 s) (mean) with a random variation with a standard deviation of 50%. These values were selected as they are similar to the rotation rate observed for the epimastigote-like 72 h induced GM6 RNAi cell line and *Leishmania* respectively. Otherwise the three populations were identical. Cell motion was simulated in three dimensions and used to generate simulated dark-field videomicrographs which were then analysed using the automated cell motion analysis tools (Section 4.4.1.3).

Procyclic (trypomastigote) *T. brucei* swimming (Figure 39A) was similar to a combination of cell motion in the tumbling (Figure 48A) and spin-stabilised simulation in which cells typically followed very straight paths (Figure 48B). Promastigote *L. mexicana* swimming (Figure 39B) was more similar to the non-spin-stabilised simulation in which cells typically followed undulating and spiral paths (Figure 48B). The original three dimensional simulation data showed these spiral/undulating two dimensional paths corresponded to helical swimming trajectories of various pitch. Average swimming speed in the tumbling simulation was very low (Figure 48D), as would be expected when lacking a propulsion force. Average swimming speed in the spin-stabilised and non-spin-stabilised swimming simulations was similar (Figure 48E-F), as would be expected from the use of identical cell propulsion and Brownian forces. Average angular persistence for the tumbling simulation was low (Figure 48G) and was very high for the simulation (Figure 48H). A combination of these two distributions is similar to the distribution of average angular persistence observed for procyclic *T. brucei* (Figure 40F). In contrast average angular persistence for the non-spin-stabilised sample was broad and monomodal with few very high persistence cells (Figure 48I), more similar to the distribution observed for *L. mexicana* (Figure 40G). Correlation of average angular persistence with average swimming speed (Figure 48J-L) confirmed that a combination of tumbling and spin-stabilised swimming is similar to the behaviour observed for *T. brucei* and non-spin-stabilised swimming is similar to the behaviour observed for *L. mexicana* (Figure 40F-G).

4.2.5.3 The *Leishmania* anterior is not axially symmetric

The spin-stabilisation model of trypanosomatid motility assumed that as a cell swims it experiences a rotational moment arising from either asymmetric hydrodynamic drag or propulsion force which makes it deviate from a straight path. Lateral attachment of the flagellum to the side of a trypomastigote cell is a large deviation from axial symmetry; this could give rise to this rotational moment. In contrast *L. mexicana* appears to be symmetrical with the flagellum protruding from the anterior tip of the cell, although some asymmetry of the anterior has previously been described (Clark, 1959). If the cell structure were perfectly symmetrical is not clear how an asymmetric hydrodynamic drag or propulsion force could be generated. The ultrastructure of the anterior of the *Leishmania* promastigote was therefore analysed in detail to determine if there were any asymmetry in structure.

Fluorescence micrographs of DAPI-stained promastigote *L. mexicana* show the kinetoplast is often positioned to one side of the midline of the cell (Figure 49A). The deviation of the centre of the kinetoplast (the centroid of fluorescence signal from the DAPI stain) from the midline of the cell (determined from the phase contrast image) was measured from 157 1K1N1F cells in an exponentially growing culture. Kinetoplast position had a broad distribution, often lying up to

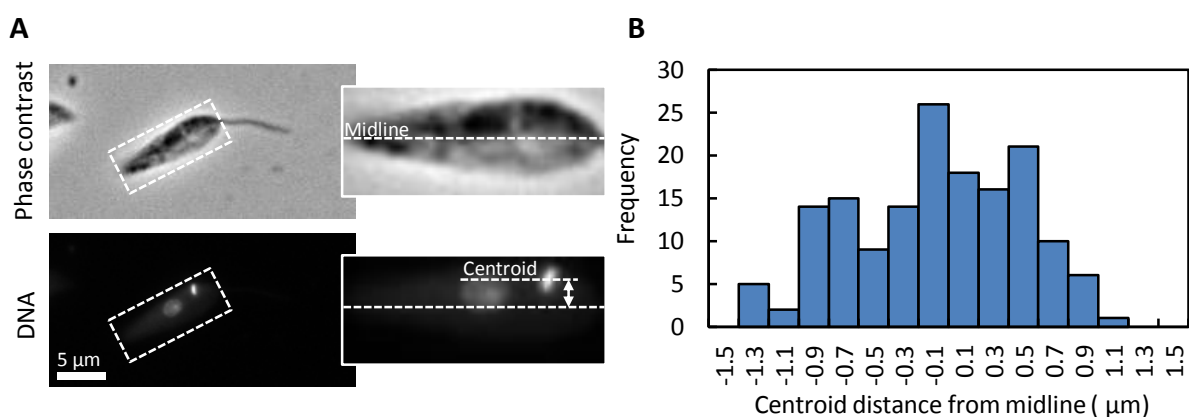


Figure 49. Kinetoplast position in the *Leishmania* anterior is asymmetric. **A.** Example light micrographs of *L. mexicana* illustrating the asymmetric kinetoplast positioning often observed. The cell midline, centroid of the kinetoplast and cell midline-kinetoplast centroid separation are indicated. DNA was stained with DAPI. **B.** Distribution of cell midline-kinetoplast centroid separation for *L. mexicana* promastigotes lying in random orientations on the slide ($n = 157$).

1.3 μm from the midline of the cell (Figure 49B). The rigid connection of the kinetoplast to the basal body via the TAC means an asymmetric kinetoplast location is indicative of an asymmetric flagellar pocket positioning.

The structure of the *Leishmania* posterior was further analysed using TEM of *L. mexicana* promastigote serial sections of cells with a single flagellum. The kinetoplast, basal body and flagellum pocket structures were consistently observed at an asymmetric position within the cell anterior, abutting one side of the sub-pellicular array. The flagellar pocket membrane and mitochondrial double membrane (enclosing the kinetoplast) at this point were typically in contact with the sub-pellicular microtubules while the far side of the kinetoplast and flagellar pocket faced the cytoplasmic lumen. This asymmetric positioning was consistently observed in cells with a single flagellum, visible in longitudinal ($n = 3$) (Figure 50A–B) and transverse ($n = 5$) (Figure 50C–E) serial section series. In addition transverse sections through the cell anterior orthogonal to the flagellum axoneme indicated the cell posterior protrudes further along one side of the flagellum than the other (Figure 50E). This protrusion consistently occurred on the opposite side of the flagellum to the PFR and on the opposite side of the cell to the position of the flagellar pocket (Figure 49E).

The propulsive force the flagellum applies to the cell is exerted through the attachment of the flagellum to the *Leishmania* promastigote by the flagellar pocket and basal body structure. As the flagellar pocket is off-centre, the propulsive force of the flagellum is likely to be correspondingly asymmetric. The leading face of a swimming cell is also asymmetric with the cell body protruding further on one side of the flagellum than the other. This may present a larger plasma membrane face for hydrodynamic drag on one side than the other. Either of these two features may cause the cell to deviate from a straight path while swimming.

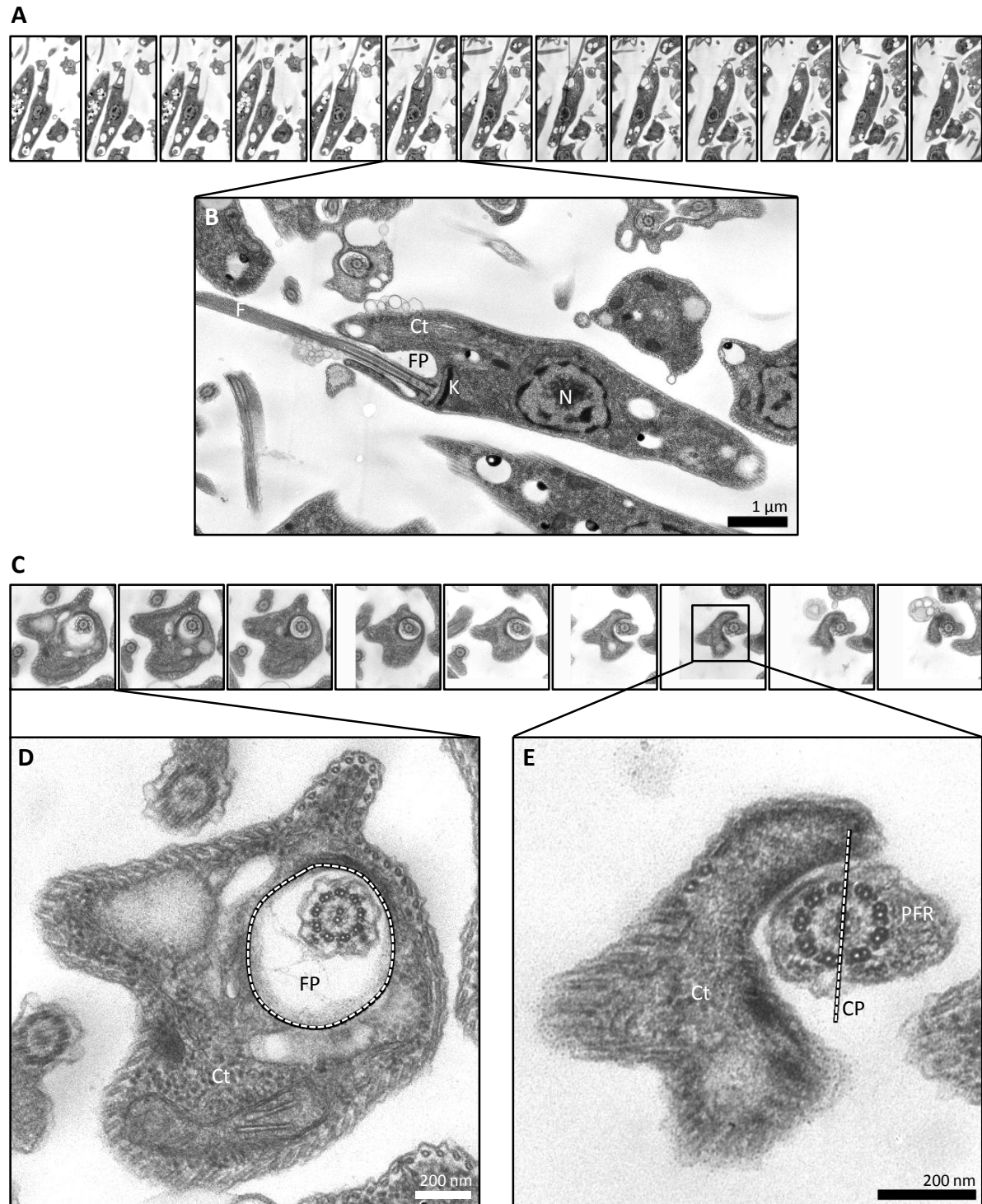


Figure 50. The *Leishmania* anterior is not axially symmetric. Electron microscopy of serial thin sections of stages of the *Leishmania* anterior, from Eva Gluenz and Amy E. Smith. **A.** Representative longitudinal electron microscopy serial sections through a single 1K1N1F *L. mexicana* cell. **B.** Detail of one section shown in **A** through the flagellum and flagellar pocket. The flagellum (F), flagellar pocket (FP), kinetoplast (K), nucleus (N) and the cytoplasmic lumen positioned on one side of the flagellar pocket (Ct) are indicated. **C.** Representative transverse electron microscopy serial sections through a single 1F *L. mexicana* cell anterior. **D.** Detail of one section shown in **C** through the anterior of the flagellar pocket. The flagellar pocket (FP) and the cytoplasmic lumen positioned on one side of the flagellar pocket (Ct) are indicated. **E.** Detail of one section shown in **C** through the anterior tip of the cell. The protruding anterior tip of the cell body (Ct), PFR and orientation of the axoneme central pair (CP) are indicated.

4.3 Discussion

These data provide insight into the functional role of the diverse range of trypanosomatid morphologies observed in motility. The mode of swimming of promastigote *L. mexicana* differed from procyclic *T. brucei*. The insect gut life cycle stage of both species had similar, broad distributions of swimming speed, but *T. brucei* featured distinct processive and tumbling swimming behaviours similar to that previously described for bloodstream form of the parasite (Uppaluri *et al.*, 2011; Zaburdaev *et al.*, 2011; Weiße *et al.*, 2012). Additionally when *T. brucei* was undergoing processive swimming it was more directional than *L. mexicana* (Figure 40), and its behaviour was unchanged upon alteration of *T. brucei* from a trypomastigote to an epimastigote-like morphology (Figure 45). In contrast the promastigote and promastigote-like parasites *L. major*, *C. fasciculata* and *P. davidii* shared a similar mode of swimming to *L. mexicana* (Figure 41). I could find no evidence to support the proposed difference in flagellar beat structure between *Leishmania* and *Crithidia* (Gadelha *et al.*, 2007) and *T. brucei* (Rodríguez *et al.*, 2009) (Figure 46) which could have given rise to different swimming behaviours. This therefore suggested the difference in swimming between *T. brucei* and these other species arises from the differences between the promastigote-like and trypomastigote/epimastigote morphologies.

4.3.1 Spin-stabilisation of trypanosomatid swimming

I proposed and tested the hypothesis that *T. brucei* uses a spin-stabilisation mechanism to undergo more directional movement when not tumbling. This hypothesis states that rapid rotation of the cell around the direction of travel averages asymmetric propulsion forces or hydrodynamic drag and allows the cell to undergo more directional movement. This concept is distinct from gyroscopic spin-stabilisation as used in gun rifling or spacecraft stabilisation which cannot occur in low Reynolds number environments where inertia is negligible. High frame-rate analysis of the *Leishmania* and *Crithidia* flagellar beats previously showed no evidence of rotation of the cell around

the direction of travel (Gadelha *et al.*, 2007) while both trypomastigote and epimastigote-like procyclic *T. brucei* completed one revolution in less than 14 flagellar beats (Figure 47), similar to that described for the bloodstream form (Heddergott *et al.*, 2011). Detailed analysis of the *L. mexicana* anterior ultrastructure revealed asymmetries through which hydrodynamic drag could cause deviation from a straight path (Figure 49 & 50), consistent curved swimming paths in the absence of spin-stabilisation. Finally simulation of spin- and non-spin-stabilised swimming confirmed this hypothesised behaviour can account for a key difference between *T. brucei* and *L. mexicana* swimming; the ability to undergo very highly directional movement (Figure 48). There is also a possibility that, in addition to the tensile force applied by the flagellum (Ralston *et al.*, 2006), longitudinal rotation of *T. brucei* may provide an additional shear force for completion of cytokinesis not experienced by *L. mexicana*.

While four species with promastigote or promastigote or promastigote-like morphology (*L. mexicana*, *L. major*, *C. fasciculata* and *P. davidii*) were analysed only one species with trypomastigote or epimastigote-like morphology (procyclic *T. brucei*) was analysed. This makes it hard to draw firm conclusions as to whether the trypomastigote and epimastigote trypanosomatid morphologies are universally associated with spin-stabilisation of swimming. Bloodstream form *T. brucei* completes one longitudinal rotation every 6 flagellar beats, (Heddergott *et al.*, 2011) and also undergoes highly directional swimming when not tumbling, both when analysed confined in 2D space (Uppaluri *et al.*, 2011) and in 3D with holographic microscopy (Weiße *et al.*, 2012). While these studies qualitatively support the spin-stabilisation hypothesis in neither of these cases was the distribution of average angular persistence presented quantitatively which could have been used to rigorously confirm it. Qualitative analysis of previously published data from epimastigote form *T. cruzi* (Ballesteros-Rodea *et al.*, 2012) suggests these cells do not rotate around their direction of travel when swimming and follow more curved swimming paths, however the cells used in that study also appeared confined to a narrow depth which may have altered normal behaviour. Further analysis of other trypomastigote

and epimastigote species and life cycle stages like *T. cruzi* and *Trypanosoma congolense* will therefore be important to confirm if the spin-stabilisation phenomenon observed for trypomastigote and epimastigote-like procyclic *T. brucei* is universal.

To my knowledge this is the first time spin-stabilisation of swimming microorganisms has been identified as a mechanism for reducing deviation from a straight swimming path. Spin-stabilisation cannot mitigate the stochastic effects of rotational Brownian motion but nonetheless this mechanism of achieving higher directionality of swimming may occur more widely. In trypanosomatids the longitudinal rotation required for this effect appeared to be generated by the lateral attachment of the flagellum to the cell body in the trypomastigote and epimastigote morphologies but other mechanisms, such as the action of a second flagellum, could also generate this effect. Bio-mimesis of the swimming mechanisms of microorganisms is of interest for generation of micro-robots, particularly for minimally invasive medical procedures (Kosa *et al.*, 2007). The phenomenon of spin-stabilisation may be of use in future biomimetic micro-electromechanical machines to achieve more directional swimming.

4.3.2 *Leishmania* morphology, swimming and the life cycle

Analysis of *Leishmania* morphology and their resultant swimming ability indicated average swimming speed correlated with flagellum length but not with cell body length (Figure 44). This supports the hypothesis that the leptomonad promastigotes (with flagellar lengths longer than the cell body) are faster swimmers than the procyclic promastigotes (with flagellar lengths shorter than the cell body) and therefore could be responsible for translocation of parasites toward the anterior of the gut following initial infection of the midgut. More generally the succession of morphologies described as life cycle substages in the sandfly are found in successively more anterior positions in the alimentary canal and have successively longer flagellum length (Rogers *et al.*, 2002; Bates, 2007). Flagellum length and swimming speed may therefore be the sorting mechanism which underlies

migration of different promastigote morphologies to different regions in the sandfly. Infection of sandflies with the immotile *L. mexicana* PFR1 deletion mutant (Maga *et al.*, 1999), in which flagellar length and the ability of the flagellum to undergo length change (in promastigote to amastigote differentiation) appears not to be altered (Section 3.2.3), could be used for testing this hypothesis.

Intriguingly during the modelling of spin-stabilisation it became apparent that the position of the flagellum on a *Leishmania* promastigote, the structure and orientation of flagellar and ciliary beats and the shape of the *Leishmania* cell body maximise two behaviours: Firstly swimming speed during flagellar beating is maximised as friction from the cell body is minimised (it is in the most 'streamlined' orientation). Secondly the ciliary beat acts to rotate the cell around one of its minor axes (Gadelha *et al.*, 2007) (around which rotational Brownian motion is at a minimum) thereby maximising the randomisation of orientation when combined with Brownian motion around the major axis (around which rotational Brownian motion is maximum). This is of interest as efficient directional swimming and reorientation behaviours may underlie a 'run-tumble' chemotaxis mechanism. A second interesting aspect of swimming pertaining to chemotaxis was the curved and undulating paths followed by cells (Figure 39B), similar to those followed by metazoan sperm, identified by analysis of *Arbacia punctulata* (sea urchin) spermatocytes (Kaupp *et al.*, 2003; Böhmer *et al.*, 2005). In this case an increase in the directionality of swimming by reduced asymmetry in the flagellar beat is the mechanism through which chemotaxis occurs (Böhmer *et al.*, 2005; Friedrich and Jülicher, 2007). If chemotaxis occurs by either of these mechanisms in *Leishmania*, the curved swimming paths mean measurement of mean time of straight line movement previously used to analyse chemotaxis in *Leishmania* (Barros *et al.*, 2006) is not a meaningful measure. In contrast the alternation between highly directional and tumbling motion in procyclic *T. brucei* was more akin to the spin and tumble swimming behaviour of *Escherichia coli* and mean time of straight line movement may be a meaningful tool for detecting the first chemotactic agents of *T. brucei*.

4.3.3 The role of the PFR in swimming

The PFR is vital for motility of trypanosomatids, including *T. brucei* and *L. mexicana* (Bastin *et al.*, 1998; Maga *et al.*, 1999; Gadelha *et al.*, 2005), however the precise function it plays is not known. The PFR had been proposed to play a role in generating a bihelical *T. brucei* flagellar beat; the paracrystalline PFR structure was proposed to provide preferred bending planes, spring effects or force amplification which could twist the flagellum into a three dimensional configuration as it beats (Koyfman *et al.*, 2011; Hughes *et al.*, 2012), although some of the structural features identified in these analyses have been contested (Höög *et al.*, 2012). A function in generating a bihelical beat was inconsistent with the planar flagellar beats observed in *L. major* and *C. fasciculata* (Gadelha *et al.*, 2007), both of which possess a PFR with a similar structure to *T. brucei* (Gadelha *et al.*, 2006). Furthermore *L. major* and *C. fasciculata* flagellum beat structure is similar to *C. deanei* which has only a small cryptic PFR (Gadelha *et al.*, 2005, 2007) in which a mechanical effect is less likely.

Through reanalysis of the *T. brucei* flagellum beat (Figure 47A), with reference to a model of the appearance of a planar and bihelical beat from different orientations (Figure 46), I could find no evidence for strong beat bihelicity. This was supported by analysis of the flagellum beat on the detached portion of the flagellum on the epimastigote-like GM6 RNAi cell line (Figure 47B) in which the flagellum could be clearly resolved. Given the proposed flagellar bihelical beat does not occur in *T. brucei* the PFR cannot have a biomechanical role in generating helicity. The portion of the flagellum laterally attached to the *T. brucei* cell body could not be resolved so the flagellum beat structure in this section could not be analysed to the same precision as the detached portion of the flagellum. The attachment of the cell body to the flagellum may perturb movement of that section of the flagellum, an effect which may give rise to longitudinal rotation of *T. brucei* which stabilises its directional swimming and give rise to the different swimming behaviour to promastigotes.

Together these data provide a comprehensive insight into how trypanosomatid morphology affects swimming and precisely how aspects of morphology (flagellum and cell body length and lateral attachment of the flagellum) correlate with swimming behaviours. On this basis it appears that the lateral attachment of the flagellum in trypomastigote/epimastigote-like procyclic *T. brucei* causes a spin-stabilisation effect allowing the cells to undergo highly directional swimming in contrast to the free flagellum of promastigote and promastigote-like cells which gives rise to swimming in curved or undulating paths. This mechanism of spin-stabilised swimming may occur more widely among other microorganisms.

4.4 Mathematics and methods development

This section covers only mathematics and method development specific to this results chapter (for standard materials and protocols see [Section 7](#)).

4.4.1 Motility analysis

4.4.1.1 Live cell microscopy sample preparation

Videomicrographs of *L. mexicana*, *L. major*, choanomastigote *C. fasciculata* and promastigote *P. davidii* were acquired from a 10 µl sample of culture in a 0.25 mm deep chamber on a slide created from a Gene Frame® (Fischer Scientific, Loughborough, UK, AB-0576). A coverslip was applied to form a sealed chamber and the sample was allowed to equilibrate to the ambient temperature of 25°C for 30 min before observation. All measurements were made on cell in M199, SDM79 or BHI ([Section 7.1](#)) with a dynamic viscosity of approximately 1.1×10^{-3} kg/ms.

For analysis of the ability of the automated tracking program to recognise defocused cells, a sample was prepared adhered to the bottom of the sample chamber. 0.5 ml *L. mexicana* culture was harvested by centrifugation at 3000 g for 3 min then washed twice by resuspension in 0.5 ml PBS and centrifugation at 3000 g for 3 min. Finally the cells were resuspended in 0.5 ml PBS and 10 µl was

loaded into the Gene Frame® chamber. The cells were allowed to adhere to the slide for 10 min then the PBS was aspirated, replaced with growth medium and observed.

4.4.1.2 Time lapse videomicroscopy

Cells were observed by dark-field light microscopy with a 10× NA 0.40 objective lens, an exposure time of 2 ms to freeze flagellar motion and a focal point midway through the sample depth. One image was captured every 200 ms (5 Hz) for at least 512 frames (1.7 min). At least two biological replicates were observed and the resulting data pooled for each sample.

4.4.1.3 Cell tracking from videomicrographs

Tracking of particle motion in the time-lapse video micrographs was performed by custom macros in ImageJ (Collins, 2007). Particles were identified by the intensity maxima algorithm following a 1 pixel Gaussian blur. Tracks for each cell were built by finding the nearest particle to the expected location of the cell based on its motion in the previous two frames. If a particle could not be found within 10 pixels (6.44 µm) the track was terminated. Cell tracks were recorded as a series of pixel coordinates and were up-sampled by linear interpolation to one coordinate every 100 ms for analysis. The track was also used to generate a series of cropped images following each individual cell over time to facilitate analysis of morphology. Tracks less than 15 s (10 s for *L. major*, *C. fasciculata* and *P. davidii*) long were excluded from the analysis. *Leishmania* cell body and flagellum length were recorded from the cropped images of individual cells. Measurements were made manually in ImageJ (Collins, 2007) and measurements were only made from frames where the whole cell body or flagellum was deemed clearly in focus.

4.4.1.4 Cell motion measurements and statistics

Cell velocity, $\mathbf{v}_t$, was calculated from the pixel coordinates, (x_t, y_t) , from each time point, t , and the next time point, $t + \delta t$:

$$\mathbf{v}_t = \begin{bmatrix} x_{t+\delta t} - x_t \\ y_{t+\delta t} - y_t \end{bmatrix}$$

The following statistics were calculated for each cell for each time point; speed, $s_t = |\mathbf{v}_t|$, change in velocity, $\delta \mathbf{v}_t = \mathbf{v}_t - \mathbf{v}_{t+\delta t}$, change in speed, $\delta s_t = |\mathbf{v}_{\delta t}|$ and angular persistence, $p = \frac{\mathbf{v}_t \cdot \mathbf{v}_{t+\delta t}}{|\mathbf{v}_t| |\mathbf{v}_{t+\delta t}|}$, then averaged on a cell-by-cell basis. In addition mean absolute velocity from total cell displacement and total tracking time was recorded.

To determine the appropriate value of δt the population average of angular persistence over all cells at all time points for values of δt from 0 to 10 s was calculated (Figure 40A), as previously described for bloodstream form *T. brucei* (Zaburdaev *et al.*, 2011). δt of 1 s was selected as an appropriate time scale for analysing cell swimming.

Histograms and averages over the populations of cells were weighted by observation time for each cell ('cell seconds') to ensure no bias against cell classes with a propensity to generate long particle tracks. Similarly point sizes in scatter plots were plotted with an area proportional to cell seconds of observation.

4.4.2 High frame-rate videomicroscopy

Samples for high frame-rate videomicroscopy were prepared as described for analysis of motility (Section 4.4.1.1) but in a 50 μm deep chamber made with adhesive tape. Following equilibration to 25°C videomicrographs were captured using a Mega Speed camera (Mega Speed Co.) at 192 Hz frame rate and phase contrast illumination. Videomicrographs were deinterlaced and rotated to give a horizontal translation of the cells as they swam. Average swimming velocity was calculated from the total displacement of the cell over the observation period, this was then used to subtract the translational movement of the cell from the videomicrograph generating videos of cells 'swimming on the spot', similar to those previously described (Gadelha *et al.*, 2007).

4.4.3 Planar and bihelical flagellum beat modelling

The model of beat structure was designed to demonstrate the shape of a flagellum undergoing a planar or bihelical beat based on a sinusoidal displacement. This is not a physically accurate model; it does not account for the active rather than passive role of the flagellum in movement, that the flagellum is inelastic and that the flagellum will experience viscous drag from the surrounding liquid and mechanical constraint from the cell body.

A flagellum undergoing a planar flagellum-type beat has a shape similar to that caused by a travelling wave on a string (Gadelha *et al.*, 2007). Displacement over time is therefore given by:

$$a = \sin(\omega t + kz)$$

where the displacement a at a point along the flagellum which extends along the z axis depends on the angular frequency, ω , and the wavenumber, k . For a planar flagellar beat the displacement occurs along a single (the x) axis. The displacement along the y axis is zero, therefore:

$$x = a \qquad y = 0$$

To model the bihelical beat the displacement of a planar beat was constrained to the surface of a cylinder of radius, r . The shape of the bihelical beat is therefore given by:

$$x = r\sin(a/r) \qquad y = r\cos(a/r)$$

The value of r defines how bihelical the beat is. As $r \rightarrow \infty$, $y \rightarrow 0$ and $x \rightarrow a$, i.e. the bihelical beat tends towards the planar beat. Therefore larger values of r indicate less bihelicity and smaller values of r indicate more bihelicity. For $r > 5$ the bihelical beat reaches negligible bihelicity; the ratio of maximum y displacement to maximum x displacement is under 10%.

Using these models of beat shape it is trivial to see that as a cell swims along the z axis there exist angles of observation (aligned to the x axis) where, if the flagellum beat is planar, the apparent

amplitude is zero. This situation can never occur with a bihelical beat except in the improbable case wavelength is much greater than flagellum length.

4.4.3.1 Calculation of bihelicity from microscopy data

The value of r can be calculated from videomicrograph data to estimate the maximum degree to which a flagellum beat deviates from the planar beat. For a bihelical beat with $r > \pi$ the maximum displacement observed in the yz plane, h , and maximum displacement observed in the xz plane, g , correspond to a sagitta and chord of an arc radius r respectively. The ratio of h/g can therefore be used to calculate r :

$$r = \frac{g}{2h} + \frac{h}{2g}$$

4.4.4 Simulation of trypanosomatid swimming

Simulation of trypanosomatid swimming incorporated a swimming force, translational Brownian motion, a rotational moment which makes the cell deviate from a straight path, rotational Brownian motion and a ‘spin-stabilising’ rotation around the long axis of the cell which lies in the direction of travel. Trypanosomatid swimming occurs at low Reynolds number:

$$R = \frac{\rho v L}{\mu}$$

Where ρ is the density of the fluid ($1.000 \times 10^3 \text{ kg/m}^3$ for water), v is mean velocity ($10 \text{ } \mu\text{m/s}$ for fast swimming cells), μ is the dynamic viscosity of the fluid ($1.002 \times 10^{-3} \text{ kg/ms}$ for water) and L is a characteristic dimension of the system (trypanosomatids are of the order of $10 \text{ } \mu\text{m}$ in length). $R \approx 1 \times 10^{-4} \ll 0$, inertia is therefore negligible and rotational and translational velocity are simply proportional to the applied moment or force.

Each cell was defined by its location, $\mathbf{l}$, and orientation, defined by three orthonormal vectors $\mathbf{d}$ (direction of travel and long axis of the cell), $\mathbf{n}$ and $\mathbf{p}$ (Figure 51). For each time step, location and

orientation were recalculated; the cell moved by δl in the direction $\mathbf{d}$, rotated orthogonal to the direction of travel by $\delta\varphi$ ($\mathbf{d}$ and $\mathbf{p}$ were rotated around $\mathbf{n}$ by $\delta\varphi$), rotated along its long axis by $\delta\theta$ ($\mathbf{n}$ and $\mathbf{p}$ were rotated around $\mathbf{d}$ by $\delta\theta$) and translated and rotated by Brownian motion (Section 4.4.4.1). Rotations were calculated using quaternions.

Cells undergoing either tumbling, spin-stabilised or non-spin-stabilised swimming were simulated in a volume of $900 \times 650 \times 100 \mu\text{m}$ with cyclic bounding conditions (i.e. a three dimensional toroidal space) at a density of 2×10^6 cells/ml. Each cell was assigned randomised starting properties: location; orientation; δl (zero, for the tumbling simulation, or $3 \mu\text{m/s}$, for the spin-stabilised and non-spin-stabilised simulations); $\delta\varphi$ ($2\pi/3 \pm 2\pi/6$ radian/s); $\delta\theta$ ($2\pi/10 \pm 2\pi/20$ radian/s, for the non-spin-stabilised simulation, or $2\pi/0.4 \pm 2\pi/0.8$ radian/s, for the spin-stabilised simulation, or zero, for the tumbling simulation). Ranges are indicated by mean $\pm$ standard deviation. Cell shape was modelled as a prolate spheroid with a minor radius of $1 \mu\text{m}$ and a major radius of $8 \mu\text{m}$. The simulation progressed in 200 ms time steps for 102.4 s (512 steps). The three dimensional coordinates were recorded for each cell and used to build a simulated dark-field image using a point spread measured from defocused *L. mexicana* (Figure 38B).

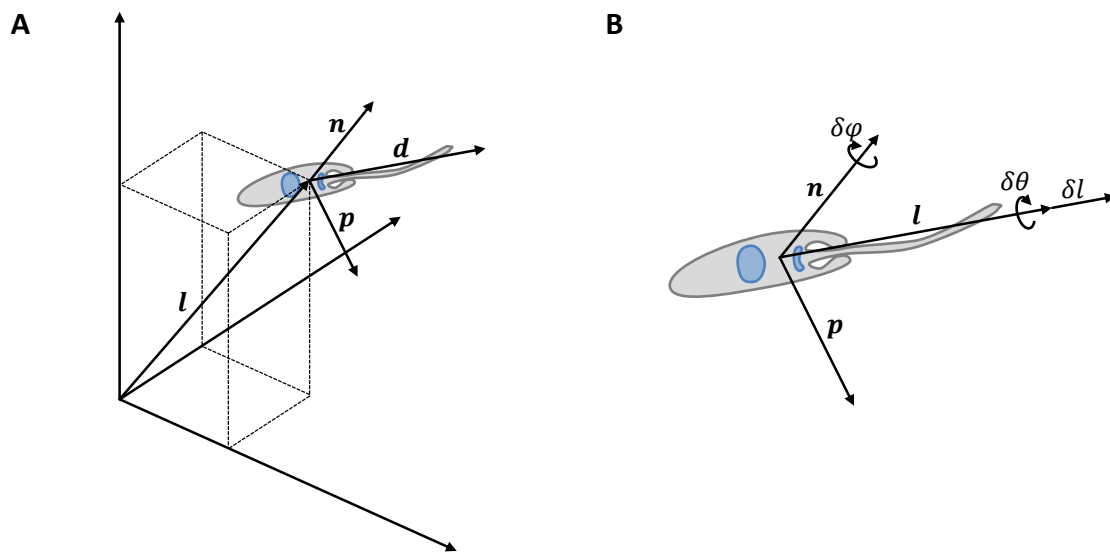


Figure 51. Coordinate and reference vector system for simulation of spin stabilised swimming. A. Diagrammatic representation of the four vectors which define cell location and orientation. B. Diagrammatic representation of the change in location and orientation per time step which give rise to swimming behaviour.

4.4.4.1 Brownian motion

Linear Brownian motion was calculated along the three orthonormal vectors defining cell orientation. Motion in each of these directions depends on the hydrodynamic radius, r , of the cell in the direction of travel; $1\ \mu\text{m}$ for $\mathbf{d}$ and $8\ \mu\text{m}$ for $\mathbf{n}$ and $\mathbf{p}$. The linear diffusion coefficient, D , is given by the Einstein relation (Einstein, 1905):

$$D = \frac{kT}{6\pi r\mu}$$

where k is the Boltzmann constant and T is the thermodynamic temperature (25°C , $298.15\ \text{K}$). Translation over the simulation time step, t , of $200\ \text{ms}$ in each direction was therefore simulated as random displacement with a mean of zero and standard deviation of $\sqrt{2tD}$.

Rotational Brownian motion was calculated around each of the three orthonormal vectors which defined cell orientation. Rotation around each axis depends on the Perrin rotational friction coefficients of a prolate spheroid around either its major ($\mathbf{d}$) or minor ($\mathbf{n}$ and $\mathbf{p}$) axes relative to a sphere of equal volume. The friction coefficients around the major, f_a , or minor, f_i , axes can be calculated from the major, r_a , and minor, r_i , prolate spheroid radii:

$$e = \frac{r_a}{r_i} \quad \xi = \frac{\sqrt{|e^2-1|}}{e} \quad S = \frac{2 \tan^{-1} \xi}{\xi}$$

$$f_a = \frac{4}{3} \frac{\xi^2}{2 - S/e^2}$$

$$f_i = \frac{4}{3} \frac{1/e^2 - e^2}{2 - S(2 - 1/e^2)}$$

The radius, r_s , of a sphere of equal volume to the prolate spheroid is given by:

$$r_s = \sqrt[3]{r_i^2 r_2}$$

The rotational diffusion coefficient, E , is also given by the Einstein relation (Einstein, 1905):

$$E = \frac{kT}{8\pi\mu r_s^3}$$

Rotation over the simulation time step around each axis was therefore simulated as random rotation with a mean of zero and standard deviation of $\sqrt{2tf_aE}$ (around ***d***) or $\sqrt{2tf_iE}$ (around ***n*** and ***p***).

5 New approaches for automating morphometric analysis of trypanosomatids

The work presented in this chapter was published in 2012; Wheeler RJ, Gull K and Gluenz E. (2012) “Detailed interrogation of trypanosome cell biology via differential organelle staining and automated image analysis”. *BMC Biol.* 10: 1. The associated image analysis tools for ImageJ (Collins, 2007) along with detailed sample preparation protocols were also published online in January 2012; <http://users.ox.ac.uk/~path0493/htiaot.html>. All work included in this chapter is my own.

5.1 Introduction

Analysis of the *Leishmania* cell cycle, its interplay with the regulation of flagellum length and the complex and diverse roles of the flagellum in the *Leishmania* life cycle have demonstrated the value of quantitative morphometric analysis in understanding the cell biology of trypanosomatids. Furthermore I have shown the enormous value of large, comprehensive morphology analysis to support mathematical and computer modelling to extract conclusions from complex cell behaviours (Section 2 & 3). The potential for this type of analysis is not limited to just the *Leishmania* flagellum; most areas of trypanosomatid cell biology could benefit from these types of analysis.

Techniques for analysis of trypanosomatid morphology are typically limited to manual measurements made from micrographs. This is slow, inefficient and subject to bias; while the human visual system has enormous capability for analysis of images, people are slow, get bored and interpretation of the image risks being altered by preconceptions. Delegating this type of analysis to a computer would therefore be advantageous and the ongoing expansion of experiments to those made up of thousands to millions of samples (such as RNAi libraries (Subramaniam *et al.*, 2006) and

screening of drug candidates (Engel *et al.*, 2010)) will eventually make it vital. Automated tools for quantifying infection levels of macrophages with *Leishmania* have also been developed (Engel *et al.*, 2010; Siqueira-Neto *et al.*, 2012) however no automated tools yet exist for the analysis of the parasite cells themselves.

Analysis of trypanosomatid micrographs inevitably comes down to analysis of the cell cycle as the majority of analyses concern cells which are undergoing exponential growth. The most basic method by which the cell cycle can be analysed is kinetoplast and nucleus number as both organelles duplicate once during the cell cycle, are easily recognised by fluorescence microscopy and can be quickly counted. Kinetoplast and nucleus DNA can also be quantified from micrographs; quantitative analysis of *T. brucei* nucleus and kinetoplast DNA content from micrographs in a semi-automated fashion has previously been shown to be accurate and effective (Siegel *et al.*, 2008). Unfortunately the presence of two large DNA-containing organelles also introduce difficulties when using standard automated image analysis tools, such as CellProfiler (Carpenter *et al.*, 2006), as these are generally optimised for use on mammalian cells which only have a nucleus (Ortiz de Solórzano *et al.*, 1999; Jones *et al.*, 2005, 2006).

While the kinetoplast and nucleus make automated analysis using standard image analysis tools difficult they also present an opportunity for image analysis and morphometry. The number, DNA content, position and morphology of these two organelles, when placed in the context of the approximately axially symmetric cell organisation of a trypanosomatid, provide a large quantity of data on the cell cycle stage and health of cells on a cell-by-cell basis. The priority of an automated analysis tool for trypanosomatids must therefore be accurate identification and analysis of the kinetoplast and nucleus. The secondary priority is then analysis of the cell morphology and the position of the kinetoplast and nucleus within the cell. I have developed new trypanosomatid staining techniques and image analysis tools which can accurately achieve both these priorities, even in difficult situations such as overlap of the kinetoplast and nucleus signal (such as in

L. mexicana (Section 2.2.1 & 2.2.3.4) or dividing *T. brucei* (Sherwin and Gull, 1989a)) or cells with heavily disrupted kinetoplast or nucleus morphology (Gluenz *et al.*, 2008). These tools, and the sample preparation, staining and image analysis techniques they are based on, will assist future analyses concerning the morphogenesis of trypanosomatids.

5.2 Results

5.2.1 Staining efficiencies of kinetoplast and nuclear DNA with different DNA stains

The kinetoplast and nuclear DNA of trypanosomatids typically have a different A-T/G-C content, with the kinetoplast being more A-T rich (Chen and Donelson, 1980; Barrois *et al.*, 1981; Kidane *et al.*, 1984; Birkenmeyer *et al.*, 1985; Macina *et al.*, 1986). It was therefore expected that different DNA stains would stain the kinetoplast and nucleus differently, and that the selection of two stains with different affinities to A-T or G-C rich DNA would allow the identification of these two organelles based purely on their A-T or G-C content. Minor groove binding (MGB) fluorescent DNA stains, including DAPI (Kapuscinski, 1995) and Hoechst (Portugal and Waring, 1988), preferentially bind to A-T rich DNA sequences (Portugal and Waring, 1988; Kapuscinski, 1995). In contrast base pair intercalating (BPI) stains, including PI and SYBR green (Zipper *et al.*, 2004), do not have a strong sequence specificity for binding.

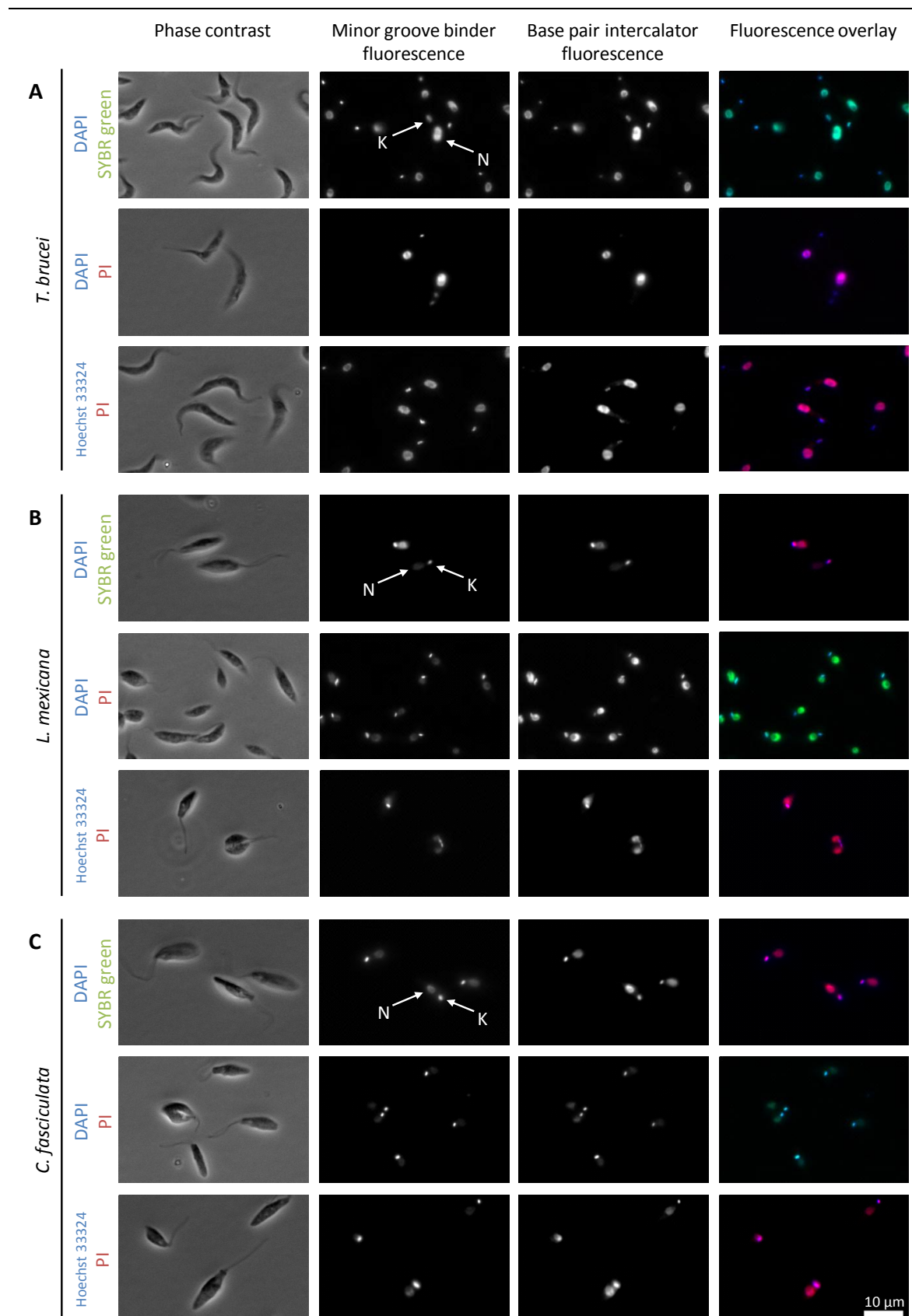


Figure 52. Labelling of kinetoplasts and nuclei with MGB and BPI DNA stains. Micrographs of three trypanosomatid species labelled with two fluorescent DNA stains, one MGB stain (DAPI or Hoechst 33324) and one BPI stain (SYBR green or PI). **A.** Procyclic *T. brucei*. **B.** Promastigote *L. mexicana*. **C.** Choanomastigote *C. fasciculata*. In each case the kinetoplast (K) is stained more strongly with the MGB stain. The converse is true for the nucleus (N).

Double staining, with one MGB and one BPI stain, of three kinetoplastid species, *L. mexicana*, *T. brucei* and *C. fasciculata*, was therefore tested to determine if the kinetoplast and nucleus would be stained differently under experimental conditions. The combinations of stain tested were DAPI with SYBR green, DAPI with PI and Hoechst 33342 with PI on fixed cells treated with RNase A (Figure 52). For each combination of MGB and BPI stain for each species a consistent effect was observed; in each case the kinetoplast was more strongly stained with the MGB stain than the BPI stain and the nucleus was stained more strongly with the BPI stain than the MGB stain. As predicted the double staining of trypanosomatids with an MGB and a BPI fluorescent DNA stain provides a method to identify the kinetoplast and nucleus based purely on the A-T/G-C content bias.

5.2.2 Separation of nuclear and kinetoplast signal from double DNA staining

A trypanosomatid cell stained with an MGB and a BPI fluorescent DNA stain allows generation of two subtly different micrographs of the DNA. The structures identified are identical in both images, as both stains label DNA; however the intensity of staining of the structures varies. This effect is illustrated in Figure 52 where the colour overlay of the two separate DNA stain micrographs yields kinetoplasts and nuclei of different colours. More rigorously the MGB and BPI fluorescence micrographs can be thought of as two colour components generating a different two dimensional colour for kinetoplasts and nuclei.

Colour deconvolution is an image processing technique used to separate features in images based on their colour. This method has been applied to quantitative histochemistry (Ruifrok and Johnston, 2001) and analysis of multi-colour fluorescence in-situ hybridisation for colour compensation (Castleman *et al.*, 2002; Choi *et al.*, 2009). It is also the image-based homolog of colour compensation used in flow cytometry (Shapiro and Leif, 2003) which flow-based imaging systems, like ImageStream (Amnis), also make use of. This technique was applied to separate the contribution of

kinetoplast and nuclear DNA to the fluorescence images to generate two separate images, one containing the image of only kinetoplast DNA, and one containing only nuclear DNA. This greatly simplified both manual and automated analysis of the micrographs as it eliminates the need to use organelle location and morphology to identify whether it is a kinetoplast or nucleus.

Colour deconvolution could only be performed after two image preparation steps. Precise alignment of the two fluorescence micrographs is required for accurate colour deconvolution so chromatic aberration (North, 2006) must be first measured and corrected. Secondly reference values of the typical signal intensity of kinetoplasts and nuclei in the MGB and BPI image must be measured. A set of analysis tools, for use in ImageJ (Collins, 2007) which automates these preparatory steps and then performs colour deconvolution was programmed. The performance of this tool set was validated on a test sample of procyclic *T. brucei* stained with DAPI and PI with 236 cells across three fields of view.

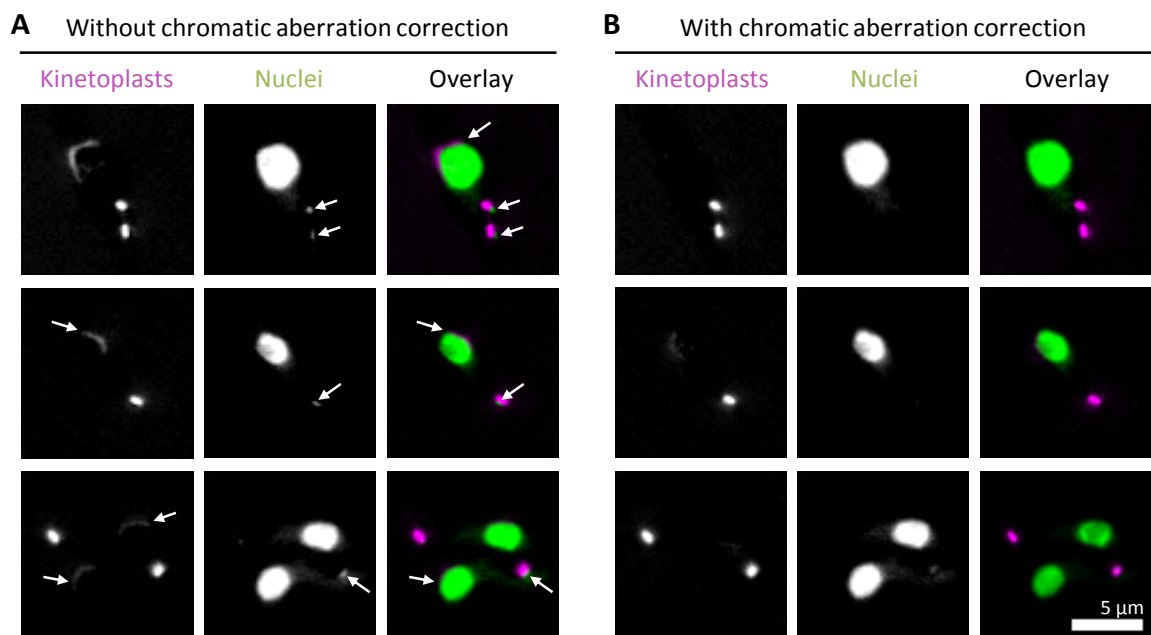


Figure 53. Colour deconvolution without correction for chromatic aberration causes colour fringes. **A.** Example images (cropped from a single field of view) of *T. brucei* stained with DAPI and PI colour deconvolved without correcting chromatic aberration. Misalignment of the fluorescence channels generates fringes around the kinetoplasts and nuclei (arrowed). **B.** The same example images colour deconvolved following correction for chromatic aberration. The fringes are no longer present.

5.2.3 Preparation for colour deconvolution

5.2.3.1 Correcting chromatic aberration

Chromatic aberration in a microscope manifests as slightly different magnifications for different wavelengths of light, including different fluorescence emission wavelengths. This effect is small but even for highly corrected apochromat lenses may disrupt precise colocalisation of fluorescence channels around the periphery of an image. The test sample of micrographs of DAPI and PI stained procyclic *T. brucei* were colour deconvolved either without (Figure 53A) or with (Figure 53B) correction of chromatic aberration. In the absence of chromatic aberration correction strong fringes around the edges of organelles were observed while with correction they were not.

As chromatic aberration manifests as a different magnification for different wavelengths of light (North, 2006) it follows that it can be corrected by the scaling of one image to account for the magnification difference. The precise strength of this effect and the centre around which to perform the scaling (the optical axis) must first be determined. An automated analysis tool was designed to perform this calibration from kinetoplasts and nuclei present in the image, the function of which is summarised in Figure 54. Bright points (kinetoplasts and nuclei) were identified in the image and their location and shift between the MGB and BPI fluorescence images was calculated (Figure 54A). Linear regression of shift in position and position in the image of the bright points provide the scaling factor and centre around which to perform scaling to correct the aberration (Figure 54B) (Section 5.4.3.1). The ability of this approach to measure and correct (so eliminate) chromatic aberration was confirmed using the test sample of DAPI and PI stained procyclic *T. brucei* (Figure 53). This method uses the fluorescent objects present in the sample, no additional calibration sample was required (unlike previously published tools (North, 2006)).

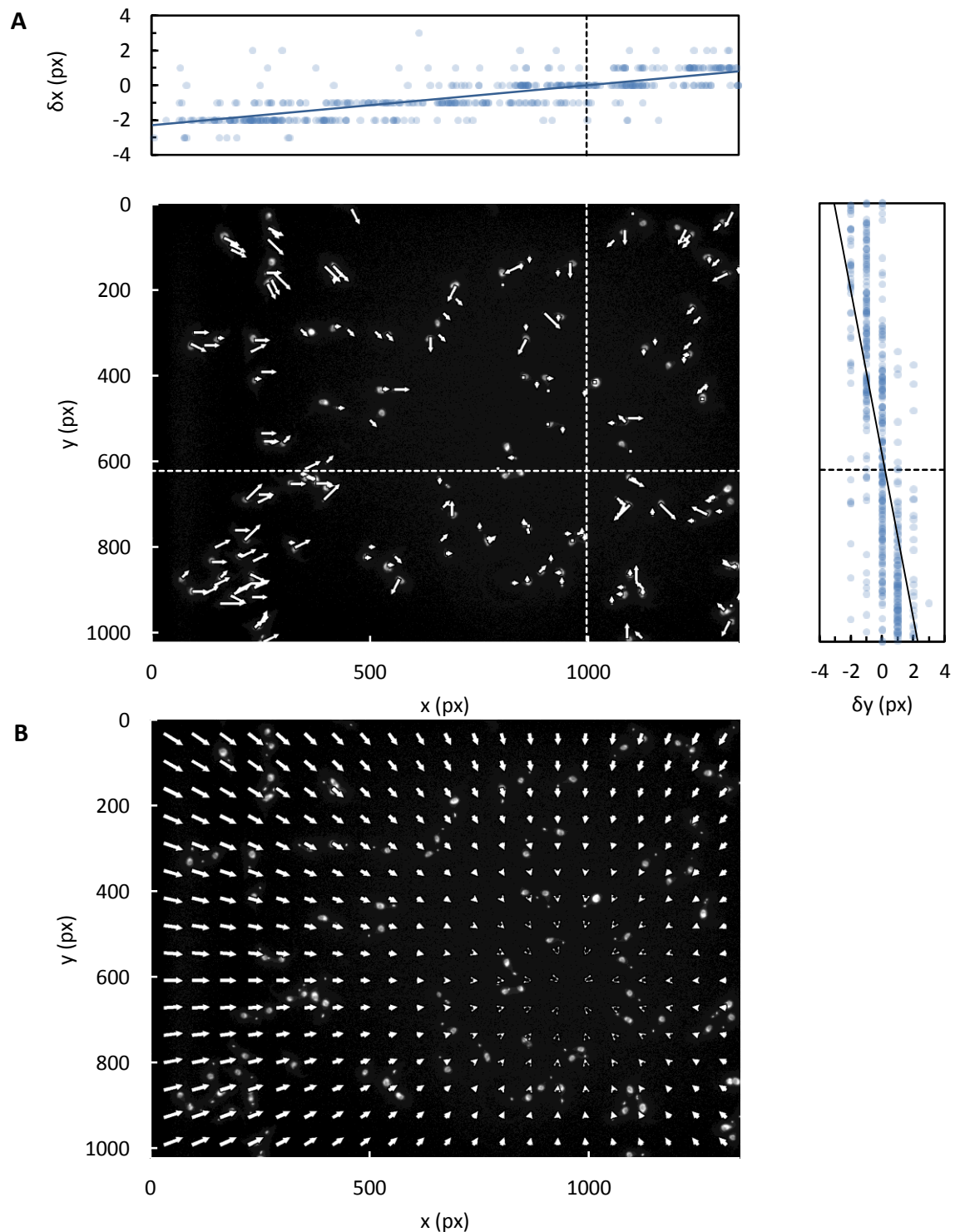


Figure 54. Automated correction of chromatic aberration for fluorescence images of kinetoplastid DNA. A. Example chromatic aberration data; the shift in position of *T. brucei* kinetoplasts and nuclei between a DAPI and a PI image. The central image shows the DAPI fluorescence image overlaid with arrows indicating the automatically measured shift (exaggerated 20 $\times$). The peripheral plots show the correlation of horizontal position with horizontal shift and the correlation of vertical position, with vertical shift. Linear regression of these data (solid blue lines) gives the centre of scaling (indicated with dotted lines) and scale factor required to correct for chromatic aberration. **B.** Graphical representation of the image transformation required to correct chromatic aberration of the DAPI image relative to the PI image. Arrows indicate the shift required at that point in the image (exaggerated 20 $\times$).

5.2.3.2 Determination of reference values

In order for colour deconvolution to successfully separate the signal from kinetoplast and nuclear DNA it requires reference values which reflect the staining properties of kinetoplast and nuclear DNA. Using the concept of the MGB and BPI images as the two dimensions of a two

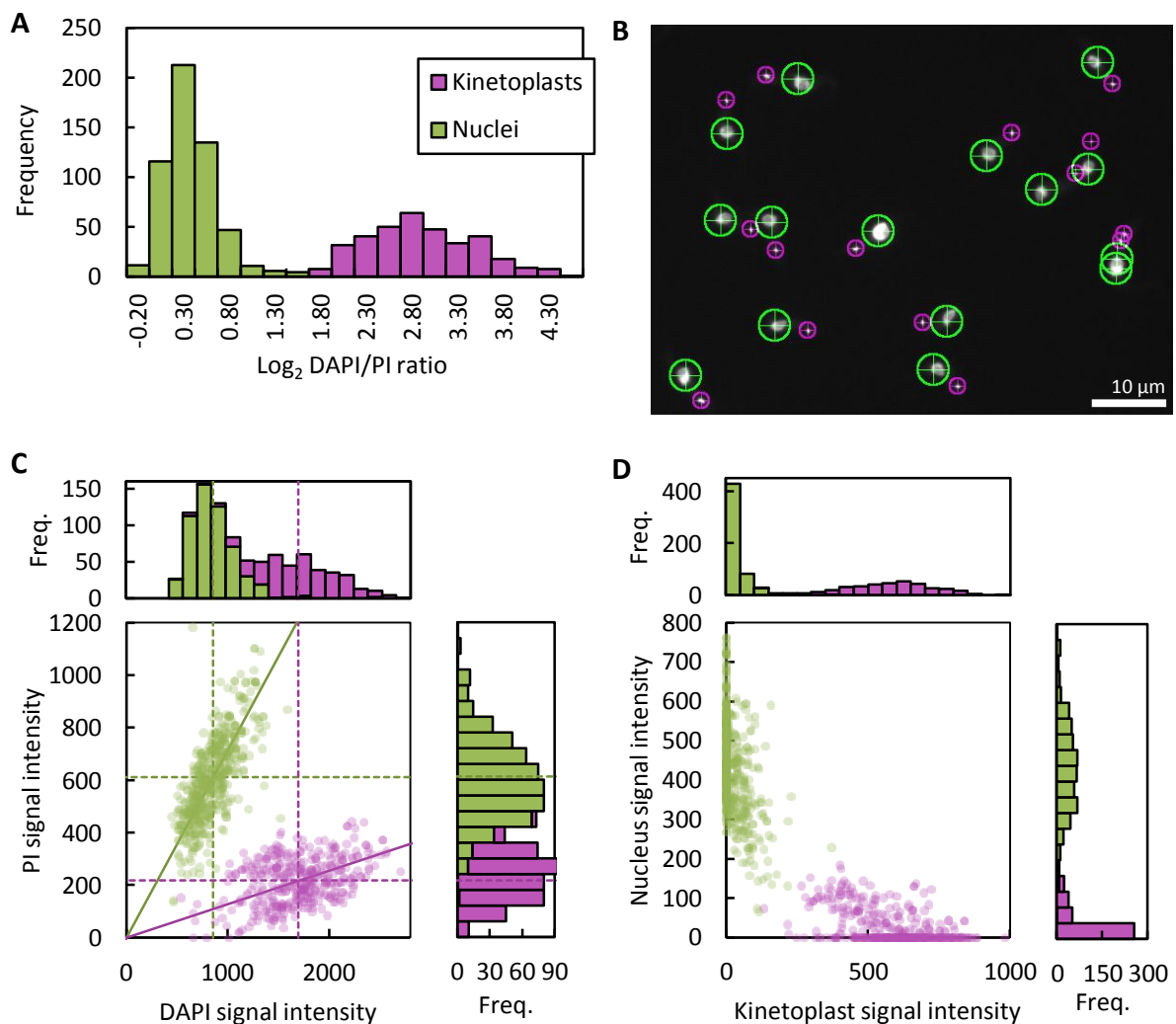


Figure 55. Automated calculation of reference values for use in colour deconvolution. A. Histogram of the $\log_2$ DAPI/PI signal ratio from 897 bright pixels automatically identified from three example images of *T. brucei* stained with PI and DAPI. Each bright pixel corresponds to a single kinetoplast or nucleus except for rare (<5%) occurrences where two or more pixels were selected from a single organelle. Pixels were automatically assigned to either high or low $\log_2$ signal ratio groups (536 and 361 pixels respectively). B. Excerpt from one of the example images used to generate the data in A. The pixels automatically selected for analysis are indicated with a coloured ring; green for low $\log_2$ DAPI/PI signal ratio, magenta for high $\log_2$ signal ratio. All pixels with a low $\log_2$ signal ratio are in nuclei and all pixels with a high $\log_2$ signal ratio are in kinetoplasts. C. Correlation of DAPI and PI signal intensity for the 897 points identified and classified A and B. Dashed green and magenta lines indicate the average DAPI and PI signal intensities for pixels from nuclei and kinetoplasts respectively. These are the reference values for colour deconvolution. The solid green and magenta lines represent the basis vectors for colour deconvolution. D. The kinetoplast and nucleus signal values for the 897 points shown in C following colour deconvolution. Colour deconvolution has used the reference values to maximise the separation of kinetoplast and nucleus signal.

dimensional colour image these reference values represent the colour of kinetoplasts and nuclei. An automated analysis tool was designed to determine these values from kinetoplasts and nuclei present in the image.

Reference values are determined by finding bright points in the fluorescence images (i.e. kinetoplasts and nuclei) and measuring the $\log_2$ signal intensity ratio between the MGB stain and the BPI image. From the test sample of DAPI and PI stained procyclic *T. brucei* this method identified 897 points. The $\log_2$ signal intensity ratios followed a bimodal distribution (Figure 55A) which were classified into two clusters, low and high $\log_2$ signal intensity ratio, by *k*-means clustering. From the staining properties of the kinetoplast and nucleus (Figure 52) the high $\log_2$ signal intensity ratio cluster were expected to correspond to kinetoplasts, and the low $\log_2$ signal intensity ratio cluster were expected to correspond to nuclei. To confirm this expectation the points identified and their classification as either nuclei or kinetoplasts were mapped back onto the source micrographs (Figure 55B). 897 points were identified and classified, of these only two were assigned incorrectly.

The average signal in the MGB and BPI fluorescence images for points classified as either kinetoplasts or nuclei was then determined (Figure 55C), yielding values for the average brightness of a kinetoplast in the MGB image, a kinetoplast in the BPI image, a nucleus in the MGB image and a kinetoplast in the BPI image. These values are the reference values for colour deconvolution and application of colour deconvolution acts to maximise the separation of signal from kinetoplasts and nuclei (Figure 55D). Importantly the process of colour deconvolution preserves signal, the output carries the same quantitative data as the input.

Through performing colour deconvolution on multiple samples it became apparent that small technical variation led to differences in the reference values for colour deconvolution. When analysing a single sample this was not an issue, however if performing a quantitative comparison of multiple samples it would become problematic. Two methods for sample staining were therefore optimised (Section 5.4.1). The first (Section 5.4.1.1) was optimised for photostability of the DNA stains and used a photoprotective mounting medium. This method was, however, too variable between samples to make quantitative comparisons. The second method (Section 5.4.1.2) was optimised for minimum sample-to-sample variation, but was more sensitive to photobleaching. To determine the typical sample-to-sample variation when using this staining approach four samples of procyclic *T. brucei* stained with DAPI and PI were prepared in parallel on a single slide and sets of fluorescence micrographs were captured from each. Using the automated image analysis tools the DAPI and PI signal intensities of 1300 kinetoplasts and nuclei were recorded from each sample (Figure 56). Variation between samples was low; the measured DAPI to PI signal ratio was 0.62 ± 0.08

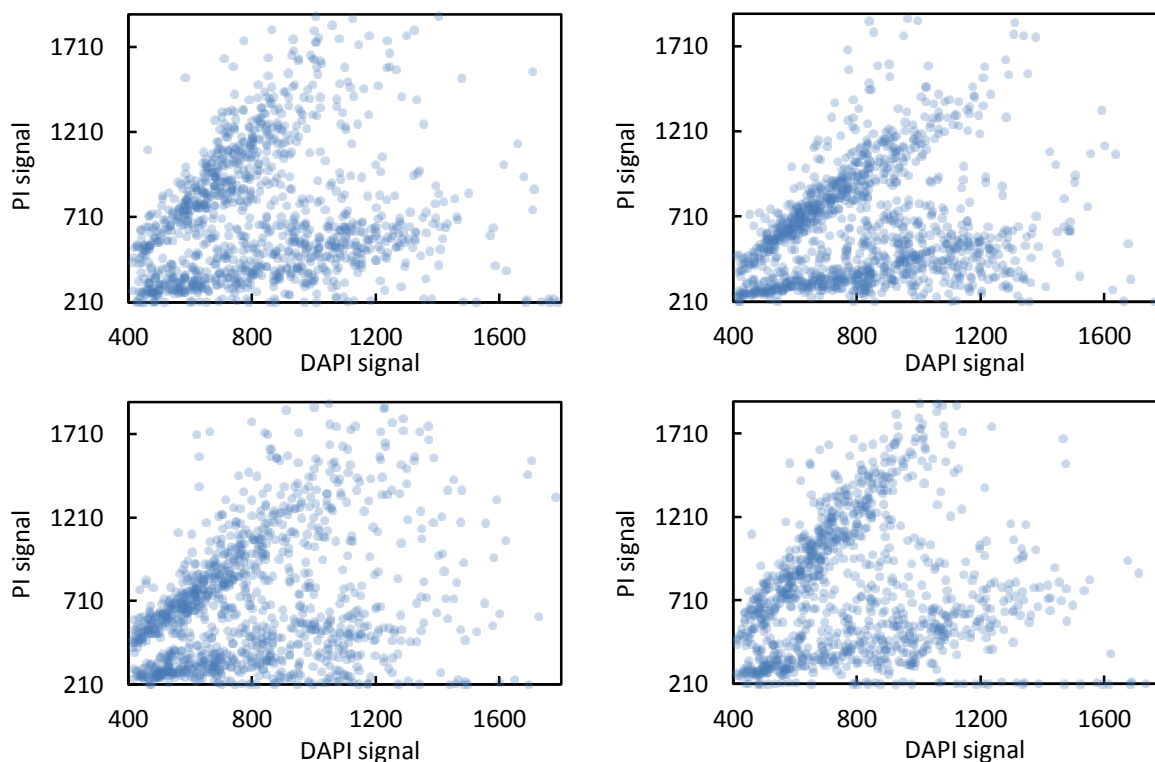


Figure 56. Staining of cells with PI and DAPI has low variation across samples prepared in parallel. Correlation of DAPI and PI signal from four sets of 30 images of DAPI and PI stained *T. brucei* captured from four samples prepared in parallel on a single slide. A random sub-selection of 1300 points is shown for each image set.

and 2.48 ± 0.24 (mean $\pm$ standard deviation across the four samples) for kinetoplasts and nuclei respectively. This variation was small enough to allow use of one sample as a calibration sample to determine the reference values for colour deconvolution then apply this to a second sample and make a quantitative comparison of the two.

5.2.4 Colour deconvolution

As shown in [Figure 55D](#) colour deconvolution takes the signal from kinetoplasts and nuclei in the BPI and MGB images and reassigns it to two new images, one containing signal only from kinetoplast DNA and one containing only signal from nuclear DNA. Signal from all combinations of DNA stain tested (DAPI with PI, Hoechst 33342 with PI and DAPI with SYBR green) could be separated into kinetoplast and nuclear DNA signal for all species tested (*T. brucei*, *L. mexicana* and *C. fasciculata*) ([Figure 57](#)). The stain combination of DAPI and PI was particularly reliable and selected for further analyses. DNA content of an organelle can be measured from fluorescence stain signal (Ruifrok and Johnston, 2001; Siegel *et al.*, 2008) and colour deconvolution preserves signal therefore DNA can be quantified from the kinetoplast and nuclear DNA images resulting from colour deconvolution. If the same staining and colour deconvolution parameters are used, multiple parallel samples may also be compared quantitatively.

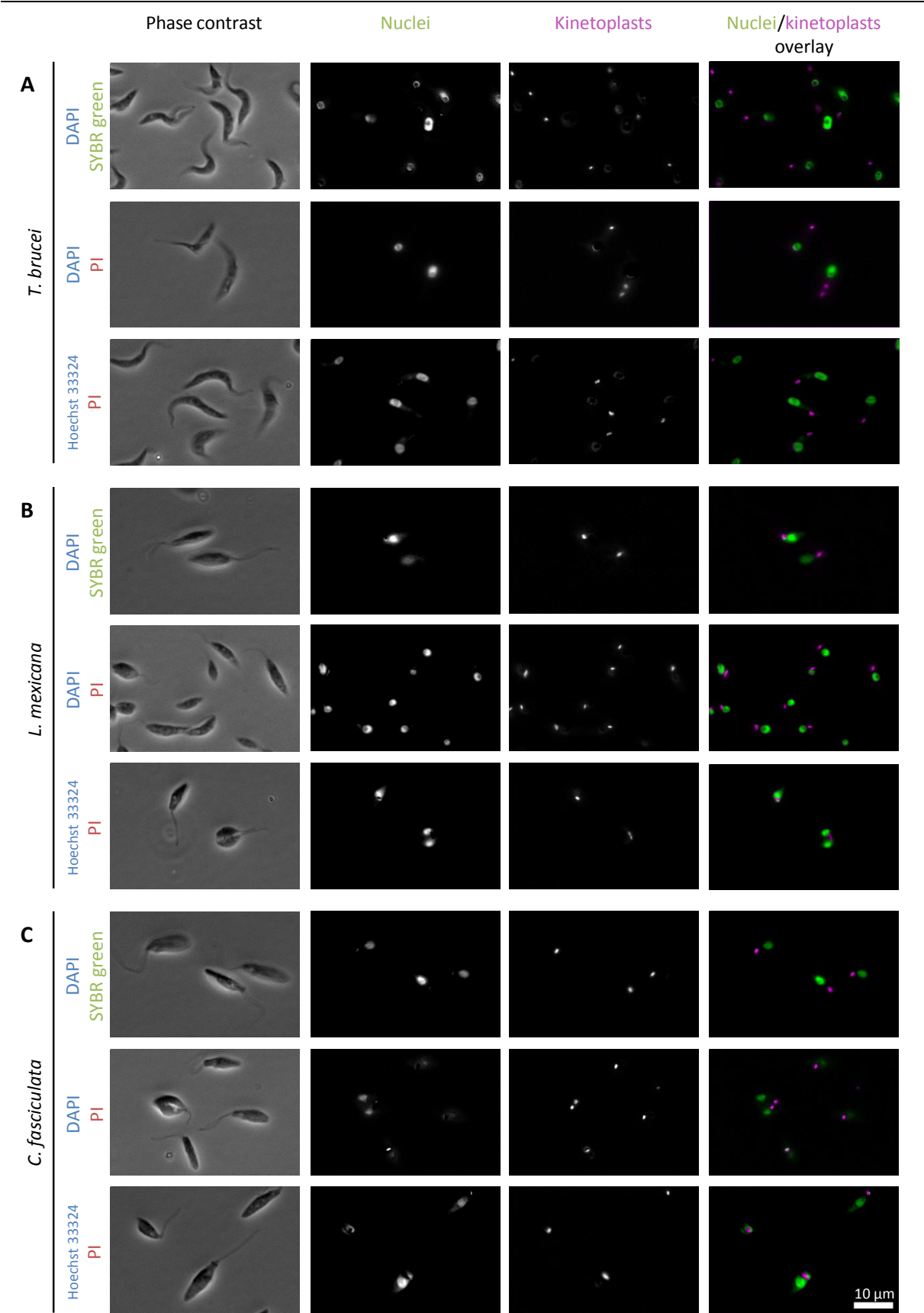


Figure 57. Separation of kinetoplast and nucleus signal by colour deconvolution. Micrographs of three trypanosomatid species labelled with two fluorescent DNA stains following colour deconvolution. The original micrographs are shown in Figure 7. A. Procyclic *T. brucei*. B. Promastigote *L. mexicana*. C. Choanomastigote *C. fasciculata*.

5.2.4.1 Colour deconvolution of aberrant kinetoplast and nucleus morphologies

The staining properties of a MGB and BPI stains are not influenced by organelle morphology, only the sequence bias of the DNA present. This implies the technique can be used for unambiguous and unbiased identification of organelles, even in cases where the morphology of the organelle or its location in the cell are disrupted. This ability would be of particular use for analysis of mutant phenotypes and drug effects on trypanosomatid cells where normal ultrastructure is disrupted.

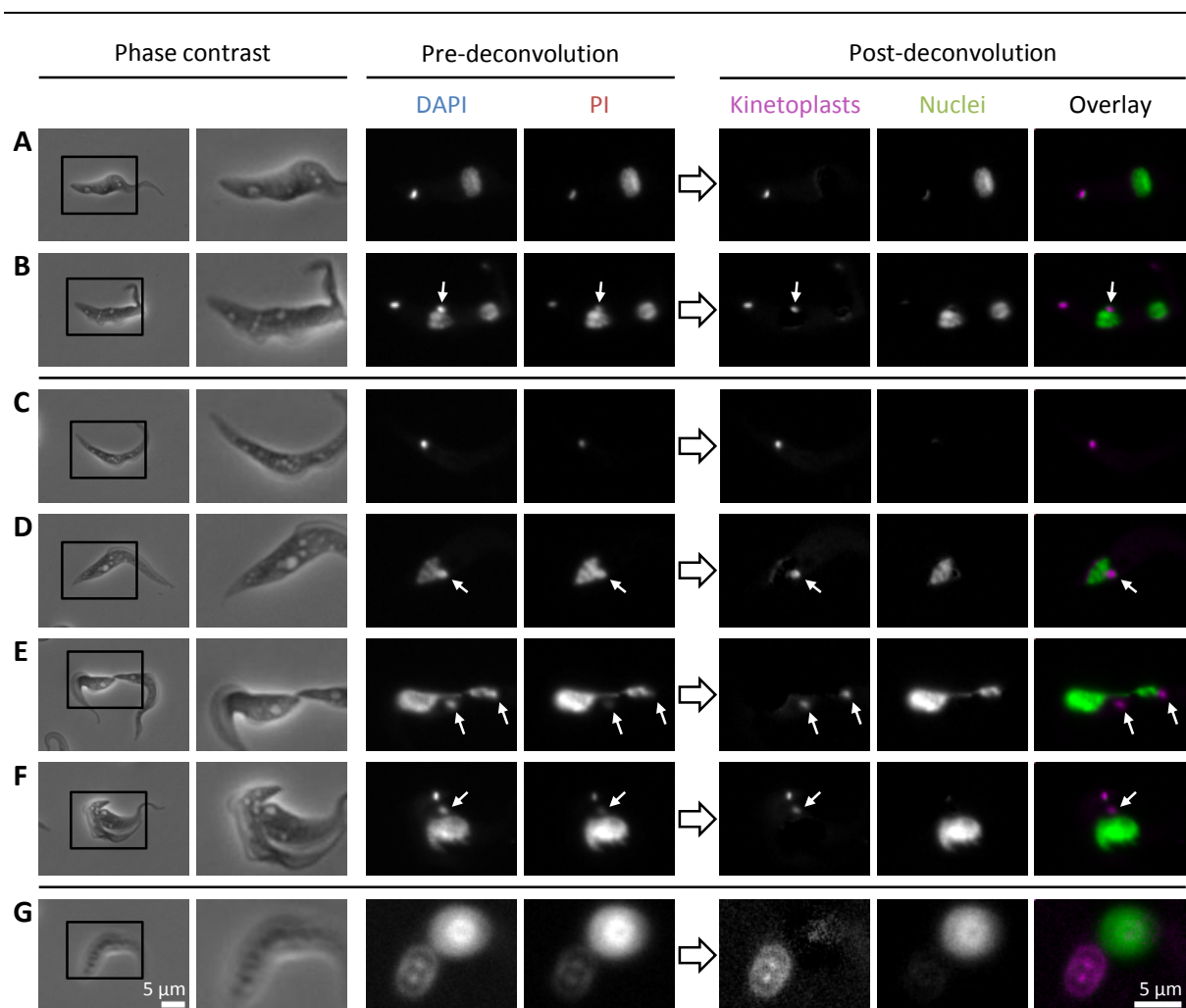


Figure 58. Separation of kinetoplast and nucleus signal, even in closely apposed and abnormal-shaped organelles. Example images of *T. brucei*, 48 h after induction of expression of sister chromatid cohesion protein 1 (SCC1)-mutAB, stained with DAPI and PI before and after colour deconvolution. **A.** 1K1N cell. **B.** 2K2N cell. Signal from the closely apposed anterior kinetoplast and posterior nucleus (arrow) can be separated. **C.** Zoid (1K0N) cell which lacks nuclear DNA. **D.** Cells part-way through abnormal cytokinesis having failed to complete mitosis. Nuclear structure is distorted but signal from kinetoplasts (arrows) and nuclei can be separated. **D.** 2K1N cell (out of focus), kinetoplast and nuclear signal can still be separated.

This application was validated using a procyclic *T. brucei* cell line in which expression of a non-cleavable sister chromatid cohesion protein 1 (SCC1-mutAB) can be induced (Gluenz *et al.*, 2008). This SCC1 protein mutant has a dominant negative impact on the onset of anaphase as it cannot be cleaved by separase. In *T. brucei* the impact of this is the generation of cells with abnormal morphology, both cells with very large nuclei and anucleate cells with a single kinetoplast (zoids) (Gluenz *et al.*, 2008). Expression of SCC1-mutAB was induced for 48 h then cells were stained with DAPI and PI and the resulting micrographs were analysed by colour deconvolution. As predicted the double staining and colour deconvolution was able to separate signal from nuclei and kinetoplasts from all cells, both cells with morphology similar to the normal stages of the *T. brucei* cell cycle (Figure 58A–B) and those with disrupted nuclei (Figure 58C–F). Even signal in out-of-focus cells could be accurately separated (Figure 58G).

The technique of double DNA staining and colour deconvolution gave insights into kinetoplast number and structure in cells that would not have been available from a conventional DAPI image. For example a closely apposed kinetoplasts and nuclei which are not easy to distinguish unambiguously in just the DAPI or PI image become clear following colour deconvolution (Figure 58B & F). Many zoids which, from just the DAPI or PI image, appeared to possess an abnormally large kinetoplast actually contain a kinetoplast with normal morphology and a fragment of nuclear DNA in the cell posterior (Figure 58D). Analysis of some very aberrant morphologies would simply not have been possible from the DAPI or PI image alone (Figure 58E).

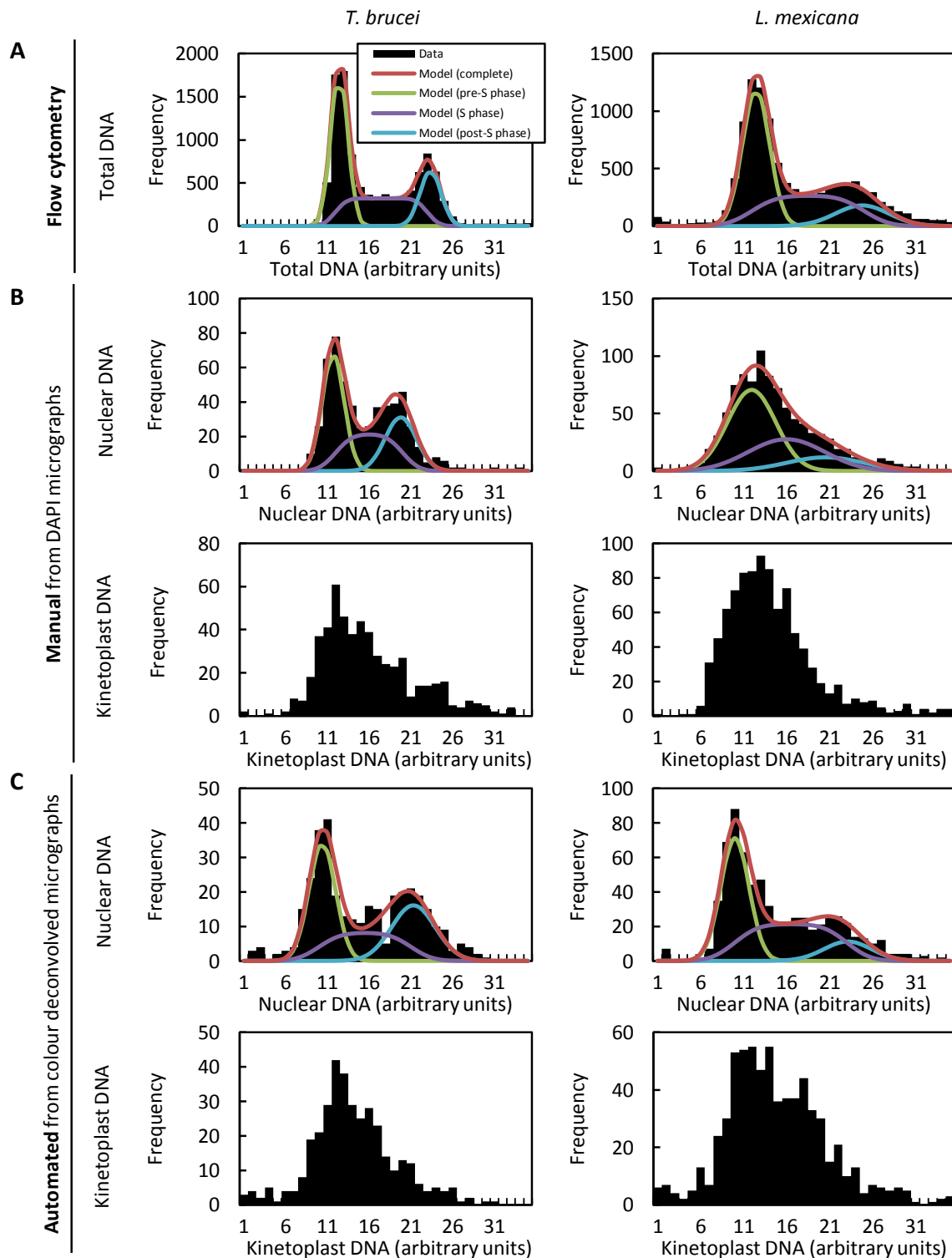


Figure 59. Three methods for quantitation of trypanosomatid DNA. *T. brucei* and *L. mexicana* were analysed by three methods. **A.** Histograms of total cellular DNA content, measured by flow cytometry of propidium iodide-stained cells, $n = 10,000$. **B.** Histograms of kinetoplast and nuclear DNA content, measured by manual analysis of signal intensity in micrographs of DAPI-stained cells, $n = 552$ (*T. brucei*), $n = 932$ (*L. mexicana*). **C.** Histograms of kinetoplast and nuclear DNA content, measured by automated analysis of signal intensity in colour deconvolved micrographs of double-stained cells, $n = 386$ (*T. brucei*, DAPI and PI stains), $n = 790$ (*L. mexicana*, DAPI and SYBR green stains). Histograms were fitted to a model of the cell cycle (Section 2.4.4) to determine the number of cells in the histogram in the pre-S phase, S phase and post-S phase stages of the cell cycle, the result of which is summarised in Figure 60.

5.2.5 Quantifying nuclear and kinetoplast DNA content from colour deconvolved micrographs

As described above the colour deconvolved images of kinetoplast and nuclear DNA preserve the quantitative nature of fluorescent staining of DNA. Kinetoplast and nuclear DNA content can therefore be quantified from colour deconvolved images and the separation of the kinetoplast and nuclear DNA to separate images greatly simplifies quantitation. Unlike with a single DNA stain there is no need to segment kinetoplasts and nuclei from the image, kinetoplast and nuclear DNA can simply be measured from the total signal intensity in the kinetoplast and nucleus image within the boundary of the cell. This approach to DNA quantitation was validated by comparing it to two conventional DNA measurement techniques; flow cytometry of PI stained cells, and quantitation of kinetoplast and nuclear DNA from cells by manual segmentation micrographs of DAPI stained cells (Siegel *et al.*, 2008). The three quantitation methods were performed on logarithmically growing procyclic *T. brucei* and promastigote *L. mexicana*. The resulting histograms of DNA content are shown in [Figure 59](#).

For both species flow cytometry gave the expected bimodal distribution of total DNA content of a logarithmically growing population of cells ([Figure 59A](#)). The two peaks correspond to the G_1 and G_2 /mitosis/cytokinesis stages of the cell cycle and intermediate DNA contents correspond to cells during S-phase. By this method it is not possible to distinguish kinetoplast and nuclear DNA. The shape of the histograms are, however, consistent with the timing of the cell cycle of both species ([Section 2](#)) (Sherwin and Gull, 1989a; Gull *et al.*, 1990; Wheeler *et al.*, 2011).

Both micrograph-based techniques for DNA quantitation are able to separately measure kinetoplast and nuclear DNA content ([Figure 59B–C](#)). Neither method was able to resolve the expected bimodal distribution of kinetoplast DNA, although the skewed histogram shapes are consistent with an underlying G_1 /S/ G_2 phase. Quantification of nuclear DNA by manual analysis gave

a clear bimodal distribution for *T. brucei* but not *L. mexicana* (Figure 59B). Automated analysis of micrographs was able to resolve the bimodal distribution of nuclear DNA for both species (Figure 59B). The data from both micrograph-based analyses are similar for *T. brucei*, however automated analysis of colour deconvolved images performed better for *L. mexicana*. This was primarily due to the close proximity of the *Leishmania* kinetoplast and nucleus during several stages of the cell cycle (Section 2.2.3 & 2.2.3.4) (Wheeler *et al.*, 2011), colour deconvolution could accurately separate the kinetoplast and nuclear DNA signal while manual segregation of the micrographs could not.

To determine the precision and accuracy of each DNA quantitation method a model of DNA content change through the cell cycle was fitted to the histograms of total DNA content (for flow cytometry) (Figure 59A) and nuclear DNA (for micrograph-based analyses) (Figure 59B–C). The result of this fitting is summarised in Figure 60. The ratio of the mean of measured DNA content of cells in G_1 to those in G_2 /mitosis/cytokinesis provides a measure of accuracy and is expected to equal 2. The standard deviation of signal for cells in the G_1 and G_2 /mitosis/cytokinesis provide a measure of precision; smaller is better. All methods had similar accuracy, with flow cytometry

Species	Data source	Ratio of means (pre-S phase/post-S phase)	Standard deviation	
			Pre-S-phase	Post-S-phase
<i>T. brucei</i>	Flow cytometry	1.83	0.08	0.10
	Manual (nucleus)	1.62	0.11	0.16
	Automated (nucleus)	1.90	0.14	0.24
<i>L. mexicana</i>	Flow cytometry	2.07	0.13	0.26
	Manual (nucleus)	1.85	0.26	0.41
	Automated (nucleus)	2.38	0.17	0.29

Figure 60. Precision and accuracy of measurement of cellular DNA content by different methods. Summary of the fitting parameters normalised to a pre-S phase DNA content of 1, from Figure 59. The ratio of the means of pre- and post-S phase gives a measure of accuracy and is expected to equal two. The standard deviation required to match the experimental error in the pre- and post-S phase peaks gives a measure of precision, smaller is better.

performing best. The precision of flow cytometry was also best with an average standard deviation of 0.17. Precision of automated analysis of colour deconvolved images was next best with an average standard deviation of 0.21 and manual analysis of micrographs was worst with an average standard deviation of 0.24. Both micrograph-based analyses have the added advantage of quantifying kinetoplast and nuclear DNA separately. Automated analysis of micrographs was both faster and more precise than manual analysis of micrographs.

5.2.6 Automated morphometric analyses built on colour deconvolution

Colour deconvolution and automated analysis of kinetoplast and nuclear DNA provide new, fast and accurate tools for analysing trypanosomatid cells. These new tools were used as a basis for extending the automated analysis to consider cell morphology and kinetoplast and nucleus location within the cell. The colour deconvolution automated analysis tools were extended to perform these automated analyses of cell morphology. Automated analysis of morphology is made up of three primary analyses; identification of cells from the phase contrast image, identification of kinetoplasts and nuclei from the colour deconvolved images and analysis of these two data sets to generate morphometric data.

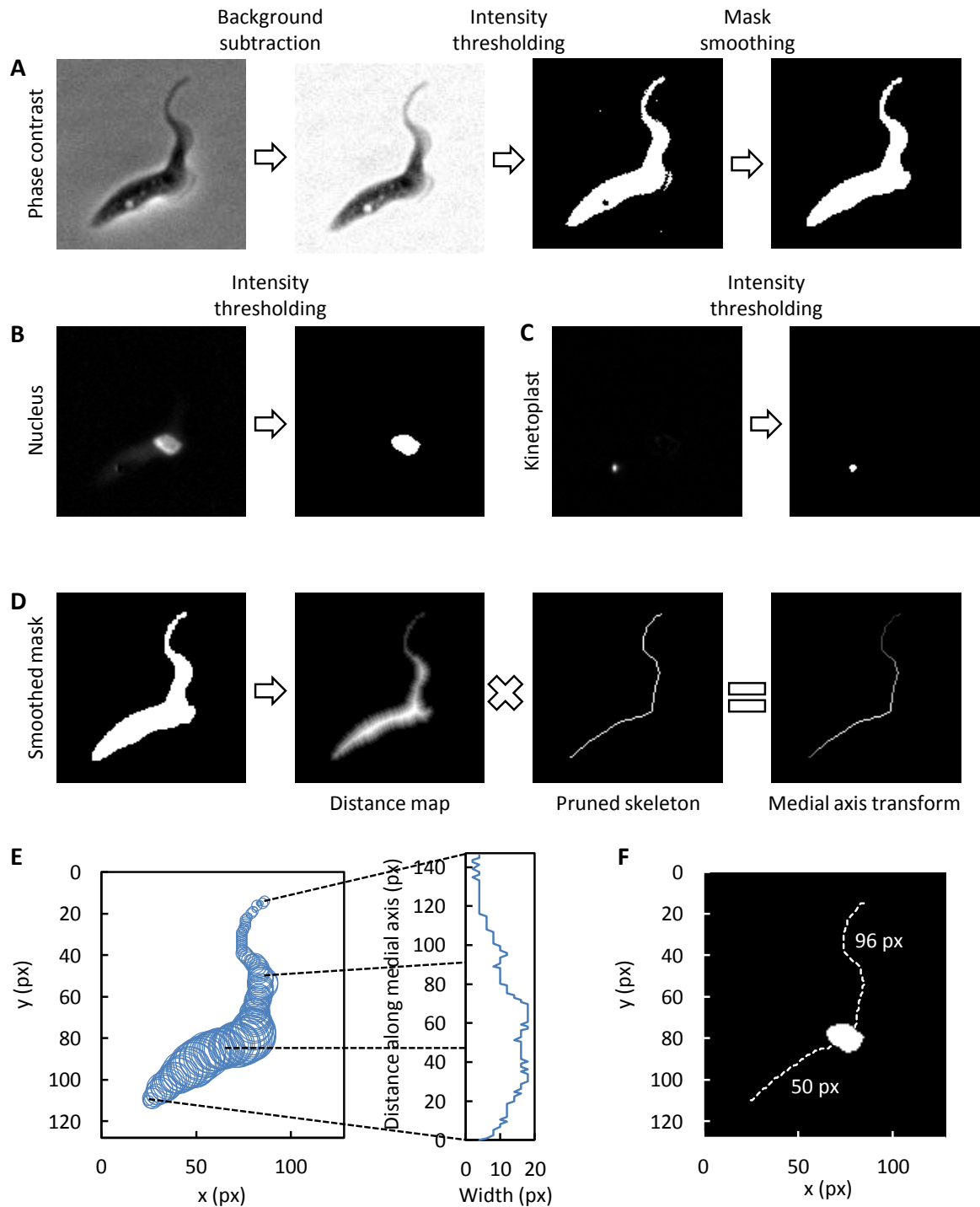


Figure 61. Automated image analysis of trypanosomatid morphology, assisted by colour deconvolution. **A.** Overview of the image processing used to identify the location and shape of cells from the phase contrast image, illustrated with a *T. brucei* cell. A rolling ball filter is used to reduce the phase contrast halo then intensity thresholding is used to generate a cell mask. Finally this mask is smoothed to remove small features. **B–C.** Nuclei and kinetoplasts are identified by intensity thresholding of colour deconvolved images. **D.** The morphology of the cell is analysed from the mask generated from the phase contrast image (**A**). The medial axis transform is generated by multiplication of the Euclidian distance map and the pruned skeleton of the smoothed cell mask. **E.** The medial axis transform encodes the shape of the cell in a single line of pixels. The shape of the cell can be reconstructed by placing a circle of the appropriate radius at every point along the medial axis (left). A plot of radius against distance along the medial axis provides the cell profile shape (right). **F.** The distance of the kinetoplast and nucleus (illustrated) from the ends of the cell can be determined from the distance of the kinetoplast/nucleus mask centroid along the medial axis.

The identification of cells from the phase contrast image is summarised in [Figure 61A](#). Phase contrast images were selected for identifying cells as the illumination technique is widely available, provides far greater contrast than bright field illumination and is simpler to analyse than differential interference contrast images. The first step is to reduce the strength of the phase contrast ‘halo’ using a rolling ball background subtraction filter (with the target of a light background) (Sternberg, 1983). The resulting image is then intensity thresholded (taking dark regions) and the resulting mask is smoothed. This mask is used to define the boundary of the cell and the visible area and total signal in the kinetoplast and nuclei images (total kinetoplast and nuclear DNA) are recorded.

Kinetoplasts and nuclei are identified by intensity thresholding of the kinetoplast and nuclei images produced by colour deconvolution [Figure 61B–C](#). The colour deconvolved images are used as the separation of kinetoplast and nuclear DNA signal to separate images simplifies identification of these organelles to intensity thresholding. Two properties of each kinetoplast and nucleus are measured; the visible area and the total signal in the kinetoplast or nuclei image respectively (the kinetoplast and nuclear DNA content for each individual organelle). The number of kinetoplasts and nuclei which fall either wholly or partially within the boundary of the cell is also recorded.

Cell morphology analysis is based on the medial axis transform (MAT) (Blum, 1967) of the cell mask [Figure 61D](#). This approach has previously been used in automated analyses of cell morphology (Narro *et al.*, 2007; Xiong *et al.*, 2010) however it is particularly suited to analysing trypanosomatids as the cells have an approximately axially symmetric structure. The MAT is generated from the multiplication of the Euclidian distance map (where each pixel has a value equal to the distance to the nearest edge of the cell mask) and the skeleton (a line of pixels along the midline of the cell, produced by terminal erosion of pixels around the edges of the cell mask). For long thin objects like trypanosomatid cells the skeleton (and therefore the MAT) is generally a single line which runs along the long axis of the shape, this approach to automated analysis can only analyse cells where

this is the case. The MAT encodes the entire shape of the cell in a single line of pixel values; to reconstruct the shape of the cell a circle is placed on each pixel of the MAT with a radius equal to the MAT value at that point **Figure 61E**. The MAT can therefore be used to measure the cell length, and profile shape **Figure 61E**. The MAT runs along the long axis of the cell and therefore provides a reference for determining the distance of organelles (including kinetoplasts and nuclei) from the cell anterior and posterior. The distance of an organelle from the midline of the cell is equal to the minimum distance between the organelle centroid and the MAT, although this is generally very small.

5.2.7 Data collection, analysis and overall workflow

These automated DNA and morphology analysis tools provide a method for extracting large quantities of data from micrographs. The DNA and morphology data is collected for every object recognised as potentially being a cell. This, along with the parameters used for analysis, can be saved making a permanent record of all data. The data collected this may include out of focus cells

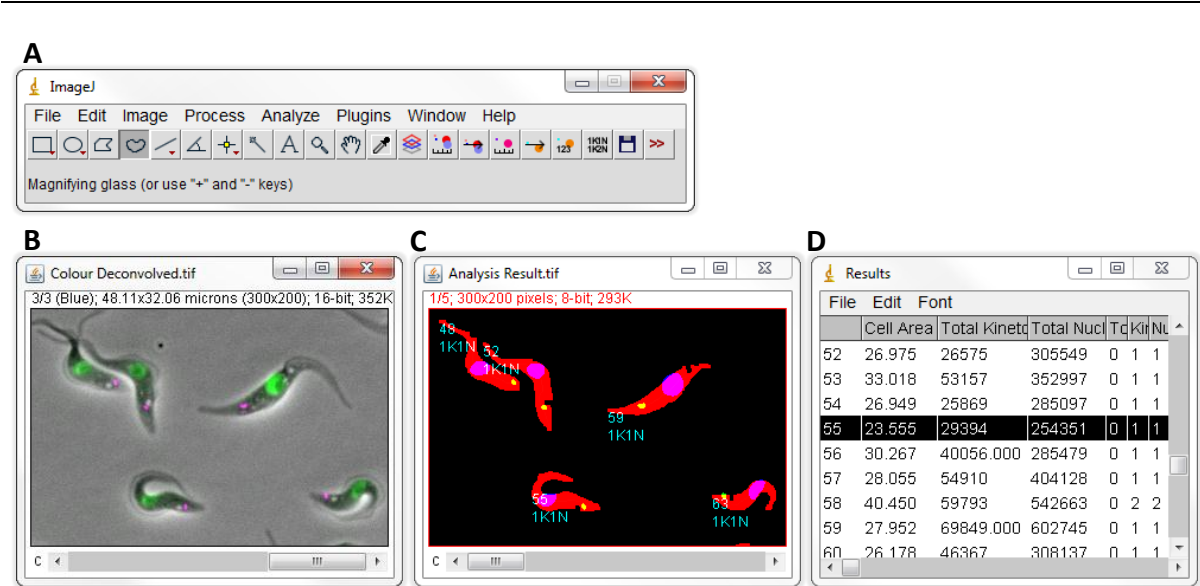


Figure 62. Screenshots of the automated image analysis tool in use. **A.** The main ImageJ window with the analysis macros installed, the eight icons on the right of the toolbar correspond to, from left to right, image setup, measure chromatic aberration, correct chromatic aberration, measure kinetoplast/nucleus reference values, colour deconvolution, cell analysis, count summariser and save analysis. **B.** Colour deconvolved source micrograph. **C.** The analysis result of the colour deconvolved image. **D.** The data output, with the data from cell ID 55 highlighted.

or debris on the slide, therefore curation is required before analysis. The data for each cell includes an ID to reference back to the original micrographs, therefore manual curation or reanalysis is possible for each cell (Figure 62).

In lieu of manual curation, simple filters may be applied to the data set to exclude objects which do not have properties typical of cells or have properties which prevent accurate analysis. Cells which are touching the image edge should be excluded as it is not known what portion of the cell is visible. These can be excluded based on dimensions and location in the image. Debris on the slide and artefacts from phase contrast thresholding should also be excluded. These were excluded based on their kinetoplast and nucleus number (OKON). Alternatively objects with very low total kinetoplast and nuclear DNA could have been excluded. The third major class of problematic cells identified was those where multiple cells lie in contact with each other. These are more difficult to exclude in an unbiased manner, especially as a mutant cells may have morphologies which appear similar. For analysis of wild-type procyclic *T. brucei* cells any cell with a branched skeleton was excluded, this was a very stringent selection which was very effective at removing problematic cells. It did, however, have a high ($\approx 20\%$) false positive rate. Therefore for analysis of mutant cell lines cells were not excluded on this basis, as an alternative manual exclusion by cross-reference to the original images could have been performed.

5.2.8 Examples of automated analysis

The performance of these colour deconvolution and automated morphology analysis tools was tested on two procyclic *T. brucei* cell lines; firstly to analyse the morphology and DNA content of wild-type *T. brucei* and secondly to analyse the changes to the DNA content of kinetoplasts and nuclei following expression of non-cleavable sister chromatid cohesion protein 1 (SCC1-mutAB).

5.2.8.1 Quantitative analysis of procyclic *T. brucei* through the cell cycle

Primary validation of the automated analysis tools used wild-type procyclic *T. brucei*. This species was selected as there are thorough previous descriptions of cell morphology and DNA content through the cell cycle. A sample of logarithmically growing *T. brucei* was prepared and analysed automatically. 30 images were analysed and 952 objects which did not touch the image edge, had at least one kinetoplast or nucleus and did not have a branched skeleton were analysed.

Kinetoplast and nucleus number were automatically determined; 70.6% 1K1N, 15.3% 2K1N, 4.8 % 2K2N and 9.3% other. This was similar to a manual count of kinetoplast and nucleus number of 500 cells from the same sample; 78.8% 1K1N, 15.0% 2K1N, 5.2% 2K2N and 0.1% other. Both are also similar to previously published data (Woodward and Gull, 1990). The larger number of ‘others’ in the

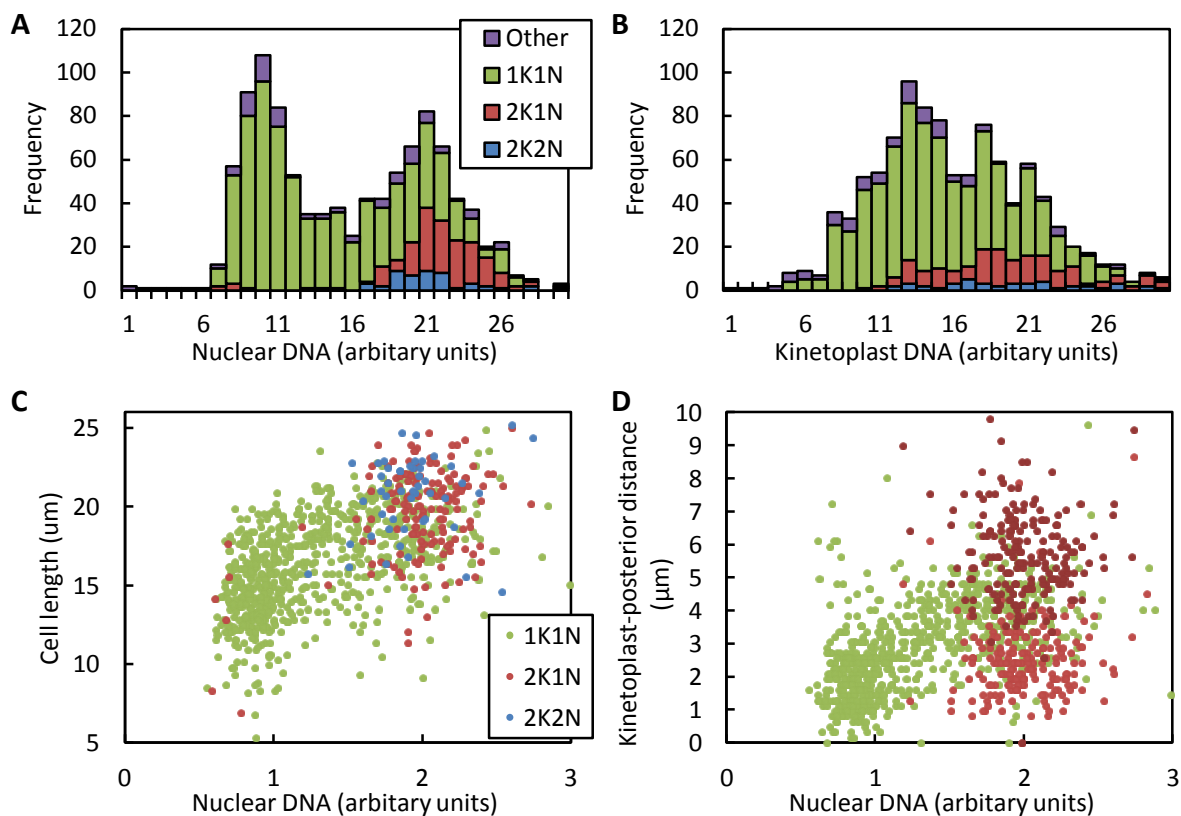


Figure 63. Automated analysis of *T. brucei* DNA content, cell shape and organelle location. Logarithmically growing cells were fixed and stained with DAPI and propidium iodide. DNA content and cell morphology were analysed by these automated tools, $n = 2720$. **A.** Histogram of nuclear DNA content, subcategorised by kinetoplast and nucleus number. **B.** Histogram of kinetoplast DNA content, subcategorised by kinetoplast and nucleus number. **C.** Scatter plot of cell body length against nuclear DNA content, kinetoplast and nucleus number categories are indicated by point colour. **D.** Scatter plot of kinetoplast position against nuclear DNA content. Kinetoplast position in 1K cells is shown in green, kinetoplast position in 2K cells is shown in red. The position of the more anterior kinetoplast in each 2K cell is shown in a lighter shade of red.

automated analysis is likely due to errors in classification, it does not have a bias towards placing hard-to-analyse cells in the expected categories of 1K1N, 2K1N or 2K2N. A histogram of nuclear DNA content featured the expected bimodal distribution (Figure 63A). Kinetoplast DNA did not, however, feature a clear bimodal distribution (Figure 63B). The automated morphology allows the breakdown of these histograms by kinetoplast and nucleus number. 2K1N and 2K2N are found towards the top end of both the nuclear and kinetoplast DNA distributions (Figure 63A–B). This is consistent with previous description of the cell cycle which showed kinetoplast and nuclear DNA replication are complete by the time the kinetoplast divides (Woodward and Gull, 1990).

The length of the cell was automatically determined from the MAT. Average lengths of cells with different kinetoplast and nucleus number were $16.3 \pm 3.1 \mu\text{m}$ (1K1N), $19.6 \pm 3.0 \mu\text{m}$ (2K1N) and $21.0 \pm 3.3 \mu\text{m}$ (2K2N) (mean $\pm$ standard deviation). This was in close agreement to a manual measurement of a subsample; the length of 50 1K1N cells manually measured from the same sample was $16.8 \pm 3.0 \mu\text{m}$. These lengths and length changes are consistent with previously published data (Robinson *et al.*, 1995). Unlike previous data sets this automated analysis data set allows correlation of cell length and nuclear DNA content to analyse morphological changes through the cell cycle in more detail (Figure 63C). This indicated that cells grow in length during G₁ from 14 to approximately 20 μm and then remain at an approximately constant length, between 19 and 21 μm , during S-phase, G₂ mitosis and cytokinesis.

Kinetoplast and nucleus position within the cell was also measured automatically from the MAT and this data set was analysed to determine the movement of kinetoplasts through the cell cycle. In *T. brucei* the kinetoplast lies closer to the posterior end of the cell, therefore the minimum distance from the kinetoplast to either end of the cell is the kinetoplast-posterior distance. Kinetoplast-posterior distance and nuclear DNA content show a distinctive correlation (Figure 63D). This revealed the kinetoplast is positioned 2 μm from the posterior during G₁. During S-phase the posterior-nucleus distance gradually increases to approximately 4 μm , after which kinetoplast

division occurs. Segregation of the two daughter positions one kinetoplast at 2 μm and the other at 6 μm from the cell posterior. This quantitative description of kinetoplast position is consistent with previous, qualitative, descriptions of basal body movement during the cell cycle (Robinson *et al.*, 1995) and division of the kinetoplast (Gluenz *et al.*, 2011).

5.2.8.2 Analysis of changes in DNA content following expression of SCC1-mutAB

Automated quantitation of kinetoplast and nuclear DNA was validated in procyclic *T. brucei* following expression non-cleavable sister chromatid cohesion protein 1 (SCC1-mutAB) of 48 h. This has previously been shown to suffer disruption of nuclear DNA as a result of failed separation of sister chromatids during mitosis (Gluenz *et al.*, 2008) however the precise effects on quantity of kinetoplast and nuclear DNA have not previously been analysed. Uninduced and 48 h induced samples were prepared in parallel on the same slide. 90 (uninduced) or 120 (induced) images were captured and analysed, from which 3201 (uninduced) or 1014 (induced) cells not touching the image

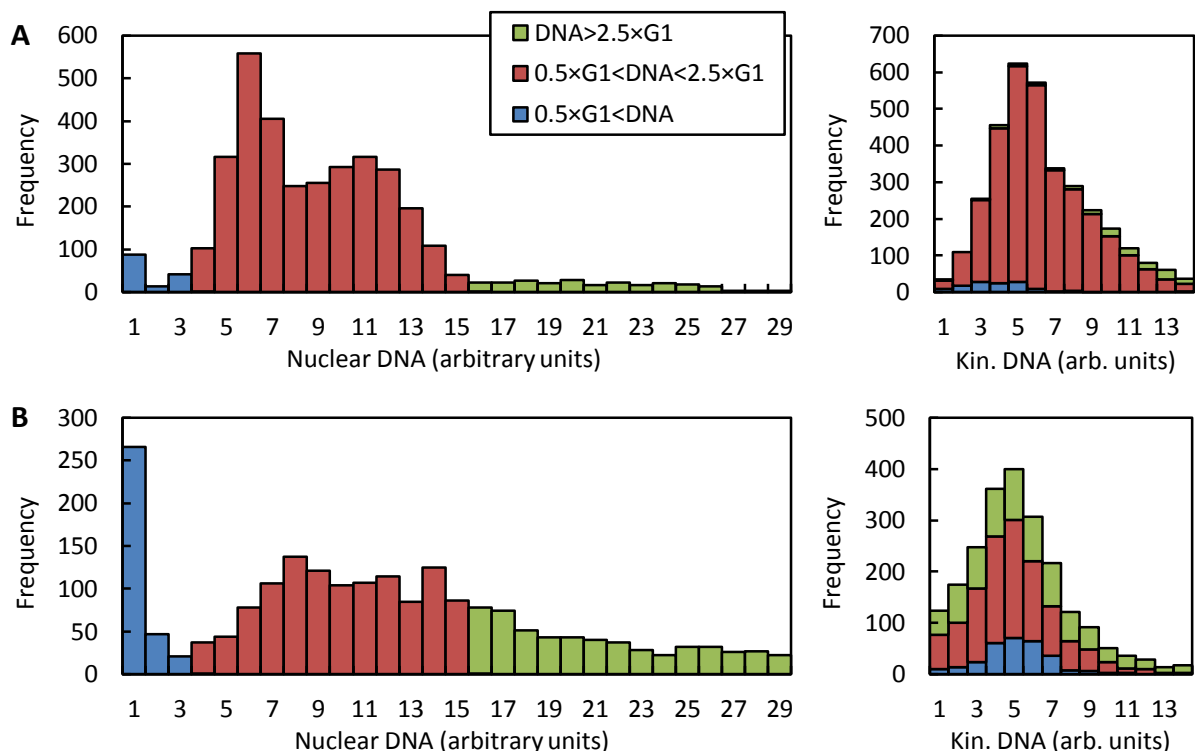


Figure 64. Loss and increase of nuclear DNA in *T. brucei* following induction of expression of sister chromatid cohesion protein 1 (SCC1)-mutAB. Histograms of nuclear and kinetoplast DNA content of *T. brucei* before and after 48 h of expression of SCC1-mutAB. DNA was quantified by automated analysis of colour deconvolved images of kinetoplasts and nuclei stained with DAPI and propidium iodide. All histograms are broken down into three categories; less than normal ($<0.5 G_1$, blue), normal ($>0.5 G_1$ and $<2.5 G_1$, red) and greater than normal ($>2.5 G_1$, green) nuclear DNA content. A. Uninduced, $n = 3508$. B. 48 h induced, $n = 2252$.

edge and possessing at least one kinetoplast or nucleus were selected for analysis. Image capture and colour deconvolution of the induced sample was performed using the same settings as the uninduced sample.

Histograms of nuclear and kinetoplast DNA as determined by the automated analysis revealed the impact of SCC1-mutAB expression on nuclear DNA content (Figure 64). The nuclear DNA content of the uninduced sample had a clear bimodal distribution similar to the wild-type (Figure 59C & 63A) however the induced sample did not. G1 DNA content was estimated from the uninduced sample and subcategorised all cells into normal (0.5 to 2.5× G1), abnormally low (less than 0.5× G1) and abnormally high (more than 2.5× G1) nuclear DNA content. Upon induction of SCC1-mutAB for 48 h the proportion of cells with abnormally low nuclear DNA content increased from 4.1% to 16.5% and the proportion of cells with abnormally high nuclear DNA content increased from 6.8% to 27.3%. Kinetoplast DNA showed little change in distribution upon induction of SCC1-mutAB and both histograms had a skewed distribution similar to seen for the wild-type (Figure 59C & 63B). Cells with abnormally high or low nuclear DNA content following induction of SCC1-mutAB did not show a bias towards higher or lower kinetoplast DNA content. Taken together these histograms confirm the previous observation that induction of SCC1-mutAB causes an increase in number of cells with abnormally low nuclear DNA content and approximately normal kinetoplast DNA content (zoids) (Gluenz *et al.*, 2008). They also quantitatively confirm the supposition that the sister cell of a zoid is able to re-enter the cell cycle and progress through S-phase to generate abnormally high nuclear DNA content. These cells, like the zoids, also possess approximately normal kinetoplast DNA content indicating division of the kinetoplast is not perturbed. The proportion of cells with abnormally low nuclear DNA content (16.5%) was lower than the proportion of zoids previously reported (38%) following induction of SCC1-mutAB for 48 h (Gluenz *et al.*, 2008). This may reflect difficulty in accurately recognising a significant nuclear DNA content of some cells with zoid-like morphology (Figure 58D) when not using colour deconvolution for analysis of kinetoplast and nucleus number.

5.2.9 Application of DNA double staining to other experimental systems

The principle of using two DNA stains on a cell to identify regions of DNA with different A-T/G-C biases is not limited to trypanosomatids. Any sample with regions of different DNA sequence of bias can be stained with a BPI and MGB stain for analysis. As an example, murine centromeric DNA is A-T rich in comparison to the rest of the genome (Waterston *et al.*, 2002). Raw264.7 (Raschke *et al.*, 1978) (a murine macrophage cell line) stained with DAPI and PI yielded the staining pattern that would be predicted in anaphase cells with the regions of chromosomes proximal to the spindle poles stained more strongly with DAPI than PI (Figure 65). In prometaphase and interphase nuclei DAPI bright loci can still be observed. These micrographs were analysed using colour deconvolution with manually specified reference values. As there are not discreet structures with DNA of different DNA sequence bias colour deconvolution yields a continuous range of A-T richness through to G-C richness which clarifies which regions of the DNA are particularly A-T or G-C rich (Figure 65). The structures deconvolution clarified in the nuclei are consistent with the expected distribution of centromeres.

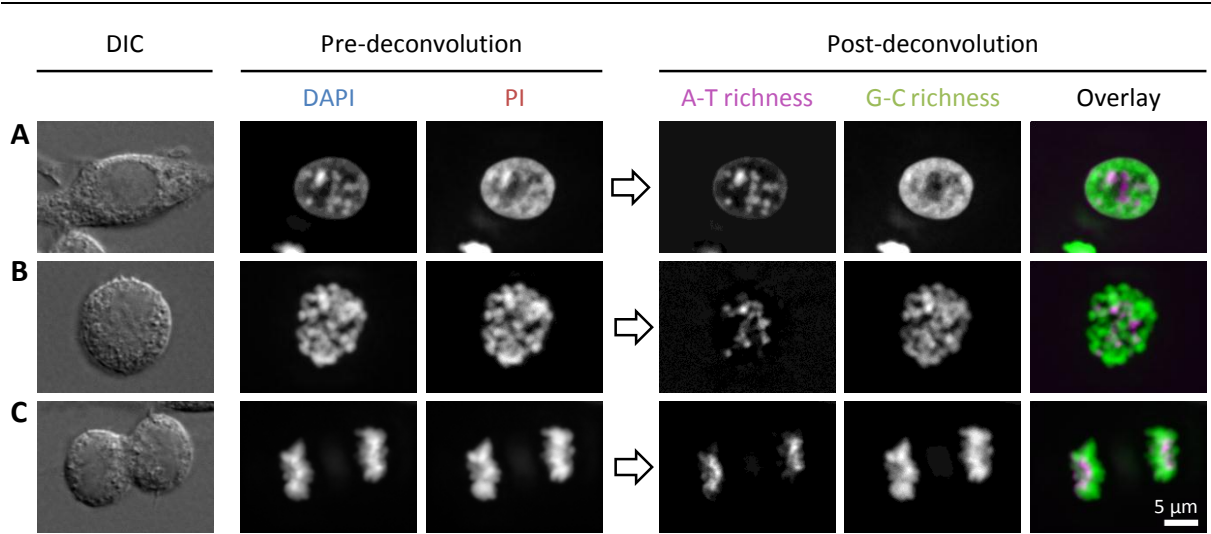


Figure 65. Behaviour of MGB and BPI DNA stains in mammalian cells. Micrographs of murine Raw264.7 macrophages labelled with two fluorescent DNA stains, DAPI and PI, before and after colour deconvolution. A. Interphase. B. Prometaphase. C. Anaphase.

5.2.9.1 Double DNA stains for live cell imaging

Membranes have low permeability for PI and SYBR green and therefore use of these stains is limited to use in fixed samples. Furthermore both stains also bind to RNA, therefore sample staining requires an RNase A treatment which also requires membrane disruption. SYTO nucleic acid stains are a family of membrane permeable cyanine dyes with a similar chemical structure to the TOTO family of (membrane impermeable) dyes which have a bis-intercalation binding mechanism (Spielmann *et al.*, 1995), although the sequence specificity this will confer is unclear. SYTO-16 primarily binds DNA (although it also binds RNA to a lesser extent) (Life Technologies and Haugland, 2010) which was tested as a live cell-compatible counterstain to Hoechst 33342. SYTO-16 bound kinetoplast DNA more strongly than Hoechst 33342 and, through overlay of the two fluorescence micrographs, allowed easy identification of the kinetoplast and nucleus (Figure 66). Colour deconvolution was not possible due to cell movement between capture of the two fluorescent channels therefore the automated analysis tools could not be used to analyse live samples stained by this method. Nonetheless this staining approach could be used to support qualitative analyses.

5.3 Discussion

The double staining approach allows the unambiguous identification of kinetoplast and nuclear DNA, purely from the DNA content of the organelles, using a simple and cost effective protocol. This

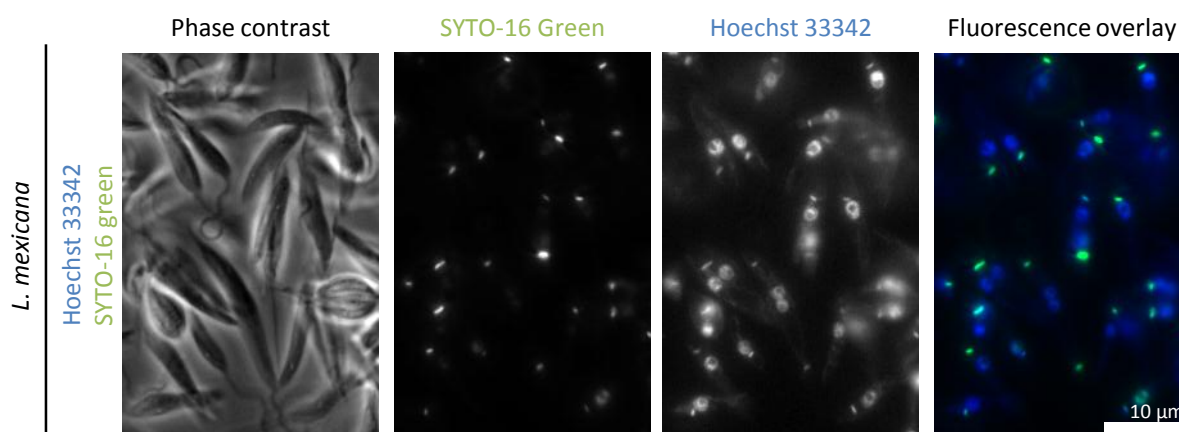


Figure 66. Live cell double DNA staining. Live promastigote *L. mexicana* stained with Hoechst 33342 and SYTO-16.

provides new opportunities for the analysis of trypanosomatid mutant phenotypes and chemical treatments and eliminates the risk of experimental bias when analysing unusual morphologies. This approach was demonstrated on three species (Figure 57) and the potential of this approach for abnormal morphologies using the SCC1-mutAB procyclic *T. brucei* cell line (Figure 58). Expression of SCC1-mutAB gave rise to kinetoplast and nucleus morphologies several of which were not possible to analyse accurately from a single DNA stain image but double staining made trivial to interpret. While it has previously been shown that cytokinesis can occur without completion of mitosis in this cell line (Gluezn *et al.*, 2008) this double staining technique provides the first evidence that this process can fragment the nucleus and generate cells which resemble zoids but include a fragment of nuclear DNA in the cell posterior. Even out-of-focus organelles can be accurately analysed (Figure 58G).

Double staining and colour deconvolution provided the foundation for a new approach to quantifying the DNA content of kinetoplastids. This method could quantify both kinetoplast and nuclear DNA in an automated (and therefore unbiased) fashion and achieved precision greater than manual analysis (Figure 59). Automation also made the process much faster than manual analysis, typically by 100×. The improved precision arose from the ability of colour deconvolution to separate signal even from very closely apposed kinetoplasts and nuclei. This was particularly advantageous for *L. mexicana* where the kinetoplast and nucleus often lie in close proximity (Wheeler *et al.*, 2011) (Section 2.2.1 & 2.2.3.4). This approach can be used for comparison of multiple samples and demonstrated this using the SCC1-mutAB procyclic *T. brucei* cell line (Figure 64). This data showed that following expression of SCC1-mutAB a cell can, following failure of mitosis, complete cytokinesis and re-enter S-phase reaching more than four times normal nuclear DNA content. It also demonstrated that kinetoplast DNA content, and not just kinetoplast number, is also largely unaffected.

Provided appropriate reference samples can be generated, the principle of double DNA staining could be applied to quantify nuclear and kinetoplast DNA in any fluorescence-based assay. For example *T. brucei* kinetoplast and nuclear DNA could be analysed separately by flow cytometry of DAPI and PI-stained samples with the use of a zoid-rich reference sample (to provide the reference values for kinetoplast DNA staining) and a dyskinetoplastid cell line reference sample (which provides the reference values for nuclear DNA staining). The contribution of kinetoplast and nuclear DNA to the DAPI and PI fluorescence signal could then be separated using the colour compensation tools found in most flow cytometry software packages.

Automated morphology analysis tools built on the double staining and colour deconvolution identification of kinetoplasts and nuclei provide a new, unbiased and fast method for collecting morphometric data. It also ensures data capture is perfectly replicable, allows archiving of the complete data set, and manual curation of data by reference to the original images. These data sets contain enormous quantities of data which may be directly analysed, used to guide more extensive manual analyses and cross-referenced back to the original micrographs. This has the potential to support analysis of very large numbers of samples, such as analysis of RNAi libraries (Subramaniam *et al.*, 2006) and evaluation of drug candidates (Engel *et al.*, 2010). Morphometric data collected about procyclic *T. brucei* by this method was consistent with both manual measurements and previously published data. Through analysis of the *L. mexicana* cell cycle I have demonstrated the value of the morphometric data contained in micrographs is huge (Section 2 & 3) (Wheeler *et al.*, 2012), and these automated morphology analysis tools provide easy access to this information.

5.3.1 Limitations and future development

These automated analysis tools were limited in some respects. Analysis of morphology by these methods was limited to cells where the MAT did not have any branches or loops, which may impact the analysis of certain mutant morphologies. It would be possible to change alter the analysis to

take into account skeleton branches and loops and allow analysis of these cells, although this was not pursued. The other primary issues arose from technical problems from sample preparation, including multiple cells lying in contact with each other, debris on the slide and uneven staining, and image capture, particularly out-of-focus cells. Image capture could be improved by focus stacking, to allow analysis of cells that would otherwise be out of focus, and thresholding could be improved by taking into account the 'texture' of the phase contrast image when thresholding to avoid including debris and phase halo artefacts.

The abilities of the automated analyses were also limited by their rigid assembly into a set of tools designed for analysing kinetoplast and nuclear DNA content and location. Given an appropriate application these automated analysis techniques are extensible to measure the location and signal intensity of any organelle within the cell. The morphology analysis tools are also extensible to measure organelle morphology, such as the length, width and curvature of a fluorescently labelled flagellum. This was for semi-automatic measurement of flagellum length of SMP1::GFP *Leishmania* cells in macrophages (Section 3.2.4.2). The potential for using the MAT as a reference axis for describing the distribution of diffuse cytoplasmic or cytoskeletal signal or for more thorough analysis of the shape of the cell were not explored. The complete code for the analysis tools is, however, available for others to use and adapt for their purposes.

5.4 Methods development and mathematics

This section covers only method development specific to this results chapter (for standard materials and protocols see Section 7).

5.4.1 Quantitative DNA staining

Fluorescence staining of microscope samples can be quantitative so long as sample preparation is performed with appropriate precautions. Paraformaldehyde-fixed slides were prepared as described for immunofluorescence (Section 7.4.1). Excess liquid was then aspirated and the cells were

permeabilised and further fixed by immersion in -20°C methanol for 30 min. Finally the slide was rehydrated and washed by three immersions in PBS. PI and SYBR green both bind to RNA as well as DNA, therefore samples were treated with RNase A. 100 μl 50 $\mu\text{g}/\text{ml}$ RNase A in PBS was added to the slide and incubated at room temperature for 1 h. The RNase solution was then removed by aspiration and the slide was stained. Two methods were optimised for staining; one (method 1) which aimed to maximise sample photostability and one (method 2) which aimed to minimise staining variation across a slide.

5.4.1.1 Staining method 1

For staining of a single sample 100 μl staining solution, 1 $\mu\text{g}/\text{ml}$ DAPI or bisbenzimidazole (Hoechst) 33342 and 40 $\mu\text{l}/\text{ml}$ PI or 1:10,000 SYBR green (Sigma Aldrich, S9430), was added to the slide and incubated at room temperature for 2 min. The slide was washed by three immersions in PBS and mounted in approximately 10 μl glycerol with 1% 1,4-diazabicyclo[2.2.2]octane and 10% 50 mM sodium phosphate, pH 8.0.

5.4.1.2 Staining method 2

For staining of multiple samples in parallel for quantitative comparison slides were immersed in staining solution, 40 ng/ml DAPI and 200 ng/ml PI, for 2 min then immediately mounted in approximately 10 μl staining solution. This method aimed to keep DNA within the sample in equilibrium with the same concentration of fluorescence DNA stain at all times, therefore minimise variation across the slide and maximise precision of quantitation.

5.4.2 Microscopy of quantitatively stained samples

Stained slides were observed by light microscopy (Section 7.5). Fluorescence staining can only be accurately quantified from regions of the slide which have not experienced photobleaching, therefore the DNA stains were not pre-exposed to fluorescence illumination prior to image capture.

5.4.3 Automated image analysis

All image analyses were performed in ImageJ (Collins, 2007). Automated analyses were performed using analysis tools written in the ImageJ macro language ([Supplementary: Script 1](#)).

5.4.3.1 Chromatic aberration correction

A two-dimensional simplification of a previously published algorithm (Kozubek and Matula, 2000) was used. A maxima finding algorithm was used to find local maxima in intensity in the fluorescence images (kinetoplasts and nuclei). Each point in the BPI image was then partnered with its nearest neighbour in the MGB image, likely to be the same kinetoplast or nucleus but offset due to chromatic aberration. For each pair of points the location in the MGB image, (m_x, m_y) , the BPI image, (b_x, b_y) , and the shift in location between the two images $(\delta_x, \delta_y) = (m_x, m_y) - (b_x, b_y)$, were calculated. Chromatic aberration in two dimensions corresponds to a linear scaling around a point (Kozubek and Matula, 2000), a transformation defined by the centre of scaling, (x_o, y_o) , and the scale factor, f .

The centre of scaling and the scale factor were derived from least squares linear regression of point location and shift between the MGB and BPI images, for the x component:

$$\delta_x = g_x m_x + c_x$$

The regression coefficient, g_x , gives the scale factor in the x direction; $f_x = g_x + 1$. The centre of scaling is given by $x_o = g_x / c_x$. The same factors can be calculated for the y component. The correction was applied by scaling up the BPI image (which is typically at a lower effective magnification as DAPI and Hoechst emit blue fluorescence) with bicubic interpolation.

5.4.3.2 Colour deconvolution

Colour deconvolution is a change in basis in two dimensions which acts to recalculate point locations in a new axis system using two new basis vectors. For colour deconvolution the old axes are MGB and BPI fluorescence signal and the new axes are defined by the measured kinetoplast and

nucleus reference values, colour deconvolution therefore moves the fluorescence images from MGB-BPI signal space to kinetoplast-nucleus signal space. The analysis tool applies the following equation to every pixel in the input images:

$$\mathbf{p}' = M^{-1}\mathbf{p}$$

Where $\mathbf{p}$ represents the value of the current pixel in the MGB, p_{MGB} , and BPI, p_{BPI} , images, $\mathbf{p} = (p_{MGB}, p_{BPI})$, and $\mathbf{p}'$ is the value of the current pixel in the destination kinetoplast, p_{KIN} , and nucleus, p_{NUC} , images, $\mathbf{p}' = (p_{KIN}, p_{NUC})$. The transformation matrix, M , is made up of the reference values which correspond to average kinetoplast and nucleus signal in the MGB and BPI images:

$$M = (\mathbf{k}, \mathbf{n}) = \begin{pmatrix} k_{BPI} & n_{BPI} \\ k_{MGB} & n_{MGB} \end{pmatrix}$$

The values of k_{BPI} , k_{MGB} , n_{BPI} and n_{MGB} are determined by the automated analysis tools. Local intensity maxima in both the MGB and BPI fluorescence micrographs, corresponding to kinetoplasts and nuclei. For every maxima the $\log_2$ MGB to BPI signal intensity ratio are calculated and k -means clustered to assign the $\log_2$ intensity ratios into two clusters; one with high $\log_2$ intensity ratio corresponding to kinetoplasts and one with low $\log_2$ intensity ratio corresponding to nuclei. The average signal intensity in the MGB and BPI images for all of the points classified as kinetoplasts and for those classified as nuclei give the values of k_{BPI} , k_{MGB} , n_{BPI} and n_{MGB} . $\log_2$ signal intensity ratio is used for classification as it is only affected by the relative signal strength, not total signal intensity; i.e. only the sequence bias of the DNA present and not the DNA quantity.

5.4.3.3 Skeleton/MAT analysis and branch pruning

Cell shape was analysed from the MAT of the cell mask shape. Skeletonisation was performed by ImageJ (Collins, 2007) by terminal erosion to an 8-connected (where connections are preserved orthogonally and diagonally between pixels) skeleton. Skeleton pixels were classified by number of neighbouring pixels; terminus (1 neighbour), branch (>2 neighbours) and normal skeleton (2

neighbours) pixels. Analysis was simplified by constraining skeleton branches to three configurations where at most one neighbour was connected orthogonally (Figure 67A) by moving one invalid pixel in a group to the adjacent square (Figure 67B). Following skeleton adjustment the length from each terminus to the next branch point was measured (Figure 67C–D) and branch lengths under the threshold were erased. Skeleton topology was then analysed; if the number of branches was zero and termini was two then the cell morphology was analysed. Using the orthogonal and diagonal length weights (Figure 67C) the cell length and organelle positioning were analysed (Section 5.2.6).

5.4.3.4 Live cell double staining

Live trypanosomatids were incubated with 10 ng/ml Hoechst 33324 (Sigma Aldrich) and 0.1 nM SYTO-16 (Life Technologies) in their culture medium for 30 min then harvested from their culture medium by centrifugation at 3000 g for 3 min. The cells were washed twice by resuspension in 0.5 ml

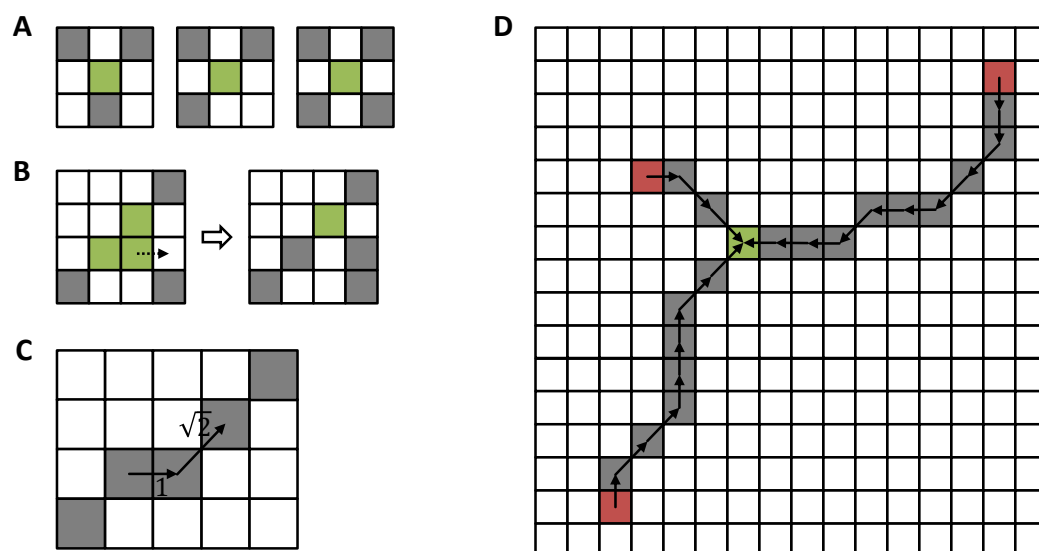


Figure 67. Analysis of cell skeleton shapes. Diagrammatic representation of different skeleton structures, adjustments and measurements. Skeletons are coloured with non-skeleton pixels in white, skeleton pixels (2 neighbours) in grey, terminus pixels (1 neighbour) in red and branch pixels (3 or 4 neighbours) in green. **A.** The three valid branch pixel configurations (or rotations of these structures) for a branch point with one or zero orthogonal neighbours. **B.** Adjustment method for invalid branch points with two orthogonal neighbours. **C.** Measurement of skeleton lengths; orthogonal neighbours contribute a length of 1 to skeleton length and diagonal neighbours contribute a length of $\sqrt{2}$. **D.** Illustration of the terminus to branch point walking to measure skeleton length.

PBS and centrifugation at 3000 g for 3 min followed by resuspension in PBS to a final volume of 50 μ l which was applied to a slide and observed by light microscopy.

6 Synthesis and conclusion

This section is a review and discussion of the conclusions of the results chapters (Section 2, 3, 4 & 5) in the context of data from the literature concerning morphological diversity of trypanosomatids. Detailed overviews and critical analysis of the conclusions from each results chapter can be found in their respective discussion sections (Section 2.3, 3.3, 4.3 & 5.3).

In this thesis I have revisited three areas of the cell biology of the *Leishmania* life cycle related to cell body morphology and morphogenesis and the function of different trypanosomatid morphologies in motility. Through these analyses I have produced the first comprehensive descriptions of cell body (Section 2) and flagellum (Section 3) morphogenesis in a promastigote, *L. mexicana*, which can be compared to morphogenesis of a trypomastigote, procyclic *T. brucei*. I have examined the implications of this for the molecular mechanisms underlying cell morphogenesis. Through analysis of motility (Section 4) I have demonstrated the impact of promastigote morphology on swimming speed within a single life cycle stage of one species and shown the role of the promastigote and trypomastigote morphologies in achieving highly directional swimming. The demands of morphometric-based analyses stimulated development of automated image analysis tools (Section 5) to assist further analyses of this type.

These advances in understanding the generation and function of morphology in trypanosomatids provide more than simply a basis for interpreting molecular and cellular biology results. By considering these mechanisms of morphogenesis and morphological function in the context of the extensive literature concerning diversity of extant trypanosomatids they provide a basis for hypotheses about the selection pressures which have generated the different morphologies in life cycle stages seen today. We can therefore begin to speculate (and sometimes answer) *why* and *how* trypanosomatid morphologies have arisen.

6.1 Evolution of trypanosomatid morphological diversity

Parasitism has evolved multiple times in the class *Kinetoplastidae* (kinetoplastids) but obligate endoparasitism is restricted to the monophyletic (Dolezel *et al.*, 2000) order *Trypanosomatidae* (trypanosomatids). The nearest exoparasitic relatives of trypanosomatids, based on phylogenies of hsp70 and 90, are the biflagellate fish exoparasites *Cryptobia* (i.e. *Trypanoplasma*) and *Ichthyobodo* (Simpson and Roger, 2004; Simpson *et al.*, 2004, 2006). The nearest relative is *Bodo saltans*, a free-living biflagellate which, like *Cryptobia* and *Ichthyobodo*, uses its long posterior flagellum for movement and surface attachment and its anterior flagellum to sweep bacteria toward its flagellar pocket and cytostome for ingestion (Mitchell *et al.*, 1988). The ultrastructure of *Bodo* is similar to that of trypanosomatids (Burzell, 1975) however the single flagellum seen nearly universally in trypanosomatids (McGhee and Cosgrove, 1980) appears to be associated with the evolution of obligate endoparasitism of this order.

Within the trypanosomatids phylogeny is not so clear. The genera were originally defined by life cycle, host species and the morphological classes attained in the life cycle (Figure 68) (Hoare, 1964; Wallace, 1966; McGhee and Cosgrove, 1980), historically regarded as a series of both evolutionary

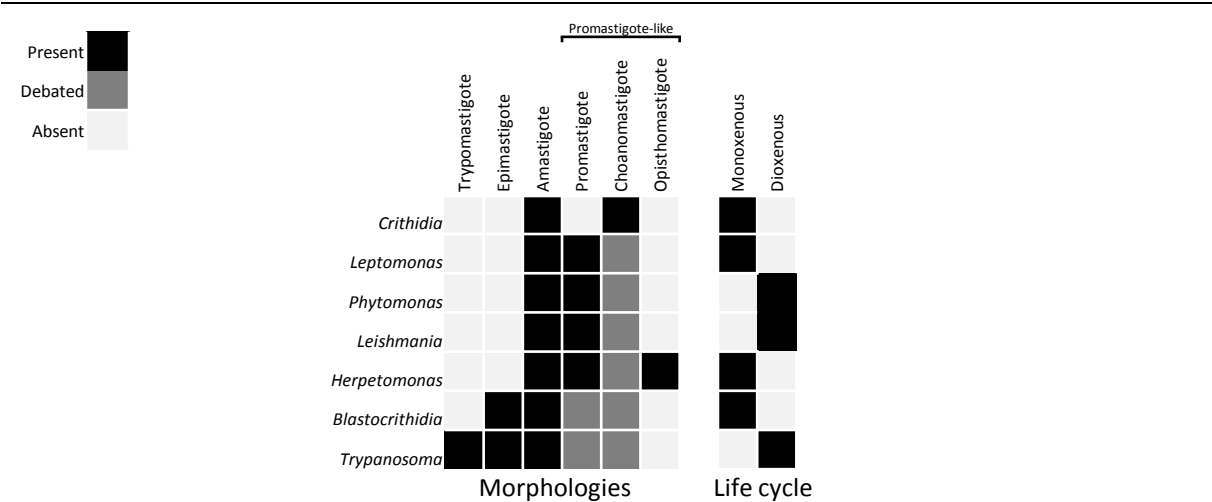


Figure 68. Tabular summary of trypanosomatid genus by morphology. Summary of the morphology and life cycle-based classification of trypanosomatids used prior to molecular phylogeny (McGhee and Cosgrove, 1980). Mixed infections of hosts led to debate over occurrence of some morphologies, for example the initial confusion between *Crithidia* and *Blastocrithidia* and the occurrence of epimastigotes and choanomastigotes in these genera (Clark, 1959; Laird, 1959). Old assumptions that all ‘lower’ morphologies (e.g. promastigotes) occurred in ‘higher’ trypanosomatids (i.e. *Trypanosoma*) (Wenyon, 1921) also led to debate.

and life cycle developmental advances (Wenyon, 1921) in line with the defunct theory of recapitulation. Modern molecular phylogeny has revealed this classification method to be inadequate as several of the genera (*Crithidia*, *Leptomonas* and *Herpetomonas*) are polyphyletic (Maslov *et al.*, 2001; Podlipaev *et al.*, 2004b; Simpson *et al.*, 2006). Nonetheless species with trypomastigote or promastigote/promastigote-like (opisthomastigote and choanomastigote) life cycle stages do cluster by lineage (Figure 69).

The morphology and life cycle of the ancestral trypanosomatid is not known with certainty and the limited fossil record indicates morphologies similar to extant species have been present in

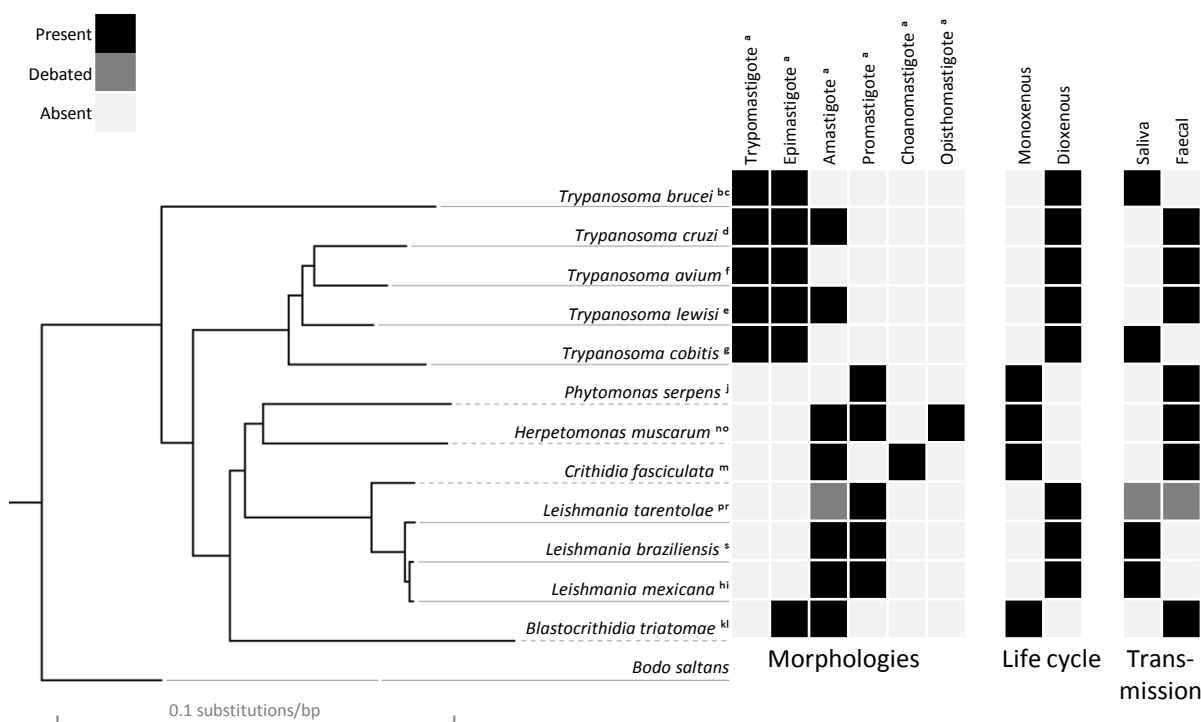


Figure 69. Tabular summary of trypanosomatid morphologies by phylogeny. **Left:** Neighbour-joining phylogenetic tree based on small subunit rRNA gene sequences (GenBank® accession numbers: M12676.1, FJ900241.2, AJ223566.1, AJ009140.1, AJ009143.1, GQ332360.1, X53913.1, M84225.1, U39577.1, Y00055.1, AF153037.2, L18872.1, DQ207573.1), rooted with the outgroup *Bodo saltans*. Solid grey lines indicate the species is a member of a monophyletic clade, dashed lines indicate it is a member of a polyphyletic genus. **Centre:** Morphologies which occur through the life cycle. **Right:** Life cycle and transmission route from the invertebrate vector/host. Relevance of the *L. tarentolae* amastigote in the life cycle (Elwasila, 1988; Raymond *et al.*, 2011) and the transmission pathway (Bates, 2007) are debated. ^a (Hoare and Wallace, 1966), ^b (Vickerman, 1985), ^c (Matthews, 2005), ^d (Goldenberg and Avila, 2011), ^e (Minchin and Thompson, 1915), ^f (Baker, 1956), ^g (Letch, 1980), ^h (Bates, 2007), ⁱ (Handman and Bullen, 2002), ^j (Jankevicius *et al.*, 1989), ^k (Kollien and Schaub, 2003), ^l (Laird, 1959), ^m (Patton, 1908), ⁿ (Drbohlav, 1926), ^o (Rogers and Wallace, 1971), ^p (Wilson and Southgate, 1979), ^r (Zhang and Leng, 1997) ^s (Bates, 2007).

insects for 100 million years (Poinar and Poinar, 2004; Poinar, 2005, 2007, 2008). Molecular phylogeny of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) suggest the dioxenous trypanosomatids, *Trypanosoma*, *Leishmania* and *Phytomonas*, arose from among monoxenous insect trypanosomatids (reviewed by (Simpson *et al.*, 2006)), supporting the hypothesis that the ancestral trypanosomatid was monoxenous too (Léger, 1904). The morphology of the ancestral trypanosomatid is not known, but has been suggested to be an epimastigote (Baker, 1963; Hoare, 1972). Modern molecular phylogeny indicates this is a parsimonious hypothesis. Epimastigotes are common in *Trypanosoma* spp. and are a defining feature of *Blastocrithidia* (Laird, 1959; Wallace, 1966), a polyphyletic genus with species widely distributed in trypanosomatid phylogenetic trees (Maslov *et al.*, 2001; Merzlyak *et al.*, 2001). Loss of the capacity for lateral attachment of the flagellum via the FAZ in multiple lineages is more plausible than multiple gains of this complex structure. Amastigotes are also found in many lineages; therefore it is plausible the ancestral trypanosomatid could also be found as an amastigote.

Trypanosoma were originally classified by a dioxenous life cycle with a trypomastigote morphology and bloodstream location for part of the life cycle in the vertebrate host (Figure 68) (Gruby, 1843). It is a monophyletic genus, assessed by molecular phylogeny of GAPDH (reviewed by (Simpson *et al.*, 2006)) and whole genome sequences (Deschamps *et al.*, 2011). *Trypanosoma* is the only genus which commonly inhabits the vertebrate bloodstream, is almost always found as a trypomastigote in this environment and is the only genus in which the trypomastigote occurs (Figure 68 & 69). While trypomastigote life cycle stages do occur outside of the blood (for example the *T. brucei* procyclic and metacyclic forms (Tetley and Vickerman, 1985; Vickerman, 1985)) it is tempting to speculate that the trypomastigote morphology evolved from a hypothetical ancestral epimastigote as an adaptation to the bloodstream.

The promastigote is common to the monophyletic (Simpson *et al.*, 2006) *Leishmania* and *Phytomonas* genera and the polyphyletic (Podlipaev *et al.*, 2004b) *Leptomonas* genus (Wallace, 1966;

McGhee and Cosgrove, 1980). Similar morphologies, the opisthomastigote and choanomastigote, characterise the polyphyletic (Podlipaev *et al.*, 2004b) *Herpetomonas* and *Crithidia* genera respectively (Wallace, 1966). Promastigote/promastigote-like morphologies, and similar surface attached forms (often termed haptomonads), are commonly found in the arthropod gut as part of either a dioxenous or monoxenous life cycle (Wallace, 1966). Promastigote-like morphologies may represent adaptation of non-*Trypanosoma* trypanosomatid species to an arthropod gut environment.

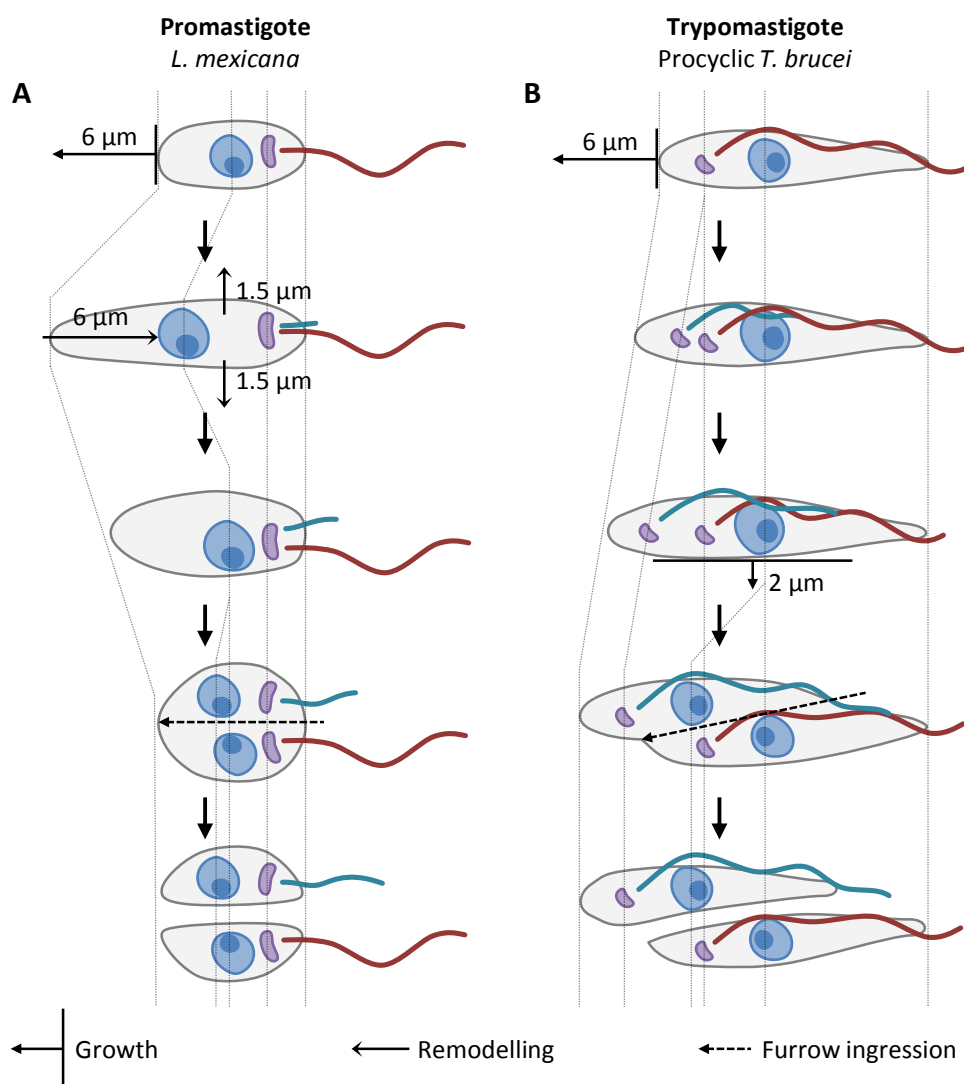


Figure 70. Cell body morphogenesis of a promastigote and trypomastigote through the cell cycle. Cell growth is indicated by arrows with a perpendicular bar, the bar indicates the region which grows. Cell remodelling is indicated by barb-headed arrows. The path of furrow ingression is indicated by dashed arrows. The old and new flagellum are coloured red and turquoise respectively. **A.** Summary of the morphological changes of an example promastigote, *L. mexicana*, through the cell cycle (Section 2) (Wheeler *et al.*, 2011). **B.** Summary of the morphological changes of an example trypomastigote, procyclic *T. brucei*, through the cell cycle (Sherwin and Gull, 1989a, 1989b; Robinson *et al.*, 1995).

6.2 Morphogenesis of trypomastigotes and promastigotes through the cell cycle

Trypomastigote and promastigote-like morphologies appear to represent two major classes of trypanosomatid cell organisation and the two may have arisen during divergence of the bloodstream-inhabiting *Trypanosoma* and other trypanosomatids from a hypothetical epimastigote ancestor. The analyses of promastigote *Leishmania* division (Section 2) (Wheeler *et al.*, 2011) and flagellum growth (Section 3) presented in this thesis, corroborated by analysis of *L. major* (Ambit *et al.*, 2011), provide the first comprehensive analysis of morphogenesis of a promastigote through the cell cycle. In combination with existing, comprehensive, analyses of morphogenesis in the procyclic *T. brucei* (a trypomastigote) cell cycle (Sherwin and Gull, 1989a, 1989b; Robinson *et al.*, 1995) this allows the first detailed comparison of cell body morphogenesis of these two major classes of trypanosomatid morphology, consideration of the impacts this has for interpretation of mutants that perturb morphogenesis and understanding morphogenesis in the life cycle.

6.2.1 Cell body morphogenesis

The growth and division of the cell body for generation of daughter cell morphology differs between promastigotes and trypomastigotes (Figure 70). Morphogenesis in procyclic *T. brucei* (Figure 70B) is linked to cell growth: two distinct types of growth occur, the cell increases in length through extension of the sub-pellicular array microtubules and, later in the cell cycle, it increases in width through insertion of microtubules into the sub-pellicular array in the central portion of the cell (Sherwin and Gull, 1989a, 1989b). Non-bilaterally symmetric cytokinesis generates two daughter cells with reduced length and width (Sherwin and Gull, 1989a). In contrast morphogenesis in *L. mexicana* (Figure 70A, summarising data from Section 2) is linked to both growth and cell remodelling: firstly the cell grows in length, presumably by the same mechanisms used by *T. brucei*, followed by rapid remodelling to achieve a decrease in length and increase in width. Bilateral

cytokinesis generates the daughter cells. Daughter cell morphology is therefore generated *during* cytokinesis for trypomastigote *T. brucei* and *prior* to cytokinesis for promastigote *L. mexicana*. The hypothesis that these two example cell cycles are representative of trypomastigote and promastigote cell body morphogenesis would be simple to test for any species which can be cultured axenically, taking advantage of the automated morphology and DNA content analysis tools I have developed (Section 5) (Wheeler *et al.*, 2012) to facilitate the analysis.

The processes of cell body growth and remodelling gives rise to more diversity in length in proliferating promastigote than trypomastigote populations; promastigote *L. mexicana* undergoes larger changes in length ($\approx 2.0\times$) through the cell cycle than trypomastigote *T. brucei* ($\approx 1.4\times$) (Figure 70). Flexibility of cell length and the small cell size immediately following division of promastigote-like morphologies may be used in genera to effect morphological change where differentiation to amastigotes occurs (*Leishmania*, *Leptomonas*, *Herpetomonas* and *Crithidia*) (Figure 69). The capacity to undergo a rapid cell remodelling may also delay the commitment point for morphological change during life cycle stage differentiation to just prior to cytokinesis. Flagellum length change during promastigote to amastigote differentiation of *L. mexicana* suggests disassembly of flagella occurs essentially independently of the cell cycle (Section 3), and the decision point for whether to grow a long or short new flagellum is likely to be the onset of new flagellum growth, just prior to cytokinesis (Section 2). This is unlike, for example, differentiation of bloodstream to procyclic form *T. brucei* where commitment is coincident with the onset of S-phase (Matthews and Gull, 1997). In the absence of detail of how the *T. cruzi* amastigote, generated by differentiation from a trypomastigote (Goldenberg and Avila, 2011), arises it is hard to examine how these hypotheses translate to amastigote generation in *Trypanosoma*.

6.2.2 Kinetoplast and nucleus positioning

Positioning of the daughter nuclei occurs asymmetrically in both *L. mexicana* and *T. brucei*; the daughter nucleus which will be inherited by the cell with the new flagellum is displaced relative to the parent nucleus. In *T. brucei* this occurs longitudinally (Figure 70B) while in *L. mexicana* it occurs laterally, with only a small longitudinal displacement (Figure 70A). Positioning of the daughter kinetoplasts also occurs asymmetrically in both morphologies; the daughter kinetoplast associated with the new flagellum is displaced relative to its parent. Movement of the kinetoplast is longitudinal and large, arising from migration toward the posterior, in *T. brucei* (Figure 70B) and lateral and small in *L. mexicana*.

These similarities in organelle repositioning suggest that, despite the overall differences in morphology, the mechanisms at the molecular level which give rise to duplication then separation of the basal bodies (and kinetoplast) and the separation of daughter nuclei are shared. Basal body duplication and maturation are similar for *T. brucei* and *L. mexicana* (Section 2) and the complex reorientation of the old and new basal body seen in *T. brucei* (Lacomble *et al.*, 2009, 2010) may also occur in *L. mexicana*. The postulated anchoring of one daughter nucleus in *T. brucei* via the nuclear membrane allowing the spindle to reposition one daughter during mitosis (Robinson *et al.*, 1995) may similarly be conserved.

6.2.3 Flagellum growth

The flagella of *T. brucei* and *L. mexicana* both have great diversity in length through the life cycle, however I have shown in the context of a single life cycle stage the promastigote *L. mexicana* flagellum has a far greater length range than trypomastigote *T. brucei* (Section 2) (Wheeler *et al.*, 2011). Mathematical modelling of *Leishmania* flagellum growth suggested it continues to grow over multiple cell cycles (Section 3) (Wheeler *et al.*, 2011), generating the flagellum length range observed, while the *T. brucei* flagellum grows to a consistent length over one cell cycle (Sherwin and

Gull, 1989a; Davidge *et al.*, 2006; Absalon *et al.*, 2008). As for cell body morphogenesis, flagellum morphogenesis therefore differs between the promastigote and trypomastigote; the trypomastigote *T. brucei* flagellum forms over a *single* cell cycle while the promastigote *L. mexicana* flagellum forms over *multiple* cell cycles.

T. brucei morphogenesis is exquisitely sensitive to perturbations of flagellum growth (Ralston *et al.*, 2009; Vaughan, 2010). In procyclic *T. brucei* the new flagellum grows laterally attached to the cell body and attached by the tip to the side of the old flagellum *via* the flagellar connector (Moreira-Leite *et al.*, 2001). Mutations which impact the growth or attachment of the new flagellum disrupt cytokinesis through misplacement of the cleavage furrow, often causing the daughter cell which inherits the new flagellum to be abnormally short and causing miss-segregation of the daughter nuclei. RNAi targeting FLA1 (LaCount *et al.*, 2002) or FAZ1 (Vaughan *et al.*, 2008) (which disrupt flagellar attachment) and RNAi targeting one of several IFT components (which disrupts flagellar elongation) (Absalon *et al.*, 2008) are typical examples of this effect. In the tsetse fly life cycle stage differentiation from a long (21 μm) procyclic promastigote to a short (9 μm) and long (26 μm) epimastigote occurs through an asymmetric division. During this division only a short new flagellum is formed which may, similar to the result of RNAi inhibiting flagellum growth, be responsible for defining the plane of cytokinesis and templating the generation of one shorter daughter cell (Sharma *et al.*, 2008). In contrast *L. mexicana* mutants with elongated or shortened flagella do not experience any problems with division (Wiese *et al.*, 2003; Bengs *et al.*, 2005; Erdmann *et al.*, 2006). Therefore while flagellum length in *T. brucei* helps *template* division and control cell size it plays no role in regulating division of promastigote *L. mexicana* in which regulation of flagellum length is *decoupled* from regulation of cell size.

Growth and division of trypomastigote *T. brucei* and promastigote *L. mexicana* therefore appear to address contrasting morphogenetic aims; *T. brucei* appears to precisely regulate cell and flagellum length and minimise length changes in the cell cycle while *L. mexicana* has decoupled flagellum and

cell body length regulation and undergoes large changes in cell length through the cell cycle. These may reflect different functional constraints on morphology arising from the environments typically inhabited trypanosomatids with these morphologies; a trypomastigote morphology constrained to a narrow size range may be required for the bloodstream environment and/or ongoing promastigote flagellum growth may provide an advantage in an insect gut. Natural diversity of promastigote-like and trypomastigote morphologies in different species and different hosts provide a route for analysis of these hypothesis (see [Section 6.3 & 6.4](#)).

6.2.4 Morphogenesis of other trypanosomatid morphologies

Little quantitative data is available concerning morphogenesis in the two other major trypanosomatid morphologies; the epimastigote and amastigote. The timing of division events have been described for *T. cruzi* epimastigotes (Elias *et al.*, 2007), and are similar to *L. mexicana* promastigote with onset of new flagellum growth, kinetoplast and nucleus division in the last 75% of the cell cycle (Elias *et al.*, 2007). The kinetoplast and nucleus do not migrate far after division, also similar to *L. mexicana* promastigotes. This initial analysis therefore suggests the morphogenesis of *T. cruzi* epimastigotes is more similar to *L. mexicana* promastigotes than *T. brucei* trypomastigotes, although more detailed analysis is required to confirm this. If the ancestral trypanosomatid was epimastigote then understanding morphogenesis of this cell configuration would be particularly

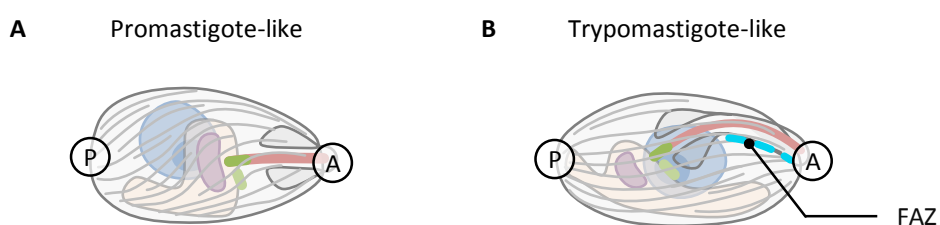


Figure 71. Possible differences between promastigote-like and hypothetical trypomastigote-like amastigote ultrastructures. Little data exists on the precise ultrastructure of *Trypanosoma* amastigotes. It is unclear where the flagellar pocket lies relative to the posterior (P) and anterior (A) sub-pellicular microtubule poles. *Leishmania* amastigotes are promastigote-like (A) (Gluezn *et al.*, 2010b) while *T. cruzi* hypothetically may be trypomastigote/epimastigote-like (B) (*confer* [Section 1.1.1](#)). The FAZ in the trypomastigote/epimastigote-like form may be so short as to make the two indistinguishable.

valuable as it may include conserved features of morphogenetic processes from which the events of the trypomastigote and promastigote cell cycles evolved.

Data concerning the amastigote is particularly limited; analysis is inhibited by the intracellular location of *Trypanosoma* (for example *T. cruzi*) and *Leishmania* amastigotes and the perception of *Crithidia*, *Blastocrithidia*, *Herpetomonas* and *Leptomonas* amastigotes (often termed ‘strap-hangers’ in older literature) as encysted life cycle stages (Wallace, 1966; McGhee and Cosgrove, 1980). More fundamentally the ultrastructure, sub-pellicular microtubule polarity and flagellum exit point on amastigotes have not been rigorously analysed; *T. cruzi* amastigotes have not been shown to be identical to *Leishmania* amastigotes in ultrastructure (Figure 71). Division of *L. major* amastigotes in macrophages appears to be bilaterally symmetric (Real and Mortara, 2012) and similar to promastigote *L. mexicana*, although it remains to be seen if cell growth preceding cytokinesis is similar to the promastigote.

Amastigotes occur as life cycle stages in species from most trypanosomatid lineages (Figure 69), including *Trypanosoma* and *Leishmania*. This indicates a long flagellum is not vital for normal morphogenesis; species which have an amastigote and a trypomastigote (e.g. *T. cruzi*) or promastigote-like (e.g. *L. mexicana*) life cycle stage can proliferate without a long flagellum under the correct circumstances. Morphologies with a long, motile, flagellum (promastigote-like and trypomastigote) commonly occur in the fly gut and bloodstream environment respectively. It therefore appears that there are selection pressures to maintain a long flagellum in these environments.

6.3 Trypomastigote diversity and the bloodstream environment

The bloodstream environment is exclusively inhabited by *Trypanosoma* spp. and the trypomastigote is observed nearly universally for these life cycle stages. Epimastigote morphologies

in the blood are described occasionally, for example *T. lewisi*, however epimastigotes are more common in peripheral tissue and parasites in the blood during high parasitemias are trypomastigote (Deane, 1969). There are no examples of promastigote-like or amastigote morphologies in the blood. The trypomastigote morphology therefore appears vital for inhabiting the bloodstream.

Several functions have been proposed for the trypomastigote motility and morphology based on analysis of *T. brucei*. This single species is, however, not representative of the diversity of trypomastigote morphology (Figure 72); there are hundreds of non-human infective species with a piscine, avian, reptile, amphibian or rodent host and an arthropod or leech vector. The selection pressure for trypomastigote morphology appears to occur in all of these hosts, therefore understanding the diversity of trypomastigote morphology will contribute to understanding why it occurs.

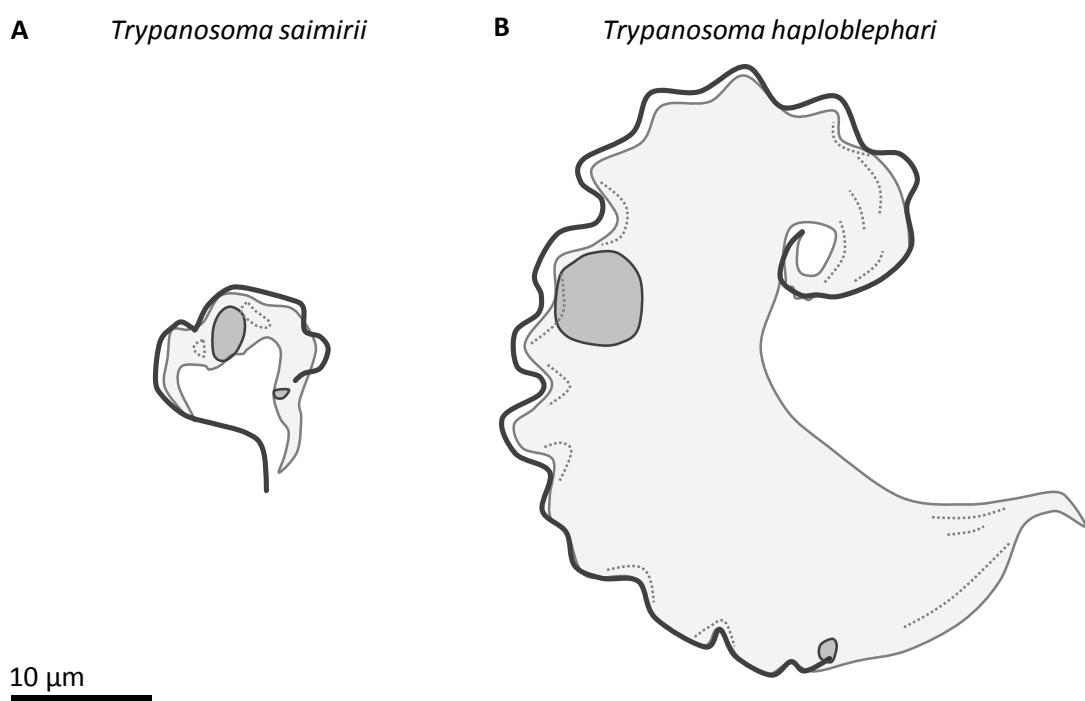


Figure 72. Examples of trypomastigote diversity. Line drawings of two extremes of trypomastigote morphology to the same scale. A. *Trypanosoma saimirii* from *Saimiri sciureus* bloodstream (Ziccardi and Lourenço-de-Oliveira, 1998). B. *Trypanosoma haploblephari* from *Haploblepharus pictus* bloodstream (Yeld and Smit, 2006).

Trypomastigote morphological diversity has been recorded through identification of new species and surveys of prevalence of trypanosomatid infections. A data set covering morphology of 109 isolates of *Trypanosoma* spp. from the blood of 72 host vertebrates (covering 47 genera distributed across *Amphibia*, *Aves*, *Chondrichthyes*, *Mammalia* and *Osteichthyes*) was generated from existing literature (Supplementary: Table 1). Parasite dimensions from isolates were recorded if the parasite cell body length and width were available, the host genus erythrocyte dimensions were available in the Erythrocyte Size Database (Gregory, 2005) and if measurements were taken from blood isolates or axenic cultures immediately derived from isolates. Free flagellum length and kinetoplast to posterior distance were recorded and mean, minimum, maximum and standard deviation of morphological measurements were recorded where available. All the following plots concerning

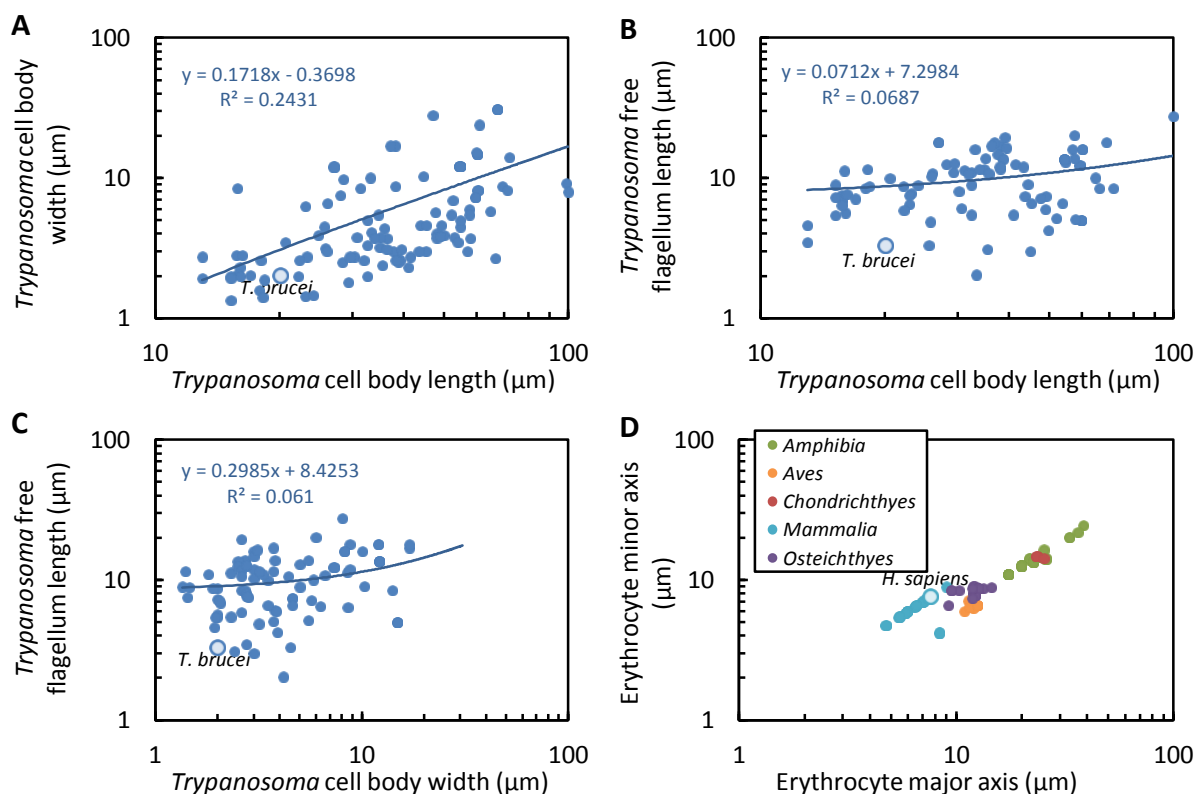


Figure 73. Morphologies of diverse *Trypanosoma* spp. and the erythrocytes of their hosts. **A.** Correlation of parasite cell body length and width of *Trypanosoma* isolates, $n = 108$. Bloodstream form *T. brucei* dimensions are indicated with an open circle. **B.** Correlation of parasite cell body length and free (overhanging the anterior) flagellum length, $n = 91$. **C.** Correlation of parasite cell body width and free flagellum length, $n = 91$. **D.** Correlation of the major axis (maximum disc width) and minor axis (minimum disc width) of erythrocytes of the host vertebrates, subcategorised by class, $n = 71$. Average *Homo sapiens* erythrocyte dimensions are indicated with an open circle.

Trypanosoma spp. morphology are derived from these data and *n* numbers indicate the number of species for which the morphometric data required for the plot was available.

Trypanosoma cell body length, width and free flagellum length are diverse and each varies by at least a factor of 10 across species (Figure 73). There is little correlation between parasite cell body length, width and free flagellum length (Figure 73A–C), although longer trypanosomes tend to be wider (Figure 73A). This diversity can be used to determine what, if any, morphology selection pressures appears to be acting across all trypanosomatids.

6.3.1 Mechanical constraints on morphology

It has been proposed that the *T. brucei* bloodstream form trypomastigote flagellum wavelength, morphology and rotation while swimming has adapted to optimise swimming among human erythrocytes (Heddergott *et al.*, 2011). If this effect is important in most *Trypanosoma* spp. then correlations between trypanosome and host erythrocyte morphology might be expected. Erythrocyte dimensions in the host species surveyed vary by a factor of 10 (Figure 73D), similar to the range of trypomastigote dimensions, but did not correlate with parasite cell body length, width or free flagellum length (Figure 74). While there is little correlation between erythrocyte and parasite dimensions other trends emerge: 98% of trypomastigotes are longer than the host erythrocyte major axis (Figure 74A) and all are longer than the minor axis (Figure 74B). 95% of trypomastigotes are also narrower than the host erythrocyte major axis (Figure 74C). Even large mammalian-infective *Trypanosoma* species, such as members of the monophyletic subgenus *Megatrypanum*, *T. (M.) cervi* and *T. (M.) theileri* (Rodrigues *et al.*, 2006), adhere to these width constraints.

Only one species which deviated significantly from the width constraint was identified; *Trypanosoma binneyi* has a large trypomastigote bloodstream form (47 to 67 μm long, 11 to 15 μm wide (Mackerras, 1959)) and can inhabit the bloodstream of the platypus (*Ornithorhynchus anatinus*)

which has small erythrocytes similar to other mammals (5.2 μm diameter (Collins *et al.*, 1986)).

T. binneyi is a member of the fish and amphibian *Trypanosoma* clade (Jakes *et al.*, 2001; Gibson *et al.*, 2005) and the platypus inhabits an aquatic environment. There is therefore the possibility that the platypus is not a normal host or is not the major host of this species.

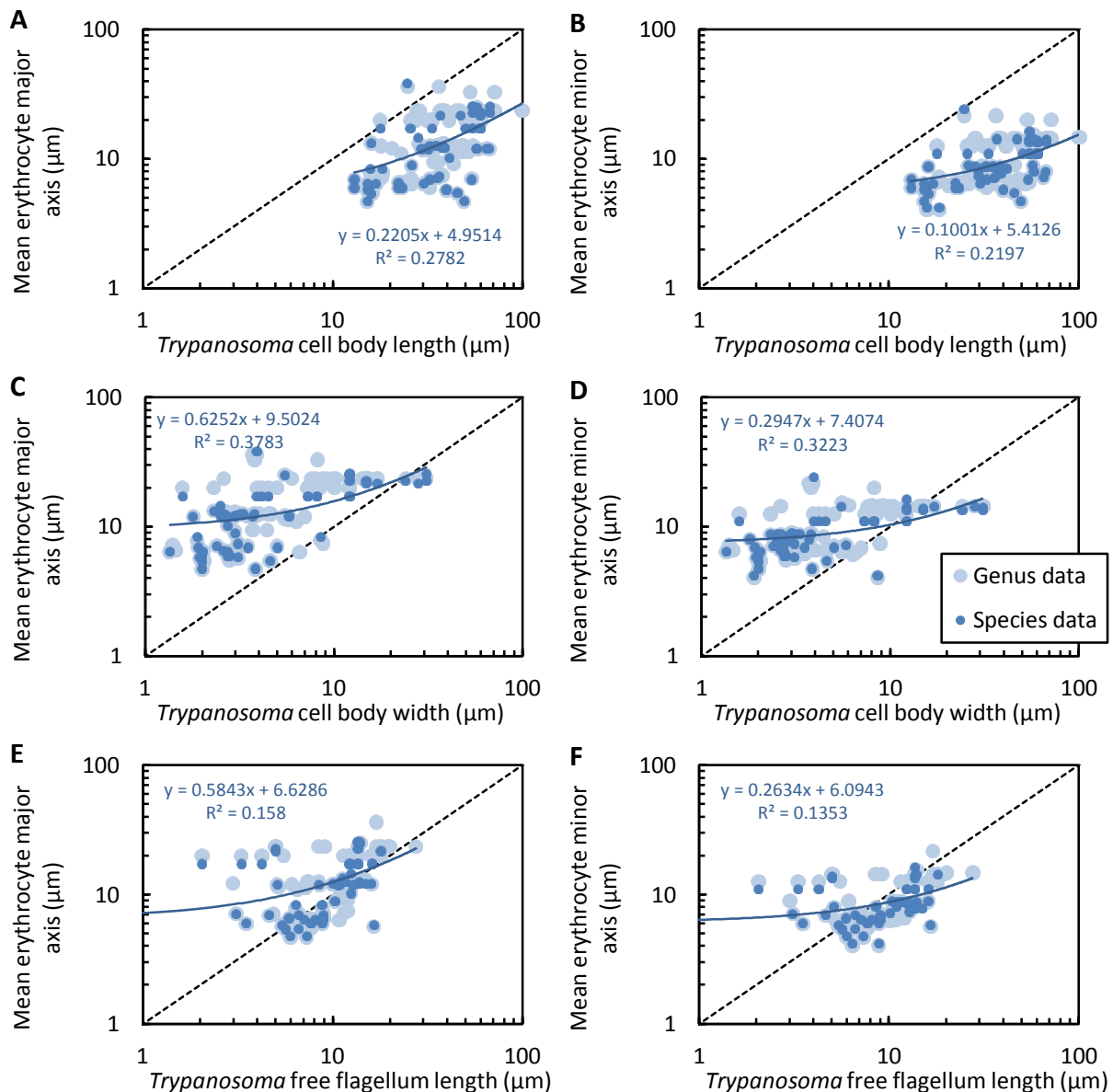


Figure 74. Correlation of *Trypanosoma* morphology with host erythrocyte dimensions. A. Parasite cell body length and erythrocyte major axis. B. Parasite cell body length and erythrocyte minor axis. C. Parasite cell body width and erythrocyte major axis. D. Parasite cell body width and erythrocyte minor axis. E. Parasite free flagellum length and erythrocyte major axis. F. Parasite free flagellum length and erythrocyte minor axis. Light blue data points indicate the average of erythrocyte data available for the host genus. Dark blue data points indicate host species-specific erythrocyte data, where available. Dashed lines indicate the line along which parasite and host erythrocyte dimensions would be equal. Genus-specific data $n = 108$ and species-specific data $n = 60$ for A, B, C and D. Genus-specific data $n = 91$ and species-specific data $n = 38$ for E and F.

In conclusion there is no evidence for a straightforward correlation between erythrocyte and trypomastigote size, but trypomastigotes are nearly always *longer* and *narrower* than the red blood cells of the host. The constraint on parasite width (Figure 74C) may be mechanical. Parasites presumably need to fit through the capillaries of the host, the diameter of which average 80% of the largest erythrocyte dimension for vertebrate species (Snyder and Sheafor, 1999). More speculatively the constraint on parasite length (Figure 74A–B) may reflect an optimisation for swimming among erythrocytes, similar to proposed for *T. brucei* (Heddergott *et al.*, 2011).

6.3.2 Hydrodynamic surface clearance and flagellar pocket position

Blood flow velocity in mammalian capillaries (Ivanov *et al.*, 1981) is at least one order of magnitude higher than maximum *T. brucei* bloodstream form swimming speed (Uppaluri *et al.*, 2011; Weiße *et al.*, 2012) making the role of swimming unclear as it is too slow to overcome the bulk flow of the blood. An alternative function has been described; hydrodynamic-assisted clearance of surface bound antibodies, which may assist avoidance of the adaptive immune response (Dean and Matthews, 2007; Engstler *et al.*, 2007). Hydrodynamic flow forces arising from swimming concentrates large surface molecules, including bound immunoglobulins, at the cell posterior (near

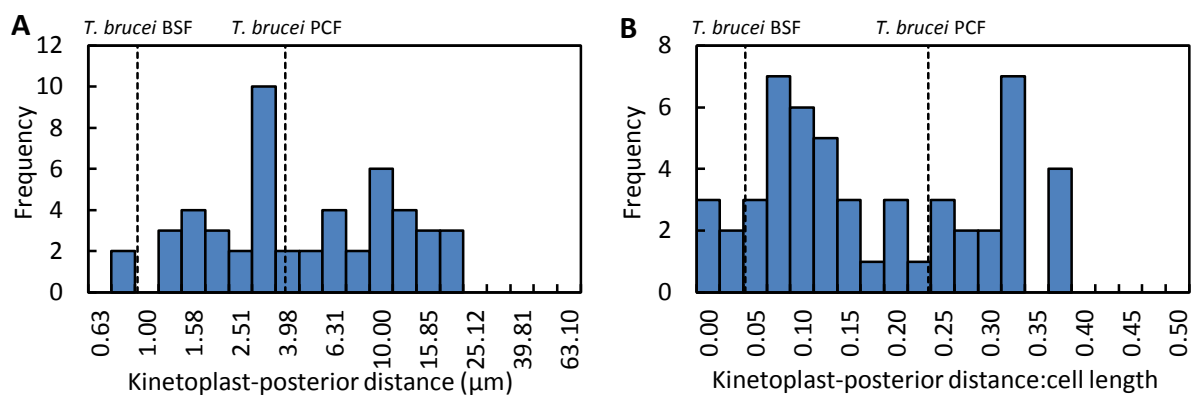


Figure 75. Kinetoplast position in bloodstream forms of *Trypanosoma* species. **A.** Histogram of kinetoplast-posterior distance, $n = 50$. **B.** Histogram of the ratio of kinetoplast-posterior to cell body length, $n = 50$. Vertical dashed lines indicate the location of procyclic (PCF) and bloodstream (BSF) form *T. brucei* on these plots.

the flagellar pocket) assisting endocytic recycling or clearance (Dean and Matthews, 2007; Engstler *et al.*, 2007). Adaptive immune responses broadly similar to mammals occur in all *Trypanosoma* host species analysed (jawed vertebrates) (Anderson *et al.*, 2004; Scott-Browne *et al.*, 2011) therefore a selection pressure for a flagellar pocket position near the posterior in bloodstream-inhabiting trypomastigotes may be expected. This is seen in bloodstream form *T. brucei* where the kinetoplast/flagellar pocket complex is very close to the cell posterior ($\approx 1 \mu\text{m}$, 5% of cell body length (Matthews *et al.*, 1995)), in contrast to the procyclic form in the insect midgut where it lies further from the posterior ($\approx 4 \mu\text{m}$, 24% of cell body length).

Histograms of kinetoplast distance from the posterior (Figure 75A) and kinetoplast distance from the posterior as a fraction of cell body length (Figure 75B) for *Trypanosoma* spp. surveyed from the literature revealed no bias for kinetoplast/flagellar pocket positioning towards the posterior of trypomastigotes. Few trypomastigotes have a kinetoplast very close to the posterior, as observed in bloodstream form *T. brucei*, and many species have a kinetoplast positioned far from the posterior, often further than for procyclic form *T. brucei*. The mechanism of hydrodynamic-assisted surface protein recycling therefore appears not to exert a selection pressure on kinetoplast positioning in all *Trypanosoma* species.

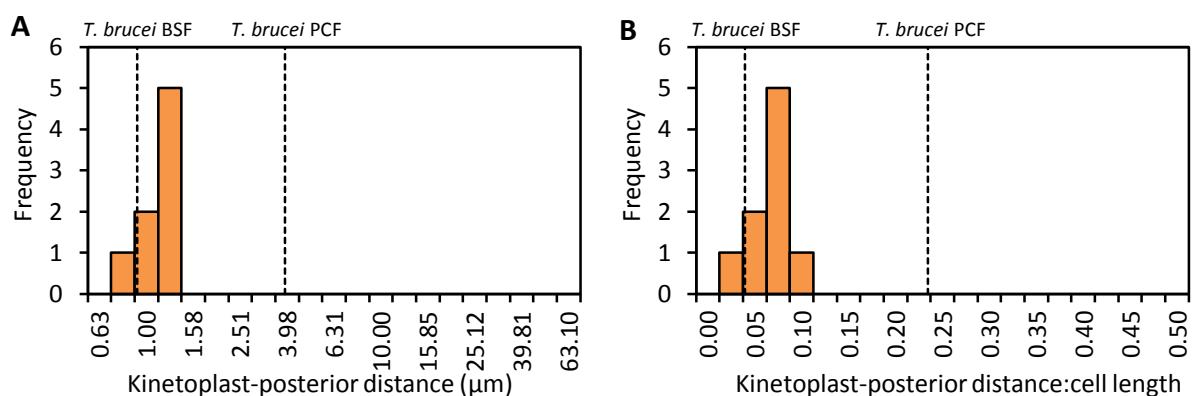


Figure 76. Kinetoplast position in bloodstream forms of African *Trypanosoma* species. Cell body length and posterior–kinetoplast distance were measured from previously published illustrations of isolates of *T. brucei*, *T. evansi*, *T. vivax*, *T. simoae*, *T. congolense*, *T. nanum*, *T. pecorum* and *T. uniforme* (Bruce *et al.*, 1909, 1910a, 1910b, 1910c, 1911a, 1911b, 1912; Bruce, 1911). **A.** Histogram of kinetoplast–posterior distance, $n = 9$. *T. vivax* falls off the left of this plot. **B.** Histogram of the ratio of kinetoplast–posterior to cell body length, $n = 9$. Vertical dashed lines indicate the location of procyclic (PCF) and bloodstream (BSF) form *T. brucei* on these plots.

While a short posterior–kinetoplast distance is not a universal feature of trypomastigote morphology in the bloodstream, illustrations of isolates of mammalian-infective African trypanosomes indicate the kinetoplast is positioned less than 2 μm ($\leq 10\%$ cell body length) from the posterior in many species (Bruce *et al.*, 1909, 1910a, 1910b, 1910c, 1911a, 1911b, 1912; Bruce, 1911) (Figure 76). Based on molecular phylogeny of *T. brucei*, *T. evansi*, *T. vivax*, *T. simiae* and *T. congolense* these African trypanosomes are members of the monophyletic *T. brucei* (Salivarian) clade (Stevens and Gibson, 1999; Hamilton *et al.*, 2004). Members of this clade have a thick surface coat in the bloodstream (Vickerman, 1969a, 1969b), and undergo antigenic variation via their GPI-anchored coat proteins (Cross, 1990). This has been analysed in detail for *T. brucei* which is coated with $\approx 10^7$ variable surface glycoprotein (VSG) molecules, and, at any time, only one variant is expressed from a library of over 1000 VSG genes (which can also be remodelled by recombination) (Barry and McCulloch, 2001; Horn and Barry, 2005; Taylor and Rudenko, 2006). The *T. brucei* VSG coat is internalised and recycled by an exceptionally high rate of endocytosis (Engstler *et al.*, 2004), and clearance of VSG bound immunoglobulins is assisted by hydrodynamic effects (Engstler *et al.*, 2007). This therefore suggests that hydrodynamic-assisted surface protein recycling for bound antibody clearance, assisted by the short posterior–kinetoplast distance, is an adaptation in all of these species to minimise antigenicity of the monotypic surface coat used for antigenic variation and applies a selection pressure to preserve the trypomastigote morphology.

6.3.3 Other effects

I have shown the procyclic *T. brucei* trypomastigote morphology is capable of highly directional motility (Section 4) but whether this spin-stabilisation of swimming also occurs in more diverse trypomastigote morphologies, including bloodstream form *T. brucei*, is not known with certainty. Furthermore in an environment where swimming speeds are slower than fluid flow, i.e. in the bloodstream, the value of spin-stabilisation is unclear. It has been proposed that directional swimming may assist penetration of *T. brucei* into tissue and the crossing of the blood-brain barrier

(Uppaluri *et al.*, 2011), however the selective advantage of this is unclear. The parasite may avoid an immune response in neurological tissue but would be less likely to be transmitted by a typical *Trypanosoma* vector; a blood-feeding invertebrate.

Several proteins are known where RNAi knockdown, deletion or overexpression in procyclic forms causes morphological change. These may be of use in further testing hypotheses concerning function of trypomastigote morphology in *T. brucei*, although phenotypes in bloodstream forms are often a greater survival disadvantage than in the procyclic (see, for example, SCC1-mutAB expression (Gluezn *et al.*, 2008) or PFR2 RNAi (Bastin *et al.*, 1998; Broadhead *et al.*, 2006)). ZFP2-C overexpression causes elongation of the procyclic posterior (Hendriks *et al.*, 2001) and GM6 RNAi causes a more anterior position of the procyclic kinetoplast (Varga *et al.*, Unpublished; Olego-Fernandez, 2010). If these phenotypes could be replicated in the bloodstream form they could be used to test the hypothesis that a short posterior–kinetoplast distance is required for surface protein recycling for bound antibody clearance. An epimastigote-like morphology from GM6 RNAi in the bloodstream form would also allow the testing of whether it is spin-stabilisation of swimming or some other morphology-mediated effect of being trypomastigote morphology which is important in the blood.

Taken together, the near universality of the trypomastigote morphology and its motile flagellum suggest it is vital in the bloodstream. The maximum width and minimum length of the trypomastigote may be constrained by the physical properties (the size of capillaries) in the bloodstream environment (Figure 74). The kinetoplast is consistently positioned near the posterior in the *T. brucei* (Salivarian) clade, consistent with the hydrodynamic-assisted recycling of surface proteins (Engstler *et al.*, 2007). Unfortunately neither of these analyses suggested a selection pressure that would apply to all *Trypanosoma* spp. which could select for trypomastigote (rather than promastigote, epimastigote or amastigote) morphology in the bloodstream. There is therefore

still enormous morphological homogeneity (trypomastigote morphology) yet diversity (size and shape) still to be understood.

6.4 Promastigote diversity and the fly gut environment

Promastigote-like morphologies, like trypomastigotes, have great diversity (Figure 77). As for the trypomastigote understanding the diversity of promastigote-like morphologies will contribute to understanding why they occur. Promastigote-like morphological diversity has, like for *Trypanosoma*, been recorded through identification of new species. A data set covering morphology of 44 isolates of trypanosomatids with promastigote-like morphology from 39 host insects (covering 33 genera) was surveyed from existing literature (Supplementary: Table 2). Parasite dimensions were recorded where parasite cell body and flagellum length were available and were taken from the initial isolate or axenic cultures immediately derived from isolates. All the following plots concerning

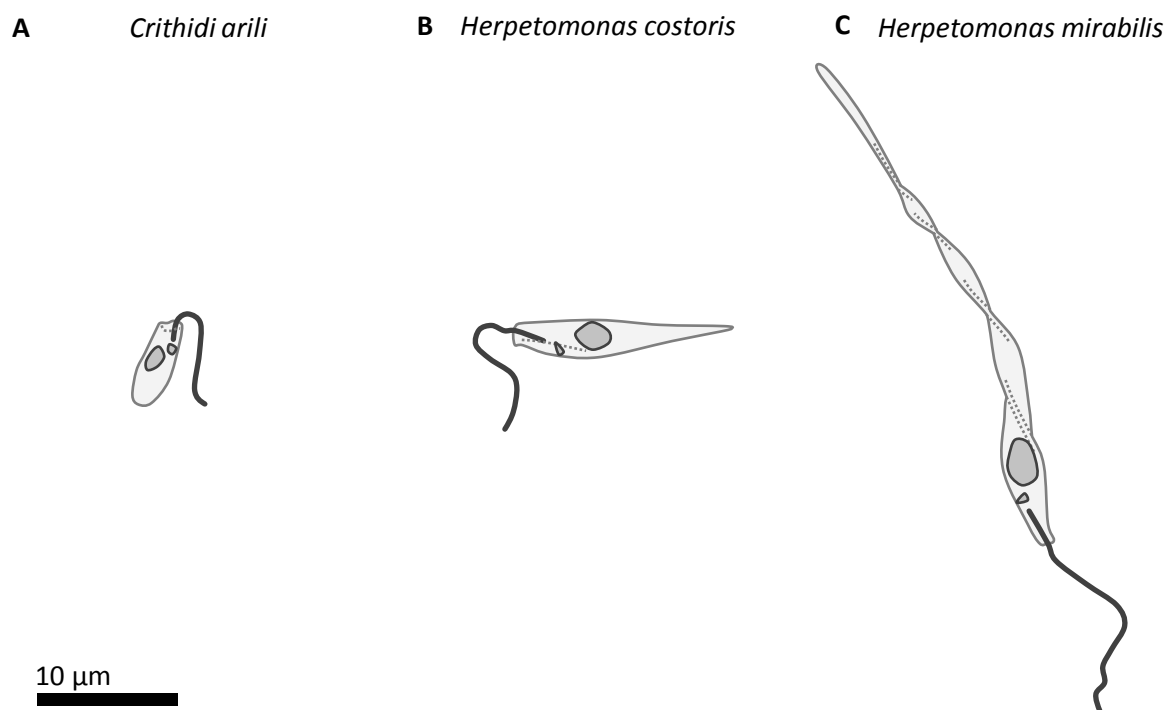


Figure 77. Examples of promastigote-like diversity. Line drawings of three promastigote-like morphologies, representative of extremes. A. *Crithidia arili* from *Arilus cristatus* (McGhee et al., 1969). B. *Herpetomonas costoris* from *Gerris comatus* (Borghesan et al., 2012). C. *Herpetomonas mirabilis* from *Cynomyopsis cadaverina* (Borghesan et al., 2012).

promastigote morphology of different species are derived from these data and n numbers indicate the number of species for which the morphometric data required for the plot was available.

Promastigote-like cell and flagellum length both vary by a factor of 10 and there is little correlation of promastigote-like cell body length with flagellum length (Figure 78). There is therefore a similar magnitude of cell length diversity across species with promastigote-like morphologies in insect hosts as trypomastigote *Trypanosoma* spp. in the bloodstream (Figure 73). The physical environment typically inhabited by promastigote-like morphologies is an arthropod intestine, quantitative descriptions of which (e.g. lumen diameter, length, convolution and typical contents flow rate) are generally not available. Correlative analyses to identify potential selection pressures are therefore not possible. Instead the properties of *L. mexicana* growth and morphogenesis in the cell cycle (Section 2), flagellum length regulation (Section 3) and capacity to swim with different cell body and flagellum lengths (Section 4.2.3) described in this thesis can be used in combination with existing descriptions of *L. mexicana* promastigotes in the sandfly vector (Rogers *et al.*, 2002; Bates, 2007) to analyse potential origins and roles of cell body and flagellum lengths for a mouthparts-transmitted promastigote in the insect host.

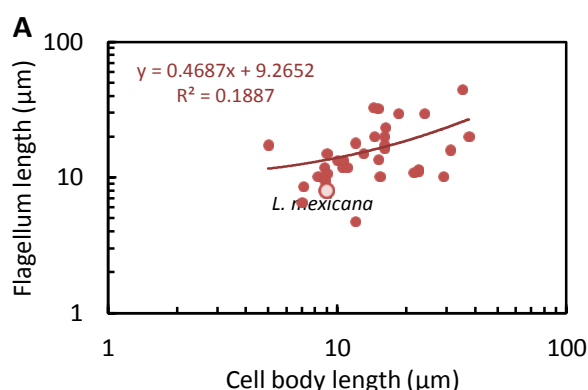


Figure 78. Diversity of cell body length in promastigote-like trypanosomatids. Correlation of cell and flagellum length of flagellate *Leishmania*, *Phytomonas*, *Leptomonas*, *Crithidia* and *Herpetomonas* (promastigote-like) morphologies, $n = 33$. Average axenic promastigote *L. mexicana* cell body and flagellum length (Section 2) (Wheeler *et al.*, 2011) is indicated with an open circle.

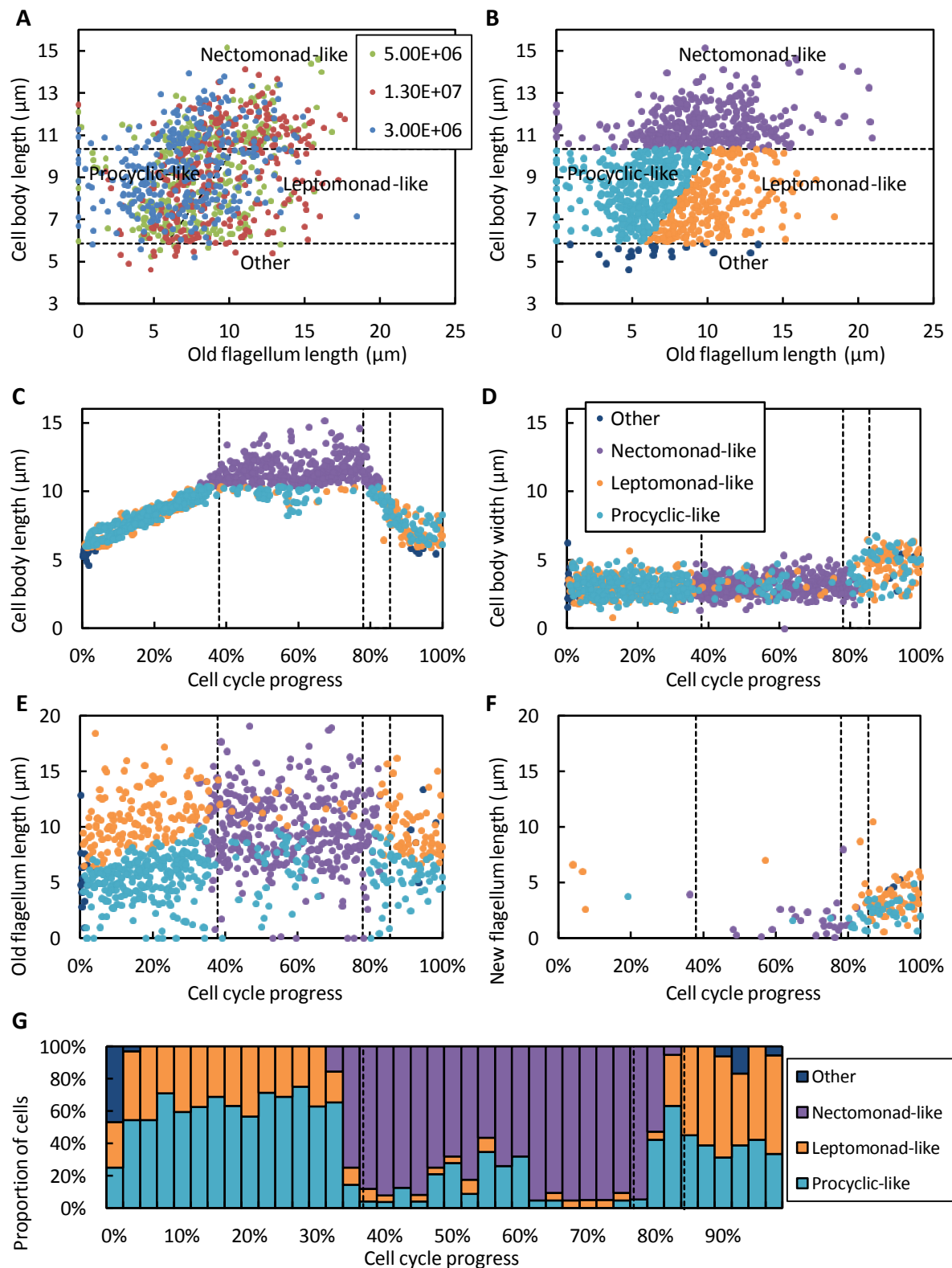


Figure 79. Occurrence of procyclic-, nectomonad- and leptomonad-like promastigote morphologies in axenic promastigote culture. **A**. Correlation of flagellum and cell body length from three logarithmic cultures at different culture densities. This is a re-plot of the data presented in Section 2.2.3. **B**. The data in **A** classified by lifecycle sub-stage morphological group (Rogers *et al.*, 2002), adjusted for 10% parasite expansion in dried-down samples (Minchin, 1909; Minchin and Woodcock, 1912). **C–F**. Correlation of cell body length, cell body width and old and new flagellum length with cell cycle stage, classified by lifecycle sub-stage morphological group. **G**. Proportion of cells of different morphological class by cell cycle progress.

6.4.1 *Leishmania* in the sandfly as a model of promastigote lifestyles

6.4.1.1 Promastigote morphogenesis in the sandfly

The *L. mexicana* promastigote has diverse cell body morphologies in the sandfly gut. It is described as progressing through a series of promastigote life cycle developmental stages (Rogers *et al.*, 2002; Gossage *et al.*, 2003; Bates, 2007), of which three (the procyclic, nectomonad and leptomonad), based on their morphological definitions (Section 1.2.1), can be identified in established, exponentially growing, axenic culture. From this comparison of my data and the existing literature a model of promastigote growth and how these life cycle substages in the sandfly arise can be proposed.

The similarity of cell cycle and some life cycle substage morphologies suggests promastigote morphogenesis in the sandfly may be associated with division (Figure 70). It is difficult to precisely compare samples which have been air dried (sandfly morphometric data (Rogers *et al.*, 2002)) and those which have been wet fixed (axenic morphometric data (Section 2.2.3) (Wheeler *et al.*, 2011)) due to expansion during drying (Minchin, 1909; Minchin and Woodcock, 1912). Nonetheless classification of axenic promastigote cell cycle morphometric data by the definitions of sandfly life cycle substage (Section 1.2.1) showed a significant majority (92.6%) of nectomonad-like cells were in S-phase (Figure 79C–E) and procyclic and leptomonad-like cells were evenly distributed through the G₁ and post-S-phases (Figure 79D–E). The short cell body of dividing parasites would classify them as leptomonad and procyclic-like, consistent with descriptions of the leptomonad and procyclic as proliferative life cycle substages in the sandfly (Gossage *et al.*, 2003; Bates, 2007).

In the sandfly the procyclic and leptomonad *L. mexicana* promastigotes are distinguished by flagellum length. In axenic culture the flagellum grows over multiple cell cycles with growth concentrated in the non-S-phase stages of the cell cycle (Section 2 & 3). Cells with a procyclic-like

morphology in axenic culture have a flagellum which was formed in the previous cell cycle and leptomonad-like cells have an older flagellum. Nectomonad-like cells in culture have always had at least one complete G_1 period of flagellum growth. Taken together my model therefore suggests that the nectomonad represents parasites during S-phase (or, speculatively, parasites stalled in G_2) and the leptomonad and procyclic morphologies represent parasites in other stages of the cell cycle. This model would be simple to test through analysis of the cell cycle stage (i.e. DNA content) of cells with different morphologies from stages of a sandfly infection. The automated morphology and DNA content analysis tools I have developed (Section 5) (Wheeler *et al.*, 2012) could facilitate these analyses.

It is recognised that many of the non-*Trypanosoma* trypanosomatids (both monoxenous and dioxenous) undergo large morphological changes, particularly in cell length, while in a promastigote-like morphology in their host (Dollet, 1984; Yurchenko *et al.*, 2008; Borghesan *et al.*, 2012). If the *L. mexicana* cell cycle is representative of normal promastigote morphogenesis, this diversity in cell length might also be partially explained by progression through the cell cycle (Figure 70).

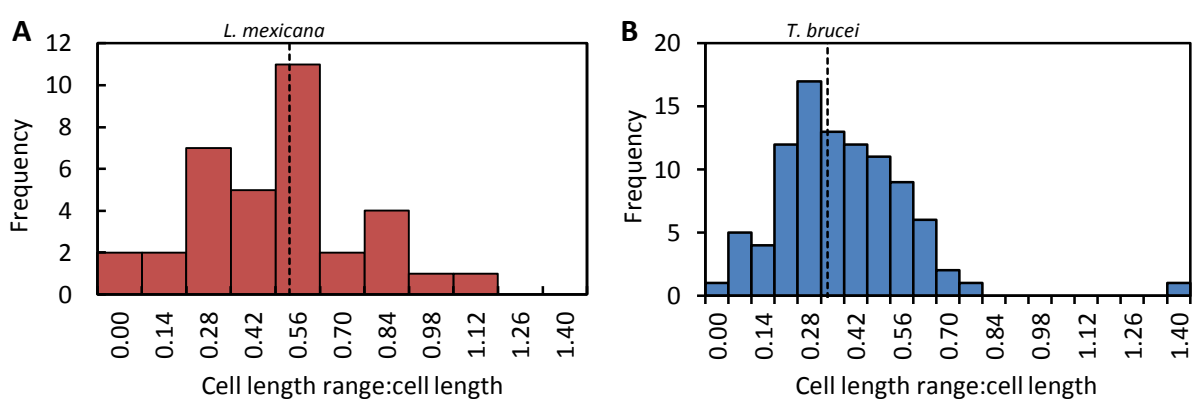


Figure 80. Range of cell body length in promastigote/promastigote-like and trypomastigote morphologies. Histograms of the ratio of reported cell body length range to average cell body length. **A.** Promastigote-like morphologies, $n = 36$. Vertical dashed line indicates the location of proliferating *L. mexicana* promastigotes (Figure 70) (6 to 12 μm , mean 9 μm). **B.** Trypomastigotes (*Trypanosoma*), $n = 93$. Vertical dashed line indicates the location of proliferating procyclic *T. brucei* trypomastigotes (Figure 70) (14 to 20 μm , mean 17 μm).

In the morphometric data of promastigote-like species surveyed from the literature (Supplementary: Table 2) the range of cell body lengths of promastigote-like parasites of a single species is broad (Figure 80A) with an average ratio of cell length range to mean cell length of 0.65 ± 0.38 (mean $\pm$ standard deviation). This is, however, broadly consistent with the range of cell body lengths which can arise during a promastigote cell cycle, based on promastigote *L. mexicana* (Figure 70) where the ratio of cell length range to mean cell length is 0.67. In contrast in the morphometric data of *Trypanosoma* species surveyed from the literature (Supplementary : Table 1) the range of cell body length of parasites from single isolates is narrower than for promastigote-like morphologies (Figure 80B) with an average ratio of cell length range to mean cell length of 0.41 ± 0.20 (mean $\pm$ standard deviation). This is broadly consistent with the range of cell body lengths which can arise during trypomastigote division, based on procyclic *T. brucei* (Figure 70) where the ratio of cell length range to mean cell length is 0.35.

These observations support, albeit weakly, the hypothesis that the different mechanisms of cell body morphogenesis of procyclic *T. brucei* and *L. mexicana* are representative of typical trypomastigote and promastigote morphogenesis respectively (Section 6.2). This opinion is also supported by existing qualitative descriptions of dividing parasites, for example *Leptomonas* (Abe, 1980). Together these also suggest the hypothesis that diversity in cell length of promastigote-like morphologies in an insect may arise (at least in part) from progression of the parasite through the cell cycle is also true in genera other than *Leishmania*.

6.4.1.2 Does promastigote motility drive life cycle progression?

Regardless of the process which generates morphological diversity in *L. mexicana* promastigotes, some mechanism must account for their distribution in the vector. Flagellum lengths tend to be longer in morphologies found towards the anterior of the gut (Rogers *et al.*, 2002; Bates, 2007) and *L. mexicana* promastigotes with a longer flagellum can achieve faster swimming (Section 4) which, coupled with evidence for chemotaxis (Barros *et al.*, 2006), may provide the explanation. A balance of

anterior-directed swimming at a speed proportional to flagellum length and posterior-directed gut contents flow which moves parasites with a short flagellum toward the posterior could sort the population with cells with a long flagellum towards the anterior (Figure 81). Cells with an insufficiently long flagellum may be cleared from the sandfly (Cuvillier *et al.*, 2003). In this model, surface attachment would help maintain the position in the gut a parasite has attained but the value, if any, of the wide range of cell body lengths is not clear.

This biomechanical model of ‘flow sorting’ could be tested using infection of sandflies with immotile *Leishmania* cell lines such as the PFR1 deletion mutant (Maga *et al.*, 1999). These mutants would be expected to be cleared from the sandfly by gut flow, especially if gut surface attachment fails in the absence of motility. Alternatively they may progress through the procyclic, nectomonad and leptomonad life cycle substages normally, but without migration toward the anterior midgut. In either case a productive, transmissible, infection of the sandfly would not be expected. *Leishmania* mutants with increased or decreased flagellum lengths (Wiese *et al.*, 2003; Bengs *et al.*, 2005; Erdmann *et al.*, 2006) might be expected to increase or decrease the capability of generating a productive sandfly infection respectively.

A role for the flagellum in migration toward the anterior of an insect alimentary canal is consistent with the transmission mechanisms and transmissive life cycle morphologies of non-*Trypanosoma* genera. Faecal transmitted species (*Herpetomonas*, *Leptomonas* and *Blastocrithidia*), with the caveat that the life cycles of many species are not known with certainty, are characterised by transmissive amastigotes stages in the hindgut and rectum (Wallace, 1966; McGhee and Cosgrove, 1980) (Figure 69) and the mechanism of generation of these cyst-like amastigotes has been analysed in detail for several species (Romeiro *et al.*, 2000; Kollien and Schaub, 2003; Malysheva *et al.*, 2006). In contrast mouthparts/saliva transmitted species (*Leishmania* and *Phytomonas*) have flagellated transmissive life cycle stages in the anterior gut or salivary glands (Camargo, 1999; Bates, 2007) (Figure 69). Metacyclic *Leishmania*, which are pre-adapted for infection of a mammalian host

(Section 1.2.1), are characterised by a particularly long flagellum. *Phytomonas* lack any amastigote life cycle stage (Camargo, 1999). This trend does not, however, extend to *Trypanosoma* spp.; for example *T. brucei* and *T. cruzi* are saliva and faeces transmitted respectively but the transmissive forms of both species are trypomastigote.

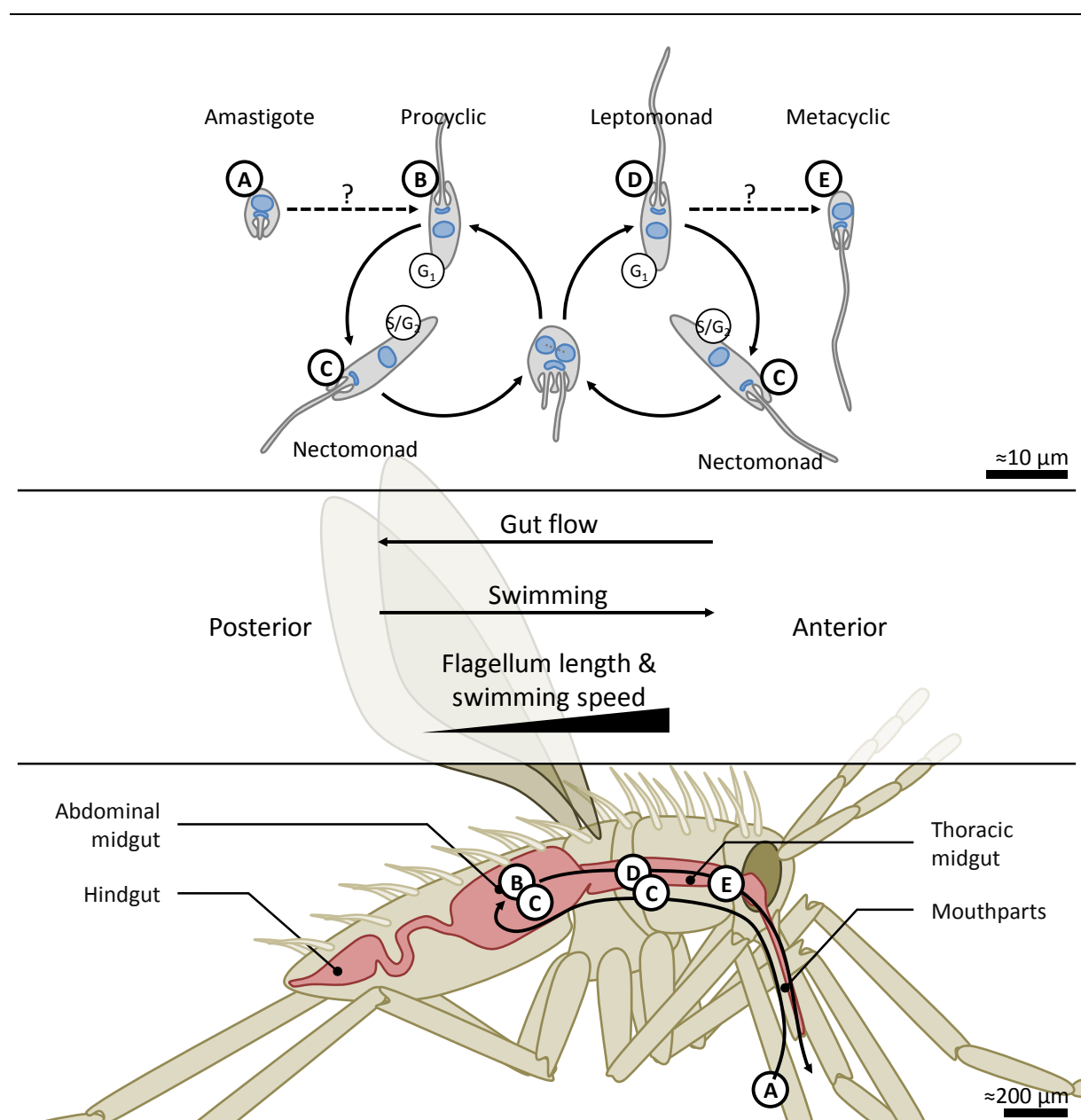


Figure 81. The flow sorting model of *Leishmania* life cycle progression in the sandfly. Top: The mechanism of possible cell cycle-linked morphogenesis of procyclic, nectomonad and leptomonad morphologies. Centre: Hypothesised biomechanical origin of sorting of *Leishmania* life cycle stages. Bottom: Approximate location of different life cycle morphologies in the sandfly (Bates, 2007), adjusted for the possible cell cycle-linked morphogenesis of some life cycle substages.

If longer flagella are universally required for migration of promastigote-like morphologies toward the anterior midgut a more thorough survey from the literature of promastigote-like morphologies and their flagellum lengths incorporating mouthpart/saliva transmitted species would be expected to show mouthparts/saliva transmitted species have longer flagella or broader ranges of flagellum length than faecal transmitted species.

6.4.2 Morphologies associated with surface attachment in the insect gut

Surface attachment in the insect host/vector (which often occurs in the gut or salivary glands) is common in most trypanosomatids (McGhee and Cosgrove, 1980) and is usually mediated via the flagellum. Attachment is associated with formation of hemidesmosome structures which have been described in detail in diverse genera; *Crithidia* (Brooker, 1971), *Leptomonas* (Laugé and Nishioka, 1977), *Trypanosoma* (Molyneux, 1969; Vickerman, 1973; Thévenaz and Hecker, 1980; Beattie and Gull, 1997; Kollien *et al.*, 1998) and *Leishmania* (Killick Kendrick *et al.*, 1974; Killick-Kendrick *et al.*, 1988; Wakid and Bates, 2004). These descriptions all concern epimastigote or promastigote-like morphologies; while the trypomastigote flagellum is capable of adhesion to a surface (its own cell body via the FAZ (Brooks, 1978)) there are no known examples of trypomastigotes adhering to external surfaces.

Trypomastigotes are uncommon as the main proliferative life cycle stages in insect vectors, instead trypomastigotes are normally associated with the forms which establish an initial vector infection (like procyclic *T. brucei*) or are transmissive forms (like metacyclic *T. cruzi* and *T. brucei*) (Section 1.1.1). Proliferation of trypomastigotes in insects certainly can occur, for example procyclic *T. brucei* is replicative (Sharma *et al.*, 2009), but there may be a selection for morphologies capable of surface attachment for the main replicative stages.

6.5 Conclusions

The combination of over a century of surveying trypanosomatid diversity, the insights of modern molecular phylogenetics and the new insights from the 21st century cell biology presented in this thesis allowed some early tests of hypotheses concerning the role of the range of morphologies trypanosomatid cells attain through their life cycles. Trypanosomatids are diverse in morphology and *Trypanosoma* appear to be under a mechanical selection pressure in the bloodstream to have width constrained by host capillary dimensions. They also appear to have a minimum length constraint through an unknown selection pressure. No single morphological selection pressure could be identified which would favour the trypomastigote over other trypanosome morphologies in the blood environment although hydrodynamic-assisted recycling of surface proteins appeared to select for a short kinetoplast–posterior distance in the *T. brucei* clade. Cell body morphological diversity in species with promastigote-like morphology appear to arise, in part, from morphogenesis through the cell cycle while flagellum range attained may be linked with ability to migrate towards the gut anterior. Occurrence of flagellar attachment to gut surfaces by different trypomastigote morphologies and occurrence of the amastigote in the life cycle are consistent with these hypotheses. My work has therefore provided a foundation for understanding the basic biology of *Leishmania* and *T. brucei*, contributed to interpretation of molecular biology results in these species and allowed speculation on the selection pressures which have given rise to different trypanosomatid morphologies.

7 Methods

7.1 Trypanosomatid culture

7.1.1 Cell lines and *in vitro* culture

Promastigote *L. (L.) m. mexicana* (WHO strain MNYC/BZ/62/M379) (referred to as *L. mexicana*) were grown in M199; M199 medium with Earle's salts and L-glutamine (Life Technologies 31100-027) supplemented with 9.1% v/v heat inactivated South American origin foetal calf serum (FCS) (Life Technologies 10500-064), 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH 7.4), 26 mM NaHCO₃ and 5 µg/ml haemin, at 28°C. Axenic amastigote form *L. mexicana* were grown in SM5.5; Schneider's *Drosophila* medium (Life Technologies 21720-024) supplemented with 18.2% v/v heat inactivated South American origin FCS and 25 mM 2-(N-morpholino)ethanesulfonic acid (MES)-HCl (pH 5.5), at 34°C with 5% CO₂.

Promastigote *L. (L.) major* (Friedlin strain V) (referred to as *L. major*) were grown in M199.

Procyclic *T. brucei* (Lister 427) were grown in SDM79 medium with 9.1% v/v heat inactivated foetal calf serum (PAA, A30411) and 5 µg/ml haemin at 28°C (Brun and Schönenberger, 1979). Bloodstream form *T. brucei* were grown in HMI9 medium with 9.1% v/v heat inactivated foetal calf serum and 5 µg/ml haemin at 37°C with 5% CO₂ (Hirumi and Hirumi, 1989).

Choanomastigote *C. fasciculata* and promastigote *P. davidii* were grown in BHI; brain heart infusion (Sigma Aldrich) supplemented with 4.5% v/v heat inactivated South American origin FCS, at 28°C.

All trypanosomatids were maintained by repeated subculture at culture densities where logarithmic growth occurred; between 1×10⁵ and 2×10⁷ cells/ml (*Leishmania* and procyclic *T. brucei*), under 1×10⁶ cells/ml (bloodstream form *T. brucei*) or 1×10⁵ and 1×10⁸ cells/ml (choanomastigote

C. fasciculata and promastigote *P. davidii*). Culture density was measured with a CASY model TT cell counter (Roche Diagnostics) with a 60 µm capillary and exclusion of particles with a pseudodiameter below 2.5 µm as debris.

7.1.2 *In vitro* Leishmania differentiation

Promastigote *L. mexicana* was prepared for *in vitro* differentiation to axenic amastigotes by subculture to 1×10^5 cells/ml followed by 5 days incubation or subculture 1×10^6 cells/ml followed by 4 days incubation (stationary phase promastigote cells). *In vitro* differentiation to axenic amastigotes was induced by subculture to 1×10^6 cells/ml in SM5.5. If the transferred volume of culture was less than 10% of the total culture volume then subculture was performed by dilution into the destination medium. Otherwise cells were harvested by centrifugation and resuspended in the destination medium.

7.2 Mutant trypanosomatid cell lines

Mutant trypanosomatid cell lines were maintained in the presence of the appropriate selection drug(s) (Figure 82). Prior to use in an experiment cells were maintained in the absence of the selection drug(s) for at least 48 h.

	<i>L. mexicana</i> promastigote	<i>T. brucei</i> procyclic
G418	40 µg/ml	15 µg/ml
Hygromycin B	32 µg/ml	50 µg/ml
Puromycin	20 µg/ml	1 µg/ml
Phleomycin	25 µg/ml	5 µg/ml
Doxycycline	n/a	1 µg/ml

Figure 82. Concentrations of drugs for supplementation of *L. mexicana* and *T. brucei* media. Final concentration of drugs used to supplement liquid culture (Barker, 2009).

7.2.1 *Leishmania* cell lines

The *L. mexicana* PFR1 deletion mutant, $\Delta pfr1::NEO \Delta pfr1::HYG$, (Maga *et al.*, 1999) was previously generated by replacement of the diploid PFR1 loci (which consist of a tandem arrays of four PFR1 coding sequences) with neomycin phosphotransferase (*NEO*, G418 resistance) and hygromycin B phosphotransferase (*HYG*, hygromycin B resistance) respectively. As previously described the PFR1 deletion mutant has defective swimming ability (Gadelha *et al.*, 2005).

The *L. mexicana* eGFP-tagged SMP1 cell line, SMP1::eGFP::TY (Gluezn, Unpublished), was previously generated using pLENT (Gluezn, Unpublished). SMP1::eGFP::TY was constitutively expressed from the endogenous locus, flanked by the endogenous 5' untranslated region (UTR) and the 3' *C. fasciculata* phosphoglycerate kinase A UTR. The cell line was maintained under selection with phleomycin.

7.2.2 *T. brucei* cell lines

Expression of non-cleavable SCC1 mutant allele (SCC1-mutAB) (Gluezn *et al.*, 2008) was induced in procyclic *T. brucei* using pDEX-377 (Kelly *et al.*, 2007) by culture in the presence doxycycline for 48 h. Expression of SCC1-mutAB had a dominant negative effect on segregation of chromosomes during mitosis causing disruption of cell division, as previously described (Gluezn *et al.*, 2008). The cell line was maintained under selection with G418.

RNAi degradation of GM6 (product of the transcripts of Tb11.57.0008 and Tb11.47.0036) in procyclic *T. brucei* was induced using p2T7 (Wickstead *et al.*, 2002) in the 2913 cell line (Wirtz *et al.*, 1999) targeting the conserved repeat region (GM6-like repeats) of the gene by culture in the presence of doxycycline for 72 h. RNAi against this target changed the trypomastigote procyclic *T. brucei* cell morphology to a more epimastigote-like morphology (Olego-Fernandez, 2010). The cell line was maintained under selection with hygromycin B, G418 and phleomycin.

7.3 Mammalian cells and culture

7.3.1 Mammalian cell lines

Raw 264.7 (murine leukaemic cell line, ATCC® Number TIB-71) macrophages were grown in RPMI; RPMI 1640 with L-Glutamine (PAA E15-840) supplemented with 9.1% v/v heat inactivated South American origin FCS, at 34°C with 5% CO₂.

Adherent mammalian cell lines were maintained by regular subculture (using a cell scraper to detach cells) at confluencies between 20% and 90% on a surface area of 75 cm² in 50 ml growth medium, corresponding to approximately 1×10⁵ to 5×10⁵ cells/ml. Density of cells following detachment from the growth surface was assessed with a Neubauer improved haemocytometer.

7.3.2 Bone marrow-derived macrophages

7.3.2.1 Generation of BMDMs

Femuri, humeri and tibiae were dissected from C57BL/6 mice, sterilised by immersion in 70% ethanol for 30 s then transferred to 4°C PBS where connective and muscle tissue was removed with forceps. The ends of the bones were removed with scissors then the bone marrow was flushed out using a syringe and 26 G needle with DMEM; high glucose GlutaMAX™ DMEM (Dulbecco's modified eagle medium) (4.5 g/l glucose) (Life technologies 10566-016) supplemented with 9.1% v/v heat inactivated South American origin FCS.

Bone marrow was homogenised by passage through a 70 µm cell strainer, centrifuged (800 g, 10 min) then resuspended in 100 µl/bone erythrocyte lysis buffer; 155 mM NH₄Cl, 10 mM KHCO₃, 125 µM EDTA, and incubated at room temperature for 2 min. Unlysed cells were washed twice by centrifugation (800 g, 10 min) and resuspension in 30 ml DMEM. Bone marrow cells were finally resuspended to a density of 5×10⁵ cells/ml in MCSF-DMEM; 72.7% v/v high glucose GlutaMAX™ DMEM and 18.2% v/v L929 conditioned DMEM. L929 is a murine fibrosarcoma cell line which

produces macrophage colony stimulating factor (MCSF) which induces differentiation of monocytes in the bone marrow into macrophages.

Bone marrow cells were differentiated into macrophages then frozen. 1.2×10^7 (25 ml) bone marrow cells in MCSF-DMEM were placed on a 14 cm diameter non-culture treated Petri dish then incubated for 6 days at 37°C with 5% CO₂. The dish was washed with 8 ml 4°C PBS, incubated in 8 ml PBS at 37°C for 5 min, resuspended in a final volume of 16 ml PBS, supplemented with 2 ml FCS and centrifuged (800 g, 10 min). The pellet was resuspended in FCS with 10% v/v dimethylsulfoxide (DMSO) then frozen at -80°C overnight and transferred to -196°C.

Macrophages were defrosted and allowed to complete differentiation before use in experiments. Samples were defrosted by incubation in a 37°C water bath and diluted to 5×10^5 cells/ml by gradual addition of pre-warmed MCSF-DMEM then placed on a non-culture treated Petri dish and incubated at 37°C for 2 days. The growth medium was then replaced with DMEM and the cells incubated for a further 2 days at 34°C. At this point differentiation was complete and the cells were maintained in DMEM with replacement of the growth medium with fresh DMEM every 2 to 3 days.

7.3.2.2 Confirmation of monocyte differentiation into macrophages

Differentiation of monocytes into macrophages was confirmed by immunofluorescence. Live cells were incubated for 20 min with one of 5 rat IgG2b antibodies; Alexa Fluor 647 anti-GR1 (clone RB6-8C5, BioLegend 108420), Alexa Fluor 488 anti-CD11b (clone M1/70, BioLegend 101219), Alexa Fluor 488 anti-F4/80 (clone BM8, BioLegend 123120), Alexa Fluor 647 isotype control (BioLegend RTK4530) or Alexa Fluor 488 isotype control (BioLegend RTK4830) diluted 1:50. Cells were then washed twice with growth medium and observed by light microscopy (Section 7.5) (Figure 83), the expected staining pattern for differentiated macrophages was GR1 negative and CD11b and F4/80 positive.

7.3.3 *In vitro* infection of macrophages with *Leishmania*

BMDMs or Raw 264.7 macrophages were resuspended and placed in a 35 mm glass coverslip-bottomed imaging plate (MatTek Corporation, P35G-1.5-10-C) with 2.5 ml growth media at 2.5×10^5 cells/plate and incubated at 37°C for at least 1 h to allow the macrophages to reattach. Exponentially growing *Leishmania* were then added at the desired MOI.

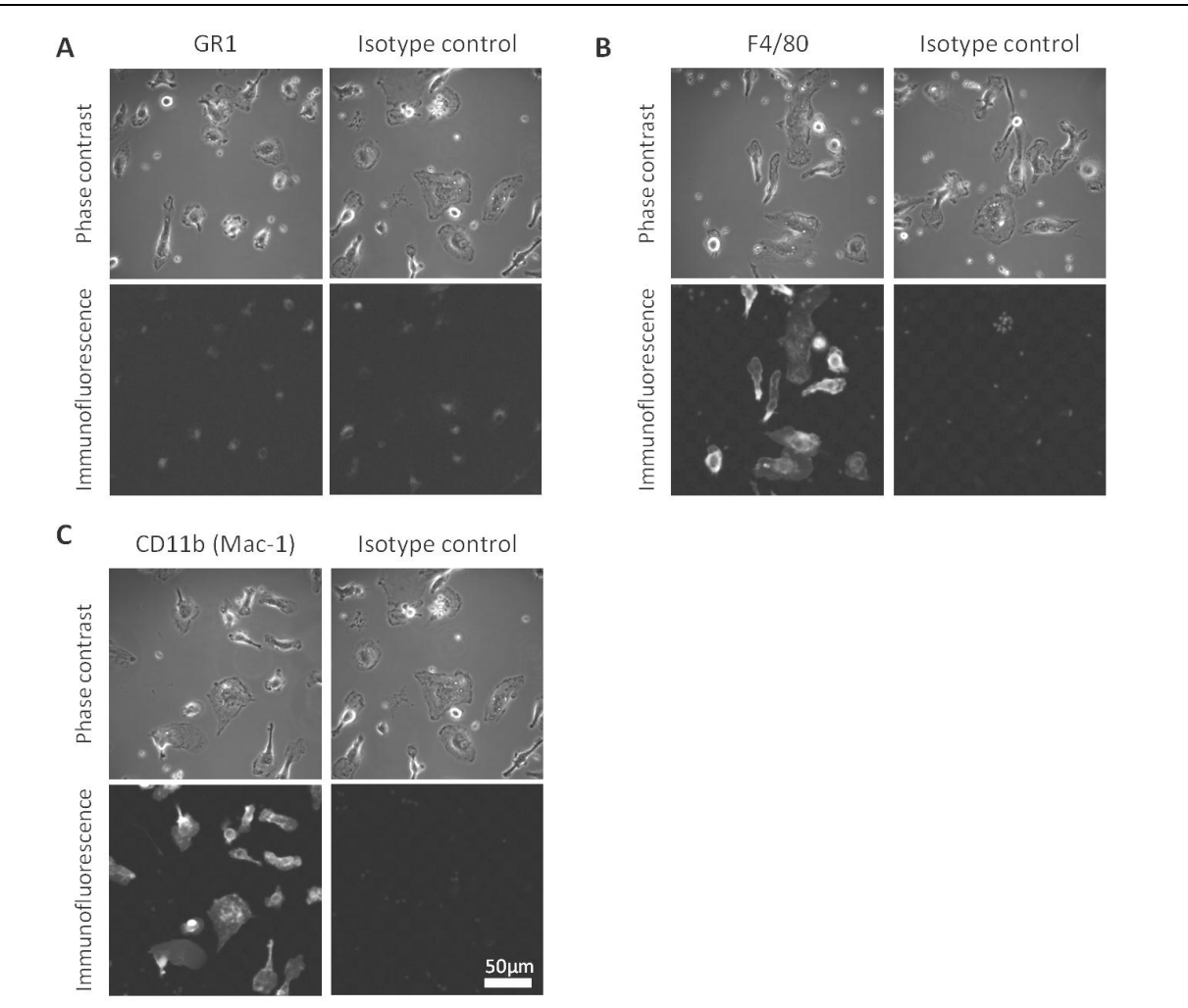


Figure 83. Confirmation of differentiation of bone marrow monocytes into macrophages. Live cell immunofluorescence of markers of macrophage differentiation. A. GR1. B. F4/80. C. CD11b.

7.4 Sample preparation for immunofluorescence

7.4.1 Sample preparation for trypanosomatid cells

5×10^6 to 1×10^7 cells trypanosomatid cells during logarithmic growth were harvested from culture medium by centrifugation (3000 g, 3 min). Cells were washed twice by resuspension in 0.5 ml PBS and centrifugation (3000 g, 3 min) followed by resuspension in PBS to a final volume of 50 μ l. The cell suspension was applied to a 1 cm² square on either a silanized slide (*Leishmania*) or a normal glass slide (other species) outlined using a fat pen (DakoCytomation S2002). Cells were allowed to settle onto the slide for 10 min and from this step onwards slides were contained in a humid chamber to prevent drying. 50 μ l 4% v/v paraformaldehyde in PBS was added to the slide (final concentration of 2% paraformaldehyde) and incubated at room temperature for 15 min. Finally slides were washed three times by immersion in PBS.

7.4.2 Sample preparation for adherent mammalian cells

Adherent mammalian cells were grown on 13 mm diameter number 1.5 coverslips in wells of a 24 well plate. Coverslips were washed three times by immersion in 0.5 ml PBS then fixed by addition of 0.5 ml 4% v/v paraformaldehyde in PBS. Finally the coverslips were washed three times by immersion in 0.5 ml PBS.

7.4.3 Immunofluorescence staining

Slides were stained by addition of 100 μ l of staining solutions to the slide. Coverslips were stained by immersion in 200 μ l staining solution. For immunofluorescence samples were incubated at room temperature for 1 h with the primary antibody/antibodies. Samples were washed 3 times by immersion in PBS then incubated at room temperature for 1 h with the secondary antibody/antibodies. Finally the samples were washed a further 3 times by immersion in PBS.

Primary antibodies used were anti-PFR2, L8C4 (murine IgG1) used 1:50 in PBS (Kohl *et al.*, 1999); anti- α tubulin, TAT-1 (murine IgG2b) used 1:10 in PBS (Woods *et al.*, 1989); anti- β tubulin, KMX used 1:10 in PBS (Birkett *et al.*, 1985); anti-tyrosinated α tubulin, YL1/2 (Rat IgG) used 1:50 in PBS (Kilmartin *et al.*, 1982; Wehland *et al.*, 1984); anti-GFP (rabbit IgG) (Life Technologies (Molecular Probes), A11122) used 1:200 in PBS. The appropriate secondary antibody was selected from the following; fluorescein isothiocyanate polyclonal rabbit anti-mouse IgG (DakoCytomation F0261), fluorescein isothiocyanate donkey anti-rat IgG (Jackson ImmunoResearch 712-095-153) or rhodamine isothiocyanate goat anti-mouse IgG (Jackson ImmunoResearch 115-025-166), all used 1:100 in PBS.

DNA was then stained by immersion in 1 μ g/ml DAPI in water for 2 min followed by three washes by immersion in PBS. Samples were then mounted in glycerol-DABCO; glycerol with 1% w/v 1,4-diazabicyclo[2.2.2]octane (DABCO) and 10% v/v 50 mM sodium phosphate, pH 8.0. The coverslip was sealed to the slide using nail varnish.

7.4.4 Labelling of synthesising DNA

Newly synthesised DNA in *Leishmania* was labelled by incorporation of modified nucleotides; live cells were incubated in growth medium supplemented with 50 μ M 5-bromo-2-deoxyuridine (BrdU) and 50 μ M 2-deoxycytidine for the desired incorporation period. Cells were prepared for microscopy on slides or coverslips (Section 7.4.1) and the samples were fixed by addition of 0.1% paraformaldehyde and incubation at room temperature for 15 min, followed by three washes by immersion in PBS. The cells were then further fixed by immersion in -20°C methanol for 15 min followed by rehydration by three immersions in PBS. Finally DNA in the sample was denatured by incubation in 1.5 M HCl for 30 min at room temperature, followed by 5 washes by immersion in PBS.

Anti-BrdU immunofluorescence staining was performed (Section 7.4.3) with anti-BrdU primary antibody, PBR-1 (mouse IgG1) (Life Technologies A21308) used 1:100 in PBS supplemented with 0.5%

v/v Tween 20 (Sigma Aldrich) and 0.5% w/v bovine serum albumen. The samples were then stained with DAPI and mounted in glycerol-DABCO (Section 7.4.3).

7.5 Light microscopy

Unless otherwise indicated light microscopy samples were observed on a DM5500 B (Leica Microsystems) upright epifluorescence microscope with an Orca (Hamamatsu Photonics) cooled CCD camera. Objectives used were either 10× NA 0.40 HC plan apochromat (Leica Microsystems 11506285), 40× NA 1.30 HCX plan apochromat oil immersion (Leica Microsystems 11506330) or 100× NA 1.40 HCX plan apochromat oil immersion (Leica Microsystems 11506211). Image capture was performed with either LAS AF (Leica Microsystems) or µManager (Edelstein *et al.*, 2010). For analyses requiring a large number of images of cells unbiased sampling was ensured by capturing in a regular grid. Images were analysed using ImageJ (Collins, 2007). Measurement of signal intensities and object sizes was performed with the measurement tools in ImageJ and, where necessary, automated using the ImageJ scripting language.

7.6 Flow cytometry of trypanosomatid cells

2×10^7 cells were harvested from culture medium by centrifugation (1800 g, 3 min), washed twice by resuspension in 1 ml PBS and centrifugation (1800 g, 3 min) followed by resuspension in PBS to a final volume of 100 µl and fixed by addition of 100 µl 0.5% v/v paraformaldehyde in PBS and incubation at room temperature for 5 min. 2 ml 4°C ethanol was added dropwise while gently vortexing the sample then incubated at 4°C for 30 min. The fixed and permeabilised cells were harvested by centrifugation (800 g, 5 min), resuspended in 4 ml PBS with 40 µg/ml propidium iodide and 100 µg/ml RNase A then incubated at 37°C for 30 min. Samples were stored at 4°C prior to analysis and were analysed with a FACSCalibur flow cytometer (BD Biosciences).

7.7 Scanning electron microscopy

Leishmania samples for scanning electron microscopy (SEM) were prepared on 13 mm diameter number 1.5 coverslips cleaned by incubation at room temperature in 1 M HCl for 10 min, three washes with distilled water and one with acetone. Coverslips were then incubated with 4% v/v 3-aminopropyltrimethoxysilane in acetone for 5 min, washed with acetone and allowed to dry.

1.2 ml logarithmic culture was incubated with 2.5% v/v glutaraldehyde for 2 h, shaking, at room temperature. Fixed cells were harvested by centrifugation (500 g, 10 min), washed three times with 1.5 ml PBS and resuspended in a final volume of 1 ml PBS. The cell suspension was allowed to settle onto the silanized coverslip for 1 h. The adhered cells were then dehydrated by successive incubation for 5 min at room temperature in distilled water ×3, 30%, 50%, 70% then 90% v/v ethanol in distilled water and finally desiccated ethanol ×3. The samples were then critical point dried, sputter coated with gold then observed with a JSM-6390 SEM (JEOL).

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9 Appendix

9.1 List of supplementary material

Supplementary: Script 1

Script 1 – HTIAoT.txt (text/plain)

Automated image analysis scripts (for use in ImageJ) for colour deconvolution and automated analysis of colour deconvolved images of kinetoplastids. These are in the form of seven separate tools: image setup, measurement of chromatic aberration, correction of chromatic aberration, measurement of K/N signal, colour deconvolution, K/N counting and summarising the K/N count results.

Supplementary: Table 1

Table 1 - Trypanosoma.xlsx (application/vnd.openxmlformats-officedocument.spreadsheetml.sheet)

Morphologies of diverse *Trypanosoma* species. Complete data set of *Trypanosoma* morphologies and their references and host erythrocyte dimensions.

Supplementary: Table 2

Table 2 - Erythrocytes.xlsx (application/vnd.openxmlformats-officedocument.spreadsheetml.sheet)

Host erythrocyte morphologies. Complete data set of host erythrocyte dimensions and their references.

Supplementary: Table 3

Table 3 - Promastigote-like.xlsx (application/vnd.openxmlformats-officedocument.spreadsheetml.sheet)

Morphologies of diverse promastigote-like species. Complete data set of non-*Trypanosoma* trypanosomatid morphologies and their references.

Supplementary: Video 1

Video 1 - Time-lapse Division Compressed.avi (video/x-msvideo)

Time-lapse of individual *L. mexicana* cells progressing through the cell cycle. Videomontage of four cells progressing through the cell cycle from time-lapse observation of *L. mexicana* cells immobilised in small fluid pockets in agarose. Still frames from cells 2 and 4 are shown in Figure 15. The four videos were realigned in time so all cells progress through the cell cycle approximately synchronously. Scale bar represents 10 µm.

Supplementary: Video 2

Video 2 - Leishmania Uptake Compressed.avi (video/x-msvideo)

Dynamics of flagellum disassembly in macrophages visualised with SMP::eGFP::TY. Videomicrograph of native eGFP and rhodamine-dextran fluorescence from a time-lapse videomicrograph of a single BMDM infected with promastigote *L. mexicana* SMP::eGFP::TY. Still frames are shown in Figure 33. Times indicates time since addition of promastigotes to the BMDMs. Scale bar represents 20 µm.