# The flagella connector of *Trypanosoma brucei*: an unusual mobile transmembrane junction

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## Summary

**Throughout its elongation, the new flagellum of the procyclic form of the African trypanosome *Trypanosoma brucei* is tethered at its tip to the lateral aspect of the old flagellum. This phenomenon provides a cytotactic mechanism for influencing inheritance of cellular pattern. Here, we show that this tethering is produced via a discrete, mobile transmembrane junction – the flagella connector. Light and electron microscopy reveal that the flagella connector links the extending microtubules at the tip of the new flagellum to the lateral aspect of three of the doublet microtubules in the old flagellar axoneme. Two sets of filaments connect the microtubules to three plates on the inner faces of the old and new flagellar membranes. Three differentiated areas of old and new flagellar membranes are then juxtaposed and connected by a central interstitial core of electron-dense material. The flagella connector is formed early in flagellum extension and is removed at the end of cytokinesis, but the exact timing of the latter event is slightly variable. The flagella connector represents a novel form of cellular junction that is both dynamic and mobile.**

Movies available online

Key words: Trypanosome, Flagellum, Morphogenesis, Cell junction, Flagella connector, Cytotaxis

## Introduction

Trypanosomatid parasites are the aetiological agents of several important human diseases, including sleeping sickness in Africa (*Trypanosoma brucei*), Chagas disease in Central and South America (*Trypanosoma cruzi*) and leishmaniasis (*Leishmania* spp.) in tropical and subtropical regions. However, in addition to the medical and economic significance of these pathogens, trypanosomatids have also proved to be important model organisms for the identification of novel biological mechanisms that have been shown to be of wider importance in eukaryotic cell biology (Ferguson, 1999; Gull, 2002; Stuart et al., 1997). More recently, trypanosomes have also emerged as useful model organisms for studying the construction and remodelling of the cytoskeleton and, in particular, the biogenesis of the eukaryotic flagellum (Gull, 1999; McKean et al., 2003; Vaughan and Gull, 2003).

The basic cellular architecture of all trypanosomatid cells is strikingly similar, with cellular morphology being defined largely by an array of subpellicular microtubules that are cross-linked to each other and to the plasma membrane (Gull, 1999). All trypanosomatids contain a precise organization of single-copy organelles, such as the nucleus, mitochondrion, kinetoplast (containing the mitochondrial genome), basal body and flagellum. In trypanosomatids, the flagellum beats from tip to base, with anterior and posterior poles of the cell defined according to their position during motility (Robinson et al., 1995).

Many detailed cytological studies on trypanosome morphogenesis have been undertaken on the procyclic (trypomastigote) forms of the African trypanosome *T. brucei* (Ogbadoyi et al., 2003; Robinson and Gull, 1991; Robinson et al., 1995; Sherwin and Gull, 1989a; Sherwin and Gull, 1989b). Although these studies can be seen as providing a model for understanding trypanosome morphogenesis per se, fundamental differences do exist between species and also between distinct developmental forms of the same species during life-cycle alternations between the mammalian host and insect vector. In trypanosomatid biology, classical descriptive terminology is largely based on the relative positioning of the kinetoplast and nucleus within the cell body (Hoare and Wallace, 1966; Robertson, 1912; Robertson, 1913). However it should be emphasized that, although the same developmental form may occur at distinct stages of the life cycle (of the same or different trypanosome species), it does not necessarily follow that such forms have identical morphogenetic or cell-cycle regulatory mechanisms.

Procyclic trypomastigote forms of *T. brucei* at the beginning of their cell cycle possess a single flagellum that exits the cell body near the posterior end through an invagination of the plasma membrane known as the flagellar pocket. Subsequently, as the flagellum extends towards the anterior end, it follows a clearly defined left-handed helical path (Sherwin and Gull, 1989a). The flagellum is, for most of its length, attached to the cell body via a specialized attachment region called the flagellum attachment zone (FAZ) (Kohl et al., 1999). The FAZ consists of a series of filaments and

membranes in association with four specialized microtubules, and we have proposed that the FAZ provides structural information required to position the cleavage furrow during cytokinesis (Robinson et al., 1995).

The trypanosome flagellum itself contains a typical eukaryotic 9+2 microtubule-based axoneme but, upon emergence from the flagellar pocket, it also acquires another large extra-axonemal structure, the paraflagellar rod (PFR). The PFR is a large lattice-like structure that runs alongside the axoneme and is linked to the axoneme between outer doublets 4 through 7 (Farina et al., 1986; Sherwin and Gull, 1989a). Both the PFR and the microtubule-based axoneme have been shown to have roles in flagellar motility in *T. brucei*. Importantly, as the new flagellum elongates, strict cytological positioning rules apply, so that the new flagellum is always located to the left of the old flagellum when the cell is viewed from the posterior end (Sherwin and Gull, 1989a). Recently, a mechanistic explanation for this conserved cytological positioning has been described, with electron-microscopy (EM) studies revealing that in procyclic (trypomastigote) forms of *T. brucei* the new flagellum is physically tethered to the old flagellum by a structure termed the flagella connector (Moreira-Leite et al., 2001). The flagella connector connects the distal tip of the new flagellum to the lateral aspect of the old via what appears to be a highly unusual cell-cell functional complex. Significantly, the position of the flagella connector is not fixed because, as the new flagellum elongates, the flagella connector migrates along the old flagellum towards the distal tip. The physical connection afforded by the flagella connector ensures that the elongating new flagellum traces the same helical path along the cell as the old flagellum and undoubtedly influences internal cytoskeletal arrangements. This reproduction of flagella high-order positioning and cell architecture by an existing cytological structure (the old flagellum) represents an example of the epigenetic phenomenon of cytotaxis (Beisson and Sonneborn, 1965; Sonneborn, 1963; Sonneborn, 1964).

The flagella connector must be a structure that can link the growing microtubules at the tip of the new flagellum to the lateral aspect of microtubules in the old flagellum and, in doing so, must entail a link across two flagellar membranes.

In this paper, we provide a detailed structural characterization of the flagella connector using high-resolution EM and also provide a conceptual model for the ultrastructural organization of the flagella connector. Furthermore, we describe the characterization of a monoclonal antibody with specificity for the flagella connector that has enabled direct visualization of the temporal events of flagella connector acquisition and loss during the trypanosome cell cycle. Both these light and EM studies indicate that the flagella connector is assembled very early during the formation of the new flagellum at a specific point before its exit from the flagellar pocket. By contrast, the direct microscopic observation of live cells undergoing cytokinesis suggests that the timing of flagella connector separation is variable. Intriguingly, despite the apparent importance of this structure to morphogenesis in procyclic-form trypanosomes, we have so far been unable to find evidence for this same structure in other developmental forms, thus raising interesting questions about the particular requirement for such morphogenetic patterning in the procyclic trypomastigote forms of *T. brucei.*

## Materials and Methods

### Culture of trypanosomes

Procyclic *T. brucei* 427 cell line was cultured in SDM 79 medium supplemented with 10% foetal calf serum at 28°C (Brun and Schonenberger, 1979). Cultures of bloodstream form *T. brucei* (Wirtz et al., 1999) were grown in HMI-9 at 37°C, 5% $CO_2$ (Hirumi and Hirumi, 1989).

### Immunolocalization studies and antibodies

Trypanosomes were settled onto poly-L-lysine-coated slides, cytoskeletons prepared by extraction with 1% NP-40 in 100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM EDTA and fixed in methanol at –20°C for 30 minutes. Cells were double labelled with anti-PFR monoclonal antibody L8C4 (IgG) and AB1 (IgM) (recognizing the flagella connector), and developed with secondary antibodies FITC-conjugated (IgG, Sigma) and TRITC-conjugated (IgM, Chemicon). Cells were embedded in Vectashield with DAPI (4,6-diamidino-2-phenylindole) (Vector Laboratories, USA). Slides were examined on a Leica DMRX microscope, captured on a CCD camera and processed in Adobe Photoshop (Adobe).

### Preparation of cells for EM

Cells were prepared for scanning EM (SEM) and transmission EM (TEM) essentially as described previously (Moreira-Leite et al., 2001; Sherwin and Gull, 1989a). Immunolocalizations at the EM level were performed as described previously (Scott et al., 1997; Sherwin and Gull, 1989b; Woods et al., 1989) except that, in some circumstances, methanol fixation or unfixed cytoskeletons were used.

### Video images of live trypanosomes

Video sequences [see Movies 1-4 (http://jcs.biologists.org/supplemental/)] were made on a Nikon Eclipse E600 microscope equipped with differential interference contrast optics using a Photometrics Coolsnap FX camera and processed in Metamorph (Universal Imaging).

## Results

### Visualization of the flagella connector structure negative-stain TEM

The major morphological events occurring during the cell cycle of procyclic trypanosomes are shown schematically in Fig. 1A. At the beginning of the cell cycle (G1), a trypanosome cell possesses a single kinetoplast, nucleus and flagellum (1K1N). As the new flagellum elongates along the length of the cell body, it follows a path defined by the old flagellum because of the physical connection afforded by the flagella connector (Moreira-Leite et al., 2001).

Further examination of dividing cells by negative-staining TEM on detergent-extracted whole-mount cytoskeletons has produced a series of high-resolution images of the flagella connector. In Fig. 1B,C, negative staining allows the visualization of three different areas of electron transparency separated by two areas of stain penetration, which describes a trilaminar structure. The overall width of this trilaminar structure is ~90nm (Fig. 1C). The central core layer 2 is ~18 nm wide and is the thickest of the three layers. The layer closest

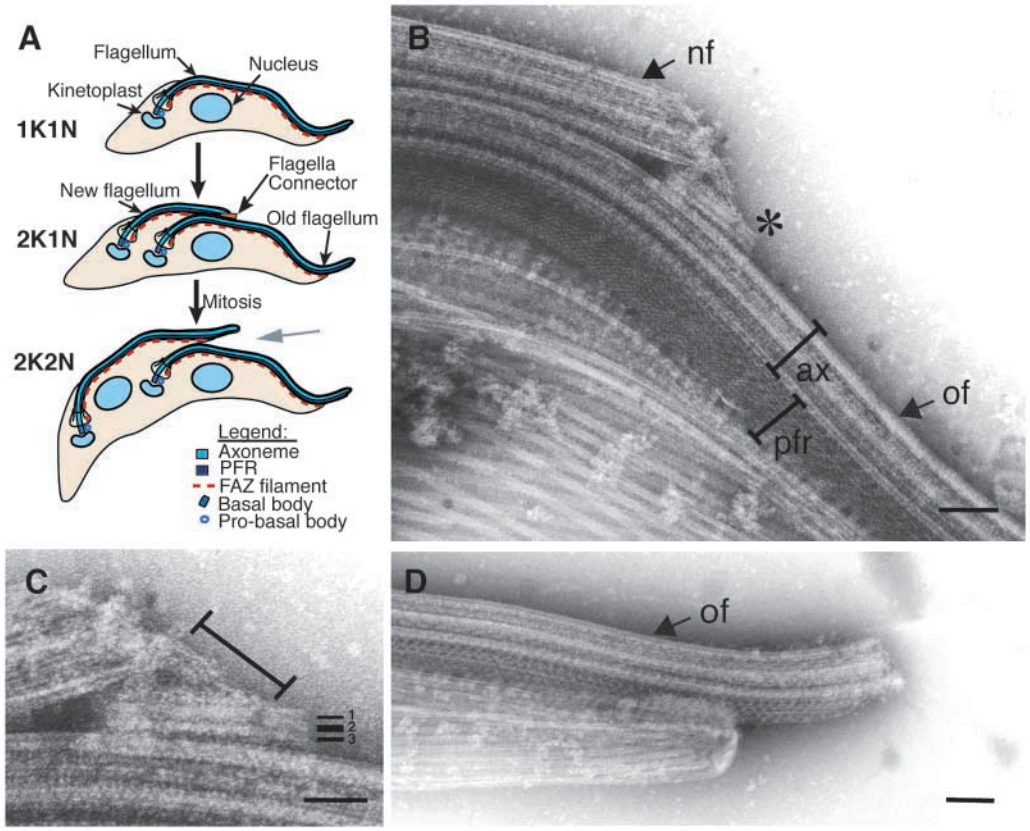


**Fig. 1.** Visualization of the flagella connector at the distal tip of the new flagellum. (A) The cell cycle of procyclic trypanosomes. At G1, trypanosomes posses a single kinetoplast and a single nucleus (1K1N) with a single attached flagellum. During the cell cycle, new flagellum elongation coincides with kinetoplast duplication and segregation (2K1N). The new flagellum is physically attached to the old flagellum via the flagella connector. Following mitosis (2K2N) and the initiation of cytokinesis, this connection is released. (B-D) Negatively stained whole-mount cytoskeletons of procyclic trypanosomes. (B) The flagella connector (asterisk) is positioned at the tip of the new flagellum (nf) and along the side of the axoneme (ax) of the old flagellum (of) and not the paraflagellar rod (pfr). (C) The flagella connector consists of a trilaminar core structure composed of three distinct layers of electron density (layers 1,2,3) and filamentous extensions (marked with a bar) extending to the tip of the new flagellum. This gives roughly an overall triangular appearance. (D) No flagella connector structure is evident at the tip of the old flagellum. Scale bars, 200 nm (B), 100 nm (C,D).

to the old axoneme (layer 3) is the next thickest layer at ~16 nm, with the layer closest to the tip of the new flagellum (layer 1) being the thinnest at ~13 nm. This layer is connected to the tip of the new axoneme by a set of filaments. There is therefore a pronounced asymmetry in the structure and interactions of the trilaminar core structure and the axonemes of the new and old flagella.

The trilaminar core structure of the connector extends for ~400 nm, running parallel to the axis of the microtubules of the old flagellum. Overall, the filaments and trilaminar structure present a roughly triangular appearance for the flagella connector in these types of negative stain preparations. Significantly this structure has only ever been observed at the end of the new flagellum; it is not present, nor have we ever observed any structural remnants, at the end of the old flagellum (Fig. 1D).

## Visualization of the flagella connector by thin-section TEM

We then asked whether it would be possible to visualize the flagella connector structure by thin-section EM. Although trypanosomes are biflagellate for approximately half of the cell cycle (possessing an elongating ‘new’ flagellum and a fully elongated ‘old’ flagellum) (Sherwin and Gull, 1989a; Woodward and Gull, 1990), there is no easy way to enrich for preparations of the flagella connector. Therefore, trypanosome cells were simply fixed and processed for analysis by thin-section EM and an extensive search undertaken for the flagella connector structure. This approach generated several images that revealed a significant set of additional features to those already recognized in cytoskeletal preparations.

Because of the strict cytological positioning rules that apply during trypanosome morphogenesis, the new flagellum is always present on the left-hand side of the old flagellum when the cell is viewed from the posterior end. In electron micrographs, this polarity can be determined because of the axial polarity of the outer microtubule doublets and associated dynein arms (Sherwin and Gull, 1989a). All micrographs in Fig. 2 are oriented such that the old flagellum is on the right and the new flagellum is on the left. The flagella connector was only observed when a section passed through the distal tip of the new flagellum (Fig. 2A). Structural clues in the micrographs allow us to know that such sections are at the distal tip of the new flagellum as there is no accompanying PFR in the new flagellum, no underlying FAZ connection to the cell body and often no full set of microtubules. These are the expected features given the timing of morphogenesis of each component determined previously (Kohl et al., 1999).

The flagella connector can be seen in Fig. 2A as a region where the membranes of the new and old flagella are juxtaposed and have a greater electron density and more linear profile. The increased electron density of the membranes is organized in three distinct zones, each associated with plate-like areas of electron density within both the old and the new flagella (Fig. 2A, Fig. 3). We observe that the three plates

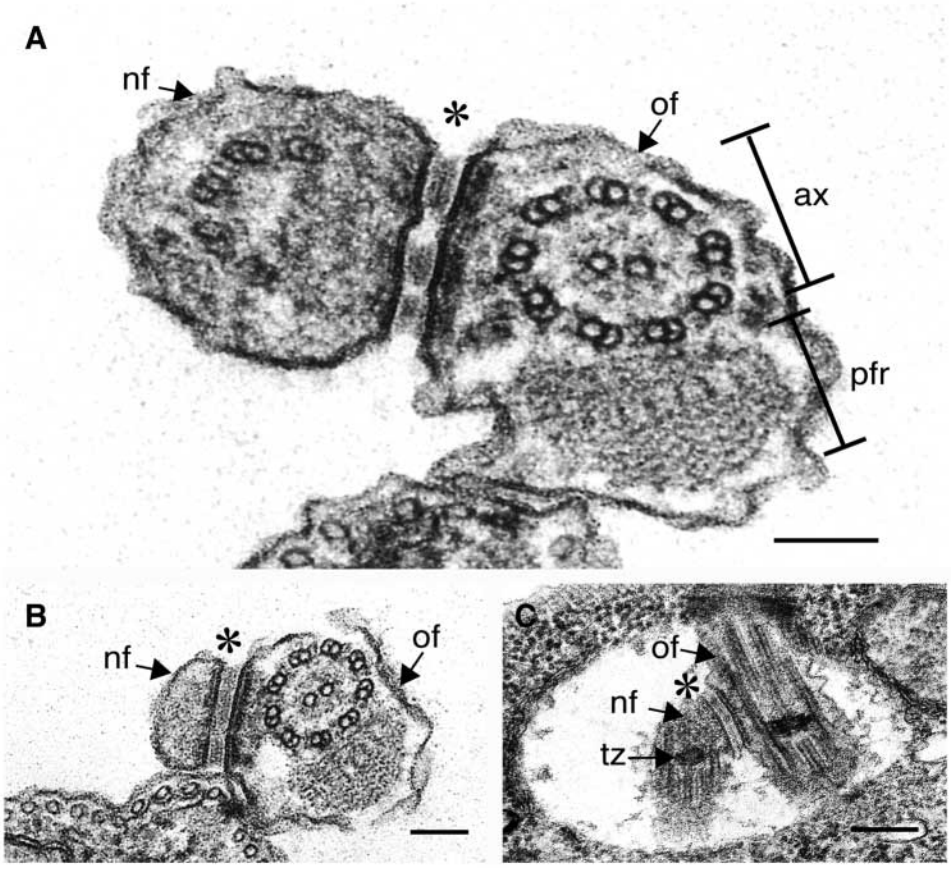


**Fig. 2.** Ultrastructure of the flagella connector viewed by thin-section EM. (A) Cross section though the flagella connector (asterisk). The new flagellum (nf) is always observed to the left of the old flagellum (of) when viewed from the posterior end of the cell. The flagella connector is composed of three discrete connection zones between two opposing flagellar membranes. These membranes display a more electron dense, linear profile, which is exemplified in (B), in which the section is cut through the extreme distal tip of the new flagellum. (C) A glance section through the flagella pocket reveals the presence of the flagella connector just distal to the transition zone (tz) at the end of the basal body, revealing its early appearance during flagellum elongation [axoneme (ax) paraflagella rod (pfr)]. Scale bars, 200 nm.

within the old flagellum are more extensive (~20 nm wide) than those of the new (~15 nm wide), again emphasizing the asymmetric nature of the flagella connector. Behind these plates, we can observe electron-dense filamentous extensions leading back to specific axonemal microtubules. Owing to the invariant linkages between specific outer doublets of the axoneme and the PFR (Sherwin and Gull, 1989a), we can unambiguously assign these connections to axonemal outer microtubule doublets 7, 8 and 9 in the old flagellum. It is more difficult to assign the connections between the three plates in the new flagellum to particular axonemal doublets because of the absence of a PFR at this region.

The intraflagellar plates and the specialized flagellar membrane zones are reflected in three further discrete zones of electron-dense material between the opposing flagellar membranes. Each of these interstitial zones is symmetrical and contains three separate layers of electron-dense material running parallel to the membranes with the central layer having a greater electron density than the two outer layers. The interstitial zone is around 32 nm wide.

Based upon the analysis of many thin section and negative staining transmission electron micrographs, we have constructed a schematic interpretation of the ultrastructure of the flagella connector (Fig. 3). Our present interpretation of the

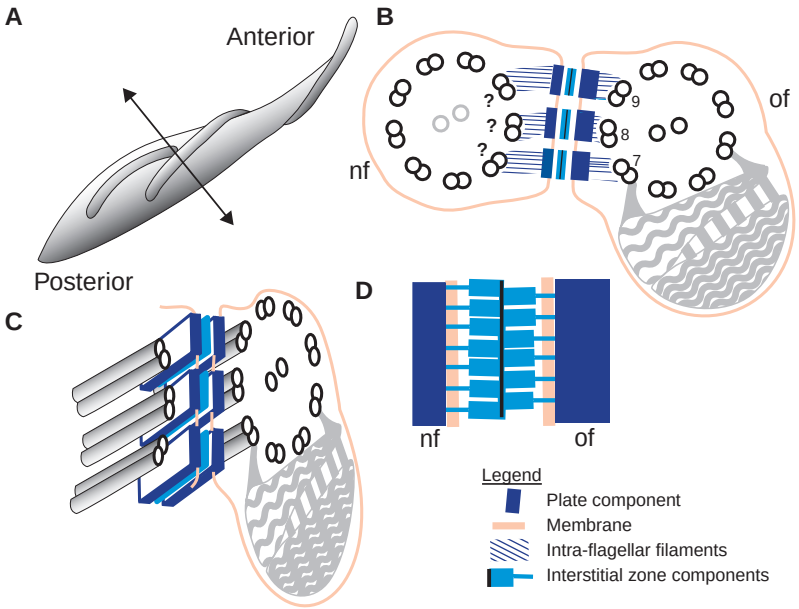


**Fig. 3.** Model illustrating the structure of the flagella connector. (A) Procyclic *T. brucei* cell. The arrow indicates a hypothetical plane of section passing through the region of the flagella connector. (B) Model based on thin-section TEM data. The axonemal outer doublets that are linked to the flagella connector are numbered. (C) Model of the flagella connector structure compatible with both negative staining TEM and thin-section TEM data. (D) Cross-section of the putative organization of protein components in the interacting membrane domains of the flagella.

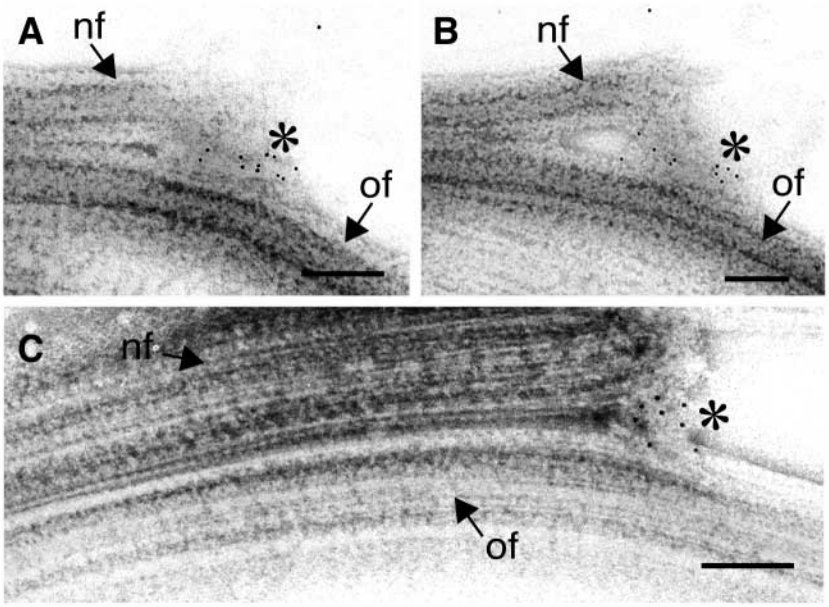


**Fig. 4.** Immunogold EM with the AB1 monoclonal antibody. Cytoskeletons fixed with methanol (A,B) and unfixed cytoskeletons (C) both show 10-nm gold staining of the flagella connector (*), which is defined by the presence of electron-dense material between the tip of the new flagellum (nf) and the side of the old flagellum (of). Scale bars, 200 nm

flagella connector structure is that the membrane of each flagellum is linked by means of intraflagellar filamentous structures to the microtubules of the respective flagellar axonemes. Between the membranes of the new and the old flagellum, we propose the existence of transmembrane protein components linking the membranes of the new and old flagellum via the interstitial central material. Our current evidence indicates that the flagella connector involves intraflagellar connections to axonemal outer doublets 7-9 of the old flagellum.

## Monoclonal antibody AB1 defines the flagella connector

In an attempt to understand further the temporal events associated with the morphogenesis of the flagella connector during the cell cycle, we produced a monoclonal antibody specific for the flagella connector structure. Over the years, experience has shown that mice often produce (natural) antibodies that detect the cytoskeletal structures of trypanosomes. These are often IgM antibodies and such an antibody was indeed recognized serendipitously in a screen for anti-trypanosomal-cytoskeleton antibodies. This monoclonal antibody was termed AB1 and has been characterized as an IgM that recognizes the tip of the new flagellum but not the tip of the old.

We then asked whether this monoclonal detected the tip of the axoneme of the new flagellum or the flagella connector structure. Whole-mount cytoskeletons were fixed and prepared using a range of methods for immunogold localization of AB1 by electron microscopy. In all preparations, the immunogold labelling shows that the antibody detects a central component of the flagella connector (Fig. 4). The fix and blocking reagents used in such whole-mount EM techniques obscure the structures involved and so we have been unable to obtain a higher definition. To date, we have also not been able to use this antibody successfully to label sections using pre- or post-embedding techniques, or in western blots (often a characteristic of these natural IgM antibodies).

## Timing of flagella connector formation

AB1 is an extremely useful probe for studying flagella connector morphogenesis during the cell cycle using immunofluorescence microscopy (Fig. 5). Trypanosome cells

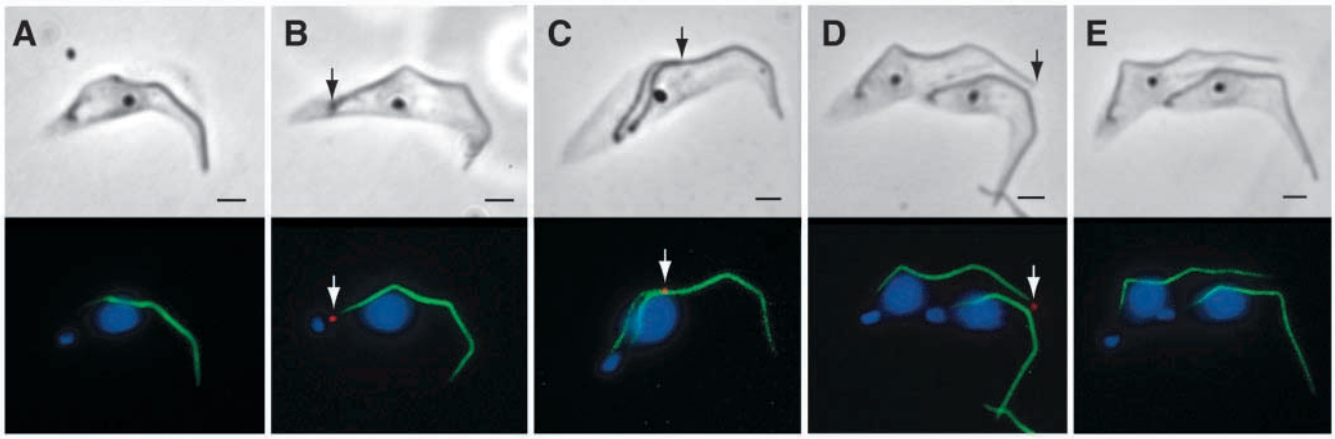


**Fig. 5.** *T. brucei* procyclic cells at various phases of the cell duplication cycle. Phase-contrast images and corresponding immunofluorescence images; DNA is labelled with DAPI (blue), the flagellum is identified by L8C4, anti-PFR antibody (green) and the flagellar connector is labelled by AB1 antibody (red). (A) An AB1 signal is not seen in most 1K1N cells. (B) AB1 is observed in a proportion of cells just above the basal body in phase contrast. (C,D) The AB1 signal is seen in cells that are elongating a new flagellum at the correct position for the flagella connector and throughout the cell cycle. (E) The AB1 signal is not seen in cells that have entered cytokinesis. Scale bars, 2 μm

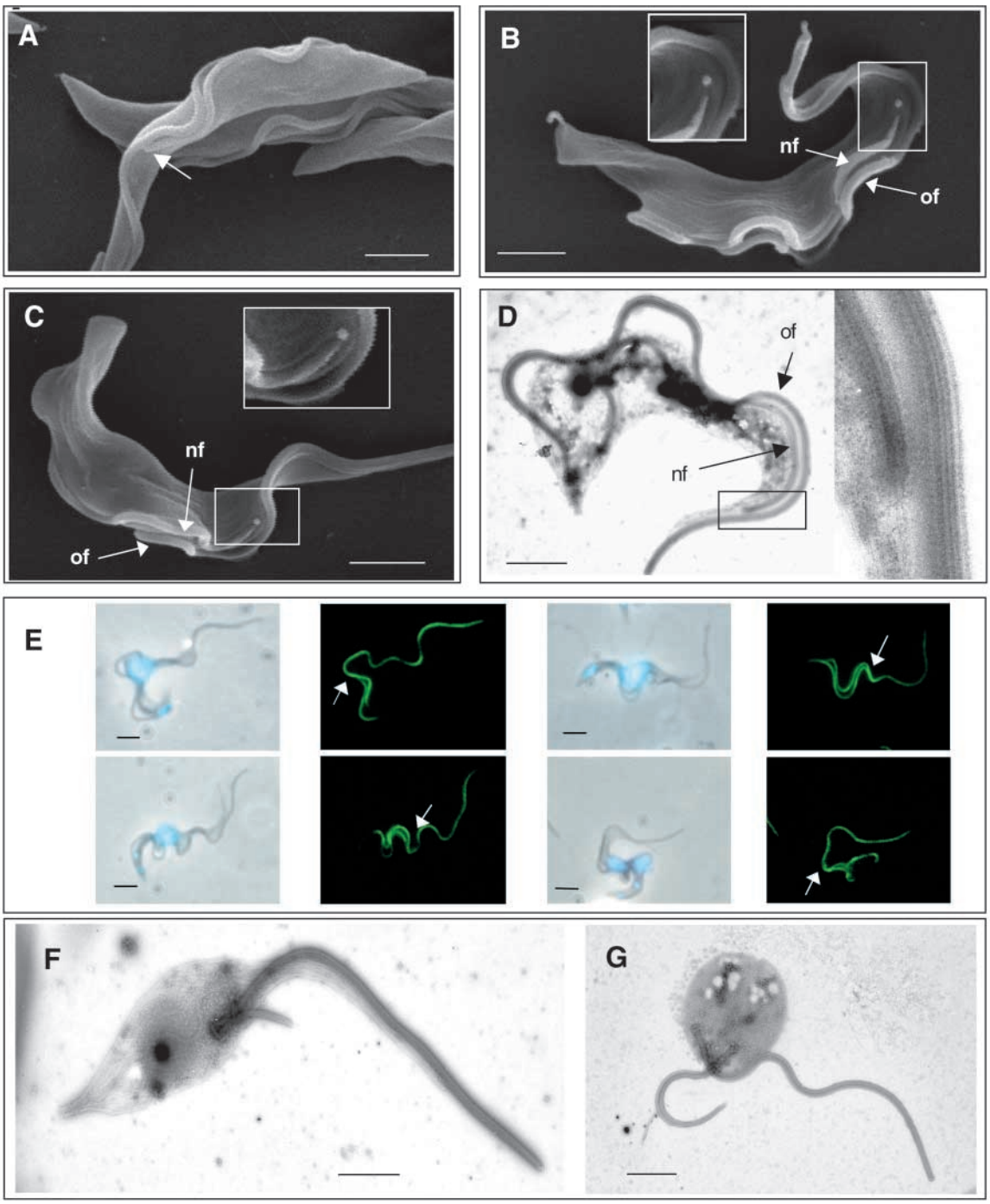


**Fig. 6.** Lack of apparent connector in other trypanosome cell types. (A) SEM of a procyclic *T. brucei* cell. The tip of the new flagellum is associated with the lateral aspect of the old flagellum, via the flagella connector, indicated by the white arrow. (B,C) SEM of bloodstream *T. brucei* forms. The tip of the new flagellum (nf) is close to the old flagellum (of) but a physical connection is not observed. (D) Negatively stained whole-mount cytoskeleton of a *T. brucei* bloodstream form. Although the tip of the new flagellum is close to the old flagellum, there is no visible flagella connector. In addition, the new axoneme lies adjacent to the old PFR. (E) Immunofluorescence images of *T. brucei* bloodstream forms. Phase-contrast and corresponding merged immunofluorescence image; DAPI is in blue, PFR is labelled with anti-PFR antibody (L8C4) in green and the flagella connector is labelled by AB1 in red. (i,ii) 1K1N cells. (iii) 2K1N cell. (iv) 2K2N cell. In no case was an AB1 signal observed at the tip of the new axoneme. (F,G) Negatively stained whole-mount cytoskeletons of *T. cuzi* epimastigote (F) and *Leishmania* promastigote (G). Both cells are in cell division and extending a new flagellum, but a flagella connector structure is not observed. Scale bars, 2 μm.

early in the cell cycle possess one nucleus and one kinetoplast (1K1N). The kinetoplast divides (2K1N) and cells finally enter mitosis producing a 2K2N cell. We observe that most 1K1N cells do not have an AB1 signal (Fig. 5A), but a proportion of 1K1N cells do possess a defined AB1 signal when viewed by immunofluorescence (Fig. 5B). A small single

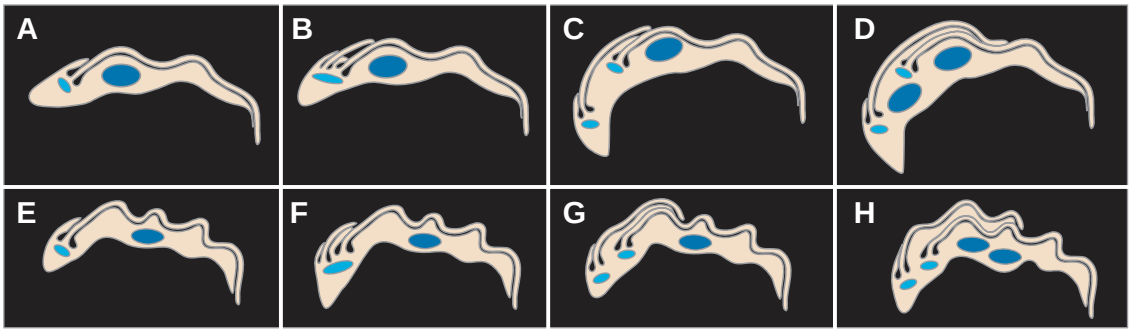


**Fig. 7.** Cartoon of the cell cycle in *T. brucei* trypomastigotes. (A-D) Procyclic form. (E-H) Bloodstream forms. Apart from differences in cell shape between the two forms, the segregation of the kinetoplast is more limited in the bloodstream form (G) than in the procyclic form (C). (D) In the procyclic form, the new kinetoplast and nucleus are located towards the posterior end of the cell, but the orientation of the nuclei in a post-mitotic bloodstream cell are not in the same configuration (H). The two kinetoplasts are located at the posterior end of the cell and the nuclei are both located anterior to the kinetoplast.

immunofluorescent signal of the AB1 monoclonal is seen near the kinetoplast at a position just above the basal body (Fig. 5B). We judge that the AB1 signal is internal to the flagellar pocket because there is a gap between the AB1 signal and the start of the PFR in the old flagellum, which runs alongside the axoneme but only after the flagellum has emerged from the flagellar pocket.

We were interested to determine whether this immunofluorescent signal was indeed an indication of the presence of the flagella connector at an early stage in the cell cycle. Hence, we conducted an extensive search using thin-section EM for sections of trypanosomes in which two flagella were present within a single flagellar pocket. Although eventually each flagellum exits from its own pocket, the newly elongating flagellum initially emerges from the same flagellar pocket as the old flagellum. Consequently, the presence of two flagella in a single pocket (Fig. 2C) represents a very early stage in the biogenesis of the new flagellum. Although this is a glancing section, it is clear that the flagella connector structure (represented by an asterisk) is present between the new flagellum and old flagellum at a position just above the transition zone of each flagellum. This indicates that the flagella connector is formed concomitant with the formation of the new flagellar axoneme or potentially even before axonemal nucleation.

In cells with an elongated new flagellum that has exited the flagellar pocket the AB1 signal is seen at the expected position of the flagella connector – at the tip of the new flagellum and alongside the lateral aspect of the old flagellum (Fig. 5C). The connector is still present in post-mitotic cells 2K2N (Fig. 5D). However, in cells that are clearly post-mitotic and in which a cytokinesis furrow is well advanced, no connector signal is visible, indicating that the flagellar connector (as defined by the AB1 signal) is removed at a late stage in the cell cycle (Fig. 5E).

## Timing of flagellum detachment during cytokinesis

In order directly to address the issue of when physical detachment of the flagellar connector occurs, we analysed live cells that were judged to be at a late stage in the cell cycle. In such cells, the cleavage furrow had already been initiated and was clearly visible between the two daughter cells. Four such movies are presented online (http://jcs.biologists.org/supplemental/). These examples show that the flagellum-flagellum detachment does not occur at a single precise point in the cell cycle but rather that there is some variation. Detachment can occur at various points between mid- to late cleavage and post-cleavage stages. In Movies 1 and 2, flagellum-flagellum detachment occurs at an early stage during cytokinesis. In these movies, the daughter with the new flagellum is always at the posterior end and it can be seen that these daughters have a highly motile anterior end, even though the new flagellum remains initially attached to the old flagellum via the flagellar connector (http://jcs.biologists.org/supplemental/). The cleavage furrow is well advanced in cells in Movies 3 and 4 but the cells remain attached at the flagellar connector. The timing of detachment is variable. Movie 1 shows two cells in which flagellum-flagellum detachment takes place before completion of cytokinesis, whereas Movie 4 shows two cells that have completed cytokinesis but remain attached by the flagella connector and only subsequently detach when the flagella connector linkage is broken.

## Flagella connector – cell-type and species-specific differences

The presence of the flagellar connector influences the shape and morphogenesis of the procyclic trypanosome cell because it is clear that, although basal bodies move apart before mitosis, the tip of the new flagellum remains attached to the side of the old flagellum (Fig. 6A). However, the morphology of different trypanosomatid cell types varies both between species and also within the same species. We therefore asked whether the strict organization of the old and new flagella also occurred in the bloodstream trypomastigote cell cycle of *T. brucei*. Both procyclic (tsetse midgut) forms and mammalian bloodstream forms of *T. brucei* are trypomastigotes. The term ‘trypomastigote’ refers to trypanosome forms with an attached flagellum for much of the cell length and a kinetoplast posterior to the nucleus. One crucial difference between procyclic trypomastigotes and bloodstream trypomastigotes is that, in procyclics, the post-mitotic nuclei occupy a position on either side of the previously segregated kinetoplasts (Fig. 7). This contrasts with bloodstream trypomastigotes, in which both the kinetoplasts are positioned posterior to the nuclei throughout division. It is evident from the scanning electron

micrographs of bloodstream-form trypomastigotes (Fig. 6B,C) that the tip of the new flagellum is located close to the lateral aspect of the old flagellum in bloodstream forms but does not appear to contact it directly as it does in procyclic trypomastigotes (Fig. 6A-C). Although the bloodstream trypomastigotes sometimes exhibited a small structure at the new flagellum tip, this was often also seen at the tip of the old flagellum (Fig. 6B), suggesting that it did not represent a flagella connector. We asked whether we could visualize the canonical flagella connector structure by negative staining of bloodstream-form whole-mount cytoskeletons (Fig. 6D). Although some amorphous material was sometimes associated with the tip of the new flagellum, it did not reveal a convincing structure with the complexity of the procyclic flagella connector. Moreover, the orientation of the new flagellum appears to be slightly different to that of the old, in that the tip of the new axoneme most often lies close to the PFR of the old flagellum (Fig. 6D) rather than close to the axoneme as in procyclic cells (Fig. 1B). We also asked whether we could detect the AB1 monoclonal signal in the bloodstream trypomastigotes, but we were unable to do so in cells at any stage of the cell cycle (Fig. 6E). Therefore, although the new flagellum of bloodstream trypomastigotes maintains a position close to the old, we cannot define the presence of a canonical flagella connector structure as defined in the procyclic trypomastigotes.

We then studied two proliferative forms of related parasitic protozoa, the epimastigote form of *T. cruzi* and the promastigote form of *Leishmania mexicana amazonensis*. In neither of these cases could we find any evidence for a flagella connector of any type (Fig. 6F,G). Electron micrographs of *T. cruzi* epimastigote cytoskeletons, which possess a semi-attached flagellum, show that the new flagellum is relatively free from the old even at an early stage of the cell cycle (Fig. 6F); this was also the case for the dividing promastigote cells of *L. mexicana*, which possess a free flagellum (Fig. 6G).

## Discussion

### Structural characterization of the flagella connector

The flagella connector of procyclic form *T. brucei* represents a complex membrane-membrane connection with some initial similarities to other cell-cell junctions that have been well described in higher eukaryotic systems such as desmosomes and tight junctions. However, although junctions such as desmosomes represent specialized morphological structures that facilitate the intercellular anchoring of adjacent cells (Burdett, 1998; Kitajima, 2002) and other metazoan junctions function as occluding or communicating junctions, the flagella connector represents a connection between two discrete membrane-bound compartments of the same cell.

Our electron microscopy of negatively stained preparations indicates that the flagella connector is composed of an asymmetric central core of three electron-dense components. Each of the two electron-dense, outside layers of this trilaminar structure is linked to axonemal microtubules via filaments. Thin section EM however, reveals that the flagellar membranes are locally differentiated within a set of three discrete regions and each of these presents a further electron-dense, interstitial structure between the two juxtapositioned flagellar membranes.

The interstitial connections could represent transmembrane components from each flagellar membrane interacting with each other at the centre of the intermembrane space, thus explaining the greater electron density found at the centre of the intermembrane space. The model would suggest that the cytoplasmic ‘tail’ of the flagella connector transmembrane components is associated with the electron-dense plates internal to each flagellum. These plates then make connections to specific microtubule outer doublets of the axoneme via filamentous connections.

The flagella connector structure is not symmetrical. When viewed in cross section, an asymmetrical relationship exists between the trilaminar core of the flagella connector and the new/old axonemes. This asymmetry might indicate that the electron-dense plates internal to the old and new flagella are composed of different structural components. These differences presumably reflect the different roles on both sides of the connector, with one side always maintaining a tight connection to the lateral aspect of the existing microtubules of the old flagellum, while the other connects to the extending tip of the new flagellum. The flagella connector is a strong tether but must also be flexible enough to cope with flagellar beating, allowing the junction to be dynamic in the membrane while anchored to the microtubules. This type of flexibility is observed in live cells [see supplementary data (http://jcs.biologists.org/supplemental/)] where the tip of the new flagellum is highly motile and often seen in different orientations, sometimes running parallel to and sometimes perpendicular to the old flagellum.

### Other axonemal tip structures

The flagella connector is linked to the distal tip of the axoneme of the new flagellum, where the ‘plus’ ends of the new axonemal microtubules are located. It is therefore suitably positioned to act as a capping structure for the new flagellum during elongation. However, several lines of evidence suggest that the flagella connector is not an axonemal cap, or at least that it does not act uniquely as a capping structure. In many systems both the central pair and the outer doublet microtubules are linked to flagellar membranes by a central microtubule cap and distal filaments. First, axonemal capping structures are linked to the ‘plus’ ends of all axonemal microtubules (Dentler, 1990; LeCluyse and Dentler, 1984). Our EM evidence suggests that the flagella connector is probably connected to a subset of axonemal microtubules because the flagella connector frequently appears to be linked only to those axonemal microtubules that are closer to the old flagellum. Second, axonemal caps are believed to have a role in controlling microtubule dynamics at the distal end of the axoneme. They form during axonemal growth but are maintained after ciliary and flagellar elongation, and are present in the mature appendages (Dentler, 1990). By contrast, both our EM and immunofluorescence data indicate that the flagella connector is not present on the mature (old) flagella of *T. brucei*, supporting its role as a cytotactic transducer rather than a structure involved per se in flagellum dynamics.

### Axonemal cross-linking at the flagella connector

The three flagella connector domains in the membrane of each

flagellum oppose each other and are linked to specific outer doublet microtubules of the flagella axonemes. In turn, each of these three domains is cross-linked to particular axonemal outer doublets, one set belonging to the new flagellum and another set belonging to the old flagellum. As a consequence, six axonemal outer doublets are involved in the flagellum-flagellum attachment. Our evidence suggests a model in which axonemal outer doublets 7-9 from the old flagellum and three (as yet unspecified) outer doublets from the new flagellum are involved in the flagella connector cross-linking.

It should be realized that some form of axonemal cross-linking has been described in other systems, often for apparent imposition of co-ordinated or synchronized beating (Dentler, 1990). In ctenophores, for instance, axonemal cross-linking is observed in both macrocilia and the ciliary swimming plates, where it aids in the generation of synchronized beating. In macrocilia, 200-250 identically oriented axonemes are directly cross-linked to each other and enclosed by a single unique membrane (Tamm and Tamm, 1985). The situation in the swimming plate is more similar to that found at the flagella connector, because the axonemes are linked to the ciliary membrane and the membranes interact with each other (Dentler, 1981).

The flagella connector appears to be localized perpendicular to the plane of beating, at least in the old flagellum, in which outer doublet microtubules 7, 8 and 9 seem to be linked to the membrane. Significantly, this does not disrupt the motility of the old flagellum, which implies that either the structure of the flagella connector is flexible enough to accommodate the sliding between doublets 7-9 or that the contacts between outer doublets 7-9 and the membrane at the flagella connector are transient.

However, an important difference between the flagella connector and the axonemal cross-linking that occurs in ctenophores is that the flagella connector mediates a singular attachment: it does not link the flagella to each other throughout their length, but solely at one specific, but different, site in each flagellum.

## Temporal events of flagella connector formation and removal

Both immunofluorescence and the EM data indicate that the flagella connector is produced very early in the trypanosome cell cycle, before the new flagellum exits the flagella pocket. Longitudinal sections through the flagella pocket show that the flagella connector is present between the new and old flagellum immediately above the transition zone of the new flagellum. The transition zone of the basal body is the region responsible for the nucleation of the axonemal central pair microtubules, C1 and C2. Although it is difficult to be categorical, the juxtaposition of the flagella connector and the transition zone suggests that flagella connector formation might be almost coincident with the nucleation of the flagella axoneme. Our model would certainly be consistent with this interpretation, although prior nucleation of the flagellar axonemal outer doublet microtubules would be required to ‘hard wire’ the intraflagellar connections.

In trypanosomes, the new flagellum is produced by the elongation and maturation of the probasal body, a structure that lies immediately alongside the mature basal body, subtending the old flagellum (Gull, 1999; Sherwin and Gull, 1989a; Vaughan and Gull, 2003). As the probasal body elongates, it protrudes through the plasma membrane of the flagellar pocket alongside the old flagellum. Although we have not visualized this event, it appears to be mechanistically reasonable that connections between the two flagella form as consequence of this initial elongation event, rather than relying upon the de novo association of two flagellar membranes within the pocket. Although a greater understanding of flagella connector formation awaits the understanding of these early-stage flagella, our present model provides a firm basis for further studies in that it gives the first overall description of the structural organization and components.

Direct microscopic observation of cells in division [see Movies 1-4 (http://jcs.biologists.org/supplemental/)] suggests that the precise timing of flagella connector removal might be variable relative to cytokinesis in *T. brucei*. Although the significance of this variation might ultimately be trivial, it is clear that daughter cells are unable to complete cell separation until the flagella connector attachment is broken. An important question to be resolved is therefore the mechanism by which this highly ordered and previously robust connection is severed. Clues might be derived from studies on cultured epithelial cells, in which desmosomal assembly and disassembly appears to be regulated by $Ca^{2+}$ acting through reversible protein phosphorylation involving both protein kinases and phosphatases (Kitajima, 2002). The involvement of protein phosphorylation in flagella connector assembly and disassembly certainly warrants further study but, as yet, the mechanism of flagella connector severing remains solely a matter of conjecture.

## Flagella connector – an unusual mobile junction

Although the flagella connector forms within the flagellar pocket of the trypanosome, the flagella connector is observed to migrate along the old flagellum as the new flagellum elongates. The EM data and the model we have constructed indicate that the flagella connector connects outer doublets of both the new and old flagellum, with outer doublets 7-9 of the old axoneme acting to guide the new flagellum along the cell axis occupied by the old flagellum. Conceptually, the flagella connector can be visualized as a railway bogey moving along a three-track line. However, at present, we are unable to determine whether the flagella connector has an active or a passive role in this process. It is possible that the flagella connector actively moves along the microtubules as a result of the activity of microtubule plus-end-directed motors such as kinesin (Kull, 2000), or movement along the outer doublets might be passive, resulting from the microtubule outgrowth of the new flagellum. We imagine that the rate of connector movement matches the extension kinetics of the new flagellum (Robinson et al., 1995). Whatever mechanism is responsible for flagella connector movement, this structure is a highly unusual junctional complex. Although cell-cell junctions are clearly not unusual in biology, such structures are not themselves directly associated with mobility.

It should also be realized that flagellum junctional complexes have previously been described in trypanosomatids, where they mediate attachment of flagella to host surfaces. At the site of contact, the flagellar membrane is observed to expand and

abundant filamentous material is present, contacting electron-dense plaques internal to the membrane and lining the region of attachment (Beattie and Gull, 1997; Tetley and Vickerman, 1985; Vickerman, 1985; Vickerman et al., 1988). Although these junctions have been referred to as 'desmosomes' or 'hemi-desmosomes', there is also no evidence to indicate that they are biochemically related to the desmosomes and hemi-desmosomes found in higher eukaryotes.

Are these flagellum attachment structures related to the flagella connector? Although certain components could be shared between the flagella connector and these flagellum attachment complexes, the two structures differ in certain key respects. For instance, the attachment complexes do not have the characteristic trilaminar core structure of the flagella connector and, more importantly, they do not exhibit the overt mobility demonstrated by the flagella connector.

## Biochemical complexity of the flagella connector

The electron micrographs presented in this study suggest that a high degree of structural sophistication exists in the flagella connector that is likely to be reflected in biochemical complexity. In addition to the filamentous connections between microtubule outer doublets and the internal face of the flagellar membrane, transmembrane linkages are also in evidence linking the two flagella. Moreover given that the flagella connector is mobile, motor proteins capable of progressing this junctional complex along the old flagellum are likely to be an important component of the flagella connector assembly.

Although the biochemical composition of the flagella connector remains unknown at present, antibodies such as AB1 do suggest a way forward with regards to biochemical characterization of the flagella connector. Unfortunately, AB1 (an IgM monoclonal) does not blot, making standard approaches (such as expression library screening) unsuitable. We are therefore currently raising new antibodies to cytoskeletal fractions of *T. brucei* and screening for flagella-connector-specific antibodies that also recognize *T. brucei* proteins in immunoblotting. The identification of constituent flagella connector proteins will of course then enable us to investigate the functional role of such proteins. The molecular ablation of *T. brucei* proteins by RNAi techniques shows great utility in trypanosome research and has already proved to be extremely informative in elucidating the structural basis of the axoneme and the PFR in *T. brucei* (Bastin et al., 1998; McKean et al., 2003).

## Species- and stage-specific assembly of the flagella connector

At present, we are unclear about why the canonical flagella connector structure that we have described for the procyclic form of *T. brucei* is not present (or is not so easily visualized) in bloodstream trypomastigote forms or in other forms such as epimastigotes or promastigotes of other trypanosomatids such as *T. cruzi* or *Leishmania*. Because the basic structure of the flagellum does not vary significantly, one explanation might lie in differences in morphogenetic patterning that exist between distinct morphological forms of trypanosomatids (Vickerman and Preston, 1976). Trypomastigote forms of *T. brucei* represent an extreme structural case in which the external flagellum is attached to the cell body for most of its length and so influences the internal cytoskeletal architecture of the cell. The components and principles of the construction of this cytoskeleton, including the contribution of templating and cytotactic phenomena, have been rehearsed previously (Gull, 1999; Moreira-Leite et al., 2001; Robinson et al., 1995; Sherwin and Gull, 1989a; Sherwin and Gull, 1989b; Woodward and Gull, 1990). Thus, in procyclic trypomastigote forms of *T. brucei*, the flagellum-FAZ complex has a central role in cell morphogenesis and so, at this stage, the flagella connector can easily be interpreted as being crucial for the inheritance of cell shape and polarity. By contrast, in *T. cruzi* epimastigotes, in which the FAZ is confined to the anterior end of the cell, flagellum attachment is not required for efficient cell division (Cooper et al., 1993). In the same manner, the promastigotes of *Leishmania* have a free flagellum that emerges from a pocket at one pole of the elongated cell.

However, trypomastigote bloodstream forms of *T. brucei* do have a long flagellum extending from posterior to anterior and connected to the cell body throughout most of its length. Curiously, although the distal tip of the new flagellum remains close to the old flagellum during cell duplication, we have found no evidence of the canonical flagella connector structure. Are there differences between bloodstream forms and procyclic trypomastigotes that explain this different requirement for the flagella connector? First, it should be realized that the shape of bloodstream and procyclic cells are strikingly different. As a consequence, expansion of the subpellicular cortex during cell duplication differs between bloodstream and procyclic forms. In particular, it appears that the cortical area between the new and the old flagella suffers less expansion during cell division of the bloodstream form. The limited remodelling and expansion of the subpellicular cortex that takes place during bloodstream-form division might allow the new internal cytoskeleton to be formed in the correct direction without the aid of a positional clue provided by the old flagellum via the flagella connector. By contrast, the flagella connector might be required to guarantee the fidelity of new cytoskeletal positioning in the context of the more extensive subpellicular cortex expansion observed in dividing procyclic cells.

Another important morphogenetic difference between procyclic and bloodstream forms of *T. brucei* relates to kinetoplast positioning during cell division. In bloodstream forms, kinetoplasts undergo only limited segregation but, in dividing procyclic forms, one kinetoplast (associated with the new flagellum) undergoes substantial migration to the posterior end of the cell. Mitosis then places one daughter nucleus into the gap opened up by this movement (see Fig. 7 for contrasting styles of organelle positioning). Attachment of the new flagellum to the old in the procyclic trypomastigote form might be required for the extensive kinetoplast/basal-body migration and internal cytoskeletal remodelling but not for the limited kinetoplast movement in the bloodstream form. These conjectures however, have the caveat that the track along the cell followed by the new flagellum in bloodstream trypanosomes is not random and there might well be a variant of the connector phenomenon operating.

These studies clearly raise important questions regarding the current terminology of cell types and morphogenesis in trypanosomes. Although both procyclic and bloodstream forms of *T. brucei* are defined as trypomastigotes, different structural

mechanisms appear to operate to safeguard the fidelity of cell shape determination and the segregation and polarized localization of cytoplasmic organelles during cell duplication. No doubt the burgeoning use of RNA interference to ablate expression of proteins and produce phenotypes will see interesting variations appear between cell cycle control and cytoskeletal morphogenesis in these different forms of the parasite.

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