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REVIEW ARTICLE

Development in the Chemotherapy of Infectious Diseases Caused by Intracellular Pathogenic Protozoa: Trypanosoma and Leishmania

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Abstract: *Trypanosoma brucei* are protozoan parasites that cause the lethal human disease African sleeping sickness and the economically devastating disease of cattle, Nagana. African sleeping sickness, also known as Human African Trypanosomiasis (HAT), threatens 65 million people and animal trypanosomiasis makes large areas of farmland unusable. There is no vaccine and licensed therapies against the most severe, late-stage disease are toxic, impractical and ineffective. Trypanosomes are transmitted by tsetse flies, and HAT is therefore predominantly confined to the tsetse fly belt in sub-Saharan Africa. They are exclusively extracellular and they differentiate between at least seven developmental forms that are highly adapted to host and vector niches. In the mammalian (human) host they inhabit the blood, cerebrospinal fluid (late-stage disease), skin, and adipose fat. In the tsetse fly vector they travel from the tsetse midgut to the salivary glands *via* the ectoperitrophic space and proventriculus. Trypanosomes are evolutionarily divergent compared with most branches of eukaryotic life. Perhaps most famous for their extraordinary mechanisms of monoallelic gene expression and antigenic variation, they have also been investigated because much of their biology is either highly unconventional or extreme. Moreover, in addition to their importance as pathogens, many researchers have been attracted to the field because trypanosomes have some of the most advanced molecular genetic tools and database resources of any model system. The following will cover just some aspects of trypanosome biology and how its divergent biochemistry has been leveraged to develop drugs to treat African sleeping sickness. This is by no means intended to be a comprehensive survey of trypanosome features. Rather, I hope to present trypanosomes as one of the most fascinating and tractable systems to do discovery biology.

Keywords: *Trypanosoma brucei*, trypanosome, kinetoplastid, parasite, African sleeping sickness, trypanosomiasis, chemotherapy, cell biology, life cycle, tsetse fly.

1. INTRODUCTION

The kinetoplastids are a highly diverse class of parasitic and free-living protozoa and have more than a billion years of evolutionary distance from humans [1]. So-called because of the condensed and concatenated 'kinetoplast' mitochondrial DNA, they include important human pathogens such as *Leishmania* spp (causes Leishmaniasis), *Trypanosoma cruzi* (causes Chagas disease) and *Trypanosoma brucei* (causes African sleeping sickness). Their evolutionary success is reflected in their ability to colonise an extraordinary diversity of eukaryotic lineages [2]. They inhabit plants (*Phytomonas* spp.), fish (*Cryptobia* spp.), insects (*Crithidia fasculata* and *Paratrypanosoma confusum*) [3] and are even found in a mutualistic relationship with amoeba (*Perkinsela*) [4, 5]. Moreover, free-living bodonid kinetoplastids, such as *Bodo saltans*, are found in a wide array of marine, fresh-water and soil habitats [2].

African sleeping sickness, or human African trypanosomiasis (HAT), is a lethal disease caused by two subspecies of *T. brucei* - *T. b. gambiense* and *T.b. rhodesiense*. African trypanosomes are transmitted between human hosts *via* the tsetse fly and are therefore constrained to the tsetse fly belt in sub-Saharan African. The first signs of infection are usually after 2-3 days, with a chancre at the site of the tsetse bite and inflamed lymph nodes at the back of the neck ("Winterbottom's sign") [6]. The first stage of disease is characterised by intermittent fever and headaches and is often overlooked. The second stage of disease is more serious and involves a disruption of the circadian rhythm that causes the sleeping disorder from which the disease is named. Other neurological symptoms include shaking hands, limb oscillatory tremors, severe behavioural changes and emotional instability. The final stage of the disease is coma, followed by death [7-9].

T. b. rhodesiense causes by far the most serious disease and can progress to the second-stage disease within a few weeks and death within 6 months. In contrast, *T. b. gambiense* causes a chronic disease lasting ~3 years, although up to 30 years until the first onset of clinical symptoms has been reported [10]. This suggests that there may be a significant, largely overlooked asymptomatic population that could complicate elimination of the disease [11]. Moreover, trypanosomiasis is a zoonotic disease [12] and causes Nagana in cattle, an economically devastating disease that reduces meat and milk yields and contributes to keeping rural farmers poor [13].

2. TRYPANOSOME CELL SHAPE IN DIFFERENT DEVELOPMENTAL STAGES

Trypanosome cells are lachrymiform, or teardrop-shaped, 18-32 μm long and 1.5-3 μm in width at their widest point, with the posterior being blunter than the anterior [14, 15] (Fig. **1**). The nucleus is either round or elongated and occupies the majority of the central volume of the cell. Their most prominent feature, the flagellum, is bound to the cell body for most of its length and describes a 180° left-handed turn around the body as viewed from the anterior pole [16]. At its proximal, end the flagellar basal body is

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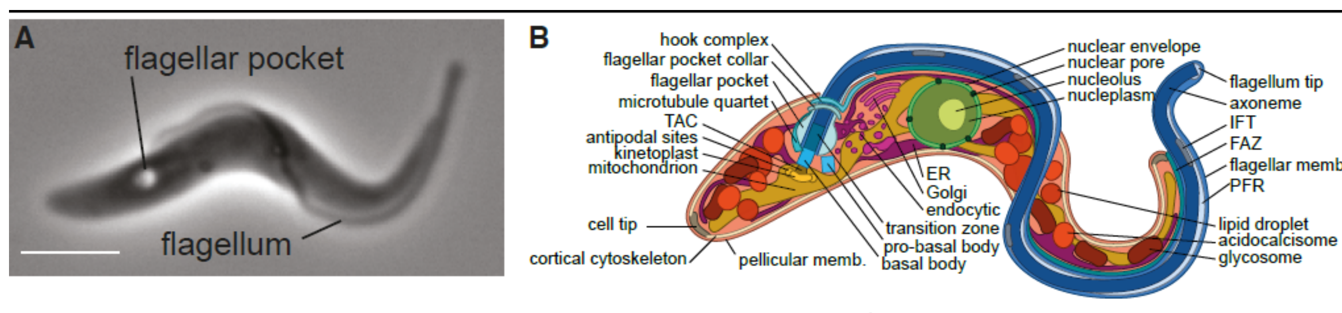

Fig. (1). Shape; Trypanosomes are lacriform. A. A phase contrast micrograph of a procyclic form trypanosome cell. Note the flagellum that is attached to the cell body for most of its length, and the phase-bright flagellar pocket. B. A cartoon of a procyclic trypanosome cell depicting most of the organelles and intracellular structures. Scale bar = 5μm. Cartoon courtesy of Richard Wheeler. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Table 1. African trypanosome lifecycle stages.

Lifecycle Stage	Habitat	Salient Features	Major Surface Protein
Slender	Blood, skin, adipose fat, organs, cerebrospinal fluid.	Proliferative, trypomastigote.	VSG
Stumpy	Blood, skin, adipose fat, organs, cerebrospinal fluid.	Cell-cycle arrested, trypomastigote, pre-adapted for differentiation and survival in the tsetse midgut.	VSG
Procyclic	Midgut lumen, peritrophic matrix.	Proliferative, trypomastigote.	Procyclin (EP1, EP2 and GPEET)
Mesocyclic	Ectoperitrophic space, proventriculus.	Cell cycle arrested, trypomastigote.	Procyclin.
Epimastigote (long and short)	Proventriculus and salivary gland epithelium.	Proliferative, epimastigote.	Brucei Alanine Rich Surface Protein (BARP)
Gamete	Salivary glands.	Haploid, undergoes cell fusion.	Unknown.
Metacyclic	Salivary gland epithelium, saliva.	Cell cycle arrested, trypomastigote, pre-adapted for survival in blood through expression of VSG.	Metacyclic VSG

attached to the kinetoplast and embedded in the cytoplasm. At its distal end, the flagellum is 'free' and extends a short distance beyond the anterior of the cell body.

Trypanosomes are digenetic parasites that alternate between a mammalian host and a tsetse fly vector. There are at least seven different developmental forms (Table **1**) that are exquisitely adapted to distinct niches within the host and vector. Most trypanosome forms are defined as "trypomastigote" and the flagellum emerges from the cell posterior to the nucleus. In the 'epimastigote' stage, the kinetoplast and flagellum are positioned anterior to the nucleus [17]. The dimensions of the cell body, flagellum attachment to the cell body and the free flagellum change in the different lifecycle stages such that the insect stages are longer and thinner. This is likely an adaptation to swimming in different environments so that the bloodstream forms are more highly adapted to overcoming the obstacle-rich blood and the insect forms swim with greater velocity to traverse the tsetse anatomy [16, 18, 19].

3. LIFE IN THE MAMMALIAN HOST

3.1. Slender and Stumpy Forms

There are two bloodstream trypanosome stages that are morphologically, biochemically and functionally distinct.

The slender form is the only proliferative stage in the blood and is therefore responsible for maintaining the infection (Table **1**). At high parasitaemia, the slender form exits the cell cycle in G1 and differentiates to the stumpy form [20, 21]. This cell cycle arrest limits the parasitaemia and preserves the lifespan of the host, thus increasing the chance of transmission.

The process whereby slender forms differentiate to stumpy forms has been the subject of intensive research for many years. Early work demonstrated that trypanosomes secrete a heat labile, low molecular weight factor that stimulates slender to stumpy differentiation [21, 22]. However, the difficulties in culturing and performing reverse genetics in trypanosomes able to differentiate to stumpy forms (termed pleomorphic cells) meant that the molecular components of trypanosome quorum sensing remained cryptic for many years. Recently, genome-wide RNAi libraries were used to identify the signalling pathway components and discovered the kinases and phosphatases that transduce the differentiation triggers [23] (Table **2**). Subsequent work identified a G-protein-coupled receptor, TbGPR89, related to bacterial oligopeptide transporters. TbGPR89 is a slender form specific surface receptor that receives the slender-stumpy differentiation signal [24]. The quorum sensing signal itself was shown to be a mixture of oligopeptides generated from a secreted protease. Interestingly, this quorum sensing signal causes cell cycle arrest in the cattle pathogen *Trypanosoma congolense*, suggesting cross-talk in mixed-species coinfections [25].

Stumpy forms are preadapted for survival and lifecycle progression in the tsetse midgut [26-31] (Table **1**). Whereas slender forms have a reduced mitochondrion and rely exclusively on glycolysis to generate energy, stumpy forms are metabolically pre-adapted to the glucose-depleted conditions of the tsetse midgut and have an elaborated mitochondrion in order to perform oxidative phosphorylation [28, 29]. Stumpy forms also express a family of PAD transporters on their cell surface that allows hypersensitivity to the differentiation trigger, citrate [32, 33] (Table **2**). Moreover, stumpy forms differentiate to insect stages in response to conditions

Table 2. Important proteins in African trypanosome bloodstream forms.

Protein	Function	Reference
VSG	VSG is a surface protein with key roles in innate and adaptive (complement) immune evasion.	Reviewed [47]
TLF1 and TLF2	Human complex that contains ApoL1, the pore-forming trypanocidal protein.	[59]
ApoL1	Human protein with trypanocidal activity.	[60]
HpHbR	Trypanosome uptake of haem and TLF1.	Reviewed [55]
SRA	Resistance to TLF in *T. b. rhodesiense.*	[66]
TgsGP	Resistance to TLF in *T. b. gambiense.*	[77]
TbGPR89	Receiving slender-stumpy differentiation trigger.	[24]
SIF response pathway	Intracellular proteins required for trypanosome quorum sensing.	[23]
PAD1	Reception of stumpy-procyclic differentiation trigger.	[32]

that rapidly lyse slender forms, such as proteases and mild acid [34, 35]. These conditions mimic the insect midgut and mean that only stumpy forms are transmissible *via* tsetse flies.

3.2. Bloodstream form Trypanosome Tissue Tropism

Unlike many of their kinetoplastid cousins, African trypanosomes are exclusively extracellular. Their link with African sleeping sickness and cattle diseases has been established for >100 years through the use of microscopy of blood smears [15]. Diagnosis of the late stage disease, after which trypanosomes cross the blood-brain barrier and are present in the cerebrospinal fluid, was done using lumbar puncture [6]. Moreover, parasitaemia assays on murine infection models are performed using blood from tail snips combined with microscopy or PCR-based methods. This focus on trypanosomes in the blood may explain why significant populations of parasites elsewhere have been largely overlooked until recently.

Trypanosomes are first inoculated into the dermis where they rapidly establish a population [36]. The presence of many trypanosomes in the skin [37] suggest that these parasites may be an important reservoir of transmissible parasites. Unlike mosquitoes that feed directly from the blood vessel, tsetse flies are telmophagus feeders and slash the skin before drinking the blood so residing in the skin may increase the chances of transmission. Importantly, skin trypanosomes have been found in sero-negative, undiagnosed individuals, demonstrating that the skin and blood should be examined when testing for trypanosome infection [11, 37].

Trypanosomes were recently shown to accumulate in murine adipose tissue soon after inoculation [38]. Given that the adipose tissue is not accessible to the tsetse fly bite, the impact of adipose tissue trypanosomes upon transmission is not clear. However, trypanosomes adapt to this environment by upregulating genes associated with fatty acid oxidation [38] and their presence could be related to the extreme weight-loss that is associated with trypanosome infection [8]. Moreover, the fatty tissue could represent an immune-privileged habitat for the trypanosomes to 'hide' from adaptive and innate immunity. Therefore, understanding more about this population could be key for understanding trypanosome pathogenesis.

In future, it will be important to determine the dynamics of trypanosome blood, skin and adipose tissue populations to understand their role in maintaining chronic infections and increasing transmissibility.

3.3. Host Immune Evasion

Their obligate extracellular lifestyle presents the bloodstream form African trypanosome with a set of challenges through which it has evolved some magnificent solutions. In the blood the trypanosome is continually exposed to the innate and adaptive immune system. Trypanosomes express a highly immunogenic variant surface glycoprotein (VSG) on their plasma membrane [39] (Table **2**). The VSG coat creates an effective barrier between the plasma membrane and complement factors to prevent parasite lysis by the complement alternative pathway [40, 41].

The VSG is highly immunogenic and stimulates a strong antibody response. At low antibody titres, the trypanosome cell removes antibodies from its surface by endocytosing the VSG-antibody complex. The complex is routed *via* the late endosomal vesicles, and possibly the lysosome, where the antibody is removed and the VSG is recycled to the cell surface [42, 43]. The efficiency of this process is facilitated by hydrodynamic flow forces that 'sweep' the antibody to the posterior of the cell where the flagellar pocket endocytic organelle resides [44, 45] (Fig. **2**).

At high titre, antibody clearance from the cell surface is insufficient and they become a target for recognition by macrophages [46, 47]. To avoid recognition by host antibodies, trypanosomes 'switch' expression to a new VSG isotype. This new isotype has different antigenicity and allows the parasite to continue to proliferate until a new antibody response is initiated and the cycle starts again. Trypanosomes express a single VSG isotype on their cell surface, but the potential repertoire encoded in their genome is vast [48, 49]. They are able to switch between 15 different telomeric VSG 'expression sites' (ES) [50] that each encodes a different VSG isotype. The active VSG open reading frame can be replaced by homologous recombination with one of the hundreds of non-ES VSG alleles to further increase the VSG availability and even make entirely new, chimeric VSG genes [51]. Most of the existing VSG repertoire is 'revealed' to the host early in the infection and new VSG mosaics become important as the infection progresses [52].

Demonstrating the long evolutionary co-existence of humans and trypanosomes, humans have evolved a trypanolytic mechanism that is effective against most African trypanosome species. Hence, trypanosomes use ApoL1 'hidden' inside different fractions of trypanosome lytic factor (TLF) [53]. Well studied and often controversial, there are two distinct types of TLF: TLF1 and TLF2. Haptoglobin-related protein (HPR) in TLF1 is bound by the trypanosome Haptoglobin-Haemoglobin receptor (HpHbR) in the parasite flagellar pocket and then internalised by endocytosis [54, 55] (Table **2**). The mechanism of entry of TLF2 is much less clear but may involve the HpHbR, low affinity binding to VSG or an as-yet unidentified receptor [55-58]. ApoL1 present in TLF is then trafficked to the lysosome where the low pH environment causes it to undergo a conformation change and form a pore. The lysosome

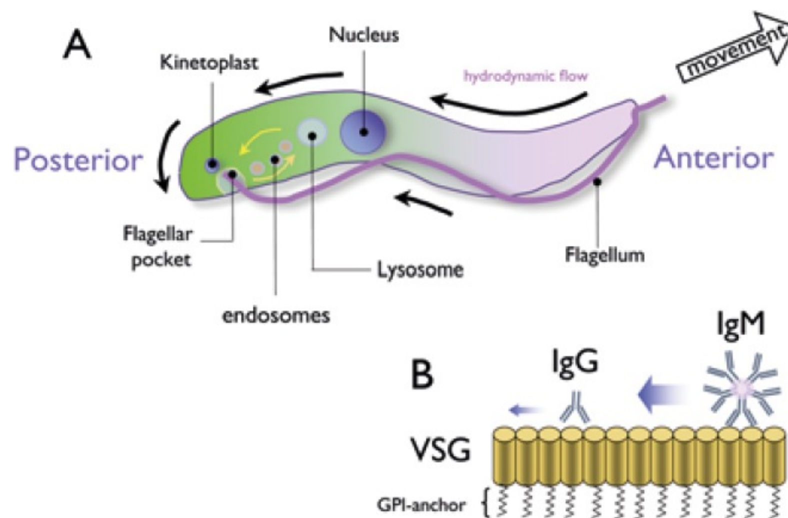

Fig. (2). Hydrodynamic flow: Antibodies are cleared from the cell surface by hydrodynamic flow forces. A. Anterior-directed motion of the parasite generates hydrodynamic flow forces that direct surface-bound antibody toward the posterior of the cell. The VSG-antibody complex is then internalized via the flagellar pocket for endocytosis and antibody removal. B. VSG (represented as vertical pillars) is attached to the parasite membrane via a glycosylphosphatidylinositol (GPI) anchor, forming a homogenous coat on the parasite surface. The GPI anchor enables free migration within the lipid bilayer, facilitating molecular flow. Antibodies bind to the VSG and are sorted via hydrodynamic flow forces, with larger molecules (IgM) moving more rapidly than smaller molecules (IgG), as indicated by the relative arrow size. Figure adapted from [44]. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

then swells through osmotic shock and kills the trypanosome through release of lysosomal proteases or *via* mechanical disruption [54, 55, 59-61]. ApoL1 may also promote trypanolysis by trafficking to the mitochondrion [62] or plasma membrane [63] where its pore forming activity kills the cell.

The two human infective trypanosome species have evolved different mechanisms to overcome ApoLI.

T. b. rhodesiense expresses a truncated VSG called serum resistance antigen (SRA) from its VSG locus [64-66] (Table **2**). Predominantly present in the endocytic system and the lysosome [60, 67-69], SRA directly binds and blocks the pore-forming activity of ApoLI in the lysosome [60, 70]. The SRA gene is essential for *T. b. rhodesiense* resistance to TLF and expressing SRA in TLF-sensitive parasites renders them resistant to lysis by TLF *in vitro* and in human serum [65, 68, 71].

T. b. gambiense does not encode SRA and instead utilises several complementary TLF resistance strategies. They express a downregulated and mutated HpHbR receptor that has reduced affinity for TLF1 and therefore reduces the uptake of ApoL1 that is present in lipid-rich fractions of TLF1 [72, 73]. This mechanism is complemented by the expression of TgsGP, a *T. b. gambiense*-specific truncated VSG [74, 75] that localises to the flagellar pocket and is necessary for resistance to TLF2 [76, 77] (Table **2**). The precise mechanism of TgsGP-conferred resistance to human serum is not fully understood but may involve imparting 'stiffness' to the trypanosome flagellar pocket membrane [78]. Although TgpGP expression is essential for *T. b. gambiense* resistance to human serum, expressing TgsGP in *T. b. brucei* does not confer resistance to TLF meaning that other factors also play a role [76-78].

4. LIFE IN THE TSETSE FLY VECTOR

There are at least five different developmental forms in the tsetse and each occupies a specific compartment of the insect (Table **1**).

4.1. Migration through the Tsetse Fly

Upon biting of an infected individual, the blood meal is taken up first into the tsetse crop and then into the tsetse midgut lumen where nutrients are extracted and digested. Slender forms are rapidly killed in the gut [79-81] and stumpy forms re-enter the cell cycle and differentiate to procyclic forms [20]. The nature and molecular mechanisms of differentiation have been the subject of intensive research and many of the pathways and triggers are now known. The *in vivo* triggers of differentiation are likely to be a combination of gut proteases, temperature drop and Kreb's-cycle intermediates in the blood-meal [32, 33, 82-85].

Upon establishment of a successful mid-gut infection, procyclic cells must cross the peritrophic matrix (PM) in order to access the ectoperitrophic space. The PM is an effective anti-parasite filter and represents a major hurdle for the trypanosomes to overcome. Comprised predominantly of chitin fibres cross-linked to structural glycoproteins [86], the PM is continuously secreted from the proventriculus [18, 86, 87] and forms a trilaminate 'sheath' with a folded and complex topology. The trypanosome genome does not encode chitinases [88] and a receptor that might facilitate crossing has not yet been found, meaning that trypanosomes are unlikely to cross the mature PM at the midgut. Instead, trypanosomes appear to cross the immature PM at the proventriculus before it hardens and while it is still fluid [89-91] (Fig. **3**). PM crossing is an active process requiring a flagellar-membrane localised phosphodiesterase, PDEB1, demonstrating an important role for the flagellar cAMP signalling pathway in tsetse colonisation [92].

In the ectoperitrophic space, trypanosomes enter G2 cell-cycle arrest, become elongated and transform to mesocyclic cells [18, 93]. Mesocyclic cells undergo a dramatic asymmetric cell division that produces a long and short 'epimastigote' [93]. The organelles and morphology of the epimastigote forms are arranged such that the kinetoplast (bound to the flagellum before it emerges from the flagellar pocket) is anterior to the nucleus [93-95] and the flagellum extends substantially beyond the anterior of the cell body. The fate of the long epimastigote is not known but, given that the short epimastigote lacks efficient forward propulsion, it has been suggested that it may facilitate migration of the short epimastigote to the salivary glands during asymmetric cell division [18]. Once at the mouth parts, the flagellum of the short epimastigote attaches to the epithelium of the salivary gland brush border by elaborate outgrowths and hemidesmosome-like attachment plaques [17, 96, 97].

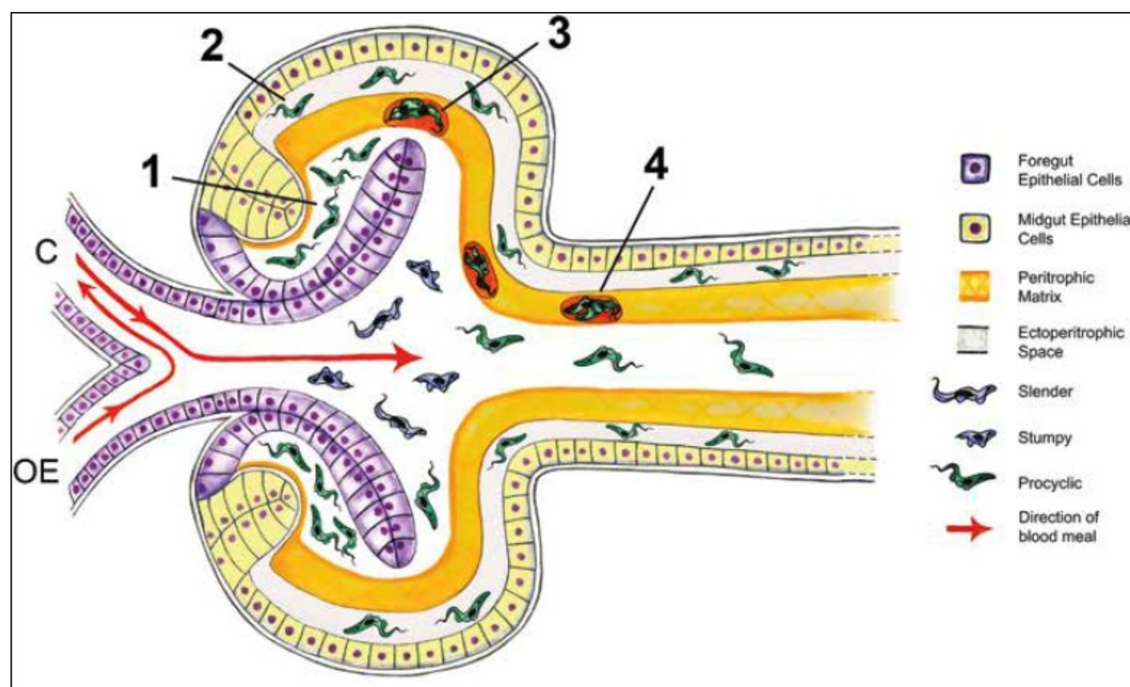

Fig. (3). Peritrophic matrix: Trypanosomes cross the immature peritrophic matrix at the proventriculus. Specialised epithelial cells in the proventricular annular pad are responsible for PM (orange) assembly and secretion. Ingested bloodstream trypanosomes (blue) transform into procyclic forms (green) within the proventricular lumen (1) and then successfully migrate to the ES through a more fluid PM in the proventriculus (2) before it maturates into a fully assembled structure as seen in the midgut. Alternatively, they may become trapped between PM layers forming cyst-like bodies (3). Parasites trapped between PM layers are carried (as on a conveyor belt) through the midgut as the PM continues to be secreted (4). OE and C represent direction of the blood flow from either the oesophagus or crop, respectively. Cartoon made by Laura Jeacock and adapted from [91]. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Salivary gland invasion appears to be a continuous process through the life of the tsetse which may promote colonisation of the salivary glands by different strains [98].

During the early stages of salivary gland colonisation, epimastigotes divide to form two identical daughter epimastigotes. In the later stages of tsetse infection, attached epimastigotes undergo another asymmetric cell division to form one daughter epimastigote and one daughter trypomastigote [99]. This "pre-metacyclic" cell undergoes further development to become the mature metacyclic cell [96, 97]. The metacyclic is cell-cycle arrested in G1 and pre-adapted for resistance to host serum by expressing a metacyclic-specific VSG on its cell surface. Subsequent release into the tsetse saliva allows the metacyclic cell to be inoculated into the vertebrate dermis [36] upon tsetse biting where it re-enters the cell cycle and differentiates to the slender form, thus continuing the trypanosome lifecycle.

The entire tsetse part of the trypanosome lifecycle can take up to three weeks [17, 100]. Once the trypanosome has successfully colonised the salivary glands the tsetse remains infective for the rest of its lifespan (up to 4 months) [101, 102].

Although largely overlooked in recent years, it has long been appreciated that trypanosomes can be transferred between hosts by tabanids or by *via* mechanical transmission of proliferative blood forms in the tsetse blood meal [103-106]. It will be interesting to investigate the relative contribution of these two distinct modes of disease transmission.

4.2. Sex

One of the earliest suggestions that trypanosomes had sex was by Muriel Robertson who suggested it happens upon reaching the tsetse salivary glands [80, 107]. However, it took nearly one hundred years to formally demonstrate that epimastigotes mated while attached to the tsetse salivary gland epithelium [108].

Genetic crosses in tsetse demonstrated that trypanosomes segregated their chromosomes according to classical Mendelian inheritance and suggested a role for meiosis [109-114]. The early meiotic stages were subsequently identified in epimastigotes attached to salivary glands by expressing conserved meiotic proteins fused to fluorescent proteins as markers [115]. Further investigation identified haploid gamete stages that were able to fuse and mix their cytoplasm [116]. Trypanosome gametes have a distinctive morphology with the nucleus in the extreme posterior and either one or two kinetoplasts [116]. Meiosis and gamete production appears to be an integral part of the trypanosome lifecycle. It starts as soon as epimastigotes colonise the salivary glands and continues throughout the life of the tsetse [98, 108, 115, 116].

Although gamete fusion is common in mixed salivary gland infections and appears to occur continuously [98], the mechanism of gamete fusion is still unknown. Cellular fusion has been demonstrated in other lifecycle stages and may well be a normal part of trypanosome biology [117]. However, this fusion is temporary does not involve the exchange of genetic material and there are likely to be fundamental mechanistic differences. Experimental trypanosome crosses have shown that inheritance of kinetoplast DNA is from both parents [118-120], meaning that the gametes' inner and outer mitochondrial membranes must either undergo fusion or the mitochondria must be broken down and reformed around the new kinetoplast DNA. Similarly, the gametes' nuclei must also either fuse or be reformed during trypanosome sex. To accommodate cellular fusion and mixing of genetic material, there must also be elaborate re-modelling of the sub-pellicular microtubule cage that underlies the plasma membrane. The flagellum has been proposed to play a role in bringing gametes into close proximity [116] and the presence of a HAP2 (a highly conserved surface protein that mediates gamete fusion) ortholog in the trypanosome genome [88] may provide future avenues for research.

Table 3. Biochemical surveys and proteomes performed on African trypanosomes using mass spectrometry (MS).

Organelle, Structure or Fraction	Description	Lifecycle Stage	Refs.
Acidocalcisome	MS using purified acidocalcisomes.	Procyclic	[274]
Whole cell, cell-cycle	Single step elutriation quantitative ten-plex tandem mass tag (TMT) proteomics.	Procyclic	[275]
Whole cell	SILAC quantitative MS comparison using bloodstream and procyclic form whole cells.	Bloodstream (slender and stumpy) and procyclic	[276]
Whole cell	SILAC comparative MS comparison using bloodstream and procyclic forms.	Bloodstream and procyclic	[277]
Flagella connector	MS using immunopurified flagella connectors.	Procyclic	[278]
Flagellum	MS using flagella purified using high salt and detergent.	Procyclic	[143]
Flagellum	MS using flagella purified using high salt and detergent.	Procyclic	[279]
Flagellum	MS using flagellum surface and matrix fractions from isolated flagella.	Bloodstream	[273]
Flagellum	iTRAQ quantitative MS between parental and mutant flagella isolated prepared using high salt and detergent.	Bloodstream	[280]
Flagellum	MS using flagella isolated using mechanical shearing.	Procyclic	[281]
Flagellum transition zone	MS using immunopurified flagellum transition zones.	Procyclic	[155]
Glycosome	SILAC labelling, purification by IP followed by MS.	Procyclic	[256]
Mitochondrial outer membrane	MS using purified mitochondrion outer membrane fractions.	Procyclic	[282]
Mitochondrial methylarginine proteins	MS using purified mitochondria to identify methylated arginine proteins.	Procyclic	[283]
Mitochondrion	MS using purified mitochondria.	Procyclic	[284]
Nucleus	MS using purified nuclei.	Procyclic	[285]
Whole cells, palmitoylated proteins	MS using palmitoylated proteins purified using biotinylation and streptavidin.	Procyclic	[254]
Paraflagellar rod	MS comparing flagella isolated from paraflagellar rod mutants and parental cells using two-dimensional difference gel electrophoresis and isobaric tags.	Procyclic	[169]
Whole cell phospho-proteins	MS identification of phosphorylated proteins from bloodstream from cells.	Bloodstream	[244]
Whole cell phospho-proteins	SILAC comparative MS of SCX and TiO2 enriched phosphopeptides.	Bloodstream and procyclic	[245]
Whole cell, stumpy form differentiation	Label-free MS using stumpy cells differentiating over 48 hours.	Slender, stumpy at different differentiation timepoints, and procyclic	[286]
Cell surface	Surface labelling, affinity purification, and MS to identify cell surface proteins.	Bloodstream and procyclic	[287]
Cell surface	Surface labelling, affinity purification, and MS to identify cell surface proteins.	Bloodstream	[288]
Glycosome	Comparative MS of glycosomes between lifecycle stages.	Bloodstream and procyclic form	[255]
Glycosome	MS on proteins and lipids of glycosomal membranes from *Leishmania tarentolae* and *Trypanosoma brucei.*	Bloodstream and procyclic	[289]
Glycosome and mitochondrion	MS comparison of differential expression of glycosomal and mitochondrial proteins between lifecycle stages.	Bloodstream and procyclic	[257]

Although gametocytogenesis is continuous in infected tsetse salivary glands [98], trypanosome sex is not an obligate part of the lifecycle. Gamete fusion and genetic exchange within a clone is much rarer than inter-clonal mating [121, 122] suggesting there is a mechanism that avoids 'selfing' through the use of mating types [123]. Furthermore, although *T. b. gambiense* is still able perform meiosis, genetic crosses in the lab have been unsuccessful [121] and genomic population studies suggest that group 1 *T. b. gambiense* reproduces clonally [124, 125].

5. CELLULAR FEATURES / ORGANELLES

Proteomics, genomics, transcriptomics and microscopy have revealed huge amounts of information about trypanosome organelle structure, composition and function (Table **3**). Furthermore, the genome-wide protein tagging project, TrypTag [126], has tagged almost all trypanosome proteins with a fluorescent protein and provided their localisation using microscopy; this will provide key insights into trypanosome cell biology and identify new cohorts of proteins that can be functionally investigated.

Their evolutionary position and parasitic lifestyle mean that trypanosomes have many fascinating aspects to their cell biology. The following overview of trypanosome organelles and cellular features is therefore not intended to be exhaustive and necessarily misses out on elements of trypanosome biology. Rather, the intention is to highlight aspects that are exemplary or characteristic of the trypanosome as a parasite.

6. SUB-PELLICULAR MICROTUBULES

Underlying the trypanosome pellicular membrane is a microtubule 'corset' that runs along the long axis of the cell and gives the trypanosome its lachrymiform shape [16, 127]. The microtubules run parallel to each other, describe a helical path and are orientated with their positive ends pointing toward the anterior pole [128, 129]. Each microtubule remains 18-20nm from the adjacent, parallel microtubule and increases in the diameter of the cell are accommodated by the addition of new microtubules [129, 130]. This close spacing is smaller than the expected diameter of a trafficking vesicle and prevents endo- and exo-cytosis at the pellicular membrane. Despite the seeming rigidity of the microtubule cage, the trypanosome body tolerates a degree of bending as it swims and can squeeze through holes smaller than its relaxed diameter [16, 131]. The microtubule array may facilitate this by adjusting its chirality and spacing to allow the trypanosome to penetrate into different host and vector tissues.

The sub-pellicular microtubules are cross-linked to each other and the overlying membrane by microtubule associated proteins [129, 132-135]. These proteins organise the microtubules into a single layer and ablating them causes severe cytokinesis defects. Several microtubule-associated proteins have stage-regulated paralogues that have arisen through gene-duplication events. The reasons for these duplications are not clear and in at least one case they demonstrate functional redundancy *in vitro* [134]. The different paralogs may instead be important for cytoskeleton remodelling during differentiation or may have specific *in vivo* functions.

A set of four microtubules termed the "microtubule quartet" (MtQ) is embedded within the corset and runs antiparallel [127] to the other sub-pellicular microtubules. The MtQ is nucleated between the basal body and pro-basal body [136], encircles the flagellar pocket [137] and runs alongside the FAZ underneath the flagellum to the anterior pole of the cell body where it terminates. The MtQ is closely apposed to the endoplasmic reticulum [39, 138]. Several MtQ components have been identified using proteomics and the TrypTag project [126, 136, 139] and the MtQ appears to have important functions in organising cytoskeletal structures and maintaining the flagellar pocket shape [136, 139].

7. FLAGELLUM

The trypanosome flagellum exhibits the conserved structural features of eukaryotic flagella and also has some lineage-specific innovations (Fig. **4**). The flagellum functions in different parts of the lifecycle include motility, host-parasites interaction and adherence to the tsetse salivary gland epithelial villi [17, 39, 140]. Importantly, flagellum function is essential for establishing and maintaining a mammalian infection and for migrating through the tsetse fly vector [141-146].

Trypanosomes have a single flagellum and a new flagellum is assembled alongside the old once each cell cycle as part of a highly orchestrated sequence of events [127, 128]. It is assembled by intra-flagellar transport (IFT), a kinesin and dynein transport system that imports components from the cytoplasm and adds them to the distal, growing end of the flagellum. The flagellum grows to a defined length before it 'locks' and further growth is stopped [147, 148]. The precise mechanism of the grow and lock model is not known but it requires the presence of the conserved protein CEP164C at the flagellum base and is likely to involve negative regulation of IFT function [148].

7.1. Flagellum Basal Body and Pro-Basal Body

The flagellum is nucleated by the flagellar basal body, a microtubule organising centre that contains nine triplets of microtubules in a circular arrangement (Fig. **4**). Electron microscope cross sections of the basal body reveal the classic SAS6 'cartwheel' and a set of fibres that link the basal body microtubules (the 'acorn') [149]. The basal body is tethered to the pro-basal body, an immature basal body that matures during cell division and nucleates a new flagellum. Transitional fibres radiate from the distal basal body triplets and anchor the flagellum to the flagellar pocket membrane. Transitional fibres also act as docking sites for IFT particles and are a platform for flagellum biogenesis and regulatory proteins [149-151].

At the distal basal body-transition zone boundary lies the terminal plate, so-called because it is the point at which one of the triplet microtubules terminates [149]. The terminal plate does not have a function ascribed but in cross-sections of paramecium basal bodies, it is evident as a ring connected to the outer microtubules [152] and the *Tetrahymena* plate has holes that IFT particles may pass through [153].

7.2. Flagellar Transition Zone (TZ)

The TZ extends ~350nm beyond the basal body, contains nine outer doublets with no central pair ("9 + 0") and is the structural intermediate between the basal body and the axoneme proper (Fig. **4**). The trypanosome TZ is a highly stable and partitioned structure that has key functions in building the flagellar axoneme [154, 155]. It templates the growth of the axonemal outer doublets and acts as a boundary separating the flagellar membrane from the rest of the cell's surface. The distal half of the TZ has "Y linkers" that extend from the outer doublets to the TZ membrane and are thought to contribute to a diffusion barrier that helps to maintain the distinct composition of the flagellar membrane [156].

A collarette with nine-fold symmetry spans the proximal half of the TZ [137, 157-159] and decorates the outside of the TZ membrane; this likely explains the rows of intramembrane particles that are observed in freeze-fracture electron microscopy as a 'ciliary necklace' [160]. Nine radial fibres enter the flagellum and underlay the proximal 80% of the TZ membrane [137]. The lumen of the TZ appears to be homogenous and translucent apart from an electron-dense filamentous structure [157]. The TZ doublets appear to be connected and short fibres extend into the TZ lumen [137]. The function of these structures is not known but is likely related to the TZ's important functions in axoneme biogenesis and maintaining the distinct composition of the flagellum.

7.3. Flagellar Axoneme

Beyond the TZ lies the axoneme. The axoneme is characterised by nine outer microtubule doublets with a central pair of microtubules ("9 + 2") that is embedded in the electron dense basal plate at the TZ-axoneme boundary [161] (Fig. **4**). The basal plate is highly conserved in motile flagella and it is essential for nucleating the flagellum central pair [154]. Electron dense outer and inner dynein arms on the outer doublets provide the mechanochemical energy that causes the flagellum to beat [162]. Radial spokes extend from the outer doublets to the central pair and associated structure and together coordinate the action of the dynein arms to ensure an efficient flagellum beat [163].

7.4. Paraflagellar Rod (PFR)

The PFR is a paracrystalline structure ~150 nm in diameter [164] that runs alongside the axoneme. It starts after the flagellum exits from the flagellar pocket and terminates just before the end of the flagellum. Electron microscope cross-sections reveal that the PFR is made of three distinct domains (proximal, intermediate and distal, defined with respect to the axoneme) (Fig. **5**). These domains are made up of filaments of different thicknesses in different

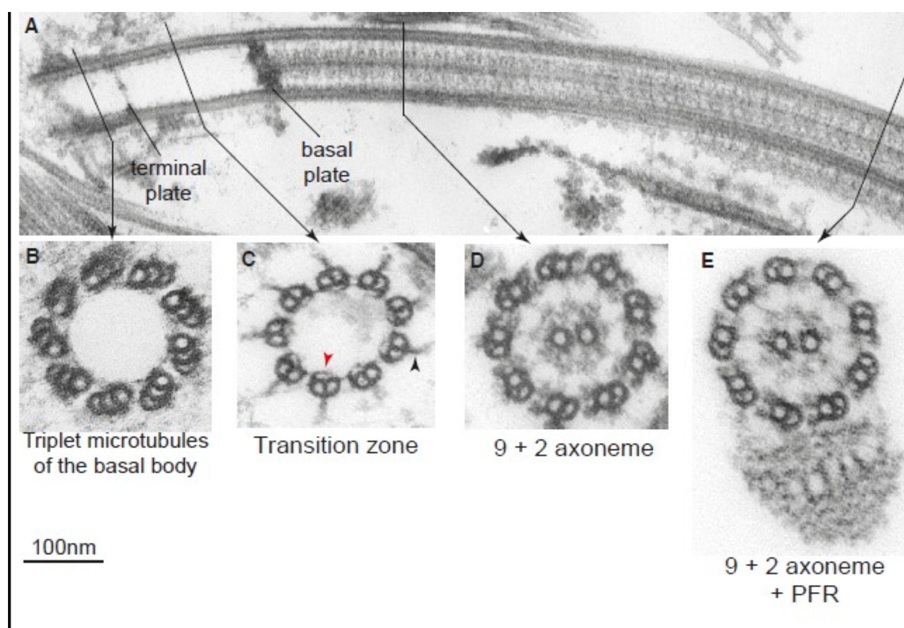

Fig. (4). Flagellum: The trypanosome flagellum exhibits canonical and divergent structural features. **A**. A longitudinal section through the procyclic form flagellum. **B**. A cross section through the basal body shows the outer triplet microtubules. **C**. A cross section through the transition zone shows the nine outer doublets with no central pair. Y linkers extend from the microtubules to the TZ membrane (black arrowhead) and short fibres penetrate into the TZ lumen (red arrowhead). **D**. A cross section through the axoneme shows clearly the 9 + 2 microtubule arrangement. E. A cross section through the flagellum after it has exited the flagellar pocket shows it is associated with the paraflagellar rod. Figure adapted from [290]. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

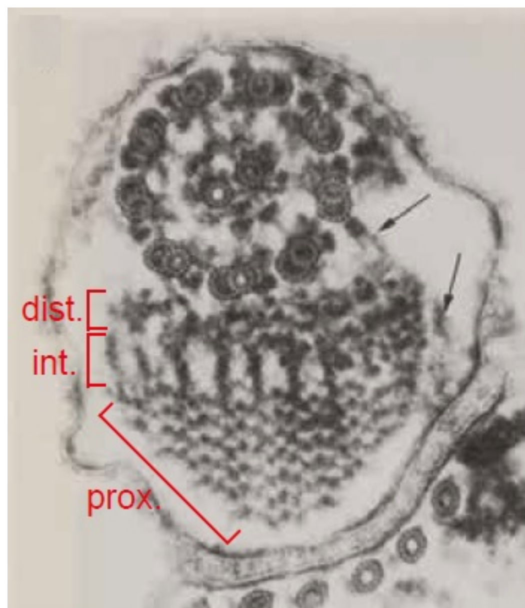

Fig. (5). PFR; Cross section EM through the flagellum showing the three structural domains of the PFR. The PFR is linked to the axonemal doublets (top arrow) and the FAZ (bottom arrow). Figure adapted from [127]. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

arrangements, each with repeating units along the longitudinal axis [39, 164-166]. The PFR's proximal domain is connected to the axonemal outer doublets 4-7 and the distal domain is connected to the underlying FAZ [39, 164].

The major structural components of the PFR are the proteins PFR1 and PFR2 [167, 168]. However, more than 150 other PFR proteins have been identified [126, 166, 169] (Table **3**) and the PFR appears to act as a platform for metabolic and signalling proteins, such as calcium binding proteins and cAMP-dependant phosphodiesterases [170-172].

The PFR is assembled predominantly at the distal tip, with only minor incorporation occurring along its length [173], suggesting a possible role for intraflagellar transport in PFR assembly. A cytoplasmic complex, PFR-AF1/2, is essential for PFR assembly [174] and indicates that at least some essential PFR components are pre-assembled in the cytoplasm. A member of the FGFR1 oncogene partner family, TbFOP1, is essential for nucleating the PFR [151] and its location at the basal body transitional fibres, almost 2 μm proximal to the start of the PFR, suggests that it facilitates import of PFR components into the flagellum. RNAi mutants with a reduced PFR exhibit partial flagellar paralysis and demonstrate an important role for the PFR in flagellum motility [175, 176]. The precise role of the PFR in motility is not fully clear but the presence of calcium binding proteins, such as calmodulin [172], suggest that it may be involved in beat regulation and it has also been proposed to act as a mechanical spring [165, 177].

8. THE FLAGELLA ATTACHMENT ZONE (FAZ)

The FAZ is a complex cytoskeletal structure that links the flagellum to the underlying cell body after the flagellum emerges from the flagellar pocket [39]. Three domains are evident in transmission electron microscope (TEM) cross sections though the cell: the flagellum domain that links the PFR to the flagellar membrane; the inter-membrane 'staples' connecting the flagellar membrane to the pellicular membrane; and a cell body domain that includes a FAZ filament [39, 127, 166, 178] (Fig. **6**).

To date, more than twenty components have been identified using different biochemical techniques (Table **3**) and several have been localised to specific FAZ domains [178]. RNAi ablation of flagellum FAZ components results in dramatic cell body shortening and repositioning of the basal body and flagellar pocket towards the anterior region of the cell [179-182], demonstrating the important role of the FAZ in cell morphogenesis. Ablating other

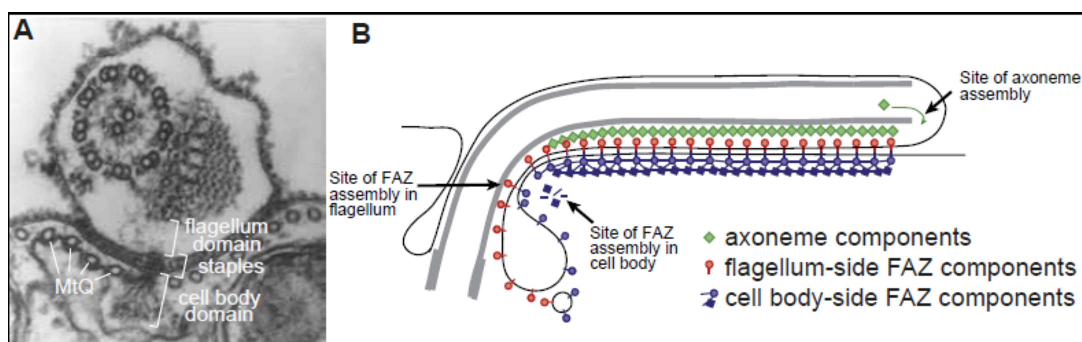

Fig. (6). FAZ; The FAZ has key roles in flagellar attachment and cell morphogenesis. **A**. Cross section TEM through the flagellum showing three different FAZ domains. The MtQ is visible running alongside the FAZ and embedded in the sub-pellicular microtubules. **B**. A cartoon depicting the proximal assembly of the FAZ, compared with the distal assembly of the flagellar axoneme. Figure adapted from [178]. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

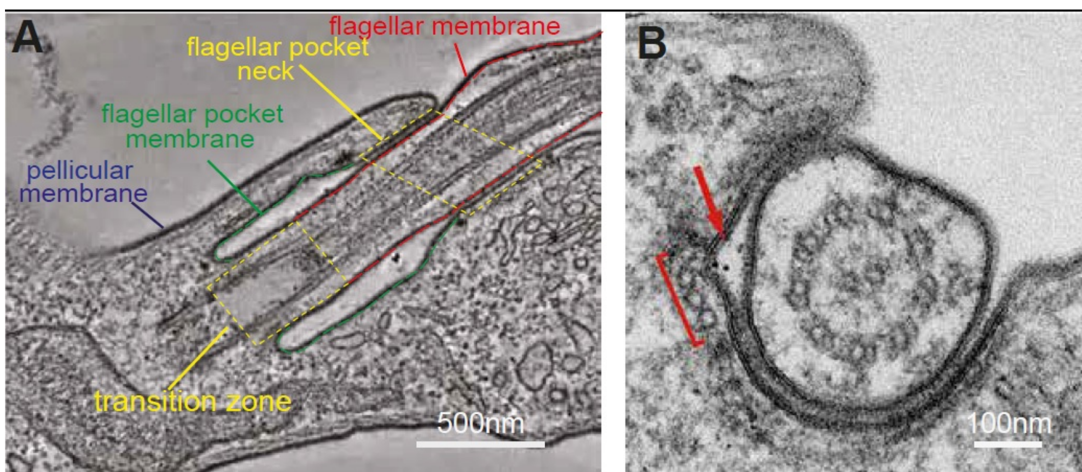

Fig. (7). Flagellar pocket; The flagellar pocket is the sole site of endocytosis and the hub of host-parasite interaction. **A**. An EM section through the slender bloodstream form flagellar pocket. The flagellar pocket membrane is demarcated from the abutting flagellar membrane and pellicular membrane by the transition zone and the flagellar pocket neck, respectively. **B**. An EM cross section through the flagellar pocket neck shows the neck channel (red arrow) conduit in and out of the flagellar pocket lumen. The bracket marks the MtQ that is proximal to the channel. Figure adapted from [160]. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

FAZ components causes flagellum detachment defects [183-185] consistent with the role of the FAZ in flagellum-cell body attachment.

Interestingly, assembly of the FAZ was shown to occur from the proximal end which is opposite to flagellum assembly [182] (Fig. **6**). This means that the FAZ must ‘slide’ along the cell as the flagellum is built, either pushed by the incorporation of new components or pulled by the tip of the growing flagellum.

9. FLAGELLAR POCKET

The flagellar pocket is an invagination of the trypanosome cell surface that appears as an asymmetric ‘bulb’ in electron microscopy sections (Fig. **7**). Apart from the anterior and posterior poles of the cell, the flagellar pocket is the cell’s only gap in the subpellicular microtubules and is therefore the site of all of the cell’s endo- and exo-cytosis [43, 186]. As the cell’s endocytic organelle, the flagellar pocket is responsible for nutrient acquisition, removal of antibody-VSG complexes from the cell surface and uptake of TLF. The flagellar pocket is therefore the hub of the parasite’s host-parasite interaction and is central to its pathogenicity. Despite comprising only 5% of the cell surface, the slender bloodstream form flagellar pocket is able to recycle the entire VSG surface coat (some 10 million molecules) every 12 minutes [43]. The flagellar pocket membrane protein composition is differentiated from the abutting flagellar and subpellicular membranes by cytoskeletal boundaries and it is enriched with many of the cell’s receptors [54, 187, 188].

In the bloodstream form trypanosome, all traffic between the flagellar pocket lumen and the extracellular milieu occurs *via* a ‘neck channel’ that is defined by the MtQ [160, 189] (Fig. **7**). Whether this channel operates passively or actively transports cargo in or out of the flagellar pocket remains to be determined. However, the extraordinarily high rate of surface membrane turnover that occurs *via* the bloodstream form flagellar pocket [43] indicates that traffic in and out of the flagellar pocket is likely to be organised.

10. FLAGELLAR POCKET COLLAR AND HOOK COMPLEX

Biogenesis of the flagellar pocket is dependent on a complex cytoskeletal structure at the flagellar pocket neck called the flagellar pocket collar [127, 190, 191]. The principal structural component of the flagellar pocket collar is a self-assembling protein called BILBO1 [192] that forms a horseshoe around the neck of the flagellar pocket. Ablating BILBO1 results in a daughter cell with no flagellar pocket and a fully detached flagellum.

A “hook complex” lies on top of the collar and extends along the FAZ ~1 μm inside the cell body [193]. The hook complex is important for endocytosis, keeping the neck channel open and maintaining flagellar attachment to the cell body [194-196]. It is

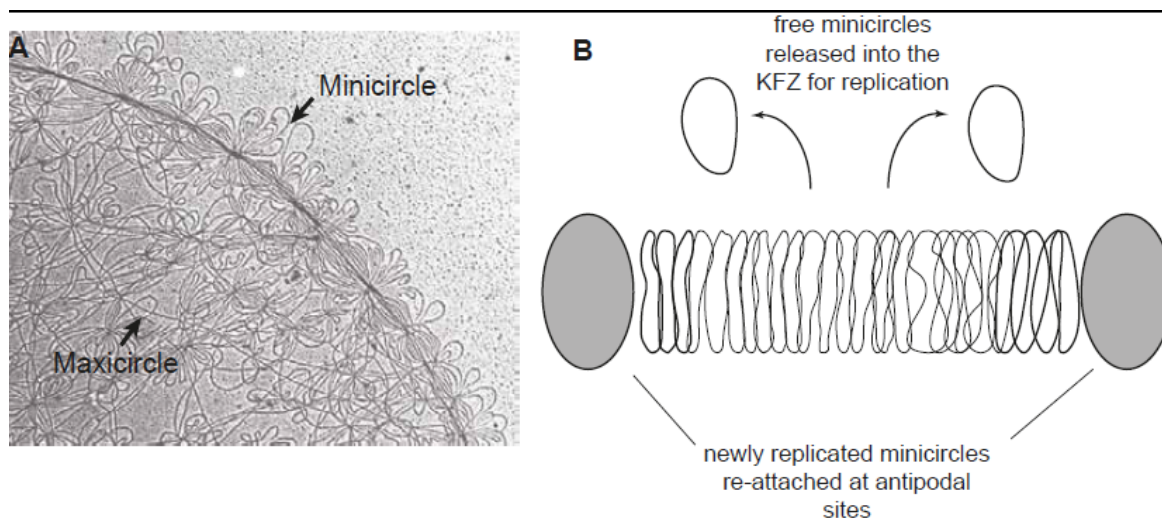

Fig. (8). Kinetoplast; The mitochondrial genome is comprised of DNA minicircles and maxicircles concatenated into a kinetoplast. **A**. Electron micrograph showing the kinetoplast minicircle and maxicircle network. **B**. During kinetoplast DNA replication, minicircles are released into the kinetoflagellar zone for replication. Once replicated, new minicircles (shown in bold) are re-attached to the kinetoplast network at the antipodal sites. Maxicircles (not shown) replicated whilst remaining attached to the kinetoplast network. Figure adapted from [199]. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

extraordinarily complex and has more than 90 components identified to date [126, 194] (Table **3**).

11. MITOCHONDRION

Trypanosomes have a single large mitochondrion that takes up at least 10% of the cell body volume. In the slender bloodstream form, this consists of a simple tubule that extends along the longitudinal axis of the cell [197]. In the insect procyclic form the mitochondrion is larger and more elaborate and the mitochondrial inner membrane has a much larger surface area that forms cristae [17]. The difference in mitochondrion size and morphology between the two lifecycle stages reflects the fact that the bloodstream form mitochondrion does not perform oxidative phosphorylation on its inner membrane, instead relying exclusively on glycolysis to generate energy. In contrast, the tsetse host is glucose-poor and the trypanosome relies on oxidative phosphorylation of amino acids for energy generation. The stumpy form mitochondrion is more elaborate than the slender form [17, 29] and is able to utilise α-ketoglutarate as an energy source [198] as a preadaptation to the tsetse midgut.

12. KINETOPLAST

The trypanosome mitochondrial DNA, termed the kinetoplast, is comprised of DNA minicircles and maxicircles that are interlocked into a disk-like structure [199, 200] that sits in a ‘pocket’ inside the mitochondrial matrix [197] (Fig. **8**). Each maxicircle is ~23kb and encodes 2 mitochondrial RNAs and 18 genes, most of which are required for mitochondrial oxidative phosphorylation. Twelve of the genes encoded on maxicircles are ‘cryptogenes’ [201, 202] that produce primary transcripts that must be ‘edited’ through the sequential addition and deletion of uradylates before they are mature and functional [203]. There are ~5000 minicircles of ~1kb in the kinetoplast that encode ‘guide RNAs’ and act as templates to direct the editing of genes encoded on the maxicircles [203, 204].

During replication of the kinetoplast, DNA minicircles are released from the kinetoplast network into the kinetoflagellar zone, a region between the kDNA disk and the mitochondrial membrane closest to the basal body, where they are replicated (Fig. **8**). Newly replicated minicircles then migrate and are reattached at opposing poles of the kDNA termed antipodal sites [205, 206]. Maxicircles replicate while still attached to the kinetoplast network.

13. THE TRIPARTITE ATTACHMENT COMPLEX (TAC)

The kinetoplast is segregated *via* the TAC [207, 208], a complex cytoskeletal structure that links the kinetoplast DNA to the basal body through the outer and inner mitochondrial membrane. The TAC has three structural domains that are visible in electron microscopy (Fig. **9**): the exclusion zone filaments (so called because electron microscopy revealed the absence of cytoplasmic ribosomes in this region [207]), the differentiated membrane (DM) comprised of the outer and inner membranes and the unilateral fibres (ULF) that links the inner membrane to the kinetoplast DNA [209, 210].

To date, eight proteins have been identified that localise exclusively to the TAC and three proteins have been shown to localise to the TAC and elsewhere in the mitochondrion [210, 211].

The TAC is a highly stable structure that is built *de-novo* each cell cycle and inherited conservatively [212]. The TAC is assembled hierarchically such that the components closest to the kinetoplast are incorporated after, and dependent upon, those that localise closer to the basal body [213] and can even be assembled in the absence of the kinetoplast DNA or a pre-existing TAC as a template [214]. The TAC’s sole function appears to be kinetoplast segregation and it is not essential for kinetoplast replication [212] or other cellular processes [214].

14. GLYCOSOMES

Glycosomes are specialised peroxisomes found in Kinetoplastiae and Diplonemida that lack catalase activity and are enriched in most steps of the glycolytic pathway [215, 216] (Table **3**). In the slender stage of the bloodstream form parasite, cells rely exclusively on glycolysis performed in the glycosomes for energy production. Glycolysis enzymes make up 95% of glycosome protein composition [217] and there are ~65 spherical glycosomes with an average diameter of 0.27 μm that are distributed throughout the cell [218, 219] (Fig. **10**). Insect stage glycosomes are elongated and, although the insect stages can produce energy *via* oxidative phosphorylation in the mitochondrion, glycolysis enzymes make up a substantial proportion of the glycosomal protein content and can still utilise glycolysis to make ATP and [220] (Table **3**).

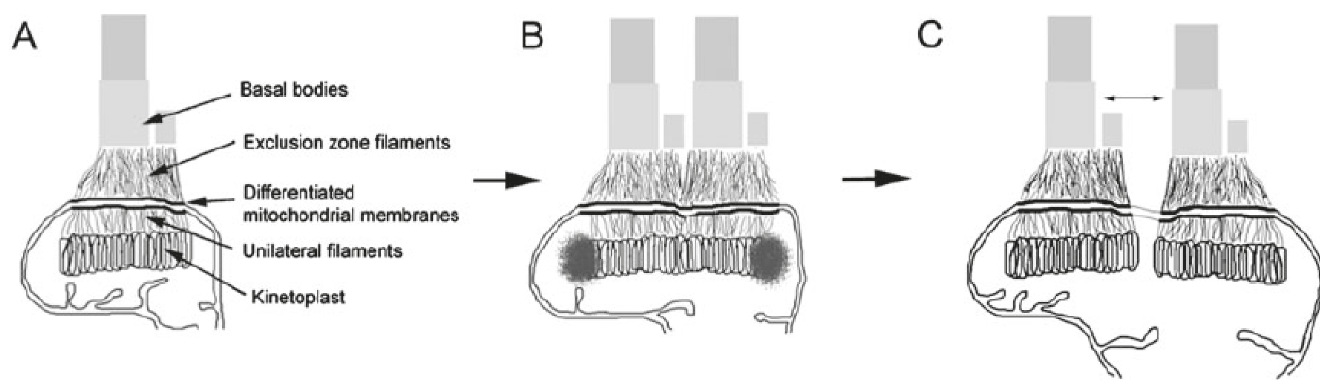

Fig. (9). TAC; The TAC connects the kinetoplast to the base of the basal body and is required for its inheritance. **A**. The TAC is composed of three structural domains: the exclusion zone filaments, the differentiated membranes and the unilateral fibres. **B**. During cell division, the movement of the basal bodies directs the segregation of the kinetoplast via the TAC. **C**. At late stage, two daughter kinetoplasts are attached to daughter basal bodies by two distinct TACs that are inherited conservatively. Figure from [207]. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

The sequestering of enzymes in glycosomes was originally thought to "turbocharge" glycolysis by bringing enzymes into close proximity with each other and their substrates [219]. However, this has largely been debunked by mathematical modelling, comparing glycolysis rates between organisms with and without glycosomes and the fact that the free-living ancestor of trypanosomes had glycosomes and lived in a glucose-poor environment [2, 215, 221-223]. Instead, glycosomes may have evolved to increase metabolic flexibility and rapid response to different host and vector niches by rapid glycosome autophagy and turnover [215, 224].

Enzymes of other metabolic pathways, such as the pentose phosphate pathway and beta-oxidation of fatty acids, have also been shown to localise to the glycosomes [225] (Table **3**) and TrypTag is likely to provide new insights into glycosome biochemistry [126]. Moreover, glycosomes facilitate the trypanosome stumpy-procyclic differentiation pathway by sequestering negative regulators of differentiation from their substrates [226].

15. DEVELOPING DRUGS TO TREAT HAT

Taking drugs from discovery to clinical use can cost $2-3B per drug and take 13-15 years [227]. Dropout rates are high due to ineffectiveness or safety concerns. Development of drugs to treat neglected tropical diseases has not been economical and there has historically been insufficient investment in drug discovery for these diseases. Not for profit organisations, such as the Dundee Drug Discovery Institute and the Drugs for Neglected Diseases initiative (DNDi), have funded early-stage research to 'de-risk' the drug development process for pharmaceutical companies.

Drug repurposing can make drug development more efficient and has been used to great success by the DNDi. Screening drugs whose pharmacological parameters and toxicity are already known can reduce the dropout rate and can reduce the time for development to ~3 years [227, 228]. This can significantly reduce preclinical costs and reduce the overall development costs to ~$300M [227].

A target product profile (TPP) outlines the desired 'profile' or characteristics to guide drug research and development for a disease, ensuring that the final product meets essential requirements. The TPP for trypanosomiasis includes the requirement that the drug is safe, effective and practical against stage 1 and stage 2 HAT [229]. The current most widely used anti-trypanosome therapy (NECT) is a combination therapy that is effective against late stage HAT but has 1.2% related mortality, significant side-effects and requires a 10-day hospitalisation to administer 7 days of twice-daily IV infusion and ten days of thrice-daily oral administration [230]. Newer drugs making their way through clinical trials, including Fexinidazole (now licenced for HAT) and Acoziborole [230-232], offer the opportunity to cure stage 1 and stage 2 HAT with a once-per-day pill, potentially eliminating the requirement for hospitalisation and representing a significant step towards eliminating HAT.

To be considered a good drug target, the Dundee Drug Discovery unit has developed a set of criteria that must be fulfilled [233]. A protein must be essential for parasite viability, preferably shown using *in vivo* infections of a trypanosome model, although a drug that interferes with an immune evasion strategy such as antigenic variation is conceivable. Ideally, inhibitors against an active site should already exist or there should be a potentially druggable active site. It should be possibly to purify the protein and establish high throughput function inhibitor assays, ideally in 384-well format. The gene encoding the drug target should be single copy to reduce the potential for drug resistance. There should not be a human ortholog to reduce the potential for toxicity through co-inhibition of the human ortholog. Finally, there should be good structural information of the target protein, ideally bound to an inhibitor, to aid development of chemicals that inhibit the target's function.

16.1. Tools and Resources to Develop Drugs against Trypanosomes

Sustained research over many years by groups world-wide has resulted in powerful tools to aid the development of drugs against HAT. RNAi libraries have been used to give each gene a 'score' of how essential it is in different lifecycle stages [141] and enable rapid triaging of gene-cohorts. These libraries have been used to great effect to determine drug resistance mechanisms, identifying transporters and receptors that allow entry of trypanocidal drugs [234, 235]. Resistance mechanisms can be further validated by reverse genetics strategies, such as RNAi and gene-knockouts, allowing the design of combination therapies that minimise the risk of cross-resistance. Over-expression libraries offer the potential to identify the target for existing trypanocidal drugs [236] and facilitate improvements to drug efficacy and allow the design of combination therapies that target different pathways.

Omics technologies have produced superb resources that have given deep insights into the biochemistry of different lifecycle stages and organelles. Transcriptomics has identified genes upregulated in lifecycle stages responsible for pathogenicity [237] and proteomics has given insights into the biochemical composition of most organelles and structures (Table **3**). The recent completion of TrypTag [126] offers unparalleled insights into organelle composition that can be leveraged for understanding trypanosome pathogenicity and mechanisms of resistance.

16.2. Druggabilty of Trypanosome Biochemistry and Cell Biology

Genome sequencing has revealed that most trypanosome genes are not conserved in humans [88]. Furthermore, their early divergence from other eukaryotes [2] means that much of their cell biology and biochemistry is either unconventional or extreme compared to that of humans, offering the potential to develop drugs with high specificity. Drugs that interfere with RNA-editing, the kinetoplast replication/segregation, gene expression, GPI-anchor synthesis, mono-allelic expression, antigenic variation and endocytosis may all provide opportunities to target specific aspects of trypanosome biology and treat trypanosome infections.

The following will consider some aspects of trypanosome cell biology and biochemistry and whether they show promise for drug development.

16.3. Kinome Inhibition

Trypanosome kinases may offer attractive therapeutic targets because of the success of kinase inhibitor libraries [238-240] and their importance in different aspects of trypanosome biology [241, 242]. Trypanosomes have a large kinase repertoire (182 kinases, ~2% of the protein-coding genome) [88, 243] and extensive protein phosphorylation [244, 245]. Although tyrosine kinases and tyrosine-like kinases are missing, trypanosomes are enriched in other kinase families and a large family of kinetoplastid-specific kinases that have not been assigned to any group may be attractive targets for development [243].

RNAi screens have established that that many kinases are essential for proliferation [242, 246-248] and at least 49 are essential for mammalian infectivity [249], meaning that trypanosome kinases fulfil many of the requirements for being good drug targets. A phenotypic screen using a Novartis kinase inhibitor library identified a trypanocidal amidobenzimidazole series that inhibited the trypanosome kinetochore protein KKT10/CLK1 [250]. This series demonstrated killing activity against other kinetoplastid species, such as *T. cruzi* and Leishmania spp., raising the prospect of a pan-kinetoplastid drug.

16.4. Palmitoylation Inhibition

Palmitoylation is a post-translational lipidation that can target proteins to specific membrane domains [251, 252] to promote sensitivity to signalling [253]. Inhibition of trypanosome palmitoylation using 2-bromopalmitate identified 124 palmitoylated proteins and strongly inhibited cell growth and viability, raising the possibility of palmitoylation as a drug target [254] (Table **3**). However, genetic ablation of the twelve individual PMTs was not deleterious to trypanosomes, suggesting functional redundancy and making the development of a specific, non-toxic trypanosome PMT inhibitor unlikely (Emmer *et al.*, 2011). Although the pan-palmitoylation inhibitor use in this study was toxic to mammalian cells, further development of 2-bromopalmitate might reduce non-specific toxic side effects.

16.5. Glycolysis and Glycosome Biogenesis Inhibition

Glycosome proteomes (Table **3**) and the TrypTag project have defined the glycosome composition [126, 220, 255-257]. Several studies have investigated inhibiting components of the glycolytic pathway [258, 259]. However, developing a specific inhibitor against trypanosome glycolysis is challenging because the glycolytic pathway is highly conserved between humans and trypanosomes and the high glycolytic flux means that such inhibition would need to be irreversible or uncompetitive [260, 261].

An alternative strategy would be to inhibit either glycosome biogenesis or import of glycosomal enzymes. This may be a viable strategy as it would lead to uncontrolled depletion of ATP from the cytosol and build-up of toxic metabolites and cell death [262, 263].

16.6. Proteasome Inhibition

The proteasome is a protein complex that plays a critical role in intracellular protein degradation through proteolysis [264]. Although the composition and structure of the trypanosome proteasome is similar to its mammalian counterpart (Table **3**), it has different substrate specificity and cleavage activity [265] and selective inhibition may be possible. Ablation of trypanosome proteasome subunits caused rapid cell death [266] and several inhibitors studies have shown trypanocidal inhibition of the proteasome [267-271]. One compound was developed and was successful in curing a stage 2 murine HAT model and also showed great promise against visceral Leishmaniasis and Chagas disease [268].

16.7. Flagellum Function and Biogenesis Inhibition

The trypanosome flagellum is a complex organelle with unique features and structures [272]. Importantly, flagellum function is essential for trypanosome infection [141, 143, 145, 146] and an inhibitor that causes defects in flagella structure or function is likely to cure trypanosome infection. The flagellum composition has been characterised by several studies (Table **3**) and is likely to contain ~1000 proteins, most of which are not conserved outside kinetoplastids. It contains several classes of druggable biochemistry, including kinases, phosphodiesterase, transporters and adenylate cyclases [143, 169, 170, 241, 273]. Furthermore, the importance of the trypanosome flagellum in tsetse stages raises the prospect of developing transmission-blocking agents.

CONCLUSION

Trypanosomes have been studied extensively for decades and there is a rich body of literature on their many unusual features and biochemistry. Their unusual cell biology and biochemistry are ripe for exploitation for therapeutic intervention, such as drugs targeting the kinetoplast, kinetochores, and kinome. Recent advances in genetic technologies and robotics are being leveraged to identify and develop drug targets and chemotherapies to treat HAT in humans. Future studies should also focus on developing treatments against Nagana in cattle to reduce the economic burden of animal trypanosomiases.

Although many researchers have undoubtedly been attracted to the trypanosome field because of their importance as human pathogens, just as many have stayed because of their fascinating and divergent biology, challenges remain, and HAT has remerged from near elimination before, but new drugs show great promise and eliminating HAT is now an achievable goal.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

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