

Investigating Intraflagellar Transport in *Leishmania mexicana* Using Dual-Colour High-Frame Rate Imaging and Genetically Encoded Fluorescent Proteins



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Thank you to my supervisor Richard Wheeler for his support and mentoring over the course of my DPhil and to my research group.

This thesis is dedicated to my partner, family and friends.

For Luna

Because I've known you

Translated and adapted from Oliver Dragojević by Ana

Find the silence and
let the moon's soft glow cover you.
For tomorrow, shadows will fade
leaving those words unspoken.
Rest beside me.

Since I've known you, I believe in angels.
Since I've known you, I believe they exist.

No destiny can be determined by the stars,
you create your own path.
Now, close your eyes and anchor yourself calmly.
Your spirit lives on,
I promise.

Eclipsing the darkness, our moon,
turning a new page
wiping tears for those left behind
the storm may rise!
But your soul is at peace.
And I know you've found your way.

Because I believe in angels,
I believe they exist.
Because I've known you.

To our ironwoman, our inspiration, our forever moon.

Abstract

The assembly, maintenance and function of motile flagella is achieved by two sets of motor protein-driven movement. Inner and outer dynein arm slide adjacent microtubules of the axoneme to produce flagellar bending, whilst the bidirectional movement of intraflagellar transport (IFT) trains along the axoneme are driven by kinesins and dyneins. Previously IFT train speeds have been quantified in mechanically immobilised or genetically paralysed flagella, limiting exploration into the relationship between IFT and flagellar motility. *L. mexicana* is a uniflagellate protozoan parasite that is highly genetically tractable. It undergoes symmetric-tip-to-base beat for forward swimming and an asymmetric base-to-tip beat for reorientation. In this project, we used high-frame-rate dual-colour microscopy and genetically encoded fluorescent tags to visualise IFT in the beating flagella of *L. mexicana*. This revealed IFT train speed is largely independent of beat type but is influenced by genetic perturbations to flagellar motility. With increased anterograde IFT train speed in slower-swimming mutants and decreased retrograde train speed in mutants with higher flagellar beating frequencies. We noted that train stalling can occur without substrate interaction, however does so less frequently than in flagella immobilised mechanically. We identified novel molecular components of the IFT system, including microtubule-associated proteins putatively acting as IFT train tracks and mitogen-activated protein kinase negative regulators of IFT train speed. In parallel, we explored the effect of light intensity, light wavelength and redox state on flagellar beating and cellular motility, with initial indications that tryparedoxin proteins with flagellar localisation could play a role in flagellar recovery from oxidative stress. Overall, this project highlights the dynamic interplay of IFT, flagellar motility and environmental regulation in the motile flagella of *L. mexicana*. It provides a foundation for future studies using advanced imaging and genetic tools to further unravel the regulatory networks governing IFT and flagellar function and their broader implications for cellular motility in other systems.

Declaration

No portion of the work referred to in this thesis has been submitted, either partially or in full, for another qualification at this or any other institute of learning.

Excluding references the text of this thesis consists of 40,000 words.

Table of Contents

Abstract.....	3
Declaration.....	4
Table of Contents.....	5
1 Introduction	12
1.1 The use of microscopy in decoding flagellar dynamics and IFT	12
1.2 <i>Trypanosoma</i> and <i>Leishmania</i>	15
1.2.1 Trypanosomatid resources.....	15
1.2.2 <i>L. mexicana</i> life cycle.....	16
1.2.3 Procyclic promastigote.....	19
1.2.4 Trypanosomatid genomic divergence.....	19
1.3 Intraflagellar transport	20
1.4 IFT train components.....	22
1.4.1 IFT-A and IFT-B	22
1.4.2 IFT kinesins	24
1.4.3 IFT dyneins	24
1.5 IFT train stalling	25
1.6 IFT train assembly and turn around	25
1.7 IFT regulation.....	26
1.7.1 Tracks	27
1.7.2 Calcium.....	27
1.7.3 Phosphorylation and kinase activity	28
1.8 The flagellum and associated structures.....	29
1.8.1 Flagellum-associated structures	29
1.9 Axonemal components.....	31
1.9.1 Central pair, microtubule doublets & their decoration	31
1.9.2 Inner & outer dynein arms.....	32
1.9.3 The N-DRC	33
1.9.4 Radial spokes.....	33
1.10 Flagellum length control.....	34
1.11 Flagella sensing.....	36
1.12 Flagellar beating	37

1.13	Regulation of flagellar beating	38
1.13.1	Calcium	38
1.13.2	ATP	40
1.13.3	cAMP	40
1.13.4	Redox.....	41
1.13.5	Phosphorylation and kinase activity	42
1.14	Project aims	43
1.14.1	The nature of IFT in beating flagella and the impact of experimental conditions.....	43
1.14.2	Interaction of IFT train speed and flagellar motility	43
1.14.3	Molecular elements of IFT system architecture	43
1.14.4	The interaction of light, redox, calcium and flagellar motility	44
2	Materials and methods	45
2.1	<i>L. mexicana</i> cell culture	45
2.2	<i>L. mexicana</i> genetic modification.....	45
2.3	Cell lines.....	47
2.4	Validation gels	50
2.5	PCR primers	53
2.6	Fluorescence microscopy	63
2.6.1	IFT analysis on adhered cell samples	63
2.6.2	IFT analysis on swimming cell samples	63
2.6.3	Motility assays.....	64
2.6.4	Image analysis	64
2.6.5	Light intensity measurements.....	65
2.7	Transmission electron microscopy	66
2.8	Comparative genomics	66
2.8.1	Basic Local Alignment Search Tool (BLAST) searches.....	66
2.8.2	Phylogenetic trees.....	67
3	The nature of IFT in beating flagella and its relationship with beat type switching.....	68
3.1	Introduction.....	68
3.2	Results	70
3.2.1	Increasing fluorescence signal for IFT train visualisation.....	70
3.2.2	IFT train speed was faster in genetically paralysed than in adhered flagella	73
3.2.3	IFT train speed decreases faster over time in adhered cells.....	75
3.2.4	High-frame rate dual-colour microscopy allows visualisation of IFT in a beating flagellum	76

3.2.5	Image analysis pipeline allows comparison of IFT train speed and flagellar motility behaviour	77
3.2.6	IFT train stalling in adhered and free flagella.....	79
3.2.7	Flagella beat type and IFT train speed are independent	80
3.3	Discussion	83
4	The effect of genetic perturbations to flagellar motility on IFT train speed	87
4.1	Introduction.....	87
4.2	Results	90
4.2.1	3xmNG::IFT172 deletion mutants display differing structural changes to their axonemes	90
4.2.2	Several 3xmNG::IFT172 deletion mutants have reduced flagellum length	91
4.2.3	3xmNG::IFT172 deletion mutants have distinctive swimming patterns	91
4.2.4	3xmNG::IFT172 deletion mutants have reduced swimming speed.....	93
4.2.5	Not all 3xmNG::IFT172 deletion mutants are able to generate wild-type beat types ...	94
4.2.6	3xmNG::IFT172 deletion mutants have distinct patterns of flagellar beating	96
4.2.7	Paralysed or reduced frequency beating flagella have faster anterograde IFT trains....	99
4.2.8	Increased frequency beating flagella have slower retrograde IFT trains.....	100
4.2.9	Investigating if proximal distal axonemal asymmetry in the flagellum alters IFT train speed	10
4		
4.3	Discussion	107
5	Novel IFT components, tracks and Regulators	111
5.1	Introduction.....	111
5.1.1	The IFT Particle	111
5.1.2	IFT Tracks.....	112
5.1.3	IFT regulators	114
5.2	Results	115
5.2.1	A novel kinetoplast IFT dynein intermediate chain	115
5.2.2	IFT dynein intermediate chain moves with IFT particle	115
5.2.3	LmxM.05.0690 has conserved localisation	116
5.2.4	PGPGXY rich protein LmxM.34.3925 is localised along the length of flagellum	117
5.2.5	LmxM.34.3925 deletion reduces IFT train speed without affecting swimming speed or axoneme structure.....	118
5.2.6	LmxM.30.0890 (CFAP97) may interact with IFT	120
5.2.7	Candidate MPKs have flagellar localisation	123
5.2.8	MPK13 and MPK14 have IFT-associated patterns of movement.....	124

5.2.9	MPK9, MPK13 and MPK14 deletion mutants have increased anterograde IFT train speed	125
5.3	Discussion	128
6	Light, redox, calcium and swimming.....	131
6.1	Introduction.....	131
6.2	Results	134
6.2.1	Light intensity alters beat type switching	134
6.2.2	There were no obvious light sensitive BLUF1 or BLUF2 in <i>L. mexicana</i>	135
6.2.3	<i>Leishmania</i> swimming is not altered by exposure to different light wavelength.....	136
6.2.4	In <i>L. mexicana</i> , oxidative stress altered swimming speed	138
6.2.5	TXNs are encoded by gene arrays across trypanosomatids.....	141
6.2.6	<i>Leishmania</i> possess TXNs with flagellar localisations	142
6.2.7	TXN deletion does not significantly impact swimming speed under normal conditions	142
6.2.8	TXN1 deletion may reduce recovery from oxidative stress-induced changes in swimming	143
6.2.9	Genetically encoded calcium sensor visible at low frame rates in beating flagella.....	145
6.3	Discussion	147
7	Perspectives and conclusions	150
7.1	Introduction.....	150
7.2	Considerations of experimental methodology.....	150
7.3	Biological findings: IFT train speed, flagellar motility and their interaction	153
7.3.1	IFT system.....	154
7.3.2	IFT speed and flagellar motility	158
7.3.3	Flagellum length, IFT and swimming.....	159
7.3.4	Flagella beat and swimming regulation	160
7.4	Avenues for future research.....	162
7.4.1	Microscopy optimisation and multi-focal plane imaging.....	162
7.4.2	Genetically encoded fluorescent sensors	162
7.5	Conclusion	163
8	References	165

Abbreviations

3×mNG – 3×mNeonGreen

ADP – Adenosine diphosphate

ATP – Adenosine triphosphate

BLAST – Basic Local Alignment Search Tool

Blast – Blasticidin-S deaminase

C. fasciculata – *Crithidia fasciculata*

C. reinhardtii – *Chlamydomonas reinhardtii*

Ca²⁺ – Calcium ions

cAMP – Cyclic adenosine monophosphate

Cas9T7 – Cas9 and T7 RNA polymerase

CILK1 – Ciliogenesis associated kinase 1

CLEM – Correlative light and electron microscopy

dDC – Distal docking complex

EM – Electron microscopy

E-value – Expected value

FAZ – Flagellum attachment zone

FOV – Field of view

GFP – Green fluorescent protein

H3 – Histone 3

Hyg – Hygromycin

IDA – Inner dynein arm

IFT – Intraflagellar Transport

IFT172 – Intraflagellar Transport Protein 172

L. amazonensis – *Leishmania amazonensis*

L. braziliensis – *Leishmania braziliensis*

L. donovani – *Leishmania donovani*

L. infantum – *Leishmania infantum*

L. mexicana – *Leishmania mexicana*

L. tarentolae – *Leishmania tarentolae*

LC – Light chain

MAK – Male germ cell-associated kinase

MAP – microtubule-associated protein

MIP – Microtubule inner protein

mNG – mNeonGreen

MOK – MAPK/MAK/MRK overlapping kinase

MPK – Mitogen-activated protein kinase

N-DRC – nexin-dynein regulatory complex

Neo – Neomycin-kanamycin phosphotransferase

NTC - Nourseothricin sulfate

OAD – Outer arm dynein

ODA – Outer dynein arm

ORF – Open reading frame

PAC – Photoactivated adenylyl cyclase

PBS – Phosphate buffered saline

pDC – Proximal docking complex

PFR – Paraflagellar rod

Phleo – Phleomycin-resistance gene

PKA – Protein kinase A

PKC – Protein kinase C

Puro – Puromycin N-acetyltransferase

ROS – Reactive oxygen species

RSP – Radial spoke protein

sgRNA – Single-guide RNA

T. brucei – *Trypanosoma brucei*

T. cruzi – *Trypanosoma cruzi*

TAC – Tripartite attachment complex

TXN – Tryparedoxin

1 Introduction

1.1 The use of microscopy in decoding flagellar dynamics and IFT

Flagella and cilia are protrusions from cell surfaces, described upon their discovery via light microscopy in the 17th century by Anthony van Leeuwenhoek as ‘incredibly thin feet’ (Mul et al., 2022; Dobell et al., 1932). In nearly all organisms, flagella are constructed and maintained by an intraflagellar transport (IFT) system, comprised of IFT trains which move bi-directionally along the flagellum carrying cargo.

Flagella have a highly conserved molecular structure believed to have evolved prior to divergence of extant eukaryotes’ last common ancestor (Cavalier-Smith, 2002; Mitchell, 2004). They possess motility, signalling and sensory functions which play an essential role in the propulsion of protists, a group containing many pathogens, and also in the development and reproduction of many multicellular organisms, including humans.

Motile flagella possess a skeleton consisting of a 9+2 microtubule axonemal structure, with 9 microtubule doublets that are suspended from a central pair of singlet microtubules by projections called radial spokes (Khan and Scholey, 2018; Ishikawa, 2017; Grigg and Hodge, 1949). Motor proteins residing on inner and outer dynein arms of the microtubule doublets power sliding relative to their adjacent doublet and control flagellar bending (Lindemann, 2007; Smith and Yang, 2004). Anterograde and retrograde IFT train movement along the microtubule doublets is driven by a second set of kinesin and dynein motor proteins. Dysfunction in motile cilia, caused by genetic mutants in genes encoding flagellar or IFT components, underlie a heterogeneous collection of human diseases known as motile ciliopathies (Wallmeier et al., 2020), the most prevalent being primary ciliary dyskinesia.

Just over one hundred and twenty years ago, Buller described the difficulty of visualising flagella using transmitted light due to their small radius, instead relying on using the cell body of echinoid spermatozoon to quantify swimming speeds and study chemotaxis (Reginald Buller, 1902). In 1949,

Lord Rothschild and Michael Swann utilised dark-field light microscopy to investigate the speed of flagella driven swimming behaviour in sea-urchin spermatozoa (Rothschild and Swann, 1949). Later in 1955, Sir James Gray used photographic recording to visualise waveforms in the motile flagellum of the same organism (Gray, 1955). High-speed phase contrast microscopy has since been used to study a range of phenomena related to flagellar motility in diverse organisms, including using multi-focal plane imaging to visualise flagellar movements in three dimensions, however it does not permit the visualisation of dynamic molecular components within the flagellum during beating (Ishijima, 1995; Mojiri et al., 2021; Cheong et al., 2015; Walker and Wheeler, 2019)

Evolving alongside light microscopy based methods of studying flagellar movement were investigations into flagellar structure. Using electron microscopy (EM), Grigg and Hodge discovered the 9+2 microtubule organisation in spermatozoa in 1949, which was shortly found to be highly conserved from algae to vertebrate animals, with some exceptions (Grigg and Hodge, 1949; Manton and Clarke, 1952; Gibbons, 1981; Challice, 1953; Bradfield, 1953). Since these initial studies, improvements in specimen preparation techniques, advancements in EM instrumentation and novel computational techniques, amongst other enhancements, have allowed for high resolution structural recapitulations of the axoneme in various model organisms, enabling characterisation of the organisation and periodicity of its components (Klena and Pigino, 2022; Piperno et al., 1990; Walton et al., 2023; Ma et al., 2019; Kubo et al., 2023; Doran et al., 2025; Imhof et al., 2019). With continuing advancement, including the emergence of cryo-electron tomography single particle analysis providing the ability to investigate protein structure at a molecular level (Mali et al., 2021; Walton et al., 2021).

IFT trains were initially observed in 1993, using differential interference contrast light microscopy in the paralysed flagellum of *Chlamydomonas* (Kozminski et al., 1993). Later correlative light and electron microscopy (CLEM) facilitated the correlation of these IFT particles with electron dense complexes we now know as IFT trains (Kozminski et al., 1995). Just before the turn of the century, the first *in vitro* fluorescence visualisation of IFT was first completed in *C. elegans* by tagging kinesin-2 with green

fluorescent proteins (GFP) (Orozco et al., 1999). In the three decades since its discovery (Kozminski et al., 1993), the molecular analysis of IFT transport machinery (Lacey et al., 2023) and advancements in structural microscopy techniques (Ma et al., 2023; Jordan and Pigino, 2021) have dramatically improved our understanding of the molecular composition of IFT machinery. The use of fluorescent labelling of IFT proteins in live cells through cloning or CRISPR/Cas9 mediated endogenous tagging of genes has allowed visualisation of tens of IFT or IFT-associated protein (Ishikawa and Marshall, 2015; Diener, 2009; Lechtreck, 2013; Blacque et al., 2006; Mukhopadhyay et al., 2007; Absalon et al., 2008; Mueller et al., 2005). However, the analysis of IFT movement has been almost exclusively carried out in immobile flagella, be this in paralysed flagella mutants, naturally immotile flagella, or flagella that have been mechanically immobilised (usually via adherence to a glass slide or coverslip). We are unaware of existing quantitative analysis of IFT train movement in a cilium/flagellum in its physiological state of beating. Thus, the nature of the intricacies of IFT machinery function – including motor protein processivity, anterograde and retrograde train speed, frequency and stalling, in motile flagella in their physiologically normal state of beating remain to be elucidated.

Advancements in technology bring with them new opportunity and permit us to explore avenues of experimental investigation previously inaccessible. In order to analyse IFT in live swimming cells, we need to visualise both tagged IFT fluorescence and flagellum position simultaneously. In 2020, Wang et al, developed high frame rate dual-colour fluorescence microscopy, showcasing its ability to visualise two fluorescently tagged structures in the beating flagellum of a swimming cell simultaneously (Wang et al., 2021). This was done by using dual-colour widefield epifluorescence microscopy in combination with dichroic mirrors, filters and a beam splitter to create two channels corresponding to green and red fluorescence which were projected in parallel onto a camera sensor. In this project, we harnessed high-frame rate dual-colour microscopy to visualise IFT in beating flagella and investigate its dynamic interaction with axonemal machinery. This work was completed in trypanosomatid *L. mexicana*, a genetically tractable model organism of flagellar motility, within which

target genes with IFT- or flagellar motility-associated function could be endogenously tagged with genetically encoded fluorophores or deleted using CRISPR/Cas9 mediated genetic modification.

1.2 *Trypanosoma* and *Leishmania*

Trypanosomatidae is a family of uniflagellate protozoan parasites which arose from an early branching in the eukaryotic lineage. As the most diverged eukaryotes, elucidating the structure and function of flagella in these organisms is of dual importance. Primarily, conserved flagellar features are likely to indicate core and eukaryote-wide features of flagella and discoveries would potentially be of consequence in research fields ranging from fundamental biology to human health and disease, particularly ciliopathies (Reiter and Leroux, 2017). Whereas non-conserved flagellar mechanisms may reveal parasite-specific adaptation critical for life-cycle transitions and transmission (Dean et al., 2017). Two genera in this family are *Trypanosoma* and *Leishmania* which contain many pathogens of humans and livestock, notably *T. brucei* and *L. mexicana* are the aetiological agents of African sleeping sickness and cutaneous leishmaniasis, respectively.

1.2.1 Trypanosomatid resources

Previous and continuing collaborations between groups researching trypanosomatids, has led to the development of a plethora of high-quality resources for informing the design of reverse genetic experiments in *L. mexicana*. *Leishmania* Genome Editing (LeishGEdit), is a CRISPR Cas9 T7 RNA polymerase online genome editing resource, which was used to inform the design of primer sequences for tagging or deleting target genes in *Leishmania* (Beneke et al., 2017). TriTryp Database (TriTrypDB), a VEuPathDB project, hosts integrated genomic data, and a selection of tools including annotated gene models, orthology and synteny, protein features and properties for *L. mexicana*, and other kinetoplastids, although information availability varies according to gene and organism (Aslett et al., 2010). For *T. brucei*, there exists primary searchable database TrypTag which holds information on genome-wide protein localisation (Dean et al., 2017). For *L. mexicana*, a genome-wide annotation

database, with additional information on protein localisation and deletion mutant phenotypes is currently being developed (LeishGEM Team, 2024).

1.2.2 *L. mexicana* life cycle

Many trypanosomatids, including species of *Leishmania* and *Trypanosoma*, are digenetic and switch between differing morphological forms (Figure 1-1A). Life cycle stages have different flagellar morphology and swimming behaviours - notably the promastigote (long flagellum, fast swimmer (Sacks and Perkins, 1984)) and the amastigote (short predominantly immotile flagellum (Gluenz et al., 2010; Gull, 2009)).

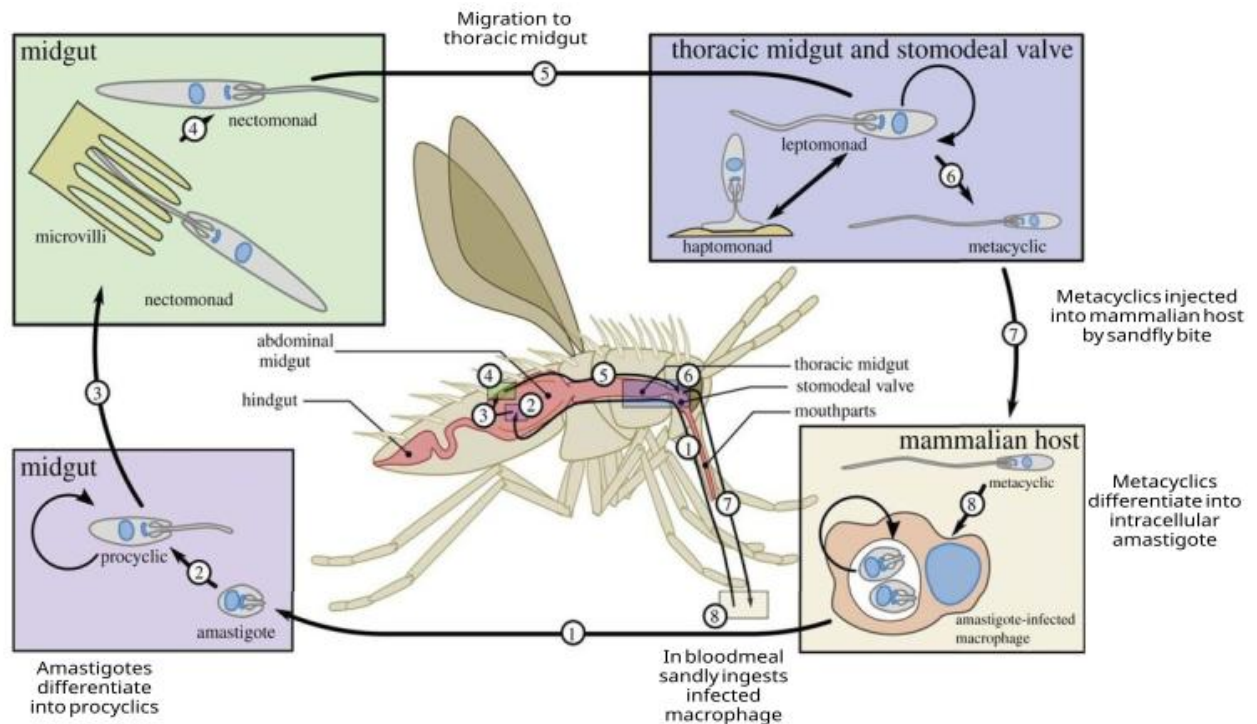
L. mexicana circulates between niches in the sandfly vector and mammalian hosts (Stevens, 2008; Barrett and Croft, 2012; Bates et al., 1992). After being taken into the sandfly abdominal gut as amastigotes, *L. mexicana* differentiate to their procyclic promastigote form (Figure 1-1B) and rapidly replicate for 24-48 hours (Kamhawi, 2006). Procyclic promastigotes swim in slow helical paths (Findlay et al., 2021). They travel anteriorly to the thoracic midgut, and adhere to the peritrophic matrix to avoid expulsion, becoming nectomonads then leptomonads with some differentiating to haptomonads (Kamhawi, 2006). Others differentiate into metacyclic promastigotes. Compared to non-infective procyclic promastigotes, metacyclic promastigotes have longer flagella and are faster swimming - undergoing a 'run and tumble' movement and chemotactic responses in the form of altering swimming speed and direction to macrophage-associated stimuli (Findlay et al., 2021). Metacyclic promastigotes also have reduced expression of protein synthesis related proteins but upregulated expression of flagellar structural components i.e. α - and β -tubulin and PFR1D (Mojtahedi et al., 2008).

In the anterior midgut, *Leishmania* secrete proteophosphoglycans which condense into a viscous promastigote secretory gel or biofilm, which has various functions including protection from the insect and mammalian immune system (Ilg et al., 1996; Stierhof et al., 1999; Rogers et al., 2002; Giraud et al., 2019; Rogers et al., 2024). *In vitro* at high viscosities, metacyclic promastigotes were more capable

of motility than procyclic promastigotes, suggesting metacyclic flagellar adaptations may aid swimming in the biofilm (Findlay, 2018). *In vitro* experiments revealed that *Leishmania* mutants with uncoordinated or paralysed flagella (Beneke et al., 2019) or with interference to their cAMP signalling through the deletion of PDEB1 enzyme (Shaw et al., 2019) were unable to travel from the posterior to anterior midgut. This highlights the importance of flagellar motility in *Leishmania* survival and transmission.

A bite from a sand fly that is *Leishmania*-infected deposits metacyclic promastigotes in the human host. Macrophages are recruited to the bite site – which phagocytose the promastigotes. Once inside the macrophages' parasitophorous vacuole, *Leishmania* cells become smaller and more spherical, diminishing its surface area and thus exposure to its novel harsh environment (Sunter and Gull, 2017). During differentiation into amastigotes, the 9+2 motile axoneme disassembles to shorten until it resides only in the flagellar pocket and loses its central pair to obtain a 9+0 arrangement (Wheeler et al., 2015). The flagellar pocket and neck are also restructured through the relocation of the flagella attachment proteins and subsequent closure of the flagellar pocket neck (Wheeler et al., 2016). In *T. cruzi*, the flagella of intracellular amastigotes underwent slow beating (~0.7 Hz) that was dependent on mitochondrial energy metabolism – namely oxidative phosphorylation (Won et al., 2023). However, *L. mexicana* 9+0 flagella possess no central pair or dynein arms – suggesting they are immobile (Alves and Bastin, 2023; Gluenz et al., 2010).

A



B

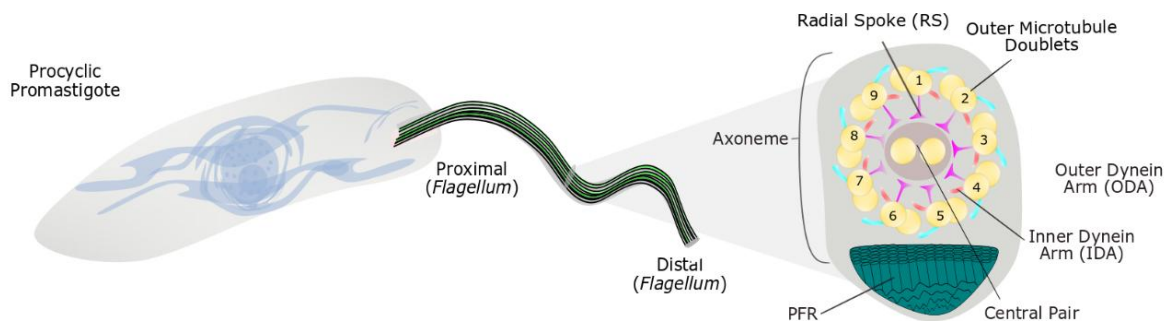


Figure 1-1, Adapted from Sunter and Gull, 2017 | Procyclic promastigotes are the *L. mexicana* life cycle stage used in this project. A. Schematic of digenetic *Leishmania* life cycle including their promastigote and amastigote forms. B. The cellular structure of an *L. mexicana* promastigote. The flagellum of *L. mexicana* promastigotes emerges from the anterior of the cell. A cross section of the distal flagellum of *L. mexicana* is shown. Visible is the 9+2 structure of the 9 microtubule doublets surrounding the central pair, extending to which are the radial spokes. Between the microtubule doublets are the inner and outer dynein arms. Running parallel to the axoneme is the paraflagellar rod (PFR).

1.2.3 Procyclic promastigote

In this research, we study *L. mexicana* in their procyclic promastigote stage. In culture, *L. mexicana* promastigotes switch between symmetric tip-to-base and asymmetrical base-to-tip waveforms for forward swimming and reorientation respectively, enabling the study of dynamic IFT and flagella function during different beat type cycles (Figure 1-2) (Wang et al., 2021; Gadelha et al., 2007). The cells have an elongated cell body with highly polarised organisation of single-copy organelles and a long motile flagellum extending from the anterior end. The centre of the cell body is occupied by the nucleus and anterior end by the kinetoplast – a structure of concatenated mitochondrial DNA (Hair et al., 2024). A filamentous tripartite attachment complex (TAC) connects the kinetoplast to the basal body (Ogbadoyi et al., 2003).

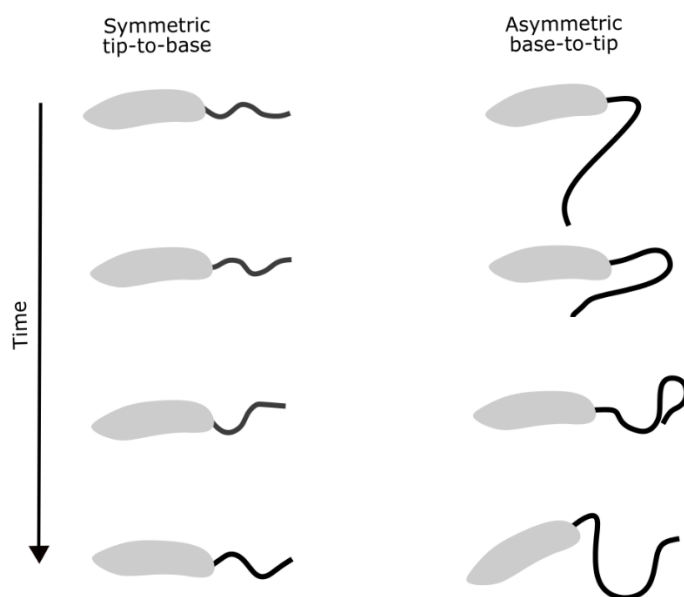


Figure 1-2, Adapted from Wang et al., 2021| *L. mexicana* undergo different beat types. The symmetric tip-to-base beat is for forward swimming and is a planar near-sinusoidal waveform. The planar, polarised, asymmetric base-to-tip beat is for reorientation and is composed of a power stroke followed by a recovery stroke.

1.2.4 Trypanosomatid genomic divergence

Trypanosomatids have evolved fundamentally different molecular processes within their cells compared to other eukaryotes (Stiles et al., 1999; Mishra et al., 2003). *Leishmania* gene expression is

largely regulated post-transcriptionally and genes are expressed as polycistronic transcripts, that can encode hundreds of proteins which are functionally unrelated (Black et al., 2023; Mishra et al., 2003; Vanhamme and Pays, 1995). These transcripts are then subject to trans-splicing and polyadenylation. mRNAs receive regulatory sequences, most commonly through protein binding sites in the 3' untranslated region which enable protein up- and down-regulation in response to environmental or life cycle changes modulating various behaviours, potentially including flagellar motility (Teixeira and daRocha, 2003; Beneke et al., 2019). In *T. brucei*, RNA polymerase II was found to be responsible for the majority of transcription, however few RNA polymerase II promoters have been found in trypanosomatids with one notable exception being the SL RNA gene promoter (Das et al., 2008; Hahn, 2004; Srivastava et al., 2018). DNA modification and histone variation are thought to be involved in transcriptional regulation, with the presence of glycosylated thymidine (Base J) found almost ubiquitously at RNA polymerase II termination sites (Cliffe et al., 2010; van Luenen et al., 2012; Reynolds et al., 2014). The generation of tandem gene arrays by gene duplication is suggested to play a role in increasing protein expression, with downstream genetic mutation of amplified genes providing a basis for functional divergence (Valdivia et al., 2015; Rogers et al., 2011). Trypanosomatid parasites are largely diploid however due to duplication have aneuploidy and polyploidy of some chromosomes; notably, in *L. mexicana* chromosome 30 is tetrasomic (Rogers et al., 2011; Black et al., 2023; Reis-Cunha and Jeffares, 2024). Given that CRISPR/Cas9 mediated genetic modification was a critical tool used during this project, the consideration of gene arrays, genomic regulation and aneuploidy was important, especially during deletion validation and biological interpretation of data.

1.3 Intraflagellar transport

The protein components of IFT are highly conserved across cilia and flagella found in eukaryotes (Pigino, 2021). Much of the early visualisation of IFT and biochemical identification of IFT proteins was completed in *Chlamydomonas reinhardtii*, a unicellular photosynthetic eukaryote with two flagella - though initially its GC codon bias caused difficulties for foreign protein expression (Behal et al., 2009;

Morga and Bastin, 2013; Heitzer et al., 2007). Since there has been a diversification in flagellated model organisms used to study IFT, with much research in *C. elegans* (Orozco et al., 1999), *Trypanosoma* (Absalon et al., 2008) and mammalian cells (Follit et al., 2006). Insights from a range of model organisms will be referenced in this thesis, with a bias towards *Chlamydomonas*, due to volume of literature, and to trypanosomatids, given *L. mexicana* is one.

The functioning of the IFT system moulds the flagellar proteome, with all flagellar proteins being synthesised in the cell body before being transported to the flagellum base and where IFT carries many as cargo along the axoneme ensuring for correct distribution (Fort et al., 2016; Webb et al., 2020). IFT trains are large macromolecular complex powered by motor proteins. They travel along the highly decorated outer face of the microtubule doublets between the outer dynein arms (ODAs) (Lacey et al., 2023). Anterograde IFT trains are pulled from base to tip by kinesin motor proteins, when they reach the flagellar tip they are reassembled into retrograde trains which are driven back to the flagella base by dynein motor proteins. In anterograde trains, IFT-A is directly below the flagellar membrane and even introduces membrane curvature, therefore it is unsurprising IFT is thought to interact with transmembrane proteins (Jordan et al., 2018; Hesketh et al., 2022).

Anterograde trains are characteristically larger, one CLEM study found the mean length of anterograde and retrograde trains in *Chlamydomonas* to be 233 nm and 209 nm respectively, composed of 16 IFT particles on average, while another found them to be ~312 nm (Stepanek and Pigino, 2016; Pigino et al., 2009; Vannuccini et al., 2016; Jordan et al., 2018). In *Chlamydomonas*, 3D CLEM revealed that bi-directional IFT transport on the microtubule doublets occurred in a double-track railway, with anterograde and retrograde transport on B-microtubules and A-microtubules respectively (Stepanek and Pigino, 2016). In *Chlamydomonas*, IFT trains are capable of travelling along any of the nine microtubule doublets (Stepanek and Pigino, 2016) whereas in *T. brucei*, IFT is largely restricted to two sets (3 to 4 and 7 to 8) of microtubule doublets (Bertiaux et al., 2018) putatively due to the large extra-axonemal paraflagellar rod (PFR) attaching at doublets 4 and 7 (Farina et al., 1986;

Portman and Gull, 2010). In *T. brucei*, focused ion beam-scanning EM revealed the average train number and length was equal for both sets of doublets with short (~108 - 306 nm) and long (~307-1337 nm) subpopulations of trains on both (Bertiaux et al., 2018). Despite much research characterising these IFT train routes, the molecular mechanisms responsible for designating IFT train movement to specific microtubules remain to be fully investigated.

1.4 IFT train components

Protein subunits of the IFT complex are highly conserved and many are required for correct functioning of the IFT system and flagellar assembly (Hao and Scholey, 2009; Wachter et al., 2019; Huet et al., 2019).

1.4.1 IFT-A and IFT-B

Anterograde trafficking is driven by kinesin-2 motor proteins in association with the IFT-B complex, while retrograde transport is powered by cytoplasmic dynein-2 and involves the IFT-A complex (Figure 1-3A) (Webb et al., 2020; Luckner et al., 2005; Taschner et al., 2016; Picariello et al., 2019). IFT-A and IFT-B are comprised of ~6 and ~16 subunits respectively (Figure 1-3B) (Cole et al., 1998; Taschner et al., 2012; Ma et al., 2023). Many IFT subunit proteins have been found to be essential for flagellum construction and IFT function, including in trypanosomatids (Wachter et al., 2019; Huet et al., 2019).

IFT-B recruits the kinesin motor proteins and carries IFT-A and its inactivated dynein motor proteins from flagellar base to tip. IFT-B is formed of IFT-B1 and IFT-B2 lobes. IFT52 is a component located along the length of IFT-B1 and provides IFT-B with structural stability (Lacey et al., 2023). IFT52 forms a heterodimer with IFT46, whilst IFT70 and IFT88 wrap around it (Lacey et al., 2023), IFT56 and a coiled-coil IFT81/74 subcomplex are bound also. IFT-B2 is composed of two large proteins IFT80, at the core of the complex, and IFT172, as well as dual pairs of coiled-coil proteins: IFT57/38 and IFT54/20, the latter mediates binding to IFT-B1 (Lacey et al., 2023).

IFT-A comprises 6 subunits and has a mass of 767 kDA (in humans), with a core set of proteins (IFT144/IFT140/IFT122) forming a stable subcomplex surrounded by noncore proteins (IFT139/IFT121/IFT43) (Behal et al., 2012).

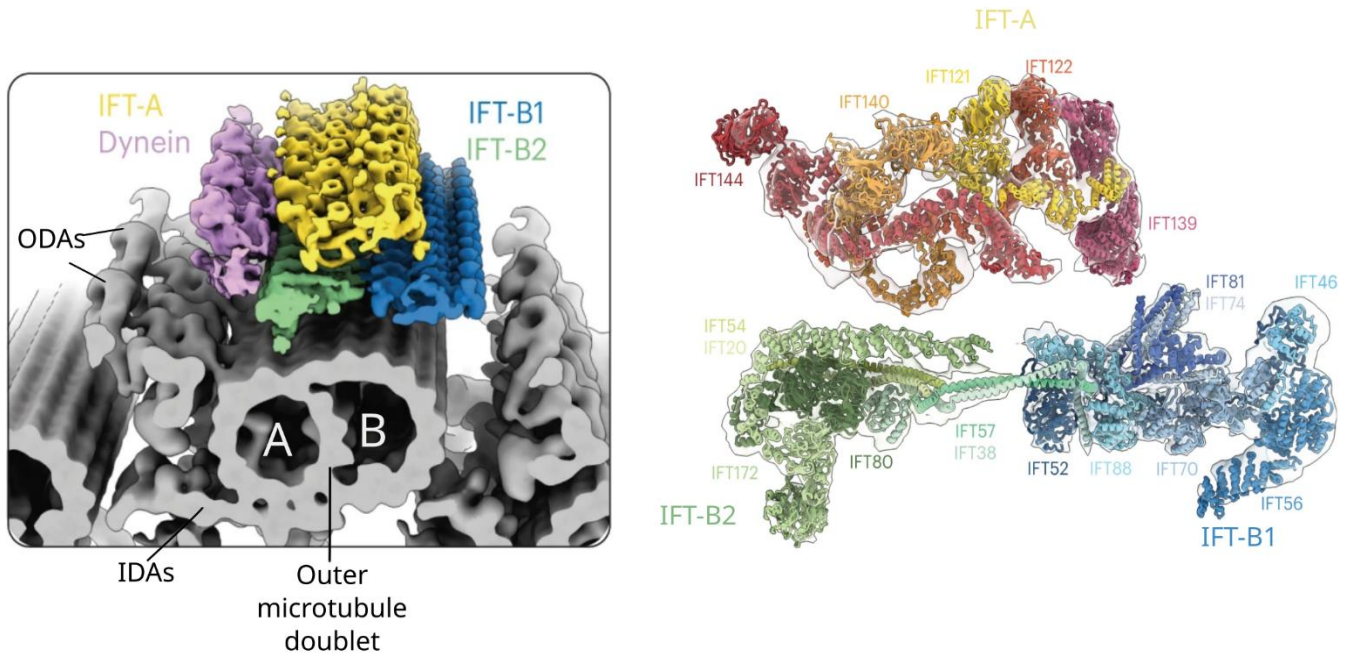


Figure 1-3, Adapted from Lacey et al., 2023 | IFT-B mediates anterograde transport, carrying IFT-A and inactivated dynein. Subtomogram averages of anterograde IFT train structure in context of the outer microtubule doublets coloured by complex, and of the IFT-A, IFT-B1 and IFT-B2 showing protein components from Lacey et al., 2023.

Alongside IFT-B and IFT-A, IFT trains possess non-integral adaptors for carrying cargo (Lechtreck et al., 2009). The Bardet-Biedl syndrome complex (BBSome) is a complex of eight core proteins (BBS1, 2, 4, 5, 7, 8, 9 and BBIP10), along with small GTPase (BBS3) which recruits it to the flagellar membrane, and a chaperone complex (BBS6, 10, 12) (Jin et al., 2010; Loktev et al., 2008). BBIP10 subunit may have a function in coupling axonemal microtubule acetylation to ciliary membrane growth (Loktev et al., 2008). *L. major* BBS1 was found to be essential for infectivity in mammalian host (Price et al., 2013). IFT-A adaptor protein TULP is essential for the flagellar localisation of several membrane proteins including Ciliogenesis associated kinase 1 (CILK1) – involved in train turn around (Hesketh et al., 2022).

1.4.2 IFT kinesins

Kinesin-2 is the driver of anterograde IFT. In *C. reinhardtii*, kinesin-2 is a heterotrimeric complex comprised of two motor proteins (FLA8/10) and an accessory subunit (KAP), the latter is putatively an adaptor protein for allowing attachment to the IFT particle (Cole et al., 1993; Wedaman et al., 1996; Cole et al., 1998; Mueller et al., 2005; Scholey, 2013). One study found an average of ten simultaneously engaged kinesin-2 motors moved IFT trains of ~200 nm in length (Laib et al., 2009; Stepanek and Pigino, 2016). In *C. elegans*, heterotrimeric kinesin-II which is responsible for anterograde transport from base to the transition zone hands over transport to homodimeric OSM-3 which is faster at ~1.3 $\mu\text{m/s}$ compared to ~0.5 $\mu\text{m/s}$ and carried trains to the flagellar tip (Snow et al., 2004; Pan et al., 2006; Prevo et al., 2015). Similarly in *T. brucei*, two kinesin-2 proteins (TbKin2a and b) contribute to flagellar lengthening. TbKin2a was required for cell cytokinesis and motility, was found to co-localise with IFT components in the flagellum and also detected in the flagellum attachment zone (FAZ) (Douglas et al., 2020). Though the exact processivity of these kinesins and whether there is a handover event in trypanosomatids remains to be elucidated.

1.4.3 IFT dyneins

IFT dyneins are essential for retrograde transport. In *Chlamydomonas*, the IFT dynein (cytoplasmic dynein 1b), referred to as cytoplasmic dynein 2 in vertebrates and other organisms, is made of a homodimeric DHC1b interacting with a dynein light intermediate chain D1bLIC, dynein intermediate chains DIC5 and DIC6 and several light chains (DLL1, Tctex1, Tctex2b, LC7b) (Rompolas et al., 2007; Hou and Witman, 2015; Hiyamizu et al., 2023). In *T. brucei*, instead of being homodimeric the dynein heavy chain proteins are heterodimeric, encoded by DHC2.1 and DHC2.2 genes (Blisnick et al., 2014). These heavy chains are stabilised by a dynein light intermediate chain (DLI1) (Blisnick et al., 2014), with an intermediate dynein chain (DIC5). DIC5, contains five WD40 repeats and is required for the localisation of the other dynein components to the flagellum base. As in *T. brucei*, *L. mexicana* possess two distinct IFT dynein heavy chains, namely two dynein-2 heavy chain isoforms (DHC2.1 & DHC2.2).

In *L. mexicana*, DHC2.2 KO resulted in cells with aberrant size and shape and immotile flagella, whilst DHC2.1 was impossible to delete homogeneously, suggesting DHC2.2 to be essential for flagella assembly and cell morphology whereas DHC2.1 is essential for *Leishmania* survival (Adhiambo et al., 2005).

1.5 IFT train stalling

Early observations in *Chlamydomonas* showed that when appressed to a solid substrate, *Chlamydomonas* displayed a “creeping movement”, which was named gliding motility (Lewin, 1952). It was later found that gliding motility was driven by transmembrane proteins carried by IFT trains interacting with the surface substrate to stall trains (Bloodgood and Salomonsky, 1989; Shih et al., 2013; Collingridge et al., 2013; Wan, 2023). IFT stalling occurs frequently in *C. reinhardtii* mutants with IFT disruption and has been noted in flagella adhered to glass for visualisation (Brown et al., 2015). During bi-directional transport such as in axons cargo spends time in a paused or static state due to a tug-of-war between motor proteins, however this has not been observed in IFT (Hancock, 2014). The nature of IFT stalling in beating flagella and the possibility of stalling without substrate interaction has not been studied.

1.6 IFT train assembly and turn around

IFT trains are assembled at the basal body from motor proteins and IFT-A recruited from the cell body, whilst IFT-B is either taken from the cell body or recycled from retrograde trains. Megadalton arrays are formed from repetitive train structures. The IFT trains bind to cargo, some directly and some through associated proteins such as the BBSome complex, ODA16 or IDA3 (Webb et al., 2020; Yang et al., 2020; Lechtreck, 2015; Nachury, 2018). Cargo of IFT particles include axoneme precursor structures like DRC4 and tubulin (Wren et al., 2013). Recently, single particle tracking has shown that in *C. elegans*, the components associate with the IFT train in a sequential manner (Mitra et al., 2024). In anterograde trains, kinesin-2 is activated, carrying inactivated dynein as cargo (Jordan et al., 2018).

In *Chlamydomonas* with full length flagella, every 1.25 seconds (on average) IFT is injected onto the flagellum and they arrive at the flagellar tip with the same frequency (Engel et al., 2009).

At the flagellar tip, cargo is offloaded (Taschner and Lorentzen, 2016) and anterograde trains are disassembled into ~thirds and converted into retrograde trains (Buisson et al., 2013). Dynein which is autoinhibited during anterograde transport to prevent binding to the microtubule, is activated at the flagellar tip to engage with microtubules and power retrograde IFT trains (Jordan et al., 2018). Advanced fluorescence microscopy and analysis of single IFT molecules showed motor proteins detach and diffuse then re-attach to another train, driving individual train turn around (Zhang et al., 2021). This turnaround process is orchestrated with the help of the flagellar tip complex (Dentler and Rosenbaum, 1977; Dentler, 1980), a central cap structure with additional plugs and filaments which connect the central pair of microtubules to the flagellar membrane at its distal end. Although, anterograde IFT trains which were stopped from arriving at the flagellar tip were able to convert into retrograde trains, suggesting remodelling was an intrinsic property driven by stopping rather than the environment of the flagellar tip (Nievergelt et al., 2022). In *T. brucei*, over 3-4 s a large anterograde train was remodelled into several smaller trains on the same side of the axoneme (Buisson et al., 2013). In *Chlamydomonas*, kinesin is not carried back on retrograde trains, instead it travels by diffusion – a process delayed as flagella increase in length, thus serving as a negative feedback mechanism for flagellum length control (Chien et al., 2017).

1.7 IFT regulation

IFT regulation is a complex process that is not yet fully understood. It likely relies on the complex interaction between axonemal microtubules and their modifications, motor proteins, IFT train components and regulatory pathways mediated by kinases.

1.7.1 Tracks

In vitro, the kinesin head and dynein stalk head shared overlapping binding sites on tubulin (Mizuno et al., 2004). Whereas *in vivo* IFT train movement is spatially segregated according to direction or restricted to certain microtubule doublets altogether. Therefore, it is likely that microtubule modifications, through post-translational modification or accessory protein activity have an important role in defining IFT trafficking routes, though the exact molecular basis of this is not fully characterised.

Microtubule properties arise from diversity in the isotypes and posttranslational modifications of tubulin. *In vitro* activity of yeast cytoplasmic kinesin and dynein was regulated by tubulin isoforms and modifications, with robust kinesin-2 activity requiring α -tubulin detyrosination (Sirajuddin et al., 2014). In sensory cilia, tubulin polyglutamylation driven by p38 mitogen-activated protein kinase (MPK) pathway influences IFT velocity (Kimura et al., 2018). In *C. reinhardtii*, the basal body and proximal axoneme had most polyglutamylated tubulin whilst in *Spermatozopsis*, it was on B-tubules, revealing tubulin modifications may be organism specific (Lechtreck and Geimer, 2000). The microtubule doublets are also decorated with proteins, including external microtubule-associated proteins (MAPs), however the interactions of these MAPs with IFT remain to be fully investigated.

1.7.2 Calcium

Calcium ions (Ca^{2+}) effect most aspects of cellular life and are important secondary messengers in trypanosomatids (Docampo and Huang, 2015). In *Chlamydomonas*, surface adhesion increased intraflagellar Ca^{2+} and initiates the movement of stalled IFT trains to prevent their accumulation (Collingridge et al., 2013; Fort et al., 2021). Though, calcium was not found to modulate IFT at the point of injection into the flagellum (Ishikawa et al., 2023). In mouse sperm cells ciliary-associated calcium-binding coiled-coil protein 1 has been shown to interact with IFT20, and is suggested to be important in IFT function during flagellum assembly (Wang et al., 2023).

In trypanosomes, Ca^{2+} can be released from intracellular stores, namely acidocalcisomes, or enter through the plasma membrane for signal generation (Prole and Taylor, 2011; Oberholzer et al., 2011). The role Ca^{2+} plays in trypanosomatid IFT regulation is unknown (Docampo and Huang, 2015).

1.7.3 Phosphorylation and kinase activity

The phosphorylation of motor proteins or associated components in mammalian cells has been shown to be important in regulating motor protein-based transport, including during ciliogenesis and flagellar beating (Cuchillo-Ibanez et al., 2008; Rotureau et al., 2009; Lukong and Richard, 2008; Smith and Yang, 2004; Walczak and Nelson, 1993). Protein kinases are enzymes that alter the function of a protein substrate by transferring a phosphate group onto an amino acid acceptor (Ardito et al., 2017) and are fundamental to a diverse range of cellular processes (Cheng et al., 2011). Phosphorylation state of proteins is mediated by the balance between kinase and phosphatase activity. In murine neurons, MPK10 (JNK3) phosphorylates the ser176 of loop8 in kinesin-1 subunit (conserved in many species) causing reduced microtubule binding (Morfini et al., 2009). The ros cross-hybridizing kinase family has several members involved in IFT regulation. In neuronal progenitor cells, CILK1 was found to be essential for cilia maintenance and ciliary transport regulation via phosphorylation of Kif3a and also regulates flagella turn around (Chaya et al., 2014; Nakamura et al., 2020). In *C. elegans* sensory neuronal cilia, Male germ cell-associated kinase (MAK) and cell cycle-related kinase were found to co-localise with the IFT particle and act in a cascade to regulate its function. Mutations to either resulted in cilia elongation and kinesin-2 ectopic localisation to the distal flagellum due to their role in stabilising the IFT particle-OSM-3-kinesin interaction, though IFT train velocity was unaffected (Yi et al., 2018). Knockdown of MAK-related kinase and MAPK/MAK/MRK overlapping kinase (MOK) resulted in increased flagellum length, interestingly overexpression of MAK-related kinase causes stumpy flagella whilst MOK did not; only MOK knockdown caused increased anterograde speeds. In *Chlamydomonas*, calcium-dependent protein kinase 1 phosphorylation of C-terminal tail of KIF3B is important for kinesin-2 dissociation from IFT-B and train turn around. It is also thought to work in

concert with protein phosphatases to mediate the flagellar entry of IFT trains (Chien et al., 2017; Liang et al., 2014). In *C. elegans*, MPK DYF-5 has been suggested to play a role in undocking IFT particles from kinesin-II and redocking to OSM-3 during handover (Burghoorn et al., 2007).

1.8 The flagellum and associated structures

In 1952, Irene Manton stated that “A cilium is manifestly a highly complex and compound organ, and . . . morphological description is clearly only a beginning” (Gibbons, 1981; Manton and Clarke, 1952). In order to decode the function of IFT we must understand how it interacts with axonemal structures and its dynamic flagellar environment.

1.8.1 Flagellum-associated structures

1.8.1.1 *Leishmania flagellar pocket*

The flagellum of *L. mexicana*, and related species, emerges from a flagellar pocket at the anterior end of the cell, which plays a key role in morphogenesis and acts as the site of all endo- and exocytotic activity (Dong et al., 2019). The flagellar pocket is divided into two domains, the proximal part being bulbous in proximity to many small vesicles and separated from the narrow cylindrical distal ‘neck’ by the flagellar pocket collar (Wheeler et al., 2016). Many other single copy organelles are also associated with the flagellar pocket including the kinetoplast (mitochondrial DNA), basal body, pro-basal body and the Golgi, which runs parallel (Lacomble et al., 2009; Gull, 2003; Gheiratmand et al., 2013).

1.8.1.2 *Basal Body*

The basal body is a barrel shaped organelle, structurally equivalent to centrioles, whose function is to support cellular motility through nucleating the flagellar axoneme (Wheeler et al., 2015). One end is physically connected to the kinetoplast, by the TAC, from the other the flagellum is formed by extension (Wheeler et al., 2011; Hoffmann et al., 2018). Tethered to the basal body is the pro-basal body, a multipotent structure which remains immature for one cell cycle and is capable of forming either a 9+2 or 9+0 axoneme depending on cellular differentiation (Wheeler et al., 2015; Hair et al.,

2023). Basal body maturation is signalled by the manifestation of transition fibres at its distal end, which facilitates IFT train docking and connects the mature structure to the flagellar membrane (Reiter et al., 2012; Kumar et al., 2021a).

1.8.1.3 Transition zone

The transition zone resides between the transition fibres of the basal body and the axoneme. It has an important role in regulating IFT protein trafficking into the axoneme and compartmentalising the flagellar cytoplasm from that of the cell body (Reiter and Leroux, 2017). The transition zone complex comprises 70+ proteins forming microtubule to membrane connections known as γ -links (Dean et al., 2016; Reiter et al., 2012; Gonçalves and Pelletier, 2017).

1.8.1.4 *Leishmania* flagella attachment

In *T. brucei*, the flagellum is attached to the cell body for most of its length by the FAZ, making flagellar waveform analysis complex. However, in *Leishmania* flagella are free for most of their length (Edwards et al., 2018), making them a good candidate species on which to complete live cell imaging for performing waveform analysis on flagella bending. Though the *L. mexicana* flagellar pocket does retain a distinct FAZ, a cytoskeletal structure, which resides near MT doublets 9, 1 and 2 and is posited to have roles in cell polarity and life cycle-related morphogenetic changes (Wheeler et al., 2016).

1.8.1.5 Paraflagellar rod (PFR)

In many parasitic kinetoplastids, the axoneme is paralleled from the flagellar pocket collar (Wheeler et al., 2016) to the flagellar tip by the PFR (Maharana et al., 2015; Mishra et al., 2003), which is connected to microtubule doublets 4 and 7 by electron dense fibres (de Souza and Souto-Padrón, 1980). The ultrastructure of the PFR is a highly organised lattice like structure around 150nm in diameter formed of interwoven cytoskeletal fibres (Maga and LeBowitz, 1999; Bastin et al., 1996). The PFR is trilaminar - with proximal, intermediate and distal domains at increasing distance from the axoneme - each structurally unique. The proteins PFR1 and PFR2 are highly abundant in the structure, with over 150 other proteins being assigned to the PFR in *T. brucei* – many of which have orthologues

in *Leishmania* species and other kinetoplastids (Portman and Gull, 2010; Gabriel et al., 2024). This increasing cohort of proteins is expanding the prospective role of the PFR, suggesting it is involved in regulating flagellar metabolism, beating and signalling.

1.9 Axonemal components

1.9.1 Central pair, microtubule doublets & their decoration

Microtubules are largely negatively charged structures formed of tubulin, a heterodimer of α -tubulin and β -tubulin subunits. Tubulin molecules possess C-terminal tails which can be subject to a range of post-translational modification, which can alter axonemal stability and protein binding (Satarić et al., 2020).

At the centre of the flagellum lies the central pair - apparatus composed of a pair of dimorphic single microtubules C1 and C2 connected by a bridge and decorated with hundreds of associated proteins per repeating unit (Dutcher et al., 1984; Goodenough and Heuser, 1985; Dai et al., 2020; Han et al., 2022). The central pair is required for flagellar beating. Paralyzed flagella protein 16 (PF16) is a central pair protein discovered in *C. reinhardtii* whose deletion causes the loss of the C1 microtubule, one of the two singlet central pair microtubules, and flagellar paralysis in many organisms (Smith and Lefebvre, 1996; Beneke et al., 2019). In *L. mexicana*, PF16 deletion produced immotile flagella, with axonemes displaying the loss of one or both of the central microtubules or an aberration in central pair orientation (Beneke et al., 2017). The central pair and its accessory proteins have a role in modulating ATP/ADP ratio, Ca^{2+} concentration and dynein function (Dai et al., 2020). For example, Pcdp1 is a central pair binding protein that interacts with calmodulin, and was shown to be essential for proper flagellar beat formation in *C. reinhardtii*.

The microtubule doublets have a distinctive structure, comprised of a complete A-tubule ring of 13- protofilaments and an incomplete B-tubule ring of 10 protofilaments. Adjacent microtubule doublets are bridged by a nexin-dynein regulatory complex (N-DRC) (Ghanaeian et al., 2023). The microtubule

doublets are highly decorated and have been reconstructed in various single-celled organisms, including in *Chlamydomonas* (Walton et al., 2023; Ma et al., 2019), *Tetrahymena thermophila* (Kubo et al., 2023), *T. brucei* (Imhof et al., 2019) and very recently in *C. fasciculata* and *L. tarentolae* (Doran et al., 2025). In *Leishmania*, the microtubule doublet decorations include luminal microtubule inner proteins (MIPs), which display periodicities of 48 nm or less and MAPs (Doran et al., 2025). These proteins are involved stabilising the microtubules against the mechanical strain generated by flagellar beating, though most MIPs were found to be non-essential for cell swimming (Ma et al., 2019; Ishikawa, 2017).

1.9.2 Inner & outer dynein arms

The dynein arms are strongly attached to the A-microtubules, and it is their rhythmic interaction with adjacent B-microtubules that drives the microtubule sliding underlying flagellar beating (Gibbons and Rowe, 1965). Over the length of the *Chlamydomonas* flagellum (~10 µm) there are 10⁴ dynein motors (Witman, 2009). The inner dynein arms (IDAs) and ODAs have distinct functions. In *C. reinhardtii* IDAs are large structures with a periodicity of 96 nm (Bui et al., 2008). The *Leishmania* IDAs have two heavy chains (1α and 1β) (Dey and Mukhopadhyay, 2015).

Mutant generation in *Chlamydomonas*, revealed ODAs are the dominant driver of flagellar waveform generation (Brokaw and Kamiya, 1987). ODAs are large (~2 MDa) structures with a periodicity of ~24 nm, consisting of a docking complex, heavy chain outer arm dyneins (OADs) and various intermediate and light chains (Harder et al., 2010; Takada et al., 2002). In *Chlamydomonas*, ODAs have three heavy OAD chains (α, β, and γ) driving motor activity, whilst have *Leishmania* have a two-headed structure (α and β) (Dey and Mukhopadhyay, 2015; Liu et al., 2008).

In *Chlamydomonas*, ODA docking complexes are formed of three proteins, two whose coiled-coil domains are wound together and a third EF-hand protein with Ca²⁺ binding properties (Koutoulis et al., 1997; Casey et al., 2003; Takada et al., 2002). Several trypanosomatid species have been found to possess distinct proximal (pDCs) and distal (dDCs) docking complexes. In *T. brucei* their localisation is

equally split along the length of the flagellum; in *L. mexicana* pDCs are only present in the first 20% of the flagellum, with dDCs along the remainder (Fort et al., 2024). The pDC is formed of pDC1, pDC2 and pDC3, all of which co-dependent for localisation and necessary for pDC assembly (Fort et al., 2024). The dDC is formed of heterodimeric dDC1 and dDC2 proteins, which are necessary for dDC assembly, as well as dDC3 (Fort et al., 2024). Studies in *L. mexicana* have revealed that the proximal distal axonemal asymmetry important for normal flagellar beat generation, is largely driven by asymmetrical proteins associated with the ODAs and that IFT is required for maintaining the asymmetric distribution (Fort et al., 2024; Edwards et al., 2018). dDC2 deletion increases the proportion of time in asymmetric base-to-tip beating, shown by electron microscopy to be due to loss of distal axoneme ODAs (Edwards et al., 2018). The deletion of LC4-like, a distal axoneme ODA-associated protein, or of pDC-associated FLAM6 gives a higher frequency symmetric tip-to-base beat (Fort et al., 2024; Edwards et al., 2018).

1.9.3 The N-DRC

The N-DRC comprises ~12 subunits and controls dynein activity through regulating microtubule sliding and ODA alignment as well as putatively mediating signalling between the central pair, radial spokes and the ODAs (Heuser et al., 2009; Gardner et al., 1994). Several N-DRC subunits that are nearly ubiquitous to motile cilia contain motifs for calcium signalling (Bower et al., 2013). Phosphoproteomic analysis in *Chlamydomonas* found that eight subunits possessed phosphorylated isoforms indicating phosphorylation as a mode of N-DRC activity regulation may play a role in flagellar motility (Lin et al., 2011).

1.9.4 Radial spokes

The radial spokes are T-shaped protein complexes which keep the axonemal diameter constant (~200 nm) through their interaction with the doublet microtubules and central pair and act as mechanochemical sensors to modulate flagellar movement. Their ~28 nm stalk region extends from where it is attached to the microtubule doublet, bifurcating at the neck and culminating in a flat head

towards the central pair (Barber et al., 2012; Pigino et al., 2011). *Chlamydomonas* radial spokes are composed of 23 proteins (Yang et al., 2006; Piperno et al., 1981). Single-particle cryo-EM characterised the radial spoke head to be a negatively charged and acidic flat surface with a rigid protein core, and as such may regulate ciliary beating through physical interactions with the central pair (Grossman-Haham et al., 2021). This radial spoke head comprises a core (RSP4, RSP6, RSP9) around which peripheral proteins protrude (RSP1, RSP2, RSP3, RSP5 and RSP10), and the neck of RSP23 intertwined with four of the head proteins (RSP2, RSP3, RSP4 and RSP6) (Grossman-Haham et al., 2021). In *C. reinhardtii*, dynamic physical coupling was observed between the second radial spoke per axonemal repeat and IDA subforms a and c, suggesting orientation changes in radial spokes can directly influence IDA dynein activity (Gui et al., 2021). Many radial spoke proteins contain signal transduction associated domains, including for secondary messengers Ca^{2+} or cAMP.

1.10 Flagellum length control

In almost all eukaryotes IFT is indispensable for flagella assembly (San Agustin et al., 2015; Sunter et al., 2018). Once built, flagella can be stable structures which are long lived, as is the case for the motile flagella of spermatozoa and many protists including *L. mexicana* and *T. brucei* or more dynamic, as in *C. reinhardtii* (San Agustin et al., 2015; Marshall and Rosenbaum, 2001). While there are numerous theoretical models proposed on flagellum length control, the precise molecular mechanisms remain to be fully elucidated and likely vary between species (Bressloff, 2006; Fai et al., 2019; Marshall, 2023; Banerjee and Banerjee, 2020; Patra et al., 2020).

In *Chlamydomonas*, variation in flagellum length between cells was found to be strongly correlated to cell diameter (Bauer et al., 2021). In *C. reinhardtii* IFT train size, frequency and speed were found to be inversely proportional to flagellum length (Engel et al., 2009). With IFT motor protein recruitment to the flagellum and IFT particle size being anticorrelated with flagellar length, with IFT trains thought to transport around 4 tubulins each (Hendel et al., 2018; Bhogaraju et al., 2014; Engel et al., 2009). However, the fluctuations in assembly and disassembly of tubulin which underlie flagella length

variation was found not to be coupled to IFT in *Chlamydomonas* (Bauer et al., 2021). Long-flagella mutants show increased IFT injection, though the rate of this was insufficient to explain length increase – suggesting other mechanisms are involved (Wemmer et al., 2020).

For organisms which maintain existing flagellum length whilst assembling a new flagellum including trypanosomatids, a grow-and-lock model has been proposed (Kohl and Bastin, 2005; Calvo-Álvarez et al., 2021; Bertiaux et al., 2018). In their insect vector, *T. brucei* have flagella of varying lengths (3 to 30 μm), in parasite isolated from tsetse flies the amount of IFT proteins correlated with flagellum length (Bertiaux et al., 2020). In *T. brucei*, CEP164C, an orthologue of mammalian centrosome protein 164, localised to the mature basal body of the old assembled flagellum during the third cell cycle, deletion of which caused elongated old flagella and short new flagella, indicating it is essential in locking stable flagellum length during new flagellum growth (Atkins et al., 2020). The flagellar Member 8 protein (FLAM8) has been proposed to act as a meta-marker of flagellum maturation state (Calvo-Álvarez et al., 2021). In *T. brucei*, once flagella were assembled, arresting IFT did not alter flagellum length however it did impact flagellar motility in the form of reduced flagellum beating and disruption to the distribution of certain proteins including protein kinase A (PKA) and FLAM8 (Fort et al., 2016).

In addition to variations based on IFT and microtubule stability, previous studies have suggested signal transduction pathways could modulate flagellum length through feedback loops for example involving protein kinases and/or secondary messengers, either in response to environmental stimuli or length deviations (Rosenbaum, 2003; Jiang et al., 2019). Mitogen-activated protein kinases (MPKs) are a conserved family of kinases who use serine and threonine as acceptors and regulate a plethora of cellular functions, from differentiation to death throughout living organisms. It should be noted that in wider literature MPKs can be used to refer to MAP Kinase Phosphatases, in *Leishmania* research MPK, or LmxMPK specifically in *L. mexicana*, is used to refer to mitogen-activated protein kinases, and will be used throughout for consistency.

Several MPKs have been shown to be required for correct flagellum length maintenance in a range of organisms; for example in *Chlamydomonas*, LF4, an ortholog of the mammalian MOK, localises along the length of the flagellum and its loss makes cilia twice as long as in the wild type (Berman et al., 2003). *Leishmania* genomes encode 17 MPKs involved in cell polarisation, cell cycle transition and proliferation (Parsons et al., 2005). Various MPKs are proposed to have a role in transducing microenvironmental signals and flagellar biogenesis in trypanosomes (Fenn and Matthews, 2007). Some of these MPKs are essential for survival – for example in *L. mexicana*, MPK4 is required for both promastigotes and amastigotes (Wang et al., 2005). While others have more specific roles in flagellum length regulation, a process paramount to *Leishmania* pathogenicity and development. MKK1 (LmxM.08_29.2320), an MPK kinase, and its target MPK3 (LmxM.10.0490) are necessary for wild-type flagellum growth, with homozygous deletion of either resulting in flagella with a length one-fifth of wild-type, in addition to aberrated vesicle and membrane fragments in the flagellar pocket (Erdmann et al., 2006; Wiese et al., 2003). In *L. mexicana*, MPK9 is a negative length regulator, with deletion resulting in significant elongation of the flagellum and overexpression in a short or non-existent flagellum (Bengs et al., 2005). Whether MPKs influence flagellum length through interaction with the IFT system or directly alter IFT train speed in *Leishmania* has yet to be characterised.

1.11 Flagella sensing

The flagellum plays a role in environmental sensing in many species, as well as being a transducer of phototactic and chemotactic responses. In *Leishmania*, the flagellum acts as an antenna for scoping the environment, responding to stimuli such as nutrients. Though genome sequencing in *L. major*, *T. brucei* and *T. cruzi* revealed that trypanosomatids widely lacked tyrosine receptor kinases and G-protein coupled receptors as well as many other common, well-characterised surface receptor classes, some flagellar membrane proteins with a role in environmental sensing have been found. Notably, a recent systematic screen of the *L. mexicana* transportome identified 312 protein components for

membrane small-molecule carriers, ion channels and pumps; the deletion of 40 of which caused a significant loss of fitness during macrophage and mouse infections (Albuquerque-Wendt et al., 2025).

For example, in *L. mexicana*, glucose transporter 1 is selectively located to the flagellar membrane and was required for transition into stationary phase in glucose depleted environments (Piper et al., 1995; Rodriguez-Contreras et al., 2015). (El-Sayed et al., 2005; Kelly et al., 2021). In *L. major* flagellar aquaglyceroporin is involved in cell volume regulation through water and solute uptake, with knockdown of the protein slowing down osmotic gradient mediated migration, with flagellar membrane restriction of the transmembrane protein dependent on phosphorylation by MPK2 (Figarella et al., 2007; Mandal et al., 2012). It is suggested that MPK mediated pathways are involved in transducing external signals in trypanosomatids (Rotureau et al., 2009). Given the importance of MPKs in flagellum length regulation in trypanosomatids, and known interaction with IFT in other organisms; could MPK mediated changes in IFT function be important for adapting to host environments during life-cycle transitions?

1.12 Flagellar beating

Disparate patterns of movement by the dynein arms generate different beat frequencies and waveforms (Riedel-Kruse et al., 2007). The central pair, radial spokes and N-DRC regulatory scaffold translates the activity of the ODAs into microtubule bending (Gui et al., 2021; Bower et al., 2013; Heuser et al., 2009; Mitchell, 2003; Wargo and Smith, 2003). The majority of flagella beat within a defined plane of bending with many undergoing planar (flat) beating, which is perpendicular to the axis of the central pair with evidence that the central apparatus modulates position of active microtubule sliding (Lindemann and Lesich, 2021; Gadelha et al., 2006). Although increasing medium fluid viscosity has been shown to convert beating from planar to helical in sea urchin sperm (Brokaw, 1966, 1975; Woolley and Vernon, 2001; Ishijima, 2019).

In *L. mexicana*, the whip-like power stroke of the asymmetrical base-to-tip beat has a fixed polarisation, which is independent of PFR presence or lateral flagellum attachment (Wang et al., 2021).

Although some beat type switching occurs stochastically, such as the transition between asynchronous and synchronous beating in *Chlamydomonas* under dark conditions or autonomous switching in eusperm, these processes still require regulation to ensure proper waveform formation (Polin et al., 2009; Shiba et al., 2014). The mechanisms underlying beat type switching have yet to be fully elucidated.

1.13 Regulation of flagellar beating

Correct generation and directional propagation of these waveforms through the flagellum is key to flagellar function, however the dynamic processes that regulate flagellar beating remains to be fully elucidated. Synchronising a single beat (generation and propagation) is likely to arise from the interaction and feedback between structures intrinsic to generating movement through driving microtubule doublet sliding (the IDAs and ODAs) and providing mechanochemical transduction and maintaining axonemal structural integrity (radial spokes, central pair, N-DRC) as well as putatively fast travelling messengers such as Ca^{2+} . In *Chlamydomonas*, forward swimming requires synchronisation of the beating of its two flagella (Polin et al., 2009). The frequency, amplitude, direction and mode of a beat may arise from the actions of these intrinsic components and their modification in response to regulatory machinery, signalling pathways and and/or environmental changes (Yang and Lu, 2011; Lindemann and Lesich, 2021).

1.13.1 Calcium

Intraflagellar calcium signalling is known to be important for flagellar motility. The oscillation of Ca^{2+} influx into the flagellum synchronises dynein arm movement to drive beating, with several studies reporting calcium-mediated regulation of flagellar motility linked to dynein activity (King, 2012; Sakato et al., 2007; Smith, 2002; Sakato and King, 2003). For example, increased cytoplasmic Ca^{2+} initiates asymmetric flagellar beating in mammalian sperm (Satarić et al., 2019; Chang and Suarez, 2011).

Whilst in reactivated *Crithidia oncopelti*, altering extracellular Ca^{2+} levels from low (<0.1 mM) to high caused a reversal of the beat from tip-to-base to base-to-tip beating (Sugrue et al., 1988).

In *T. brucei* (FS179) and *L. mexicana* (LmxM.33.0480), there exists a putative Ca^{2+} channel localised to the flagellar membrane of the FAZ, which could be involved in flagellar sensing and signal transduction. Across five species of *Leishmania* a predicted IQ-like calmodulin binding motif on the N-terminal of the inner-arm 1α and outer-arm α dynein heavy chains, similar to *C. reinhardtii* γ heavy chain, were conserved and are calmodulin binding, suggesting calcium signalling-based regulation (Dey and Mukhopadhyay, 2015). Protein kinase C (PKC), a membrane associated enzyme responsive to secondary messenger Ca^{2+} and diacylglycerol, is proposed to have a role in flagellar motility regulation in sperm and *Helisoma trivolvis* embryos, and in modulating macrophage interaction in *L. amazonensis* (Christopher et al., 1999; Rotem et al., 1990; Vannier-Santos et al., 1995). Radial spokes have various calcium-responsive subunits including the calmodulin LC8 complex and nucleoside diphosphate kinase (NDK), suggesting radial spokes play a role in transducing calcium levels into flagellar bending (Yang et al., 2001; Patel-King et al., 2004; Dymek and Smith, 2007). The PFR contains several calcium-associated proteins with EF-hand domains and calmodulin is required for PFR assembly in trypanosomes (Wang et al., 2021; Docampo and Huang, 2015; Portman et al., 2009; Ginger et al., 2013).

Is it possible for Ca^{2+} flux to control beating at the timescale of a single beat cycle? In cytosol, microtubules act as a conduit for Ca^{2+} ions propagation at speeds faster than free diffusion to increase ion conduction (Minoura and Muto, 2006; Cantero et al., 2019; Priel et al., 2008; Satarić et al., 2019; Sekulic et al., 2016). Similarly, it is posited that in the flagellum the microtubule doublets with their negatively charged surfaces may act as charge-storing devices and facilitate accelerated movement of the calcium ions along the concentration gradient, similar to the process of ion propagation in neurons to allow rapid fluctuations in calcium concentrations to regulate flagellar beating (Satarić et al., 2020;

Poznanski et al., 2017). Though fluorescent Ca^{2+} indicators and genetically encoded Ca^{2+} sensors have been used on immobilised flagella, we are not aware of such studies in beating flagella.

1.13.2 ATP

The relationship between ATP and flagellar beating is complex. In spermatozoa, isolated flagella contain ATPases and flagellar beat activity can be reactivated by exogenous ATP in cells with selective membrane permeability, with ATP controlling beat frequency (Hoffmann-Berling, 1955). As the movement is driven by dynein ATPases, ATP consumption increases with higher beat frequencies (Chen et al., 2015; Takano et al., 2020). However, ATP is also thought to have an inhibitory effect on dynein function. In sea urchin sperm, magnesium-ATP acts as a substrate for ATPases whereas free ATP causes inhibition (Hayashi, 1976). A study into *Chlamydomonas* mutants paralysed by loss of central pair or radial spokes showed flagella can generate low frequency beats in low ATP (<50 μM) concentrations and were responsive to Ca^{2+} concentration, and in an enriched solution with 200 μM ATP axonemes lacking the central pair and radial spokes could generate ~20 Hz beating (Omoto et al., 1996; Yagi and Kamiya, 2000; Wakabayashi et al., 1997; Frey et al., 1997; Yoshimura et al., 2007). This suggests that the central pair and radial spokes may be involved with ATP-based inhibition.

1.13.3 cAMP

cAMP is a near universal secondary messenger synthesised from ATP by adenylyl cyclases, often associated with the intracellular domain of transmembrane proteins. Research has shown that adenylyl cyclase activity is likely important for motility and life cycle transitions in trypanosomatids. *T. brucei* possess around ~65-80 transmembrane proteins with adenylate cyclase domains, whereas *L. mexicana* only have six (Kelly et al., 2021; Durante et al., 2020). In *T. brucei*, cAMP signalling, mediated by stage-specific receptor adenylate cyclase is thought to regulate social motility and migration in the insect vector (Lopez et al., 2015). Interestingly, in trypanosomes with a single-host life cycle, many receptor adenylate cyclases became pseudogenised – implying their main role is in host adaptation

(Lopez et al., 2015). There is evidence that vector-derived ligands are recognised by receptor adenylate cyclases and promote life cycle differentiation (Bachmaier et al., 2019; Fraidenraich et al., 1993). Oxygen-sensing adenylate cyclases have been expanded in *Leishmania* species in association with extracellular lifestyle and complex migration involving life-cycle changes through the insect vector (Durante et al., 2020). cAMP has been found to induce reversal of flagellar beating through PKA – a phenomenon discussed further in 1.13.5 (Vassella et al., 1997; van den Abbeele et al., 1995).

1.13.4 Redox

Many cellular activities are subject to redox-based regulation. Reactive oxygen species (ROS) are byproducts of metabolism and are used by all aerobic organisms as signalling molecules, to coordinate cellular responses to environmental changes (de Almeida et al., 2022). ROS are also important in the regulation of protozoan infection.

In phototactic organisms, light-driven motility responses are in part modulated by redox-poise. Specifically in *C. reinhardtii*, the chloroplast's production of reduced NADPH and consequent impact on cytosolic enzyme activity is thought to be important in photokinesis (Wakabayashi and King, 2006; Wakabayashi et al., 2011). ODA subunits LC3 and LC5 have thioredoxin motifs that are redox active (Patel-King et al., 1996; Sakakibara et al., 1993; Harrison et al., 2002). However, there were no clear changes in redox state of thioredoxin-related LC3 and LC5 or DC3 in response to light (Wakabayashi and King, 2006). Similarly, although the ODA DC3 subunit had EF-hands only bound to Ca²⁺ in the presence of dithiothreitol suggesting the binding is redox-sensitive, disruption of their Ca²⁺ did not alter swimming or photoshock response (Casey et al., 2003). In this way, the exact mechanisms by which redox-poise alters the function of axonemal components driving beating is unknown.

Decoding motile responses to redox in non-light reactive cells is important for understanding the response of protozoan parasites to host immune systems but also for human health conditions. In human cells motile cilia are rich in thiol-dense and thiol-regulatory proteins which keep the environment in a reduced state (Blackburn et al., 2017; Ostrowski et al., 2002). The loss of function

mutation in thioredoxin motif containing TXNDC3 is a cause of primary ciliary dyskinesia, implicating redox regulation as important in motile cilia function in humans (Price and Sisson, 2019). Ubiquitous to almost all eukaryotes are the glutathione and thioredoxin pathways based disulfide reductase systems, which are important in maintaining cellular redox homeostasis, defending against oxidative stress and transducing redox-based signalling pathways (Ren et al., 2017).

Interestingly, trypanosomatids lack thioredoxin and glutathione reductases and instead have a trypanothione reductase system to allow redox metabolism. Part of the trypanothione reductase system pathway is tryparedoxins (TXNs), which are thiol disulfide oxidoreductases, that work with Tryparedoxin Peroxidase to reduce peroxidases (Fiorillo et al., 2012). To our knowledge, the role that the trypanothione reductase system may play in redox-poise mediated flagellar beating in trypanosomatids has not been investigated.

1.13.5 Phosphorylation and kinase activity

Several kinases and phosphatases have been shown to be anchored to the axoneme and involved in initiation or regulation of flagellar motility (Bracho et al., 1998; Yang and Sale, 2000). Phosphoprotein analysis in *C. reinhardtii* revealed 126 phosphorylation sites on 32 flagellar proteins, many of which are implicated in regulation of flagellar beating (Boesger et al., 2009). For example, in *Chlamydomonas* casein kinase I is anchored to the axoneme and phosphorylates the 138-kDa intermediate chain of the IDA to regulate dynein activity, inhibition of which allowed microtubule sliding in radial spoke lacking mutants (Yang and Sale, 2000). In *L. mexicana*, PKA was shown to be involved with flagellar beat regulation, with catalytic subunit 3 implicated in bi-directional regulation of swimming speed and subunit 1 required for symmetric tip-to-base waveforms (Fochler et al., 2023; Mukhopadhyay and Dey, 2016). In *Chlamydomonas*, PKA is anchored by RSP3, suggesting radial spokes have a role in cAMP-dependent signal transduction (Gaillard et al., 2001).

1.14 Project aims

The aim of this project was to explore the nature of IFT in freely beating flagella, specifically in *L. mexicana*. This was done using genetically encoded fluorescent tags, advanced microscopy and a novel image analysis pipeline.

1.14.1 The nature of IFT in beating flagella and the impact of experimental conditions

We set out to evaluate the challenges and limitations of visualising IFT in a beating flagellum using dual-colour high-frame rate microscopy. We investigated the optimum frame rate and fluorescent markers to do so and developed an image analysis pipeline which allowed IFT particle tracking in videos taken from a beating flagellum. We then compared the nature of the IFT observed in beating flagella with that in genetically and mechanically adhered flagella, revealing significant differences in IFT train speed and stalling between experimental conditions.

1.14.2 Interaction of IFT train speed and flagellar motility

We set out to investigate if natural beat type switches undertaken by *L. mexicana* flagella or longer term alterations to flagellar motility influenced IFT train speed. To do this we measured IFT train speed in flagella undergoing different beat types and in the flagella of a cohort of deletion mutants with genetic perturbation to their flagellar beating to decode if short or long-term changes to flagellar motility and/structure alter IFT train speed.

1.14.3 Molecular elements of IFT system architecture

We set out to identify previously uncharacterised proteins involved in the IFT particle, IFT track formation or IFT regulation and investigate their function. We utilised genome-wide subcellular protein maps and fluorescent tagging to identify a putative novel IFT dynein intermediate chain which may be essential for *L. mexicana* survival. We investigated the localisation of, and where possible the

effect of deletion, of three MAPs (two SHIPPO-domain containing proteins and a CFAP97 orthologue) and a cohort of MPKs on IFT train speed.

1.14.4 The interaction of light, redox, calcium and flagellar motility

We explored the effect of light intensity, light wavelength and ROS levels on swimming speed and flagellar beat type switching to test if light or redox-poise based flagellum regulation occurred in *L. mexicana*. We identified two TXN proteins with putative involvement in mediating flagellar motility recovery from oxidative stress, which deserve further investigation. Finally we used genetically encoded calcium sensor GCaMP3 to complete an initial investigation into the possibility of visualising Ca^{2+} activity in a beating flagellum.

2 Materials and methods

2.1 *L. mexicana* cell culture

L. mexicana (strain MNYC/BZ/62/M379) procyclic promastigotes that stably express both Cas9 and T7 RNA polymerase (designated Cas9T7) were cultured at 28°C in M199 medium (Life Technologies). The medium was supplemented with 10% foetal calf serum, 5 mM HEPES (pH 7.4) and 0.005% hemin. The media was filter-sterilised through a 0.22 µm membrane (Corning, Millipore).

The cultures were kept in exponential growth phase through dilution, namely at cell densities ranging from 10^5 to 10^7 cells per millilitre. Measurements of culture density were taken using an Improved Neubauer haemocytometer.

2.2 *L. mexicana* genetic modification

Cas9T7 were subjected to CRISPR-based genome editing techniques (as described in Beneke et al., 2017, and Beneke and Gluenz, 2019). Cas9T7 and Cas9T7-derived cells were maintained in the presence of 50 µg/ml nourseothricin sulfate (NTC) and 32 µg/ml hygromycin B (Hyg) (Cambridge Bioscience Ltd) to maintain Cas9T7.

For endogenous tagging, constructs were generated using two PCRs. The first PCR used the pLPOT plasmid series as the template in a long primer PCR. The amplified section of the pLPOT plasmid encodes a fluorescent tag and a drug selectable marker (Dean et al., 2015). LeishGEdit was used to design primer sequences, which in the PCR would add homology arms for the desired target site flanking the drug selectable marker and the genetically-encoded fluorescent protein. The PCR was performed according to established protocols (Beneke et al., 2017; Dean et al., 2015): an initial 3 minutes at 95°C, followed by 35 cycles of: 15 seconds at 94°C, 30 seconds at 65°C, and 60 seconds at 72°C, and finally extension for 1 minute at 72°C.

The second PCR generates the template for the single-guide RNA (sgRNA) which is to be transcribed *in vitro* by the T7 RNA polymerase in Cas9T7 and Cas9T7-derived cell lines. The PCR product formed is an sgDNA scaffold containing the T7 polymerase promoter, a 20 nucleotide sequence homologous to the target locus and a section homologous to the sgRNA scaffold. The PCR consists of 33 seconds at 94°C, then 35 cycles of 15 seconds at 94°C, 30 seconds at 65°C, and 60 seconds at 72°C, followed by 2 minutes at 72°C. One primer encodes the gene-specific protospacer adjacent motif site and T7 promoter, while the other primer encodes the remaining portion of the sgRNA.

To delete genes, we utilized constructs created through a PCR (cycle as described for pLPOT) with pT Blast, pT Puro, pT Phleo or pT Neo as plasmid templates (Beneke et al., 2017).

For precipitation of the PCR products, 10 µl of 3M Sodium Acetate (NaCH₃COO) and 300 µl 100% ETOH are added and agitated before being incubated for 20 minutes at -80°C. PCR products are then centrifuged at 14000 RPM (21910g), 4°C, for 30 minutes. After discarding supernatant, 300 µl of 70% ETOH is added before an additional 14000RPM (21910g), 4°C, for 30 minutes. Supernatant is removed and pellet is left in hood until any remaining ETOH evaporates, leaving a glassy pellet, which is resuspended in 10 µl of water.

For transfection, purified PCR products are resuspended in calcium chloride transfection buffer. *L. mexicana* promastigotes were harvested from cultures of densities between 1×10^6 and 1×10^7 and centrifuged for 3 minutes at 2200 RPM. The cell pellet was resuspended in buffer with the PCR product in 2 mm cuvettes, and subjected to X-100 programme of electroporation in the Amaxa Nucleofector II before being returned to pre-warmed FCS-supplemented M199 medium (Dean et al., 2015).

Cell lines that were successfully modified were selected and maintained using a combination of 5 µg/ml blasticidin, 40 µg/ml neomycin, 20 µg/ml puromycin and 25 µg/ml Phleomycin (from Cambridge Bioscience Ltd) for the Blasticidin-S deaminase (blast), Neomycin-kanamycin phosphotransferase (neo), Puromycin N-acetyltransferase (puro) and phleomycin-resistance (phleo) selectable markers, respectively. After drug selection, genomic DNA was extracted from the mutant deletion cell line using

the DNeasy kit from Qiagen, following the manufacturer’s protocol. Deletion was validated using three PCRs with the following conditions: an initial 3 minutes at 95°C, followed by 35 cycles of 15 seconds at 95°C, 15 seconds at 58°C, and 30 seconds at 72°C, ending with 1 minute at 72°C. The three PCRs and their products are detailed with the validation gels in 2.4. The primers used for deletion validation were designed using Primer3, as previously described (Beneke et al., 2019). The presence or absence of PCR products was determined by agarose gel electrophoresis.

If needed, cell lines were subcloned into 96-well plates, with 200 µl of cell suspension per well at a density of 0.5 cells/ml and incubated at 28°C in 5% CO₂. Cells from wells that tested positive were then transferred to flasks to undergo validation.

The details of the cell lines generated (2.3), the validation gels (2.4), and list of validation primers (2.5) are presented below – ordered by the chapter in which they first appear.

2.3 Cell lines

Chapter 3

Cell Line Name	Fluorescent tag on IFT172 (LmxM.21.0980)	Tagging Drug Marker	ID of Gene with both alleles deleted	Name of deleted gene	Deletion Drug Marker
eGFP::IFT52	N/A (Tag on IFT52)	Blast	N/A	N/A	N/A
3xmNG:IFT172	3xmNeonGreen	Blast	N/A	N/A	N/A
mNG:IFT172	mNeonGreen	Puro	N/A	N/A	N/A
3xmNG:IFT172 ΔPF16	3xmNeonGreen	Blast	LmxM.20.1400	Paralyzed flagella protein 16	Puro & neo

Figure 2-1, Adapted from Gray et al., 2024 | Table of cell lines generated present in Chapter 3.

Chapter 4

Cell Line Name	Fluorescent tag on IFT172 (LmxM.21.0980)	Tagging Drug Marker	ID of Gene with both alleles deleted	Name of deleted gene	Deletion Drug Marker
3xmNG:IFT172 ΔH3	3xmNeonGreen	Blast	LmxM.10.0990	Histone 3	Puro & neo

3xmNG:IFT172 ΔdDC1	3xmNeonGreen	Blast	LmxM.15.0540	Distal docking complex protein 1 (dDC1)	Puro & neo
3xmNG:IFT172 ΔdDC2	3xmNeonGreen	Blast	LmxM.31.2900	Outer dynein arm docking complex protein 2	Puro
mNG:IFT172 (N) ΔLC4-like	mNeonGreen	Neo	LmxM.01.0620	Beat Regulation Protein / LC4-like	Blast
mNG:IFT172 (P) ΔLC4-like	mNeonGreen	Puro	LmxM.01.0620	Beat Regulation Protein / LC4-like	Blast
3xmNG:IFT172 ΔLC1	3xmNeonGreen	Blast	LmxM.24.1030	Light Chain 1	Puro & neo
3xmNG:IFT172 ΔOADβ	3xmNeonGreen	Blast	LmxM.13.1650	Outer Arm Dynein β	Puro & neo
3xmNG:IFT172 ΔRSP4/6	3xmNeonGreen	Blast	LmxM.13.0430	Flagellar radial spoke protein 4/6	Puro & neo
3xmNG:IFT172 ΔPF16	3xmNeonGreen	Blast	LmxM.20.1400	Paralyzed flagella protein 16	Puro & neo
3xmNG:IFT172 ΔdDC2 pDC1::mCh	3xmNeonGreen	Blast	LmxM.31.2900	Outer dynein arm docking complex protein 2	Puro

Figure 2-2 , Adapted from Gray et al., 2024 | Table of cell lines generated presented in Chapter 4.

Chapter 5

Cell Line Name	Fluorescent tag	ID & name of gene tagged	Tagging Drug Marker	ID & name of gene with both alleles deleted	Deletion drug marker
mNG::LmxM.18.1460	mNeonGreen	LmxM.18.1460	Blast	N/A	N/A
mNG::Lmx.05.0690	mNeonGreen	Lmx.05.0690	Blast	N/A	N/A
mNG::LmxM.34.3925	mNeonGreen	LmxM.34.3925	Blast	N/A	N/A
3xmNG::IFT172 ΔLmxM.34.3925	3xmNeonGreen	LmxM.21.0980 (IFT172)	Blast	LmxM.34.3925	Puro & neo
mNG::LmxM.30.0890	mNeonGreen	LmxM.30.0890	Blast	N/A	N/A
ΔLmxM.30.0890	N/A	N/A	N/A	LmxM.30.0890	Puro, neo, phleo, blast

3xmNG::IFT172 ΔLmxM.30.0890	3xmNeonGreen	LmxM.21.0980 (IFT172)	Blast	LmxM.30.0890	Puro, neo, phleo, blast
mNG::MPK3	mNeonGreen	LmxM.10.0490 (MPK3)	Blast	N/A	N/A
mNG::MPK9	mNeonGreen	LmxM.19.1080 (MPK9)	Blast	N/A	N/A
mNG::MPK13	mNeonGreen	LmxM.34.5010 (MPK13)	Blast	N/A	N/A
mNG::MPK14	mNeonGreen	LmxM.27.0100 (MPK14)	Blast	N/A	N/A
mNG::LmxM.29.0600	mNeonGreen	LmxM.29.0600	Blast	N/A	N/A
3xmNG::IFT172 ΔMPK9	3xmNeonGreen	LmxM.19.1080 (MPK9)	Blast	LmxM.19.1080 (MPK9)	Puro & neo
3xmNG::IFT172 ΔMPK13	3xmNeonGreen	LmxM.34.5010 (MPK13)	Blast	LmxM.34.5010 (MPK13)	Puro & neo
3xmNG::IFT172 ΔMPK14	3xmNeonGreen	LmxM.27.0100 (MPK14)	Blast	LmxM.27.0100 (MPK14)	Puro & neo

Figure 2-3 | Table of cell lines generated presented in Chapter 5.

Chapter 6

Cell Line Name	Fluorescent tag	ID & name of gene tagged	Tagging Drug Marker	ID & name of gene with both alleles deleted	Deletion drug marker
SMP1::KillerRed	KillerRed	LmxM.20.1310 (SMP1)	Blast	N/A	N/A
mNG::TXN1	mNeonGreen	LmxM.08_29.1160 (TXN1)	Blast	N/A	N/A
mNG::TXN2	mNeonGreen	LmxM.08_29.1150 (TXN2)	Blast	N/A	N/A
nMG::TXN-like A	mNeonGreen	LmxM.08_29.1140 (TXN-like A)	Blast	N/A	N/A
nMG::TXN-like B	mNeonGreen	LmxM.08_29.1130 (TXN-like B)	Blast	N/A	N/A
mNG::TXN3	mNeonGreen	LmxM.30.1960 (TXN3)	Blast	N/A	N/A

mNG::TXN4	mNeonGreen	LmxM.30.1970 (TXN4)	Blast	N/A	N/A
ΔTXN1	N/A	N/A	N/A	LmxM.08_29.1160 (TXN1)	Puro & neo
ΔTXN2	N/A	N/A	N/A	LmxM.08_29.1150 (TXN2)	Puro & neo
ΔTXN-like B	N/A	N/A	N/A	LmxM.08_29.1130 (TXN-like B)	Puro & neo

Figure 2-4 | Table of cell lines generated presented in Chapter 6.

2.4 Validation gels

Images were taken of the deletion validation PCR products separated by gel electrophoresis (Figure 2-5 - Figure 2-8). For each open reading frame (ORF), three PCR samples were used, with genomic DNA and primers labelled accordingly (Figure 2-5 – Figure 2-8). The first lane represents a control for the primers of the deleted ORF, showing that they can form a product when the gene is present in genomic DNA of the parental cell. The second lane is the deletion mutant genomic DNA with the primers of the deleted ORF. No product will be present if the ORF is successfully deleted. The third lane is the product of a PCR with the deletion mutant genomic DNA and primers to an ORF that has not been detected, to show that mutant genomic DNA can be used as a template for PCR.

Chapter 3

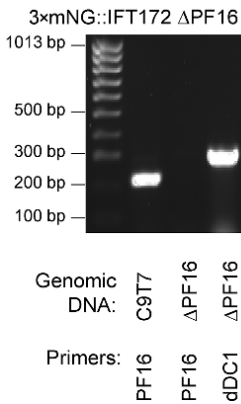


Figure 2-5, Adapted from Gray et al., 2024 | Confirmation by PCR of deletion of gene ORF in in Chapter 3.

Chapter 4

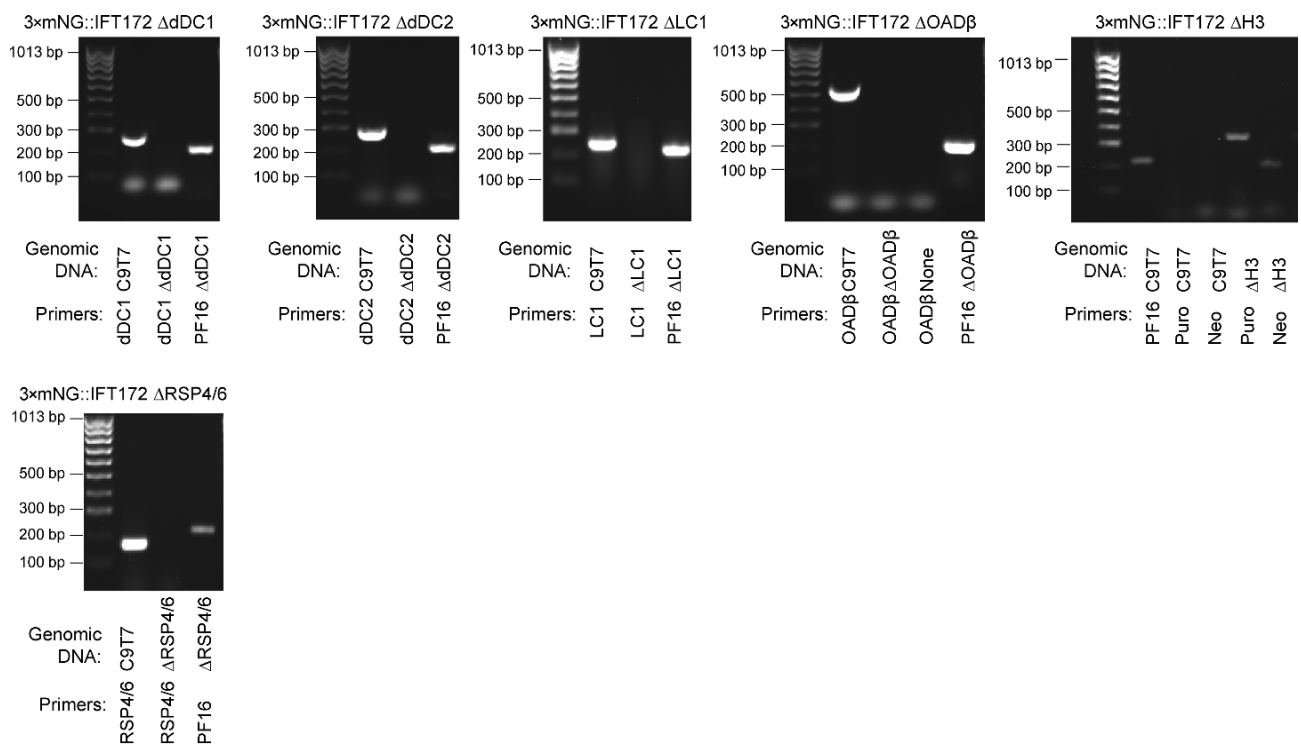


Figure 2-6, Adapted from Gray et al., 2024 | Confirmation by PCR of deletion of gene ORF in Chapter 4. For the validation of 3xmNG:IFT172 ΔH3, the presence of the neo and puro drug markers was used as the H3 gene has many repeats with identical coding, and it was the insertion of the drug makers that were a required negative control for the deletion mutant cohort. The LC4-like deletion mutant cell line is from (Edwards et al., 2018).

Chapter 5

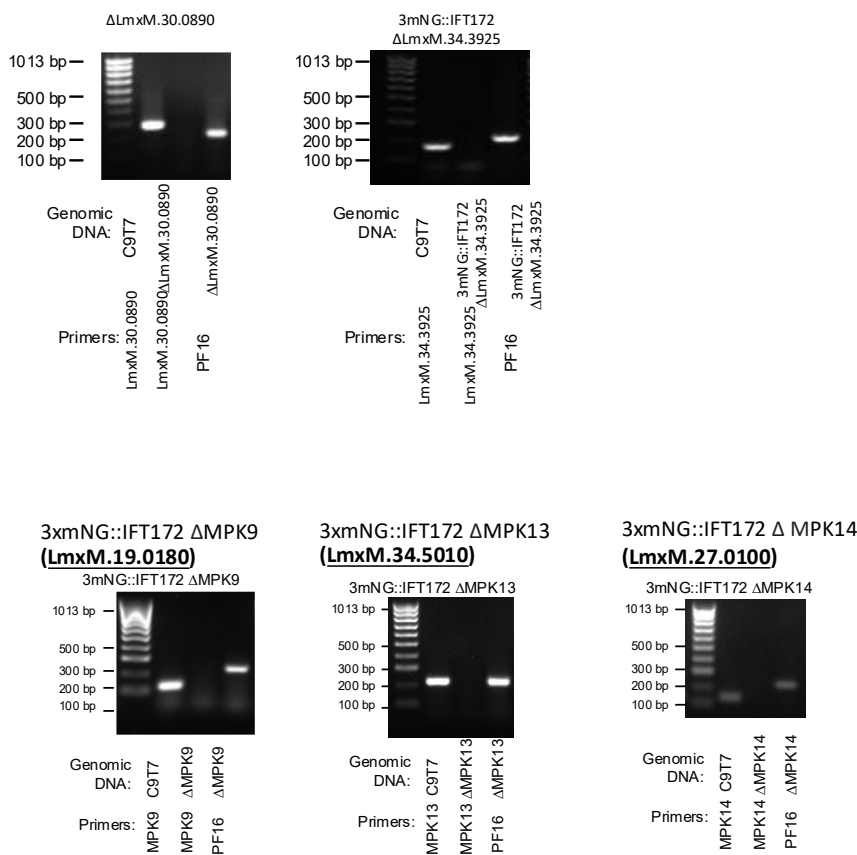


Figure 2-7 | Confirmation by PCR of deletion of gene ORF in Chapter 5.

Chapter 6

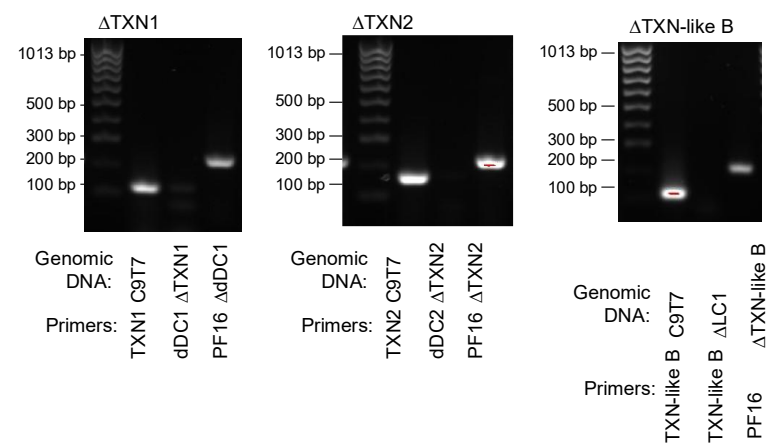


Figure 2-8 | Confirmation by PCR of deletion of gene ORF in Chapter 6.

2.5 PCR primers

Chapter 3

Gene ID	Gene name	Primer Category	Primer Sequence
LmxM.21.0980	Intraflagellar Transport Protein 172 (IFT172)	Upstream Forward	ACACACACACACACGCACAGAGGTGCCCTgtataatgcagacctgtgc
LmxM.21.0980	Intraflagellar Transport Protein 172 (IFT172)	Upstream Reverse	CATGATGTTCTGGTAAACTGTACCTGCATactacccgatcctgatccag
LmxM.21.0980	Intraflagellar Transport Protein 172 (IFT172)	5' sgRNA	gaaattaatacgactcactataggGCAACGGTATAGGAGAGAAGgttttagagctagaaatagc
LmxM.21.0980	Intraflagellar Transport Protein 172 (IFT172)	Downstream Forward	GGCGCGGCGGCGAGCCCCAACTTCAAGGTGggttctggtagtgttccgg
LmxM.21.0980	Intraflagellar Transport Protein 172 (IFT172)	Downstream Reverse	CTTTTTTATTGGCCCCCTCCCCTCCCGCccaatttgagagacctgtgc
LmxM.21.0980	Intraflagellar Transport Protein 172 (IFT172)	3' sgRNA	gaaattaatacgactcactataggTCCACGCCACCCTGCGGGTgttttagagctagaaatagc
LmxM.20.1400	Paralyzed flagella protein 16 (PF16)	Upstream Forward	CCGCGCTACACAGTGGCCCTCTTCCCTCCTgtataatgcagacctgtgc
LmxM.20.1400	Paralyzed flagella protein 16 (PF16)	Upstream Reverse	GAAGGTTTGCAGAATAACCCGATTTCGACATactacccgatcctgatccag
LmxM.20.1400	Paralyzed flagella protein 16 (PF16)	5' sgRNA	gaaattaatacgactcactataggCTTTGTTTGGCGGACGGGAGgttttagagctagaaatagc
LmxM.20.1400	Paralyzed flagella protein 16 (PF16)	Downstream Forward	AAGATCGAGAACTACCACGTGCAGCAGCACggttctggtagtgttccgg
LmxM.20.1400	Paralyzed flagella protein 16 (PF16)	Downstream Reverse	CGAGCAGCGTGCATGGGCGTGACTGTGCCGccaatttgagagacctgtgc
LmxM.20.1400	Paralyzed flagella protein 16 (PF16)	3' sgRNA	gaaattaatacgactcactataggCGGATGCTCAGCGGGCCTTTgttttagagctagaaatagc
LmxM.20.1400	Paralyzed flagella protein 16 (PF16)	Forward (Validation)	CCAGCACTGTGTCTACCTACCC
LmxM.20.1400	Paralyzed flagella protein 16 (PF16)	Reverse (Validation)	TGATGATCTGGATGGACTCGGC

Figure 2-9, Adapted from Gray et al., 2024 | Table showing primer sequences used in Chapter 3 for protein tagging, gene deletion, and deletion validation. Cell line eGFP::IFT52, as described in Wheeler et al. (2015), was utilized for this study (Wheeler et al., 2015).

Chapter 4

Gene ID	Gene name	Primer Category	Primer Sequence
LmxM.10.0990	Histone 3	Upstream Forward	GCCACCACTACCCACCCACCCACCCACCCgtataatgcagacctgtgc
LmxM.10.0990	Histone 3	Upstream Reverse	CGCGCGGGCGGTCTCCTTGGTGCGGGACATactacccgatcctgatccag
LmxM.10.0990	Histone 3	5' sgRNA	GaaattaatacagactcactataggCGGTGTGGGGGGGAGGGAGTgttttagagctagaaatagc
LmxM.10.0990	Histone 3	Downstream Forward	CTGGCGCTGCGCCTGCGCGGTGAGCGCCATggttctgtagtggttccgg
LmxM.10.0990	Histone 3	Downstream Reverse	TCCTGGTTTCACCACTGCTGCACCGCACCAccaatttgagagacctgtgc
LmxM.10.0990	Histone 3	3' sgRNA	gaaattaatacagactcactataggTACCCTCTATCTCTGTGCGgttttagagctagaaatagc
Puro	Puro	Forward (Validation)	GGTTCGTCTTGCTACGCGAGACGAT
Puro	Puro	Reverse (Validation)	GCCGGCTCCTTAGGTCTATGTGGTG
Neo	Neo	Forward (Validation)	AGGTTCTCCGGCCGCTTGGGTGGAG
Neo	Neo	Reverse (Validation)	GAACGCCCCGTCGTGGCCAGCCACG
LmxM.15.0540	Distal docking complex protein 1 (dDC1)	Upstream Forward	GCACGCACACACACACGGCTGACGCGCACCCgtataatgcagacctgtgc
LmxM.15.0540	Distal docking complex protein 1 (dDC1)	Upstream Reverse	GGCGATGTTCTCACTCGCAGTGCCGTTCAactacccgatcctgatccag
LmxM.15.0540	Distal docking complex protein 1 (dDC1)	5' sgRNA	GaaattaatacagactcactataggGTTTCTTTGTTTCCTACGTGTgttttagagctagaaatagc
LmxM.15.0540	Distal docking complex protein 1 (dDC1)	Downstream Forward	CAGCAGCGACGCAACAAGGAAGAAATGGTGggttctgtagtggttccgg
LmxM.15.0540	Distal docking complex protein 1 (dDC1)	Downstream Reverse	AGCTGAGAGCGAGGTTGAGGGGGTGGGGTGccaatttgagagacctgtgc
LmxM.15.0540	Distal docking complex protein 1 (dDC1)	3' sgRNA	gaaattaatacagactcactataggGGGGGGGGGAGAGACGCAGgttttagagctagaaatagc
LmxM.15.0540	Distal docking complex protein 1 (dDC1)	Forward (Validation)	GATGCAAGCGACTATGGCCTTC
LmxM.15.0540	Distal docking complex protein 1 (dDC1)	Reverse (Validation)	GCAGTTACGCAGCAGATCGATC

LmxM.31.2900	Distal docking complex protein 2 (dDC2)	Upstream Forward	CTCAATTCGTTGTTCGACAGGACTATCCTgtataatgcagacctgtgc
LmxM.31.2900	Distal docking complex protein 2 (dDC2)	Upstream Reverse	GTCCTTCTTTTTGGCAGCCACCACTGACATactacccgatcctgatccag
LmxM.31.2900	Distal docking complex protein 2 (dDC2)	5' sgRNA	GaaattaatacgaactcactataggCAAGTCCGCCAATGTACGTCgttttagagctagaaatagc
LmxM.31.2900	Distal docking complex protein 2 (dDC2)	Downstream Forward	AAGAAGAAGGACGCAGGTCAGAAGGCCAAGggttctggtagtgttccgg
LmxM.31.2900	Distal docking complex protein 2 (dDC2)	Downstream Reverse	CTCTCTGCTTTCACCTGGCTGTGACTGCCGccaatttgagagacctgtgc
LmxM.31.2900	Distal docking complex protein 2 (dDC2)	3' sgRNA	gaaattaatacgaactcactataggGTGACCGCATGTGAGCAACAgtttttagagctagaaatagc
LmxM.31.2900	Distal docking complex protein 2 (dDC2)	Forward (Validation)	CTGTGGAAGGTGAGGAGAGGTG
LmxM.31.2900	Distal docking complex protein 2 (dDC2)	Reverse (Validation)	TCCACCTCCCAGTCTTCTACCA
LmxM.24.1030	Light Chain 1 (LC1)	Upstream Forward	TACGATTTATTGTCCGCACCATCAGTGCCAgataatgcagacctgtgc
LmxM.24.1030	Light Chain 1 (LC1)	Upstream Reverse	GGCCTCCTTGATGCTCGTCGATGACGACATactacccgatcctgatccag
LmxM.24.1030	Light Chain 1 (LC1)	5' sgRNA	GaaattaatacgaactcactataggGAAGGGAGAGAAGATCAAGGgttttagagctagaaatagc
LmxM.24.1030	Light Chain 1 (LC1)	Downstream Forward	GAGGAAAAAGAGGAGGCGGACCGCCGTGCGggttctggtagtgttccgg
LmxM.24.1030	Light Chain 1 (LC1)	Downstream Reverse	ACACACACACACACCAACACACACTCACccaatttgagagacctgtgc
LmxM.24.1030	Light Chain 1 (LC1)	3' sgRNA	gaaattaatacgaactcactataggAGAGGTGTGGGGAGCATCTGgttttagagctagaaatagc
LmxM.24.1030	Light Chain 1 (LC1)	Forward (Validation)	ATGGACAAGGAGATCTCGACGC
LmxM.24.1030	Light Chain 1 (LC1)	Reverse (Validation)	TCATGTACAGCACCCGAAGTCC
LmxM.13.1650	Outer Arm Dynein β (OAD β)	Upstream Forward	GAAACTGCCTTCTCGCACTGCGCCGGTCAGgtataatgcagacctgtgc
LmxM.13.1650	Outer Arm Dynein β (OAD β)	Upstream Reverse	TGGCGCGTCATCCTTGTGCGCCCTTCCCCATactacccgatcctgatccag
LmxM.13.1650	Outer Arm Dynein β (OAD β)	5' sgRNA	GaaattaatacgaactcactataggTTGACCTGAGTGTGTTGTCTCgttttagagctagaaatagc
LmxM.13.1650	Outer Arm Dynein β (OAD β)	Downstream Forward	GGGGTGGGTCTGTTGTTAGACGTCGTGGAGggttctggtagtgttccgg
LmxM.13.1650	Outer Arm Dynein β (OAD β)	Downstream Reverse	TGTTTCGTCAGGCACGTGTCGAGCACGCCGccaatttgagagacctgtgc

LmxM.13.1650	Outer Arm Dynein β (OAD β)	3' sgRNA	gaaattaatacgaactcactataggTAACCAGACTCGGACACACGgttttagagctagaaatagc
LmxM.13.1650	Outer Arm Dynein β (OAD β)	Forward (Validation)	CTGTACTCGGTGAAGTCGCTCA
LmxM.13.1650	Outer Arm Dynein β (OAD β)	Reverse (Validation)	GTTGTTGTAGAAGCCGGACTGC
LmxM.13.0430	Radial spoke protein 4/6 (RSP4/6)	Upstream Forward	ACGGTCGCGCTCTCTCGAGCCGTCGAATTGgtataatgcagacctgtgc
LmxM.13.0430	Radial spoke protein 4/6 (RSP4/6)	Upstream Reverse	CTCCGCCGCAGAAAGCGCTGTTGAGGCCATactaccgatcctgatccag
LmxM.13.0430	Radial spoke protein 4/6 (RSP4/6)	5' sgRNA	gaaattaatacgaactcactataggAGCTCATTCTCCGCCGACTAgtttttagagctagaaatagc
LmxM.13.0430	Radial spoke protein 4/6 (RSP4/6)	Downstream Forward	CCGGAAGAGCTGGAGGAAGAGGAGGACAGCggttctgtagtggttccgg
LmxM.13.0430	Radial spoke protein 4/6 (RSP4/6)	Downstream Reverse	GAGGAAGGGGAAAGGGAGGTTGGATACCCGccaatttgagagacctgtgc
LmxM.13.0430	Radial spoke protein 4/6 (RSP4/6)	3' sgRNA	gaaattaatacgaactcactataggAAAGAGGTTCCGCGGGTGCGgttttagagctagaaatagc
LmxM.13.0430	Radial spoke protein 4/6 (RSP4/6)	Forward (Validation)	GACTACTTTGTTCTCCGCACGC
LmxM.13.0430	Radial spoke protein 4/6 (RSP4/6)	Reverse (Validation)	ATGCGGTTACCCAGAACGTGTA

Figure 2-10 Adapted from Gray et al., 2024 | Table showing primer sequences used in Chapter 4 for protein tagging, gene deletion, and deletion validation. LC4-like deletion mutant cell line (generated using blast) was used from Edwards et al., 2018.

Chapter 5

Gene ID	Gene name	Primer Category	Primer Sequence
LmxM.18.1460	N/A	Upstream Forward	GCGGAGAACACAACCAGAGGCTCGTTGATCgtataatgcagacctgtgc
LmxM.18.1460	N/A	Upstream Reverse	CAGCCGGCGCTCCTCCTCGCTCTCCGGCATactaccgatcctgatccag
LmxM.18.1460	N/A	5' sgRNA	gaaattaatacgaactcactataggCTTTTTCCCTTCTCTCCCTCgttttagagctagaaatagc
LmxM.18.1460	N/A	Downstream Forward	GTCCACTCGGAGCGCGACGAGCGTTGGATCggttctgtagtggttccgg
LmxM.18.1460	N/A	Downstream Reverse	CATCATCACAATCCGCCAACCTACACCAccaatttgagagacctgtgc

LmxM.18.1460	N/A	3' sgRNA	gaaattaatacgactcactataggAGAGCGAAAGATGATCTCTGgttttagagctagaaatagc
Lmx.05.0690	TZP250	Upstream Forward	TATCAAGGCCCTCCCCCTCTCCACACCCgtataatgcagacctgctgc
Lmx.05.0690	TZP250	Upstream Reverse	GTACGCCGCGTAAGAGGGGGAGGACGGCATactaccgatcctgatccag
Lmx.05.0690	TZP250	5' sgRNA	gaaattaatacgactcactataggTGTGGTGTGTGTGTGTGTGttttagagctagaaatagc
Lmx.05.0690	TZP250	Downstream Forward	GCCACAGCCACCGACAGCCGAGCACTGGGggttctggtagtgttccgg
Lmx.05.0690	TZP250	Downstream Reverse	CAGCGACGGCGCACACGTACACGGATCCCGccaatttgagagacctgtgc
Lmx.05.0690	TZP250	3' sgRNA	gaaattaatacgactcactataggTGAAGGGCACGCGCTTCATCgttttagagctagaaatagc
LmxM.34.3925	N/A	Upstream Forward	GGGTCCGTATCGCTTTGTGTCAGGTTTCCAgataatgcagacctgctgc
LmxM.34.3925	N/A	Upstream Reverse	CTTGGCTCGAAGCAAGGGGAGTTCCTCCATactaccgatcctgatccag
LmxM.34.3925	N/A	5' sgRNA	gaaattaatacgactcactataggGACAAGAGCACTGGAGCACTgttttagagctagaaatagc
LmxM.34.3925	N/A	Downstream Forward	ACGATCGGCTCCGACACTGCGTGGATTGAGggttctggtagtgttccgg
LmxM.34.3925	N/A	Downstream Reverse	TCGGCCGTCGCTATCTTACATCCAGTTCCccaatttgagagacctgtgc
LmxM.34.3925	N/A	3' sgRNA	gaaattaatacgactcactataggGCGCAAGCAAAGACACAGCAgttttagagctagaaatagc
LmxM.34.3925	N/A	Forward (Validation)	GGACCGTTGCCAACTTTAGTCG
LmxM.34.3925	N/A	Reverse (Validation)	AGCGAGAGGATTCCGACACAAA
LmxM.30.0890	N/A	Upstream Forward	CACACACACTTCGCGATTCTCCAACCCgtataatgcagacctgctgc
LmxM.30.0890	N/A	Upstream Reverse	CGCCATGTTGGCGCTCCTCTTCGGCTCCATactaccgatcctgatccag
LmxM.30.0890	N/A	5' sgRNA	gaaattaatacgactcactataggAGAATTCAGTGAGCAGGTGGgttttagagctagaaatagc
LmxM.30.0890	N/A	Downstream Forward	CCCTACCAGCCCATGGACAGGACGAGCTACggttctggtagtgttccgg
LmxM.30.0890	N/A	Downstream Reverse	GGGTATAGCATCTCCCCGAATAGTCGCCGccaatttgagagacctgtgc
LmxM.30.0890	N/A	3' sgRNA	gaaattaatacgactcactataggAAGTTGCTATTTACTGCTCTgttttagagctagaaatagc

LmxM.30.0890	N/A	Forward (Validation)	CATCTCGAACGGTTGAACAGGC
LmxM.30.0890	N/A	Reverse (Validation)	CTTCGAATTCTCCTGCGCGATC
LmxM.10.0490	MPK3	Upstream Forward	TCAGTCTCTTTTTTATTTCTGTTACCCGgtataatgcagacctgtgc
LmxM.10.0490	MPK3	Upstream Reverse	CACGCTCAGCTCCTGGTTGCTCTTGTGCATactacccgatcctgatccag
LmxM.10.0490	MPK3	5' sgRNA	gaaattaatacgactcactataggACTGGCTGGTGTGGCTACTGgttttagagctagaaatagc
LmxM.10.0490	MPK3	Downstream Forward	AGCTCAAAGAACGGTAGCGGTCACCATCACggttctggtagtgttcgg
LmxM.10.0490	MPK3	Downstream Reverse	CCTGCGTGAGCGGGATGGCACACACAGTCGccaattgagagacctgtgc
LmxM.10.0490	MPK3	3' sgRNA	gaaattaatacgactcactataggAAACACAACACGACGGCGCCgttttagagctagaaatagc
LmxM.10.0490	MPK3	Forward (Validation)	TCGAGGTTCCGTCCAAGTACAC
LmxM.10.0490	MPK3	Reverse (Validation)	CGCGACACTTCTTGATGGACAC
LmxM.19.0180	MPK9	Upstream Forward	AGTCTTTTTTTGGTTTTGGTTGCTTTTTgtataatgcagacctgtgc
LmxM.19.0180	MPK9	Upstream Reverse	CAGCTGGCCCATCACCGTGTAGCGCTCCATactacccgatcctgatccag
LmxM.19.0180	MPK9	5' sgRNA	gaaattaatacgactcactataggACTCTTGTCGCGCCCTCCTgttttagagctagaaatagc
LmxM.19.0180	MPK9	Downstream Forward	GCAGCGGACAGCGACGACGAGTTCAACTTCggttctggtagtgttcgg
LmxM.19.0180	MPK9	Downstream Reverse	CGCGCACACAGGAGAACAAGAAGAAAACCAccaattgagagacctgtgc
LmxM.19.0180	MPK9	3' sgRNA	gaaattaatacgactcactataggCCCCTCTCCCCTGCTCACGTgttttagagctagaaatagc
LmxM.19.0180	MPK9	Forward (Validation)	GTGAAGCTGAAGGAAGTGGTGC
LmxM.19.0180	MPK9	Reverse (Validation)	ATTGGGCCGCTCATTTATTGG
LmxM.34.5010	MPK13	Upstream Forward	CTTCCCGTTTCTTCCCGCCTCGTGTTCCTgtataatgcagacctgtgc
LmxM.34.5010	MPK13	Upstream Reverse	CTTCTTGCCTAAGATTTGGTACTTCTGCATactacccgatcctgatccag
LmxM.34.5010	MPK13	5' sgRNA	gaaattaatacgactcactataggGCACACCACTGATGAGAAGTgttttagagctagaaatagc

LmxM.34.5010	MPK13	Downstream Forward	CTAGTCGCCCCGAAGCCTGCCAAAGCTGATAggttctggtagtggttccgg
LmxM.34.5010	MPK13	Downstream Reverse	CCCCAAAAAAGTGTACCAGACACGGCCAccaatttgagagacctgtgc
LmxM.34.5010	MPK13	3' sgRNA	gaaattaatacgactcactataggAGTCACTGTCATGTGTGCGCgttttagagctagaaatagc
LmxM.34.5010	MPK13	Forward (Validation)	CACCCGAACATTGTTGACCTGG
LmxM.34.5010	MPK13	Reverse (Validation)	CACCCGAACATTGTTGACCTGG
LmxM.27.0100	MPK14	Upstream Forward	CTTGTTGCCTCTCTCGATTCCCTTCCCTgtataatgcagacctgctgc
LmxM.27.0100	MPK14	Upstream Reverse	GATTTGAGCGAGAATTTTCATATTTGTCCATactaccgatcctgatccag
LmxM.27.0100	MPK14	5' sgRNA	gaaattaatacgactcactataggGGTCTAAAATAAAGTAAAGGgttttagagctagaaatagc
LmxM.27.0100	MPK14	Downstream Forward	AAGCACAAGACGAGTGGTCTGGCGTTCCTGGgttctggtagtggttccgg
LmxM.27.0100	MPK14	Downstream Reverse	CACACCACAGAACAAAAAGCGAAAAGACGAccaatttgagagacctgtgc
LmxM.27.0100	MPK14	3' sgRNA	gaaattaatacgactcactataggCCATGAAGAGAAACGGAAGCgttttagagctagaaatagc
LmxM.27.0100	MPK14	Forward (Validation)	GTACACCCTCTACTGCACCGTT
LmxM.27.0100	MPK14	Reverse (Validation)	GGTTTTCCGGCTTCATGTCTCG
LmxM.29.0600	N/A	Upstream Forward	GGCTGCGGTGTCTTGTTACCGGTTTTCAAgataatgcagacctgctgc
LmxM.29.0600	N/A	Upstream Reverse	CGCGTAGCCTACATTTTTGCGAGAGTGCATactaccgatcctgatccag
LmxM.29.0600	N/A	5' sgRNA	gaaattaatacgactcactataggAAGCCAGTGTACAGGCCATTgttttagagctagaaatagc
LmxM.29.0600	N/A	Downstream Forward	GGCCACGCACTGAAAGACGATCACAAGGTGggttctggtagtggttccgg
LmxM.29.0600	N/A	Downstream Reverse	TAGGGTACGCAGCCTCTCACAGCGACACCTccaatttgagagacctgtgc
LmxM.29.0600	N/A	3' sgRNA	gaaattaatacgactcactataggGTGCGCATATGCGCACGTATgttttagagctagaaatagc
LmxM.29.0600	N/A	Forward (Validation)	TCGCACATCCTTCATCGTGACT
LmxM.29.0600	N/A	Reverse (Validation)	GTGTCCGCCAACTCCTTACTCT

Figure 2-11 | Table showing primer sequences used in chapter 5 for protein tagging, gene deletion, and deletion validation.

Chapter 6

Gene ID	Gene name	Primer Category	Primer Sequence
LmxM.20.1310	SMP1	Upstream Forward	TTATTGACGGGTCTCCCCTCCCCCTTCCCTgtataatgcagacctgctgc
LmxM.20.1310	SMP1	Upstream Reverse	GCCGTTCTCAGACGAAGCACCGCAGCCCATactacccgatcctgatccag
LmxM.20.1310	SMP1	5' sgRNA	gaaattaatacgaactcactataggACACACAGGTGTGCGCGCTGgttttagagctagaaatagc
LmxM.20.1310	SMP1	Downstream Forward	TACCGCCAGCGTCAGGGGGAGAAGGAAAAGggttctggtagtgttccgg
LmxM.20.1310	SMP1	Downstream Reverse	CTAAAGAAACACTCTCATCTACTTTCACCTccaatttgagagacctgtgc
LmxM.20.1310	SMP1	3' sgRNA	gaaattaatacgaactcactataggAAGGTCGACAACGAAAAAAGgttttagagctagaaatagc
LmxM.08_29.1160	TXN1	Upstream Forward	TCCCTTTTCGAACTGTTCTTTGTACTTCCTgtataatgcagacctgctgc
LmxM.08_29.1160	TXN1	Upstream Reverse	GTCCCCCAAGTGCTTCCTGACACCGGACATactacccgatcctgatccag
LmxM.08_29.1160	TXN1	5' sgRNA	gaaattaatacgaactcactataggGCGCGCGGGGAGGAACAGAGgttttagagctagaaatagc
LmxM.08_29.1160	TXN1	Downstream Forward	GAAGGCGCGCAGTTCCCGTGGAGAGACGAGggttctggtagtgttccgg
LmxM.08_29.1160	TXN1	Downstream Reverse	CTAGCGCAGCAGGGTGGGGGGTCAAAGCCTccaatttgagagacctgtgc
LmxM.08_29.1160	TXN1	3' sgRNA	gaaattaatacgaactcactataggCGACTGTGCGCTTGAGCCACgttttagagctagaaatagc
LmxM.08_29.1160	TXN1	Forward (Validation)	GAGCTCTCTATCCGGCAAGACC
LmxM.08_29.1160	TXN1	Reverse (Validation)	CTTCGAAACGTGGTGCTTGTC
LmxM.08_29.1150	TXN2	Upstream Forward	TTTGGCTGTCCTTCGTCTCTCGTACACTTCgtataatgcagacctgctgc
LmxM.08_29.1150	TXN2	Upstream Reverse	GTGAGGGAAGAACTTCTCAAGCCTGACATactacccgatcctgatccag
LmxM.08_29.1150	TXN2	5' sgRNA	gaaattaatacgaactcactataggGTGTCCGTGCCTGACATCTTgttttagagctagaaatagc
LmxM.08_29.1150	TXN2	Downstream Forward	GAGTTCCCGTGGCCCGACGTGTGCGGAGAAGggttctggtagtgttccgg

LmxM.08_29.1150	TXN2	Downstream Reverse	GCGTACCTCATCGTGC GTTCACACTCCCCAccaatttgagagacctgtgc
LmxM.08_29.1150	TXN2	3' sgRNA	gaaattaatacgactcactataggTGCACCGTATACACCAGTGCgttttagagctagaaatagc
LmxM.08_29.1150	TXN2	Forward (Validation)	TCACTCCACCAGCTTCCTCAAG
LmxM.08_29.1150	TXN2	Reverse (Validation)	CGACTTCGCGTGTGTCTTGTAG
LmxM.08_29.1140	TXN-like A	Upstream Forward	CACGCAGAGGCTGCAGCTGCTCTCTCTCCGtataatgcagacctgtgc
LmxM.08_29.1140	TXN-like A	Upstream Reverse	GAATGGACCGCCCTTGTGACGCATGGACATactacccgatcctgatccag
LmxM.08_29.1140	TXN-like A	5' sgRNA	gaaattaatacgactcactataggTGGCTGAGCGTCTCTGTATAgtttttagagctagaaatagc
LmxM.08_29.1140	TXN-like A	Downstream Forward	TGTGGTGTTTACTACTACTTTTGGTATTATggttctggtagtgttccgg
LmxM.08_29.1140	TXN-like A	Downstream Reverse	TCATTTTGCTAGTACTACCCACTTCTGCCGccaatttgagagacctgtgc
LmxM.08_29.1140	TXN-like A	3' sgRNA	gaaattaatacgactcactataggAGGAGGTTGCCTCAGCGAGGgttttagagctagaaatagc
LmxM.08_29.1130	TXN-like B	Upstream Forward	CAGAGACACGCGCTGGGCACACCCCGACCGgtataatgcagacctgtgc
LmxM.08_29.1130	TXN-like B	Upstream Reverse	TAGATTAGACCACTGGCCAAAGTAGTTCATactacccgatcctgatccag
LmxM.08_29.1130	TXN-like B	5' sgRNA	gaaattaatacgactcactataggCTGTGTTTAATTGGGCGCGAgtttttagagctagaaatagc
LmxM.08_29.1130	TXN-like B	Downstream Forward	CGGACTACTTTGAAGACACAGGCAGCGCTGggttctggtagtgttccgg
LmxM.08_29.1130	TXN-like B	Downstream Reverse	CTTCCTCAACTCCACCCCATCCCCTTCCCTccaatttgagagacctgtgc
LmxM.08_29.1130	TXN-like B	3' sgRNA	gaaattaatacgactcactataggGCAACGCAGGCCGGCGTGAGgttttagagctagaaatagc
LmxM.08_29.1130	TXN-like B	Forward (Validation)	AGCTGCGACATCACACATCTCT
LmxM.08_29.1130	TXN-like B	Reverse (Validation)	TTCATCACCATCGTCTTGCCCT
LmxM.30.1960	TXN3	Upstream Forward	TGTTCTCTCCCCGCTCCTCGCTCTCGTTGgtataatgcagacctgtgc
LmxM.30.1960	TXN3	Upstream Reverse	CCCCGAGTTATTGAAAAAGTTGGGCTCCATactacccgatcctgatccag
LmxM.30.1960	TXN3	5' sgRNA	gaaattaatacgactcactataggCACGCAGGGATACGTACCCTgttttagagctagaaatagc

LmxM.30.1960	TXN3	Downstream Forward	AGCAGGCGCAGTTCGTGGGGTACGCTGAGCggttctggtagtggttccgg
LmxM.30.1960	TXN3	Downstream Reverse	CCATCGAGCGCTTGCCTCCCACTACCCAccaatttgagagacctgtgc
LmxM.30.1960	TXN3	3' sgRNA	gaaattaatacgactcactataggGCAAACGGATCGGTCAACTAgttttagagctagaaatagc
LmxM.30.1970	TXN4	Upstream Forward	TTTTTTTCTTCACTGAGCCGCTCCGCCCCgtataatgcagacctgctgc
LmxM.30.1970	TXN4	Upstream Reverse	GACTGCCGCATTCTCGAAAAGATGCGACATactaccgatcctgatccag
LmxM.30.1970	TXN4	5' sgRNA	gaaattaatacgactcactataggAGCCCGAGGAGAGGTGCGTGttttagagctagaaatagc
LmxM.30.1970	TXN4	Downstream Forward	GCGATCCTCCTGCTGACTTTGCTCATCCAGggttctggtagtggttccgg
LmxM.30.1970	TXN4	Downstream Reverse	AGGAATAAGCAGCACTATGGGCTCGCACCTccaatttgagagacctgtgc
LmxM.30.1970	TXN4	3' sgRNA	gaaattaatacgactcactataggTTGGACGCGGGAAACAGAACgttttagagctagaaatagc

Figure 2-12 | Table showing primer sequences used in chapter 6 for protein tagging, gene deletion, and deletion validation.

2.6 Fluorescence microscopy

L. mexicana procyclic promastigote cells were taken from cultures with a cell density of 5×10^6 to 1×10^7 in exponential growth phase and imaged live. Axio Observer A1 (Zeiss) was used with a 63× or 100× NA 1.40 Ph3 objective, and samples were kept at ambient temperatures, around 22°C.

2.6.1 IFT analysis on adhered cell samples

1 ml sample of cells was taken and washed in PBS thrice before being resuspended in 50 µl of PBS; 10 µg/ml Hoechst 33342 was added during the second wash to stain the DNA. 1 µl was taken from this sample prep and placed on a glass slide before a coverslip was added. Through this process the live cells and their flagella became adhered to the slide. Then Zeiss Zen-2.6 (Blue Edition) Hamamatsu Flash 4 camera was used to capture images.

For fluorescent protein localisation, phase contrast and widefield epifluorescence images were taken. For visualising IFT, mNeonGreen (mNG) signal was captured at 5 Hz widefield epifluorescence for all cell lines, except 2.5 Hz for IFT52::eGFP.

2.6.2 IFT analysis on swimming cell samples

1 ml sample of cells taken and centrifuged before being resuspended in 100 µl of media. We used dual-colour, high-frame rate (100 – 200 Hz) microscopy to visualise IFT in swimming cells. This optical setup is similar to the one described in Wang et al., 2020. On an Axio Observer A1 Zeiss microscope, we used a red filter (ThorLabs Astronomy red) on transillumination (100W halogen bulb) phase contrast. The epi-illumination is provided by a 120V metal halide light source (Zeiss, HXP 120 V), which passes through a custom filter cube (Chroma) with a dual-pass excitation filter (Chroma Technology, 59022x), delivering blue/yellow wavelengths to the sample. Light emitted from the sample passes back through the custom filter cube and is subject to a dual-pass green/red dichroic mirror (Chroma Technology, 59022bs) to deliver green/red wavelengths to the Optosplit II optical splitter (Cairn Research). The optical splitter filter cube has a 565 nm dichroic filter (Chroma Technology, T565lpxr)

which splits light to the green reflected (Chroma Technology, ET520/40m) and red transmitted (Chroma Technology, ET63260m) light filters. These two light paths are then reflected for projection onto the Andor camera sensor (Andor Neo 5.5 - Andor Solis version 4.22.3) in parallel.

2.6.3 Motility assays

An 8 μ l sample of *L. mexicana* in exponential growth phase were taken and placed on a slide with an adhesive gel frame (Fischer Scientific), then covered by a coverslip (Wheeler, 2017). A 512-frame 5Hz dark illumination video of cells swimming in M199 media, was captured using a Zeiss microscope, with a 10 $\times$ NA 1.40 Ph3 objective and a Zeiss Zen-2.6 (Blue Edition) Hamamatsu Flash 4 camera. A custom ImageJ particle tracking macro, that had been previously used by the lab, was employed to trace cell movement automatically. The traces are then drift corrected to account for any net movement of fluid during imaging. Unless otherwise stated, for each cell line six videos were taken - each containing 1000+ traces, from which the mean cell speed and velocity were calculated, as described in Beneke et al., 2019 and Wheeler, 2017.

2.6.4 Image analysis

ImageJ Version 1.53k (Schneider et al., 2012) was used for analysing IFT in both adhered and free cells. In general, lab-designed custom ImageJ macros were used as sequential functional modules throughout the image analysis pipeline. The flagella were traced (as described below) and the coordinates used to straighten the flagella fluorescence signal intensity, which was plotted per frame in the form of an IFT kymograph. The diagonal fluorescence lines on the IFT kymographs which represent anterograde and retrograde IFT trains were manually traced using the line tool and their coordinates were recorded, to allow for calculation of IFT train speed.

For adhered cells, the flagella were manually traced using the ImageJ segmented line tool. For free cells, images were taken with phase-contrast in both the red and green channels to act as a reference. The relative position and thus offset of the two channels was measured using this reference image to

the nearest pixel – this allowed for the generation of a composite image showing the position of the green fluorescence in the phase contrast cells. The red-phase contrast channel, or red fluorescence, was used for flagella tracing. Automated tracing was completed utilising a custom ImageJ script, for each image in the sequence through intensity thresholding, followed by skeletonization, as described in Walker and Wheeler, 2019.

The coordinates of the flagellum were then used to generate tangent angle and IFT kymographs. The tangent angle kymographs display the angle of the flagellum tangent relative to the flagellum base along its distance, as detailed in previous publications (Walker and Wheeler, 2019; Wang et al., 2021). To analyse flagellar beat type, the polygon tool was used to select sections of flagellar movement, which were then categorised into one of the five beat types: symmetric tip-to-base, asymmetric base-to-tip, uncoordinated, low frequency symmetric tip-to-base or static (the latter two added categories for mutant cell lines). An automated script was used to map IFT train speeds to their respective beat types for comparison.

Given the timescale of flagellar beating and IFT train movement are different, a Fast Fourier transformation with a 128-frame rolling window could be used on the tangent angle data to generate beat power spectrum, showing beat frequency over time (Walker et al., 2019).

2.6.5 Light intensity measurements

Light intensity measurements for the trans- and epi-illumination sources were taken using a microscope power sensor (100mW, 350nm -1100nm, S170C, ThorLabs) feeding back to an optical power and energy meter console (PM400, ThorLabs). The microscope was set up for measurement of desired light conditions, and the beam focused on correct focal plane at the centre of the microscope power sensor. Light intensity could then be read off of the digital screen of the meter console.

2.7 Transmission electron microscopy

L. mexicana were fixed in media using 2.5% glutaraldehyde (Electron Microscopy Sciences - EM grade glutaraldehyde 25% stock solution) for 10 minutes at room temperature. 10mL was centrifuged for 5 minutes at 16,000 g and supernatant was discarded. Next 4% PFA (Electron Microscopy Sciences – EM grade 16% stock solution) and 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) were added to the pellet for 2 hours minimum. OsO₄ (Taab Laboratories Equipment, 1%, from 4% stock aqueous solution) was used to stain cells before samples underwent serial dehydration using ethanol solution. Embedding and polymerisation in low-viscosity resin Agar 100 (Agar Scientific, UK) for 24 hours at 60°C. Ultrathin 80-90 nm sections were cut using a Leica EM UC7 ultramicrotome, and then placed on nickel grids and stained at room temperature for 5 minutes with uranyl acetate (Electron Microscopy Sciences - uranyl acetate dihydrate, 1%, w/v) and then with lead citrate (80 mM Reynolds solution, Reynolds, 1963). TEM images were captured with a Gatan OneView camera on a JEOL 2100 Plus 200kV microscope.

For axoneme cross-section averaging, the centre of each outer doublet A tubule was marked to fit an ellipse, which was perspective corrected to be circular and standardised, before nine-fold rotational averaging (Gadelha et al., 2006). An ImageJ StackReg plugin was used to align the rotationally averaged axoneme for an overall average. From these, per-pixel electron density difference maps showing statistical significance between control parental cells and deletion mutants as calculated by Mann-Whitney U test (with multiple comparison correction).

2.8 Comparative genomics

2.8.1 Basic Local Alignment Search Tool (BLAST) searches

For trypanosomatids, protein sequences for the query were obtained from TriTrypDB, and BLAST Protein (BLASTP) searches were performed using the inbuilt function in the TriTrypDB database. For other species, protein query sequences were obtained from the NCBI Protein Database and BLASTP

completed through UniProt BLASTP function (UniProt Consortium, 2019; Altschul et al., 1990). Annotations were often incomplete for the *C. reinhardtii* genome, meaning a search for protein names in this species using Ensembl Plants, UniProt, Phytozome and NCBI Protein Database largely yielded no results. Instead, protein sequences were obtained through scientific papers in which they featured. For BLASTP searches, hits with an expect value (E-value) smaller than 0.01 were considered as significant hits and putative homologue/orthologue matches.

2.8.2 Phylogenetic trees

For phylogenetic tree construction sequences were found as described about for BLAST searches. Phylogenetic trees were constructed using phylogenetic analysis pipeline by ETE3 (Huerta-Cepas et al., 2016). This calculates evolutionary distances using the maximum likelihood method on substitution rates.

3 The nature of IFT in beating flagella and its relationship with beat type switching

Some work outlined in this chapter was published in Gray et al., 2024; Gray, S., C. Fort, and R.J. Wheeler. 2024. Intraflagellar transport speed is sensitive to genetic and mechanical perturbations to flagellar beating. *Journal of Cell Biology*. 223:e202401154. doi:10.1083/jcb.202401154. All work completed in this chapter was my own, with the exception of the generation and imaging of the eGFP::IFT52 cell line (Figure 3-2), and generation and still imaging taken of the 3xmNG::IFT172 SMP1::mCherry (mCh) cell line (Figure 3-5A) which was completed by Richard Wheeler.

3.1 Introduction

The motor proteins powering IFT and beating interact with the axoneme outer microtubule doublets, use ATP as an energy source, and occupy the same cellular environment. Therefore, one may suspect spatial, energetic or cross-regulatory interaction between them.

Spatial interaction is plausible as anterograde IFT trains are around 50 nm wide - roughly the same width as the microtubule doublets themselves, therefore only tightly fitting between the ODAs on either side (Jordan et al., 2018; Lacey et al., 2023; Webb et al., 2020). The entire axoneme is encapsulated in a flagellar membrane, housing many proteins. The nature of these spatial constraints may change as the axoneme bends during beating, with flagellar bending for different beat types periodically generating a different geometric environment.

The beating of a flagellum is an energy intensive process. For example, a demembranated sea urchin sperm flagella uses 2.3×10^5 ATP per beat (Chen et al., 2015), or $\sim 5 \times 10^6$ ATP per second at 20 beats per second. This ATP consumption has a linear relationship with beat frequency (Chen et al., 2015; Takano et al., 2020).

It is likely that IFT is much less energy intensive. In *Chlamydomonas*, anterograde trains likely have a kinesin every 6 nm (Lacey et al., 2023; Jordan et al., 2018), an average length of $\sim 233 - 312$ nm (Jordan

et al., 2018), a speed of $\sim 2.5 \mu\text{m/s}$ and a frequency of 1.6 trains/s (Engel et al., 2012). Kinesin, similar to dynein, has a step distance of $\sim 8 \text{ nm}$ for each hydrolysed ATP (Coy et al., 1999; Toba et al., 2006). Therefore, an estimate of total ATP usage by anterograde IFT would be $\sim 3 \times 10^4$ per second. The retrograde IFT train structure is not as well characterised, but ATP usage would likely be comparable to anterograde, thus landing total consumption by IFT at $\sim 6 \times 10^4$ ATP per second. This is orders of magnitude lower than the consumption rate of beating. Cytoplasmic ATP diffusing rapidly from base to tip of flagella (Takao and Kamimura, 2008) is likely the major energy source for both sets of flagella motor proteins.

Calcium signalling has been shown to regulate both IFT and flagellar beating, raising the question of whether Ca^{2+} could be used to allow flagellar beat and IFT co-regulation, though experimentally disentangling their individual contributions remains highly challenging. In *Chlamydomonas*, IFT transport capacity scales inversely with flagellum length to allow length control (Engel et al., 2009). Is it possible that flagellar beating has an interaction with IFT movement to allow flagellum length control based on movement?

Understandably, visualising the movement of IFT trains within flagella that are free to beat is extremely challenging. When forward swimming *L. mexicana* flagella beat at around $\sim 25\text{Hz}$ (Gadelha et al., 2007; Wang et al., 2021; Wheeler, 2017). Therefore, in order to visualise IFT during flagellar beating, videos with very high framerates of 100 to 400 Hz are necessary to reduce flagellum movement happening in each image of an image sequence. This would equate to roughly 4 to 16 frames per beat cycle, thus capturing 4 to 16 different flagella positions during each beat. However, by using high frame rates of $> 100 \text{ Hz}$ we are decreasing the exposure time to $< 10 \text{ ms}$. During this very short timeframe, the constraint will be collecting enough photons emitted by a fluorescently tagged IFT protein within the sample to visualise the localisation of the IFT trains – this is our photon budget. Therefore, the frame rate needs to be high enough to reduce motion blur of the flagellum in a given

image for its position to be traceable, but also long enough for our photon budget to allow visualisation of the fluorescence signal.

In this chapter, we investigate the use of genetically encoded fluorescent proteins, dual-colour high frame rate microscopy and a bespoke image analysis pipeline for IFT visualisation in swimming cells, and compare the observed IFT train speed and stalling to that in immobilised cells. We then compare the IFT train speed in flagella undergoing different beat types to identify if there is a change upon beat type switching. Overall, we demonstrate that IFT trains can be visualised and their speed quantified in freely beating flagella and that IFT train speed is independent of beat type.

3.2 Results

3.2.1 Increasing fluorescence signal for IFT train visualisation

Previously in the lab, analysis of IFT in *L. mexicana* has been completed in phosphate buffered saline (PBS)-washed cells with flagella immobilised via adherence to slides, using an mCherry tag on diacylated membrane protein small myristoylated protein-1 (SMP1) and an eGFP tag on IFT52, for visualisation of the flagellar membrane and of IFT respectively (Wheeler et al., 2015). For visualising the IFT trains at the high frame rates required to capture flagellar movement, we need to increase fluorescence signal as much as possible through selecting both the fluorophore and the taggable gene that will give the brightest signal.

Therefore, candidate IFT proteins amenable to tagging were assessed on their brightness in *T. brucei* using TrypTag genome-wide protein location, leading to the selection of IFT172 tagging. IFT172 is a large protein in the IFT-B2 complex (Lacey et al., 2023) containing a pair of tandem WD domains neighbouring C-terminal TPR motifs. In anterograde trains, interaction with fellow IFT-B2 proteins IFT57 and IFT80 is mediated by the N-terminal WD40 repeat domain of IFT172, whilst in retrograde formation it is membrane binding (Taschner et al., 2016; Petriman et al., 2022; Wang et al., 2018). The TPR domain at the C-terminal of IFT172 has mutually exclusive interactions TPR helices of IFT144 and

IFT140, putatively involved with mediation of anterograde and retrograde IFT train architecture (Zacharia et al., 2025). There is also data supporting the interaction of IFT172 with dynein-2 in human cell lines (Hiyamizu et al., 2023).

Initially, mNG, a photostable fluorophore (Shaner et al., 2013) that is up to three times brighter than eGFP (Hostettler et al., 2017) was selected to N-terminally tag IFT172. To increase the signal even further, a plasmid containing a triple tandem repeat of mNG, joined by GS linkers, (3×mNG) was created by the lab to generate a 3×mNG tagging construct for use in *L. mexicana*, equivalent to one previously published for endogenous tagging in *Trypanosoma* (Paterou et al., 2023).

Genetically modifying cells can affect their swimming behaviour and tagging IFT proteins with fluorescent marker can perturb flagellum structure. Therefore the swimming speed and flagellum length of mNG::IFT172 and 3×mNG::IFT172 cell lines were compared to a Cas9T7 control (Figure 3-1).

To investigate cell swimming, I took low magnification dark-field videos of Cas9T7, mNG::IFT172 and 3×mNG::IFT172 free to swim in M199 media. From these videos, particle tracking with a lab-designed custom ImageJ macro was used to automatically trace cell movement. Cell path maps displaying the reconstructed path of these swimming cells were generated (Figure 3-1A). Not all cells will be traced for the full length of the video – for example they will not be traced if they move out of frame or out of the focal plane of imaging. *L. mexicana* procyclic promastigotes have curved swimming paths with low directionality (Wheeler, 2017), when forward swimming cells tend to take loose helical paths. The cell path maps of Cas9T7, mNG::IFT172 and 3×mNG::IFT172 were largely similar, each with the majority of cell paths indicating forward swimming. The distance moved over time for each cell trace in a field of view was used to calculate a mean speed for each video. It is also important to note that although the cells are free to move in three dimensions during imaging, the tracing, resulting cell path map and swimming speed is calculated in two dimensions. 3×mNG::IFT172 cells swam significantly slower than both its parental Cas9T7 and mNG::IFT172 cells (Figure 3-1B).

High magnification phase contrast stills of Cas9T7, mNG::IFT172 and 3×mNG::IFT172 cells were captured and each flagellum manually traced to calculate their length (Figure 3-1C). Parental Cas9T7 flagellum length was $14.3 \pm 4.4 \mu\text{m}$ ($n=68$, mean $\pm$ standard deviation), mNG::IFT172 was not significantly different at $13.0 \pm 3.5 \mu\text{m}$ ($n=61$, $p > 0.05$, two-tailed t-test), whilst 3×mNG::IFT172 had significantly shorter flagella at $10.9 \pm 4.1 \mu\text{m}$ ($n=53$, $p < 10^{-5}$, two-tailed t-test).

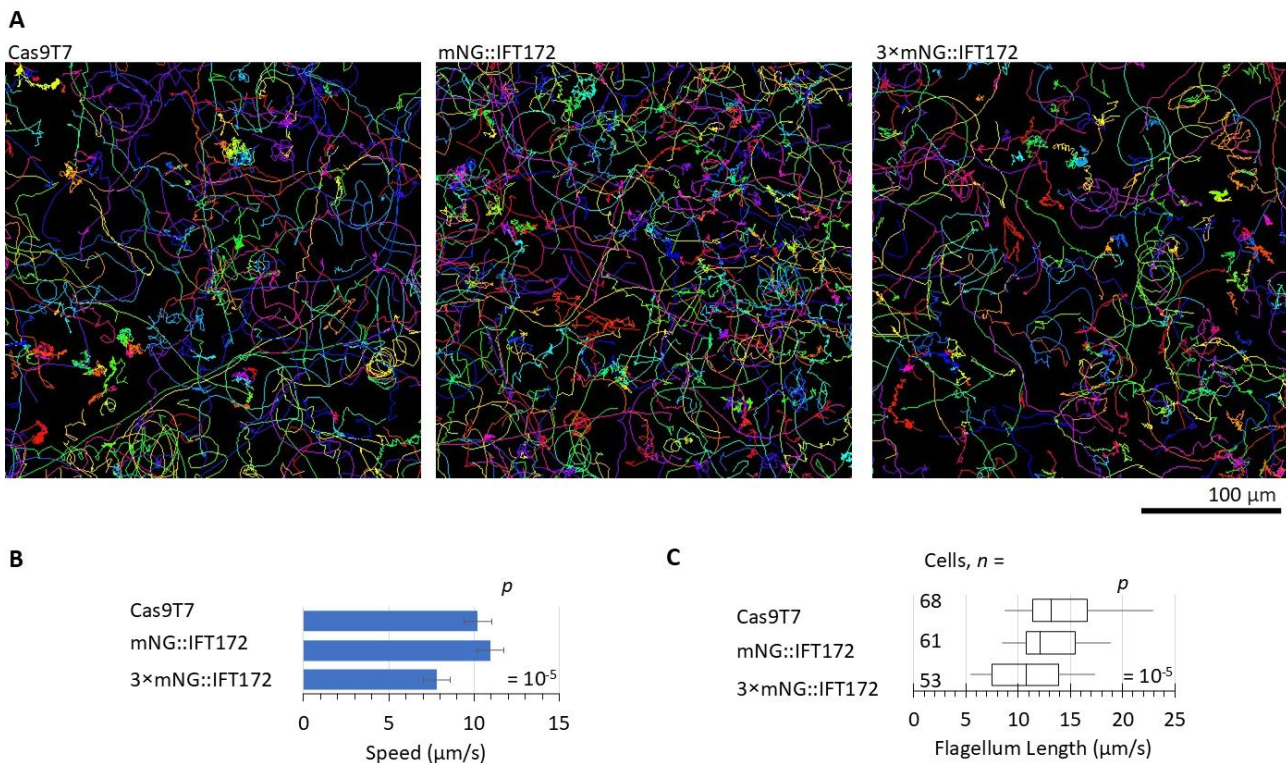


Figure 3-1 developed from Gray et al, 2024. | 3×mNG::IFT172 has reduced swimming speed and shorter flagella. A. Cell path maps showing traced cells for a portion of the field of view (FOV) for the full duration of the 10× dark-field 5 Hz image sequences. Each trace, presented in different colours, represents a single cell. The scale is indicated by a scale bar. B. Bar chart of swimming speed for Cas9T7, mNG::IFT172, and 3×mNG::IFT172 cell lines. The error bars show the standard deviation of the six replicates. Each replicate comprises the average swimming speed of over 1000 cells analysed. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented. C. Box plot showing the flagellum length for Cas9T7, mNG::IFT172, and 3×mNG::IFT172 cell lines. The boxes illustrate the lower quartile, median, and upper quartile, while the whiskers represent the 5th and 95th percentiles. P-values from t-tests comparing each cell line are indicated on the right side of the graph if statistically significant ($p < 0.05$).

3.2.2 IFT train speed was faster in genetically paralysed than in adhered flagella

Given that the use of a larger fluorescent tag shortened flagellum length it is important to evaluate the effect of mNG and 3xmNG on IFT train speed. In addition, we wished to investigate whether genetic paralysis and adherence to glass, both traditional methods of IFT visualisation, had any effect on IFT train speed. A 3xmNG::IFT172 mutant with deletion of both alleles of PF16, a protein essential for central pair microtubule stability and flagellar motility, was generated as a cell line with flagellar paralysis.

Epifluorescence microscopy was used to visualise IFT in mNG::IFT172, 3xmNG::IFT172 and 3xmNG::IFT172 Δ PF16 cells adhered to glass in PBS and in free but paralysed 3xmNG::IFT172 Δ PF16 in media. From the low frame-rate videos, the flagella of cells were manually traced, then digitally straightened to generate IFT kymographs (Figure 3-2A), using lab-designed ImageJ macros. Data of the eGFP::IFT52 cell line, collected by Richard Wheeler, is presented for comparison in Figure 3-2.

Kymographs represent the movement of particles over time along an axis. For an IFT kymograph, this shows the movement of trains along the length of the flagellum (x-axis) over time (y-axis). IFT trains are represented as diagonal lines. The movement of trains in the IFT kymographs confirmed canonical IFT behaviour (Figure 3-2), similar to the classic model *Chlamydomonas* (Dentler et al., 2009; Iomini et al., 2001) and the *Leishmania*-related parasite *T. brucei* (Morga and Bastin, 2013). From these kymographs, the IFT trains were identified and traced, then IFT train speed was calculated for each trace (Figure 3-2B).

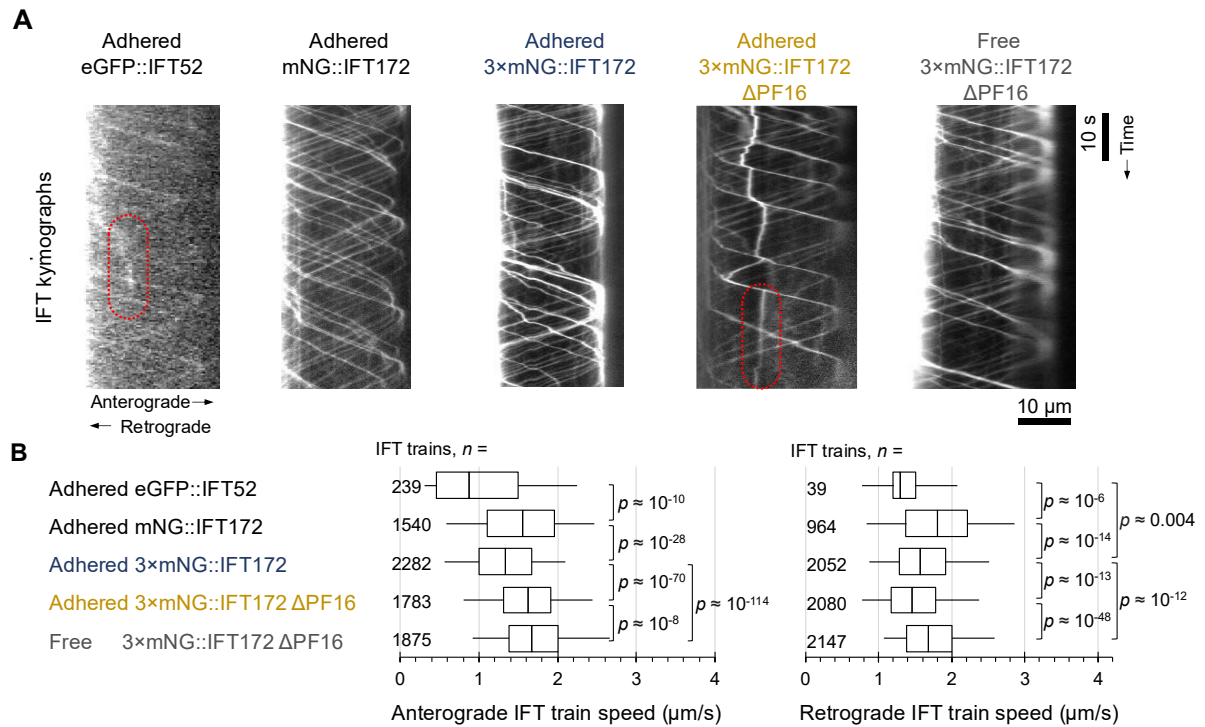


Figure 3-2 developed from Gray et al, 2024 | In Leishmania, the speed of IFT trains is influenced by both the type of fluorescent tag used and the method of flagellum immobilisation. A. Example kymographs of intraflagellar transport (IFT) from cell lines expressing a fluorescently tagged IFT protein, including those immobilised by adherence to glass, those with PF16 deletion-induced paralysis, or both conditions. Instances where IFT trains stall midway along the flagellum are circled in red. B. Box plots displaying anterograde and retrograde IFT train speeds, derived from 102.4-second videos of glass-adhered flagella for IFT52::eGFP, mNG::IFT172, 3×mNG::IFT172, 3×mNG::IFT172 ΔPF16, and free flagella of 3×mNG::IFT172 ΔPF16. The boxes show the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. P-values from t-tests comparing each cell line are provided on the right if significant ($p < 0.05$).

In adhered cells, IFT trains were markedly more visible in cell lines expressing mNG::IFT172 or 3×mNG::IFT172 in comparison to eGFP::IFT52 (Figure 3-2A) and had faster recorded train speeds in long term (≈ 100 s), low framerate videos (5Hz) (Figure 3-2B). Given that IFT52 is located along the length of IFT-B1 and integral to IFT-B stability (Lacey et al., 2023), reduced IFT train stability with IFT52 tagging may be involved with contributing to slower IFT train speed measurements in the eGFP::IFT52 cell line.

In addition, in 3×mNG::IFT172 ΔPF16 when flagella were free rather than adhered, anterograde and retrograde IFT train speeds were significantly higher. Interestingly in adhered flagella, deletion of PF16 caused a small increase in anterograde and decrease in retrograde train speed relative to the parental

3×mNG::IFT172 cell line (Figure 3-2B). However, to thoroughly investigate this phenomenon, IFT train speeds should be measured in flagella that are free to beat if able to do so.

3.2.3 IFT train speed decreases faster over time in adhered cells

While eGFP intensity decreased markedly with photobleaching, making trains less and less visible throughout the imaging period, the photostability of the 3×mNG signal permitted long-term imaging of IFT trains. I used this to test the effect of long-term immobilisation by flagellum adhesion on IFT train speed.

IFT train speed decreased markedly in speed over time in all samples, with a stronger decrease in speed of retrograde than anterograde trains (Figure 3-3). However, immobilisation via genetic paralysis was less detrimental than adherence on IFT train speed, with the decrease in IFT train speed in free 3×mNG::IFT172 ΔPF16 flagella being markedly less than in the adhered 3×mNG::IFT172 ΔPF16 flagella. Therefore, the process of PBS-washing and flagella adhesion has a time-dependent detrimental effect on IFT train speed during imaging on the timescale of minutes.

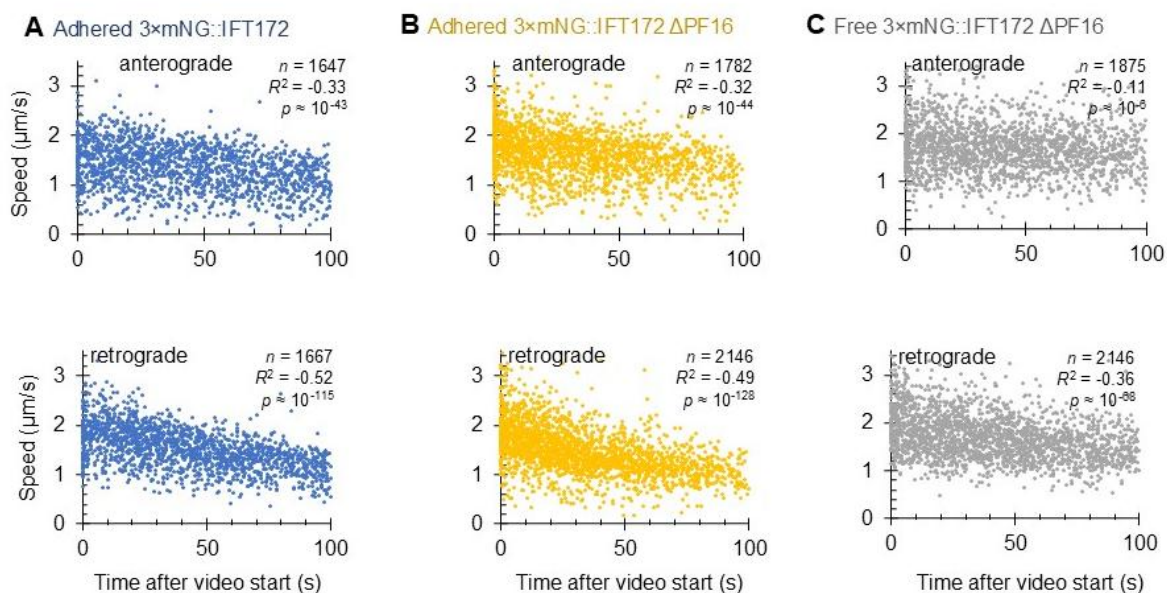


Figure 3-3, developed from Gray et al, 2024. | IFT trains in adhered flagella decrease in speed over time. A. Scatter plots showing the speed of anterograde and retrograde IFT trains over time in flagella of 3×mNG::IFT172 cells with flagella adhered to glass. Each data point represents an individual IFT train. The number of IFT trains

(n) and Pearson's correlation coefficient (R^2) are provided, along with the p-values for these correlations. B. As for A, however for 3xmNG::IFT172 Δ PF16 cells with flagella adhered to glass. C. As for A, however for the free flagella of 3xmNG::IFT172 Δ PF16 cells.

3.2.4 High-frame rate dual-colour microscopy allows visualisation of IFT in a beating flagellum

To achieve the faster framerate analysis for visualising IFT in beating flagella, increased IFT fluorescence signal is needed. The excitation source, used for live cell imaging (epi-illumination with dichroic filter cube) had a 156 mW intensity on the sample, giving a rough median wavelength as 506nm the photons per second delivered to the sample would be 3.97×10^{17} photons per second (ph/s). mNG has an extinction coefficient (light absorbing ability) of $116 \text{ mM}^{-1} \text{ cm}^{-1}$ and quantum yield (proportion of absorbed photons emitted as fluorescence) of 0.80 Φ (Shaner et al., 2013). So it could be estimated that under 156 mW excitation light intensity each mNG particle would emit roughly 2.3×10^{13} ph/s, whilst 3xmNG would give 6.9×10^{13} ph/s, or 1.7×10^{11} per frame even at 400 Hz. Swimming samples will be visualised at 63 $\times$ and images taken using an Andor Neo 5.5 CMOS camera which has a quantum efficiency of 95% and 6.5 μm pixel size. Each IFT train is a polymeric structure composed of IFT-A and IFT-B subunits, this each will be tagged with multiple fluorophore molecules. Therefore, utilising the 3xmNG tag on IFT172 should give orders of magnitude higher fluorescent signal than the background, even at high frame rates, allowing IFT trains to be visualised and their speed quantified in beating flagella. Estimating IFT train length based on fluorescence signal in beating flagella is unlikely to be accurate with this set-up, due to limitations in spatial resolution and motion blur. Additionally, given that flagella are moving during video capture, the position of the fluorescent signal of tagged IFT trains relative to the focal plane may fluctuate, leading to changes in light scatter and intensity with de- and re-focusing.

For the majority of my work, I used green fluorescence and red phase contrast. A notable exception is the use of red and green fluorescence for investigations into proximal-distal IFT asymmetry using the 3xmNG::IFT172 Δ DC2 pDC1::mCh cell line in 4.2.9. These distinct channels were created using an optical path similar to one previously described (Wang et al., 2020) and detailed in 2.6.2 to capture a

high-frame rate (100-200 Hz) image sequence (4.75 s) (Figure 3-4). An image generated from each of the two channels for each frame is projected onto the camera sensor side by side. Multiple still reference images are taken with phase contrast in both channels to allow alignment of the channels downstream in image processing.

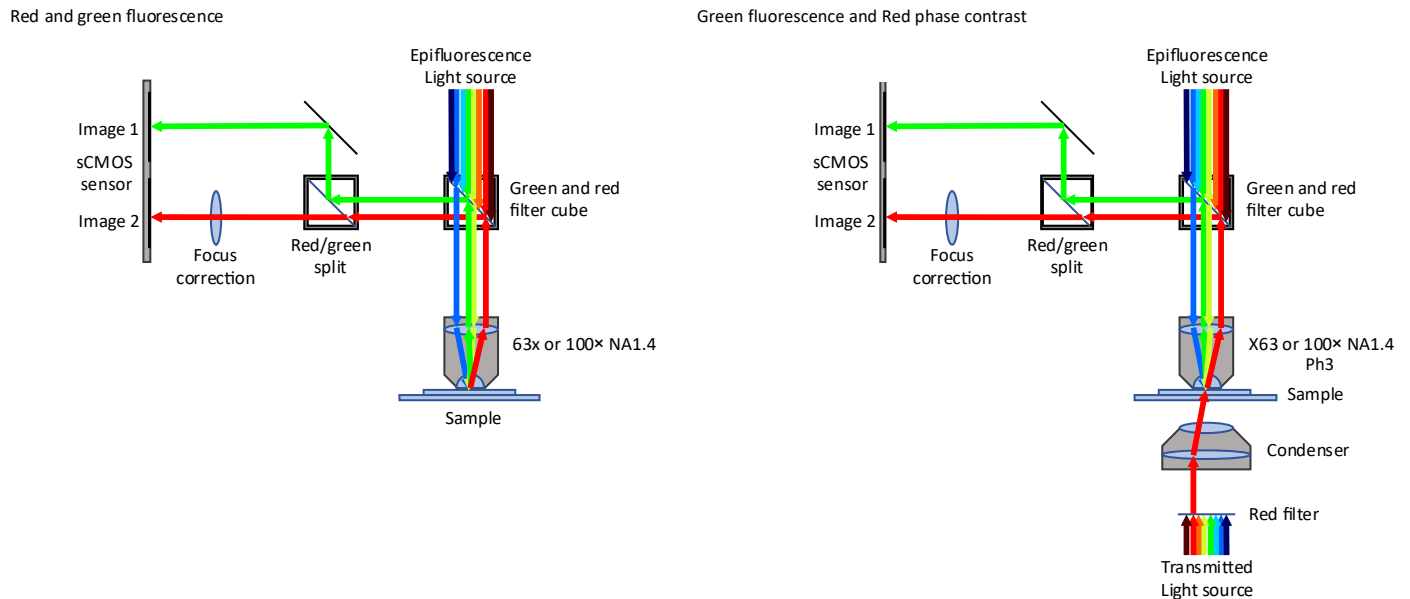


Figure 3-4, adapted from Wang et al. (2020) and published in Gray et al, 2024 | A schematic diagram displaying the flexible optical setup for dual-colour imaging. The light path has a transmitted light source that can be fitted with a red or green filter. Light is first filtered through a dual-band excitation filter cube (59022bs; Chroma Technology), which covers 450–490 nm (blue) and 550–590 nm (yellow/green) wavelengths before reaching the sample. The sample's emitted green (500–545 nm) and red (600–660 nm) light is reflected by a dual-band dichroic mirror in the custom filter cube into an optical splitter. A 565-nm dichroic filter (T565lpxr; Chroma Technology) then divides the light into two paths: one passes through a red transmitted filter (ET632/60 m; Chroma Technology) and the other through a green reflected filter (ET520/40 m; Chroma Technology), based on wavelength. These two light paths are then directed by mirrors and projected in parallel onto the camera sensor.

3.2.5 Image analysis pipeline allows comparison of IFT train speed and flagellar motility behaviour

In order to be able to calculate IFT train speed in a beating flagellum, IFT kymographs must be generated. The image analysis required to do this was completed using a lab-designed ImageJ macro-based image analysis pipeline detailed in 2.6.4.

In the dual-colour videos of cell lines expressing either 3×mNG::IFT172 alone or 3×mNG::IFT172 and SMP1::mCh, flagellum position is visible from either red-light phase contrast or SMP1::mCh signal and is automatically traced (Figure 3-5A-C). Using the offset of the two channels, calculated from the phase contrast reference images, the coordinates of the traced flagellum in each video frame can be used for per-frame digital straightening of the 3×mNG::IFT172 signal in the green channel (Figure 3-5D). A tangent angle kymograph can be generated from the coordinates and curvature derived from the flagellum tracing (Figure 3-5F). This shows the flagellum curvature along the length of the flagellum (x-axis) over time (y-axis).

As the flagellum beat occurs at much shorter timescales to IFT, it is possible to further summarise flagellar movement as a power spectrum over time (Figure 3-5G) (Walker and Wheeler, 2019). For this a Fourier transform converted the data displayed in tangent angle kymograph on flagellum curvature change to a ‘frequency’ of beating – roughly correlating to flagellar beating frequency over time. However, I rarely used this downstream, as the ‘frequency’ on the power spectrum was highly abstracted and it is unknown how this correlates with the biology of flagellar beating such as functioning of components driving beating i.e. the dynein arms or ATP consumption. Additionally, though it provides a reproducible value for wildtype beat types, it is unable to provide valid frequencies for the irregular beat types of mutants with genetic disruption to beating who we will meet in later chapters.

The kymographs describing flagellar movement (Figure 3-5F-G) can be directly compared to IFT kymographs (Figure 3-5H) generated from the same free to move cell and with a free to beat flagellum. Thus, allowing us to analyse the interaction of flagellar movement with IFT train movement within a cell.

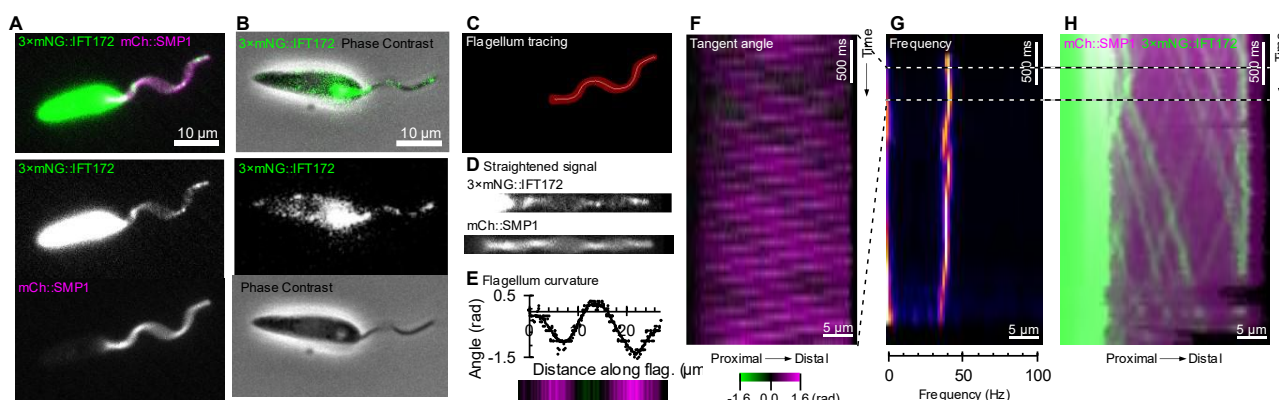


Figure 3-5 presented in Gray et al, 2024 | Pipeline for Quantitative Analysis of IFT in Beating Flagella. Previously detailed in 2.6.4. A. Cell line generated and captured by Richard Wheeler. Still frame taken from a high-frame-rate, 200 Hz dual-colour video showing a cell expressing 3xmNG::IFT172 (IFT marker) and SMP1::mCh (flagellar membrane marker). B. Still frame from a 200 Hz dual-colour, high-frame-rate video of a cell expressing 3xmNG::IFT172, displaying both fluorescence and phase contrast images. C. SMP1::mCh image used for flagellum tracing (marked in red) and for determining the flagellum midline (indicated by the white line) through thresholding. D. Digitally straightened view of the flagellum from the frame shown in A, using the midline determined in B. E. Graph and colour-coded bar representing the tangent angle along different distances of the flagellum, for the frame shown in A. F. Kymograph of the flagellum's tangent angle for a 100-frame segment of the dual-colour, high-frame-rate video. G. Power spectrum over time, obtained from the kymograph of the flagellum tangent angle, covering the entire video length. H. Kymograph illustrating the fluorescent signals of 3xmNG::IFT172 and SMP1::mCh over time for the full video, clearly showing IFT trains.

3.2.6 IFT train stalling in adhered and free flagella

In free flagella, we observed some stalled IFT trains. To compare IFT train stalling, stalled IFT trains were traced in IFT kymographs derived from adhered and free flagella of 3xmNG::IFT172. Aware of the effects of long-term imaging, the first 4.75 s of the adhered flagella IFT kymograph was used for comparison with the 4.75 s-long free flagella videos. Adhered flagella had twice as many stalled trains per flagellum (Figure 3-6A). Moreover, the nature of stalling was very different. In free flagella, stalled trains tended to stay immobile for at least the full 4.75 s, whereas in adhered flagella the trains exhibited intermittent stop-start behaviour (Figure 3-6B).

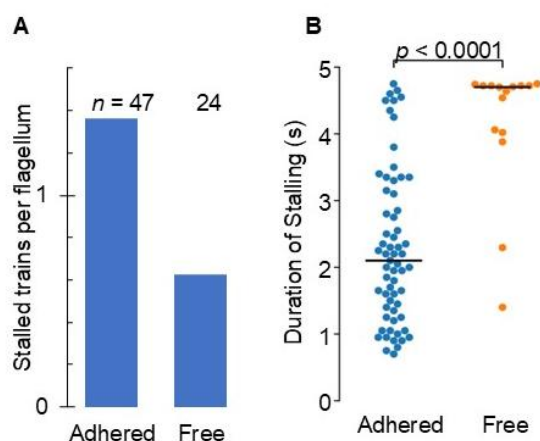


Figure 3-6, developed from Gray et al, 2024 | IFT train stalling is more frequent and of more varied durations in adhered cells. A. Bar chart of the average number of stalled IFT trains per flagellum, based on the first 4.75 s of the IFT imaging video from adhered and free flagella. The number of flagella (n) is described. B. Bee swarm plot showing the duration of IFT train stalling in adhered and free 3×mNG::IFT172 flagella. Each dot represents a stalled train, traced from the n, flagella depicted in A. The p-value from a Mann–Whitney U test comparing the two conditions is provided.

3.2.7 Flagella beat type and IFT train speed are independent

In culture, *L. mexicana* undergo faster (20 to 25 Hz) symmetric tip-to-base for forward swimming and lower (5 Hz) frequency base-to-tip asymmetric beats for reorientation, as well as aperiodic uncoordinated movements (Gadelha et al., 2007; Holwill and McGregor, 1975). In order to determine whether IFT train speed was influenced by beat type, we first measured IFT train speed before and after beat type switching to assess changes in individual train speeds. Additionally, we independently measured IFT train speed and categorised them by beat type to see if trains generally had different speeds in flagella undergoing distinct beat types.

These beat types (Figure 3-7A) can be recognised downstream in the image analysis pipeline - by the patterns generated from the periodic changes in curvature along the flagellum displayed in the tangent angle kymographs (Figure 3-7B), and following Fourier transform the frequency in a beat power spectrum (Figure 3-7C). This can then be compared to the IFT kymograph of the same flagella captured simultaneously (Figure 3-7D).

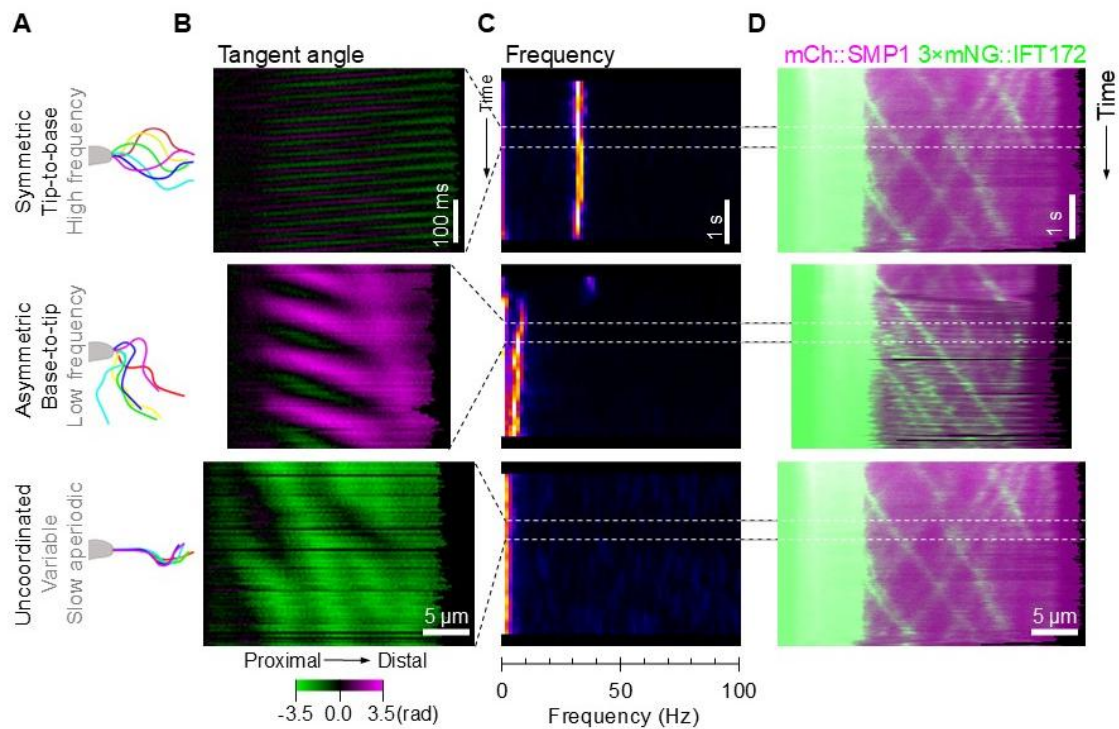


Figure 3-7, Adapted from Gray et al., 2024 | Tangent angle and IFT kymograph generation allow the comparison of flagellar beating and IFT within the same cell. A. Diagrammatic reconstructions of flagella undergoing symmetric tip-to-base beating, asymmetric base-to-tip beating and uncoordinated slow movement. B. Tangent angle kymographs of symmetric tip-to-base beating, asymmetric base-to-tip beating and uncoordinated slow movement. C. Beat power spectrum of symmetric tip-to-base beating, asymmetric base-to-tip beating and uncoordinated slow movement. D. IFT kymographs from flagella undergoing symmetric tip-to-base beating, asymmetric base-to-tip beating and uncoordinated slow movement.

We identified examples of flagella which switched movement over the course of imaging and directly observed whether IFT train speed changed upon transition between beat types (Figure 3-8). To maximise the sample size, this analysis was completed for 3xmNG::IFT172 and two independently generated 3xmNG::IFT172 SMP1::mCh cell lines. Then a paired measurement of IFT train speed in a flagellum before and after it switched beat type was completed. Even during the short period of imaging IFT train speed decreases, therefore the average rate of decline in train speed measured during the duration of a video was calculated in flagella undergoing a symmetric tip-to-base beat ($0.071 \mu\text{m}/\text{s}^2$ ($n = 58$)) and used to calibrate paired measurements. The paired measurements revealed that there was a very small but significant decrease in IFT train speed as flagella switched from symmetric tip-to-base to asymmetric base-to-tip beating (Figure 3-8B).

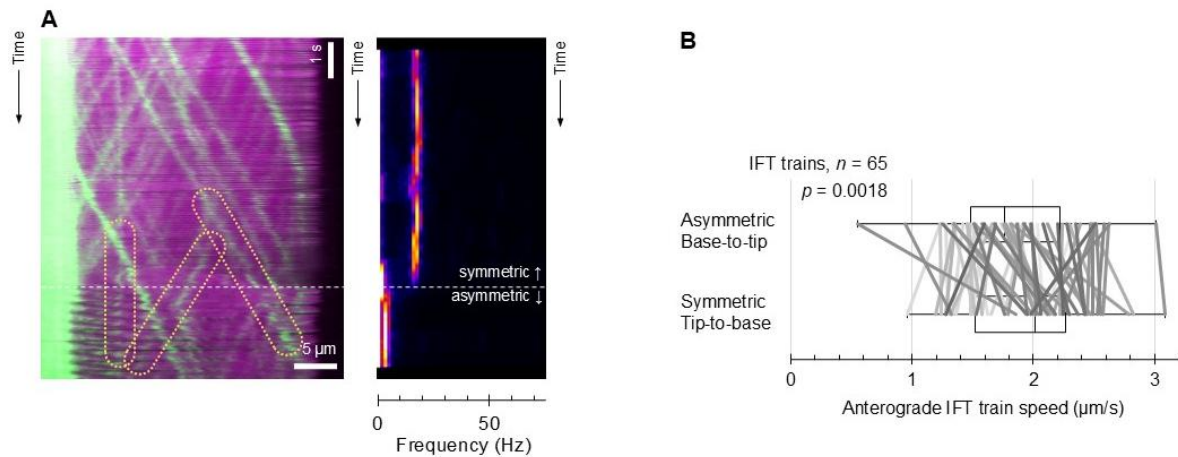


Figure 3-8, Adapted from Gray et al., 2024 | On average, IFT train speed decreases slightly as flagella switch from symmetric tip-to-base to asymmetric base-to-tip beating A. Kymograph displaying the fluorescent signals of 3×mNG::IFT172 and SMP1::mCh, along with the power spectrum over time, for a flagellum undergoing a transition from symmetric tip-to-base to asymmetric base-to-tip beating. Notable examples of anterograde, retrograde, and stalled IFT trains observed during this transition are highlighted. B. Analysis of individual anterograde trains where the beating pattern changed, combining data from 3×mNG::IFT172 and 3×mNG::IFT172 SMP1::mCh. The box-and-whisker plots illustrate statistical measures, with lines connecting paired data points. The p-value from a two-tailed paired t-test is provided.

The second method of quantification was a blinded analysis of IFT train speeds in cell lines expressing mNG::IFT172, 3×mNG::IFT172 or 3×mNG::IFT172 and SMP1::mCh. Independently IFT trains were traced from IFT kymographs and the polygon tool in ImageJ was used to demarcate beat types in tangent angle kymographs. For each trace a train speed was calculated and mapped to its flagellum beat type: symmetric tip-to-base, asymmetric base-to-tip or other/uncoordinated. This revealed no significant difference in the speed of either anterograde or retrograde IFT train speeds in flagella undergoing symmetric, asymmetric or uncoordinated movement (Figure 3-9). We therefore conclude that IFT train speed and beat type may have a weakly dependent relationship, however, are not strongly co-regulated.

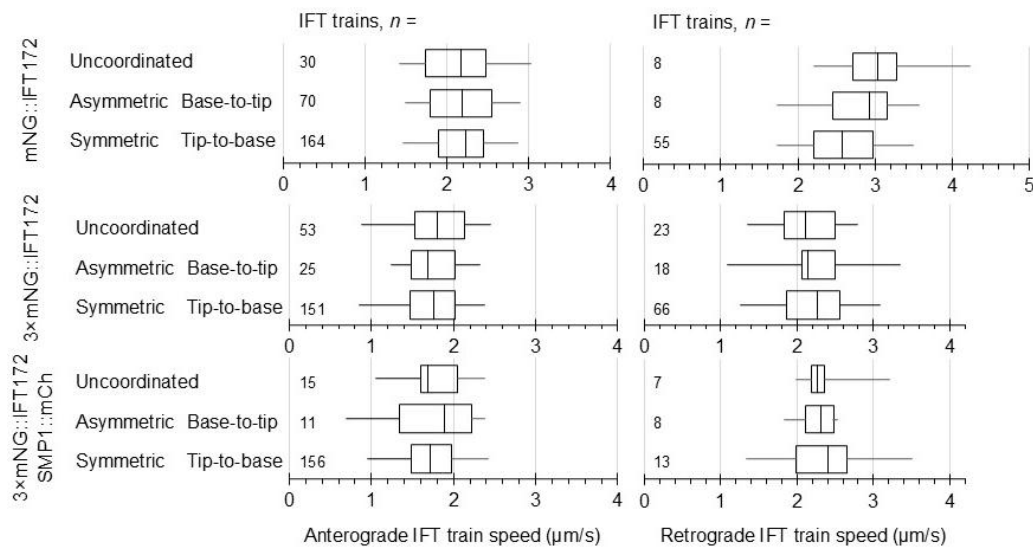


Figure 3-9, Adapted from Gray et al., 2024 | IFT train speed is not different in flagella undergoing symmetric, asymmetric or uncoordinated movement. Box plots of anterograde and retrograde IFT train speeds in the flagella of mNG::IFT172, 3xmNG::IFT172, and 3xmNG::IFT172 SMP1::mCh, generated using fluorescence signal data from flagella undergoing different beat types. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. The number of IFT trains (n) is presented. No significant differences ($P > 0.05$, ANOVA) were observed in the speeds of anterograde or retrograde IFT trains in flagella undergoing different beat types.

3.3 Discussion

We utilised high-frame rate, dual-colour microscopy to visualise and quantify IFT train movement in beating flagella for the first time since its discovery over three decades ago (Figure 3-4, Figure 3-5) (Alves and Bastin, 2024; Kozminski et al., 1993). IFT in motile flagella in their physiological state of beating is broadly similar to that observed in immobilised flagella, but with a propensity for higher IFT train speeds and fewer stalled IFT trains (Figure 3-6A-B). Quantifying IFT train speed in freely beating flagella allowed us to reveal the experimental flaws in analysing IFT in immobilised flagella, whether mechanical or genetic.

In *L. mexicana*, adherence of flagella to a slide post PBS-washing has clear detrimental effects on IFT train speed, with retrograde IFT trains particularly strongly affected, and the speed decreasing over time (Figure 3-2, Figure 3-3). Though PBS is isotonic and non-toxic, it lacks the nutrients found in M199, therefore extended exposure could have metabolic effects, including reduced ATP production causing

slowed IFT train speeds over time. Disruption to IFT could equally be due to the process of mechanical adhesion itself (discussed more below in the context of stalling trains) or the effect of extended light exposure on cell processes. Photobleaching of fluorescent markers may reduce recorded IFT train speed by making faster moving IFT trains less visible. Regardless of the underpinning process, this highlights the importance of minimising immobilisation time through experimental design.

Intriguingly, genetic paralysis of flagella had a different confounding effect – an increase in the speed of IFT trains (Figure 3-3). There are many plausible reasons this could occur, for example with energy intensive flagellar beating no longer occurring in culture there could be increased ATP availability for hydrolysis by the motor proteins powering IFT. Alternatively, the loss of one or both of the central microtubules that occurs with PF16 knock out could alter axonemal structure in a way that increases space for IFT trains to progress. An additional consideration is that following PBS-washing it is possible some cells in the FOV of visualization would be incompletely adhered to the glass slide. In the low frame rate videos, incompletely adhered 3×mNG::IFT172 flagella are automatically excluded from analysis by virtue of flagellar beating rendering them untraceable, whilst incompletely adhered flagella of 3×mNG::IFT172 ΔPF16 would be largely indistinguishable from adhered ones. The effect of genetic disruption of flagellum beating on IFT will be explored further in beating flagella in the following chapter.

The reduced swimming speed (Figure 3-1A), flagellum length (Figure 3-1B) and IFT speed (Figure 3-2B) of 3×mNG::IFT172 cells, compared to mNG::IFT172 and parental Cas9T7 reveals that although the tandem repeats of mNG are advantageous for increasing IFT train signal for visualisation of trains at high-frame rates in beating flagella, it affects IFT train speed. This could be due to the effect of the N-terminally attached 3×mNG altering N-terminal domain WD40 repeat inter-IFT-B2 and membrane interaction in anterograde and retrograde trains respectively, or altered dynein-2 interaction. Additionally, given structural data on IFT172 positioning relative to the microtubule doublets (Lacey et al., 2023), it is likely that the 3×mNG molecule is oriented towards the outer microtubule doublets

along which the train is walking, potentially also affecting train progression. Interestingly, eGFP::IFT52 had much lower observed IFT train speeds, likely due to the importance of IFT52 in IFT-B complex stability and the de-stabilising effect of introducing a ~27 kDa tag to its N-terminus. The variation in recorded IFT train speed depending on fluorescent marker used and the IFT protein tagged (Figure 3-2B) showcases the importance of considering tag size and orientation, subunit chosen for tagging and maintaining consistency of tagging method to ensure appropriate experimental controls.

The incidence of stalled trains in freely beating flagella was notable, although the incidence and variability of length of stalling was higher in adhered flagella (Figure 3-6). In many species, IFT trains have been shown to interact with and even carry membrane-associated and intra-membrane proteins (Liew et al., 2014; Williams et al., 2014; Huang et al., 2007; Qin et al., 2005), therefore, it may be possible that adhesion of a proportion of the flagellar membrane to a glass slide could reduce the fluidity of membrane interacting protein movement and in some way contribute to IFT train stalling – or intermittent halting. In *Chlamydomonas*, completely blocking IFT through a silicone wedge consistently led to IFT turnaround (Nievergelt et al., 2022). In our kymographs, we did not see frequent incidence of premature turnaround, likely due to the adhesion to glass not resulting in complete mechanical blockage.

In surface-adhered *Chlamydomonas*, IFT particles carrying transmembrane components are thought to interact through the flagellar membrane with the substrate or adherent surface to stall train movement, where it can lead to gliding motility (Bloodgood and Salomonsky, 1989; Shih et al., 2013). We have never observed gliding motility in adhered *L. mexicana*, however, it is notable that we observed IFT trains can stall without the possibility of mechanical interaction through the membrane with a solid substrate. Possible reasons for IFT train stalling in free flagella could include regulation of motor protein activity, interactions between cargo or train subunits and surrounding proteins, disruption due to opposing forces or external loads exceeding the stall force or other obstacles to

progression for example microtubule track specialisations or post-translational modifications (Prevo et al., 2017; Toropova et al., 2017; Yeyati et al., 2017; Xie et al., 2020; Gennerich et al., 2007).

The ability to visualise IFT in freely beating flagella is necessary to understand any interaction or co-regulation of IFT with flagellar motility. We anticipated that the greater flagellum curvature during asymmetric base-to-tip beats might alter IFT train speed. This would provide a mechanism for chemotactic responses to influence flagellum length, composition, and maintenance. We found that though IFT train speed may be weakly dependent on natural switches in beat type, the pair did not exhibit strong co-regulation (Figure 3-8, Figure 3-9). This suggests that any intraflagellar secondary messengers controlling beat type switching are unlikely to also be major regulators of IFT speed. This allows us to exclude several hypotheses about the regulation of flagellar biology through the *Leishmania* life cycle. It is unlikely that the changes in flagellum length and composition associated with life cycle transitions between amastigote, procyclic promastigote and metacyclic promastigote stages is controlled by flagellar beat change mediated alterations in IFT. In addition, this indicates flagella curvature or direction of wave propagation does not cause differing spatial hindrance to IFT trains travelling in either direction. This could be a result of IFT trains being restricted to microtubule doublets that are less affected by the planar movement of flagella – as occurs in *T. brucei* (Bertiaux et al., 2018).

4 The effect of genetic perturbations to flagellar motility on IFT train speed

Some work outlined in this chapter was published in Gray et al., 2024; Gray, S., C. Fort, and R.J. Wheeler. 2024. Intraflagellar transport speed is sensitive to genetic and mechanical perturbations to flagellar beating. *Journal of Cell Biology*. 223:e202401154. doi:10.1083/jcb.202401154. All work completed in this chapter was my own, with the exception of capture and analysis of the TEM presented in Figure 4-1 which was completed by Cecile Fort, on a cell line generated by myself.

4.1 Introduction

In the previous chapter, we explored how IFT speed in beating flagella is largely independent of beat type. Interestingly, we found that free and adhered 3×mNG::IFT172 ΔPF16 cells had higher anterograde train speeds than adhered 3×mNG::IFT172 (Figure 3-2). Since the discovery of IFT in paralysed flagella, genetically-driven flagella paralysis has frequently been used to straighten flagella and minimise their movement to allow IFT visualisation (Dentler, 2013, 2005; Kozminski et al., 1995; Pazour et al., 1998; Kozminski et al., 1993). Across flagellated species, there is a lot of research involving not only genetically-induced paralysis but also genetic dissection of flagellar motility (Hoffman and Goodenough, 1980; Huang, 1986; Vyklicka and Lishko, 2020), including in *L. mexicana* (Beneke et al., 2019). Although the use of paralysed flagella in IFT research is standard practice and motility-challenged mutants are widely studied; the effect of paralysis or genetic perturbation to flagellar movement on IFT train movement has not been thoroughly investigated - especially not in swimming cells.

Therefore we aimed to assess whether the alteration in IFT speed in ΔPF16 cells was a result of genetically induced permanent disruption to flagellar movement and further decode the interaction between flagellar motility and IFT train movement. To do so I engineered a cohort of 3×mNG::IFT172 cell line deletion mutants, with known or expected perturbations to flagellar beating (dDC1, RSP4/6,

OAD β , LC1). To confirm that any effects on swimming behaviour, flagellar beating or IFT train speed were not a result of drug selectable marker expression, a control cell line was used: a diploid deletion of one gene in the histone 3 (H3) tandem array with the same drug selectable markers as the other deletion mutants. The deletion mutant cohort was selected to decipher whether structural or functional changes to ODA or ODA-associated structures, or to non-ODA axonemal structures have the capacity to influence IFT train speed. The motivation for selecting each deletion mutant is outlined below.

As discussed in 1.9.2, dDC1 is predicted to form a heterodimer with dDC2, and deletion of either disrupts outer dynein arm assembly in the distal axoneme (Edwards et al., 2018; Fort et al., 2024). dDC1 and dDC2 deletion mutants will allow us to investigate whether loss of ODA in the distal flagellum and altered beat type impacts IFT speed. In *Chlamydomonas*, both outer arm dynein components light chain 1 (LC1) (Sakato-Antoku and King, 2023) and outer arm dynein beta chain (OAD β) are required for normal beating, however OAD β , but not LC1, is required for outer dynein arm assembly (Kamiya, 1988; Liu et al., 2008; Mitchell and Rosenbaum, 1985). In *T. brucei*, OAD β and LC1 are required for both normal flagellar beating and ODA assembly (Ralston et al., 2011; Baron et al., 2007). If LC1 and OAD β in *L. mexicana*, are more similar to in *Chlamydomonas*, we can expect that the extent to which Δ LC1 and Δ OAD β phenocopy Δ dDC1 or Δ dDC2 to give us insight into whether it is the alteration in flagellar movement or the loss of the ODAs themselves that are having an impact on IFT train speed.

The effect of flagella paralysis through the disruption of non-ODA structures was tested using Δ PF16 and Δ RSP4/6. As aforementioned, PF16 deletion is well characterised in many species, including several *Leishmania* (Espada et al., 2021; Martel et al., 2017; Yagoubat et al., 2020). In *Chlamydomonas*, radial spoke proteins (RSPs) RSP4 and RSP6 are required for the assembly of the radial spoke head (Wei et al., 2010), therefore it is predicted that deletion of RSP4/6 in *L. mexicana* will result in the loss of this structure.

In addition, to exploring how disrupted beating influences IFT train speed, it is important to investigate the effect of increased beating frequency on IFT train speed. To do this, we analysed IFT train speed in a cell line with deletion of the distal axoneme ODA-associated LC4-like.

The possibility of proximal-distal changes in IFT train speed in beating flagella has not been investigated. This may occur for several reasons. For example, in *C. elegans* there is a handover from slower kinesin-II to faster processive OSM-3 at the transition zone, if this is the case in *L. mexicana*, who possess orthologues of the Kin2a and Kin2b proteins found in *T. brucei*, we may expect to proximal IFT train speeds being slower than distal ones. Additionally, many proteins have an asymmetric distribution in the axoneme, including those which have direct interactions with the microtubule doublets along which the IFT trains travel. In *L. mexicana*, one example is the docking complexes – with the pDCs and dDCs occupying the proximal 20% and the distal 80% of the flagellum respectively (Edwards et al., 2018). The deletion of dDC1 or dDC2, mainly affects dynein function and flagella structure in the distal flagellum. If structural changes or localised energetic changes contribute to IFT train speed changes, then the changes in IFT train speed in the Δ dDC2 mutant would be expected to be asymmetric along the flagellum. Comparing proximal-distal changes in IFT train speeds in 3×mNG::IFT172 and 3×mNG::IFT172 Δ dDC2 may give further insights.

The following chapter explores in detail the axonemal structural changes, swimming behaviour, flagellar beating patterns and anterograde and retrograde IFT train speeds in the above-described deletion mutants. As well as the existence of proximal-distal asymmetry in IFT train speeds.

4.2 Results

4.2.1 3xmNG::IFT172 deletion mutants display differing structural changes to their axonemes

For the 3xmNG::IFT172 deletion mutant cell lines for which structural changes were previously uncharacterised (RSP4/6, OAD β , LC1), we confirmed by electron microscopy the expected structural changes in the axoneme which are displayed alongside transmission electron micrographs from Fort et al., 2024 for dDC1 and dDC2 deletions (Figure 4-1). The electron micrographs were taken by Cecile Fort, who also completed the rotational averaging. These show LC1 deletion mutants have no major changes in ODA structure whilst OAD β deletion mutants lost their ODAs, similar to in *Chlamydomonas* (Figure 4-1). As expected, the deletion of RSP4/6 caused the loss of the radial spoke heads (Figure 4-1).

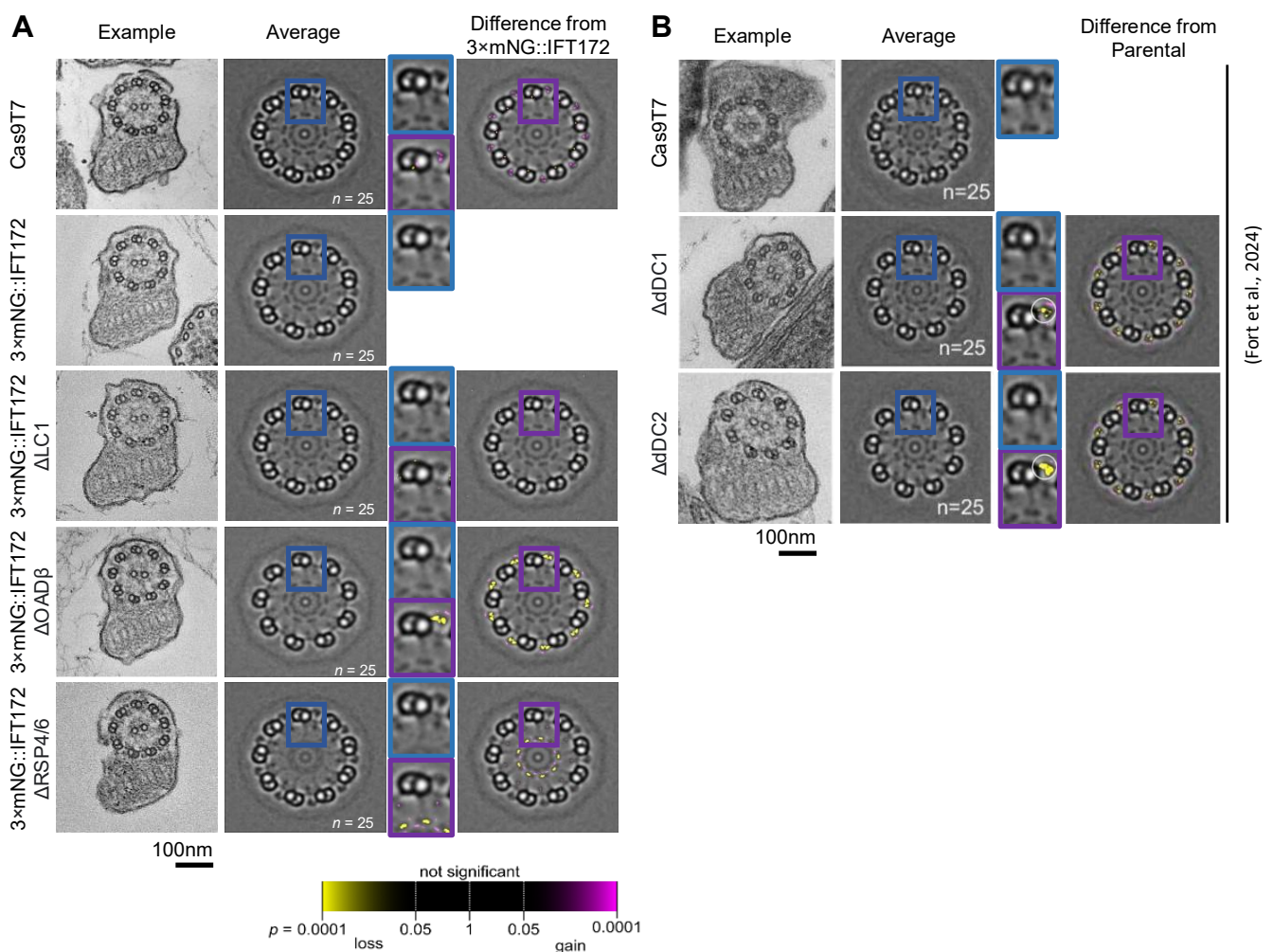


Figure 4-1, Adapted from Gray et al., 2024 and Fort et al., 2024 | Transmission electron micrographs of axonemal cross sections revealing deletion mutants had ultrastructural changes. A. Electron microscopy completed and analysed by Cecile Fort. The images are as described: the first column displays an example transverse section of the flagellum, showing both the axoneme and paraflagellar rod. The second column displays an average of 25 nine-fold rotationally averaged axonemes, with an enlarged view of the area highlighted in blue showing a single microtubule doublet and its associated complexes. The third column compares the electron density to that of 3×mNG::IFT172 using a per-pixel Mann–Whitney U test, with an enlarged view of the purple boxed area highlighting a single microtubule doublet. B. Transmission electron micrographs of dDC1 and dDC2 deletions from Fort et al., 2024.

4.2.2 Several 3×mNG::IFT172 deletion mutants have reduced flagellum length

To determine if deletion mutants had altered cellular morphology or flagellum length, I visualised live cells at 63× (frames from phase contrast videos available in 4.2.5). The cells of the deletion mutants were largely similar to the parental 3×mNG::IFT172, with no obvious or extreme changes in cell body size or shape. The flagella of visualised cells were manually traced to calculate the flagellum length revealing several of the mutants have significantly reduced flagellum length, the most severe shortening of the flagellum was seen in $\Delta OAD\beta$ (Figure 4-2).

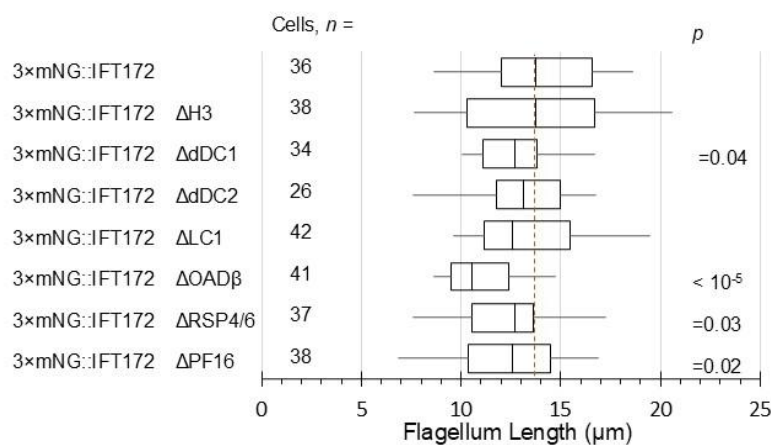


Figure 4-2 | 3×mNG::IFT172 deletion mutants have the same length or shorter flagella. Boxplots displaying the length of the flagellum the parental and slower-swimming mutant cell lines. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. The number of cells (n) is presented. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented.

4.2.3 3×mNG::IFT172 deletion mutants have distinctive swimming patterns

To characterise swimming patterns of the deletion mutant cohort, low magnification dark-field videos were captured and cell path maps generated (Figure 4-3). In the cell path maps for the 3×mNG::IFT172

and 3×mNG::IFT172 Δ H3, the majority of cells have loose helical paths traces depicting forward swimming cells. In 3×mNG::IFT172 Δ dDC1, 3×mNG::IFT172 Δ dDC2 and 3×mNG::IFT172 Δ LC1 there is an increased proportion of fairly static cells, and cell paths have less directionality. In the 3×mNG::IFT172 Δ OAD β , 3×mNG::IFT172 Δ RSP4/6 and 3×mNG::IFT172 Δ PF16 cell lines, there are no or very few cells travelling significant distances or taking loose helical paths associated with forward swimming, instead they remain largely static or move in a zig-zag pattern.

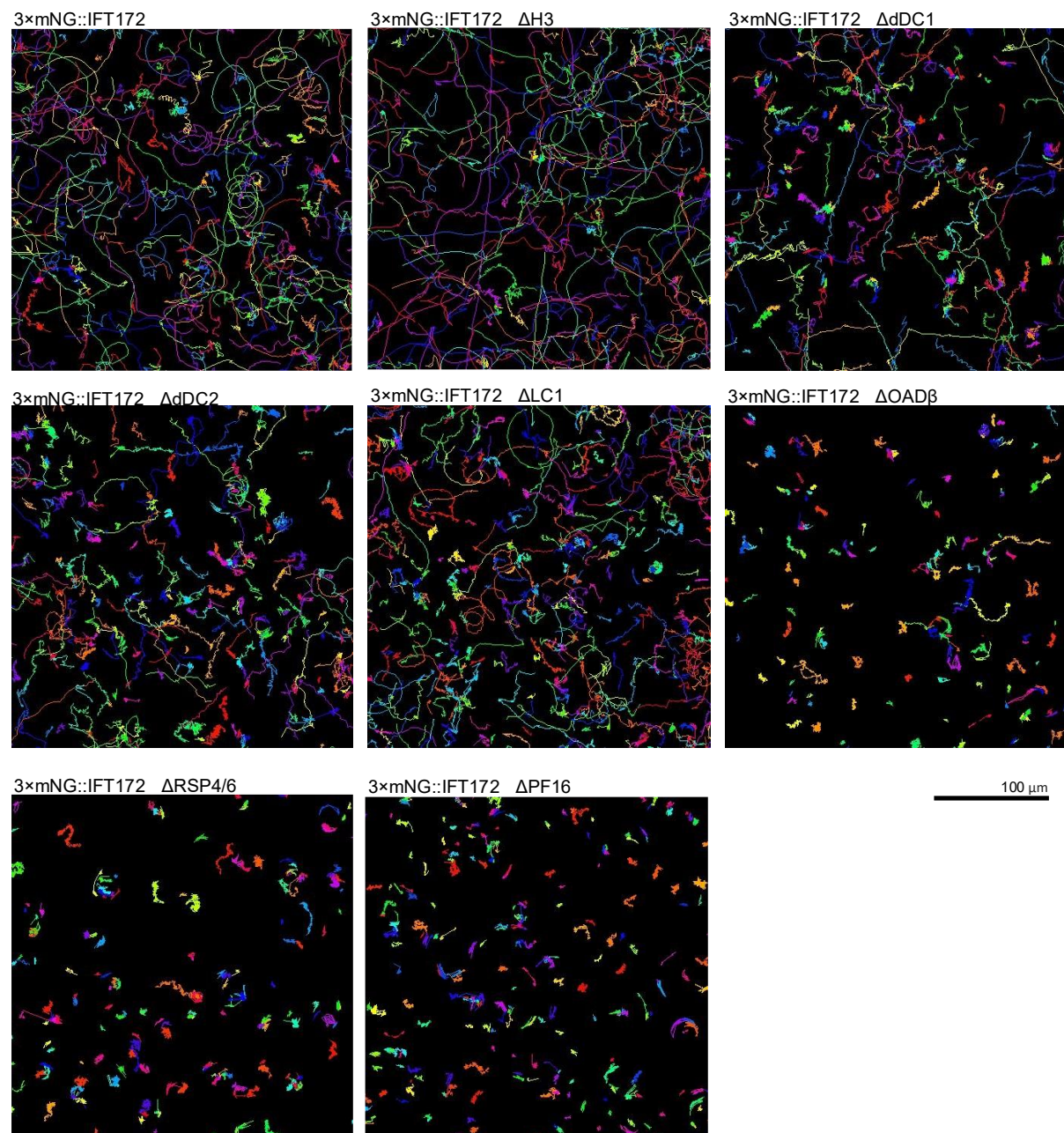


Figure 4-3 | The cell path maps of 3×mNG::IFT172 deletion mutants show different patterns of swimming. Cell path maps showing traced cells for a portion of the FOV for the full duration of the 10× dark-field 5 Hz image sequences. Each trace, presented in different colours, represents a single cell. The scale is indicated by a scale bar.

4.2.4 3×mNG::IFT172 deletion mutants have reduced swimming speed

Cell traces were used to calculate the swimming speed for each mutant and the parental cell line (Figure 4-4). There was no significant difference between the swimming speeds of 3×mNG::IFT172 and 3×mNG::IFT172 ΔH3. However, every other deletion mutant had a significantly reduced swimming speed compared to the 3×mNG::IFT172 parental cell line, with the reduction in swimming speeds of 3×mNG::IFT172 ΔOADβ and 3×mNG::IFT172 ΔPF16 the most extreme.

The swimming speed for 3×mNG::IFT172 ΔRSP4/6 is higher than may be expected given the traces on its cell path map, potentially due to the tight zig-zag pattern of the traces with its non-directional movement being less efficiently drift corrected.

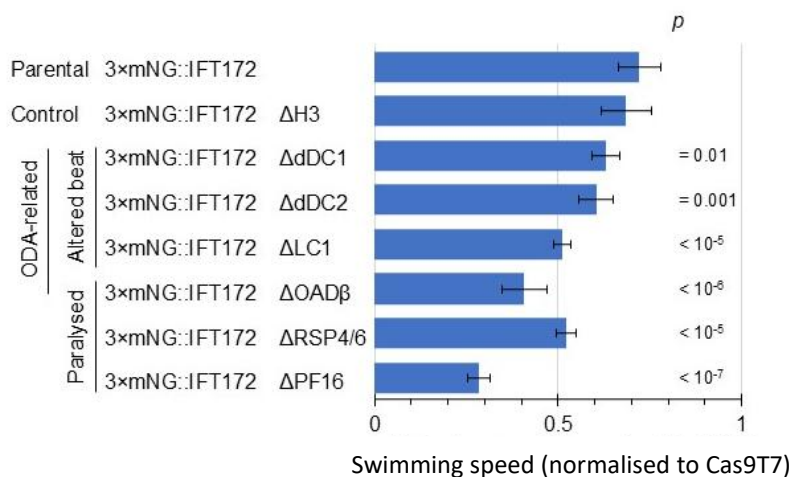


Figure 4-4, Adapted from Gray et al., 2024 | 3×mNG::IFT172 deletion mutants have slower swimming speeds. Bar chart depicting the average swimming speed of slower-swimming deletion mutants, derived from 3×mNG::IFT172. The error bars show the standard deviation of six replicates. Each replicate comprises the average swimming speed of over 1000 cells analysed, normalized against a sample from the Cas9T7 cell line (the (grand)parental line) taken on the same day. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented.

4.2.5 Not all 3xmNG::IFT172 deletion mutants are able to generate wild-type beat types

In order to investigate beating behaviour and IFT train speed (latter discussed in 4.2.7), I visualised freely swimming cells from the deletion mutant cohort at 63× in phase contrast and green fluorescence in a high-frame rate videos. For each cell line, phase contrast stills of a cell undergoing one beat cycle of a symmetric tip-to-base beat if able to do so is presented (Figure 4-5). These stills were taken at frames 1, 4 and 8 or 1, 8 and 16, depending on beat frequency undertaken by the cell's flagellum.

3xmNG::IFT172, 3xmNG::IFT172 Δ H3, 3xmNG::IFT172 Δ dDC1, 3xmNG::IFT172 Δ dDC2 and 3xmNG::IFT172 Δ LC1 were all able to generate the sinusoidal symmetric tip-to-base beat, although the latter three tended to have lower beat frequencies. Whereas 3xmNG::IFT172 Δ OAD β , 3xmNG::IFT172 Δ RSP4/6 and 3xmNG::IFT172 Δ PF16, were largely unable to do so.

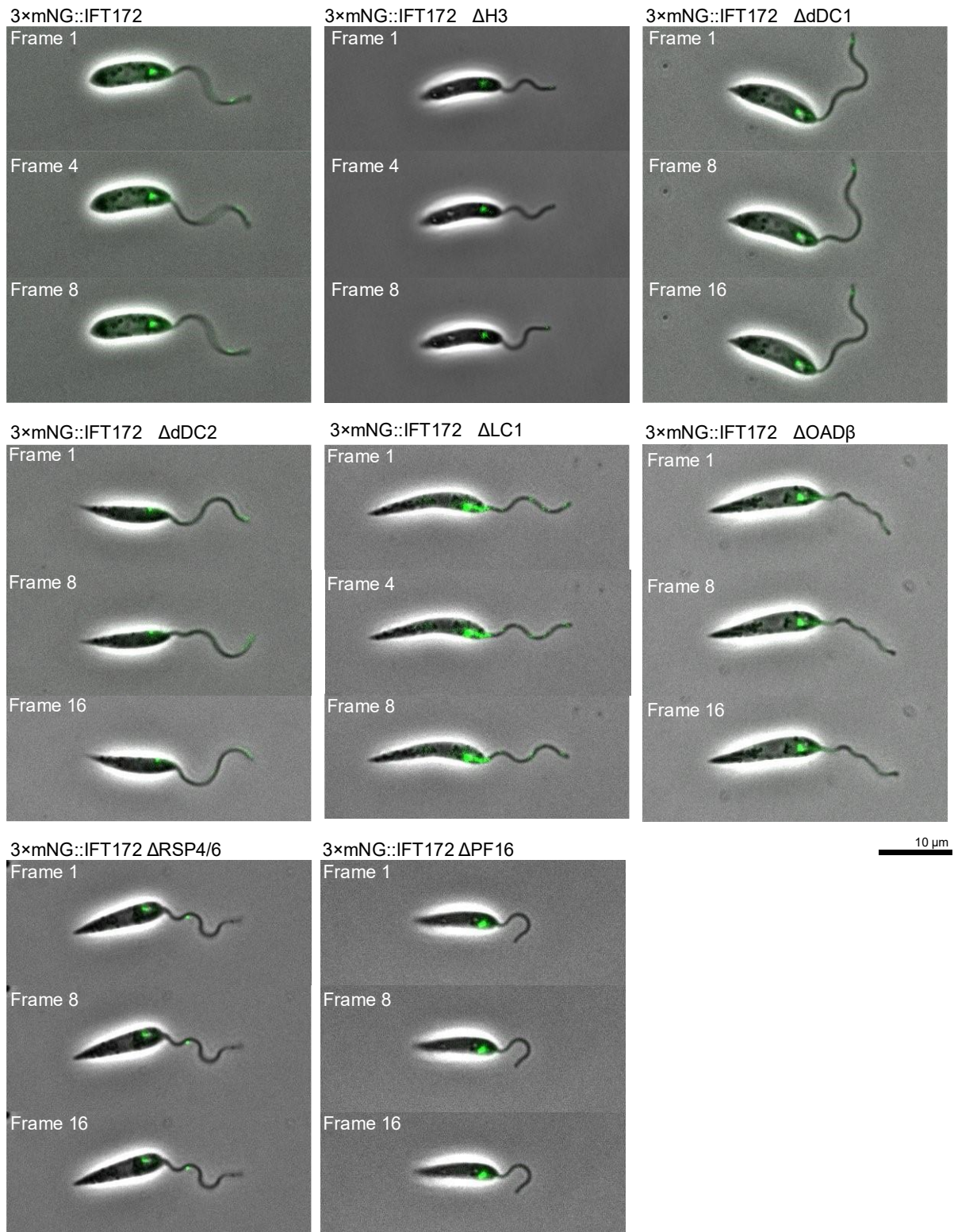


Figure 4-5 | Not all deletion mutants can generate wild-type symmetric tip-to-base beat. Three frames taken from high-frame rate (200 Hz) 63× phase contrast videos of an example cell of each of the cell lines to show one beat cycle of a symmetric tip-to-base beat if cell line is able to do so. The number of the frame is displayed on each still. The scale is indicated by a scale bar.

4.2.6 3×mNG::IFT172 deletion mutants have distinct patterns of flagellar beating

To investigate the type of beating undertaken by these slower swimming mutants, tangent angle kymographs were generated from the 63× videos. An example representing typical beating behaviour for each cell line is presented in Figure 4-6. Our strict requirements that the cell stays in focus, within the field of view and that the flagellum can be traced in an automated way, introduced some biases. For example, in cells undergoing exaggerated ciliary beat type, the flagella cannot be traced for frames where they flagella fold back to the point of crossing over itself. An instance of this is visible in the example tangent angle kymograph for 3×mNG::IFT172 Δ dDC1, in which some black areas representing no tracing are surrounded by a dotted white line. Whilst an example of a cell swimming out of the field of view at the end of the video can be seen in 3×mNG::IFT172 Δ H3 encircled in a terracotta dotted line.

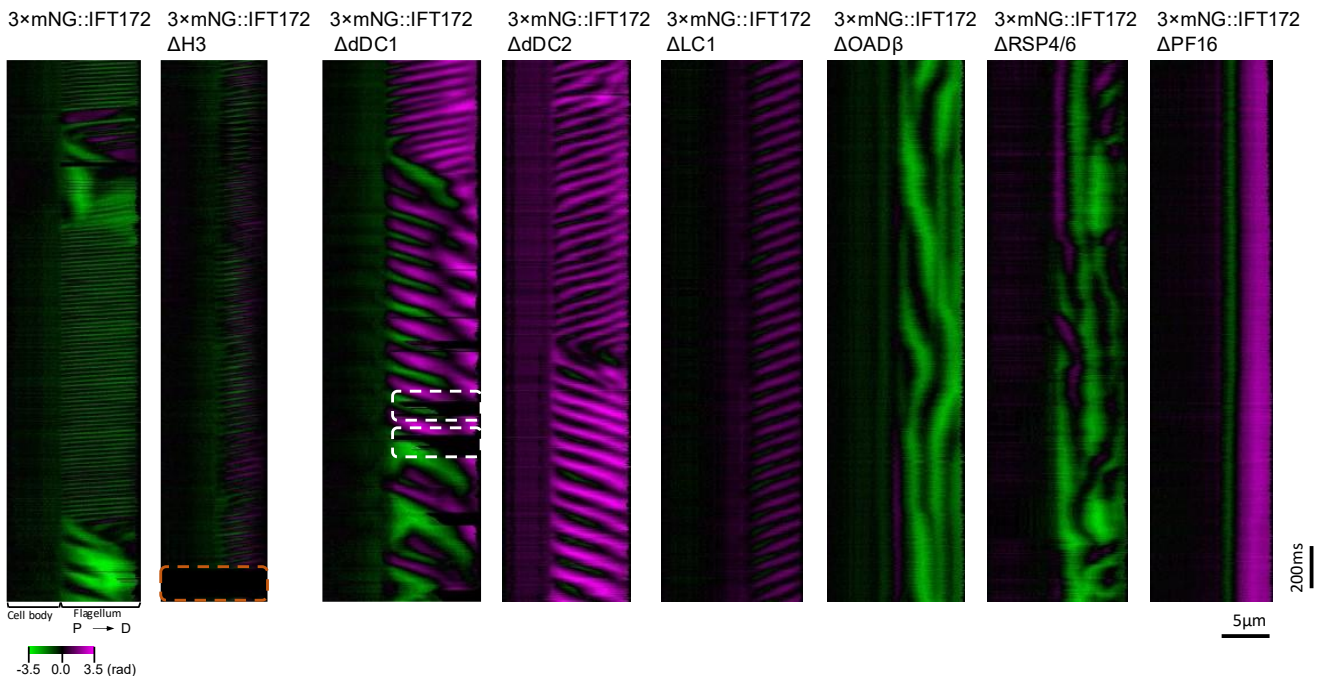


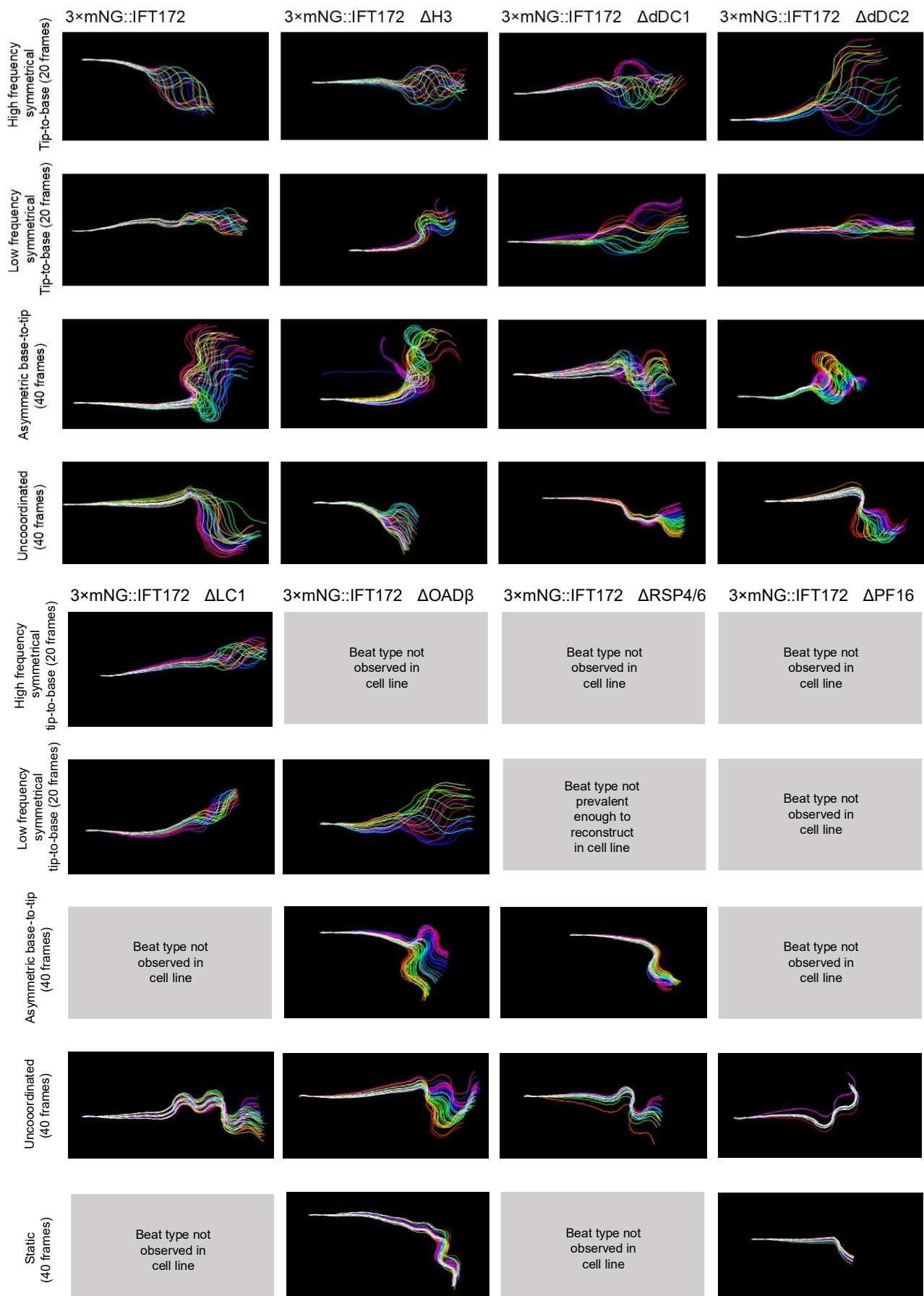
Figure 4-6 | 3×mNG::IFT172 deletion mutants have distinct flagellar beating patterns. Cell lines are labelled above their tangent angle kymographs. A tangent angle in radians of the entire length of the cell relative to the flagella base, over the length of the video is represented by its colour. For the first kymograph, the cell body and flagellum are labelled. The cell body does not bend a lot, whereas the flagellum has high curvature. The proximal (P) and distal (D) ends of the flagellum are labelled. The dotted terracotta line encircles an area where the cell

was not traced as it left the FOV. The dotted white line encircles an area where the flagellum was not traceable due to it circling back to touch the cell. The scale of time and distance is indicated by scale bars.

The tangent angle kymographs show patterns of flagellar beating for each cell line. For previously characterised cell lines, the results were largely replicative of previous results (Beneke et al., 2019; Gadelha et al., 2007; Wheeler, 2017). However, the LC1 deletion mutant showed a different swimming phenotype than the TbLC1 RNAi previously described in *T. brucei*, exhibiting a persistently lower frequency tip-to-base beat rather than a reversal of beat direction.

As well as the three normal beating behaviours (high frequency symmetric tip-to-base, asymmetric base-to-tip and uncoordinated) observed in wild type and in our parental tagged cell lines, I noted two additional beat types in mutants: low frequency base-to-tip and static.

For each beat type observed in each cell line, beat reconstructions were made using a lab-designed ImageJ macro (Figure 4-7). This is a representation of the flagellum position during twenty frames (for high and low frequency symmetric tip-to-base) or forty frames (for uncoordinated, asymmetric base-to-tip and static) of the 200 Hz video, with time represented by the distance of the colour of the flagellum trace along the red-green-blue colour spectrum. Twenty and forty frames were selected as this provides a complete beat cycle for the majority of the wild-type beats for symmetric tip-to-base and asymmetric base-to-tip respectively. However, it should be noted that for deletion mutants - the beats they generate can be irregular, and often slower than their parental cell lines. There is variation in the frequency and amplitude of beating within each beat type category. In particular, for the rare occurrence of categorisable beating behaviour in the RSP4/6 deletion cell line, the beats were much slower and more irregular than in their parental line.



10 μm

Figure 4-7 | 3×mNG::IFT172 deletion mutants beat cycle reconstructions display diverse beating behaviour. Cell line is labelled above beat cycle reconstructions and arranged in columns. The rows are labelled with the beat types and number of frames used for the reconstruction. A beat type reconstruction is generated for each beat type observed in each of the parental and deletion cell lines. It is noted where the beat type was not observed or reconstruction was not possible. The scale is indicated by a scale bar.

To enable comparison of beating behaviour between the slower swimming mutants, the time spent in each of the five beating behaviours for each of the cell lines was quantified (Figure 4-8). Δ dDC1 and Δ dDC2 had a decreased propensity for symmetric tip-to-base flagellar beats, consistent with previous results (Edwards et al., 2018). The flagella of Δ LC1 cells were largely unable to generate an asymmetric base-to-tip beat and spent the most time in low frequency tip-to-base beating. Both Δ OAD β and Δ RSP4/6 had an increased proportion of uncoordinated flagellar movement compared to parental control, making this their predominant form of flagellar movement. Δ PF16 was largely static – with some uncoordinated movement, though this could be a consequence of the movement of the media they were in.

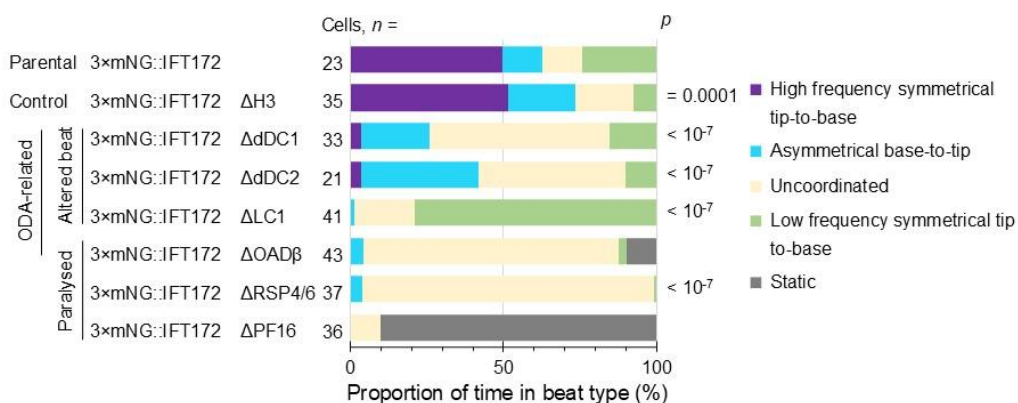


Figure 4-8 Adapted from Gray et al., 2024 | Deletion mutants spend a different proportion of time in each beat type to parental cells. A stacked bar graph representing the proportion of time spent in the different beat types for each deletion mutant cell line with reduced flagellar movement and parental 3×mNG::IFT172. The number of cells (n) is presented. For significant differences (chi-squared test, $P < 0.05$) P-values are provided. For Δ PF16 and Δ OAD β chi-squared test is invalid due to the novel 'static' category.

4.2.7 Paralysed or reduced frequency beating flagella have faster anterograde IFT trains

From the green channel of the 63× videos, anterograde and retrograde IFT train speeds were quantified in the free flagella of slow-swimming mutants (Figure 4-9). For Δ H3 the IFT train speeds were unchanged (Figure 4-9). Whilst for all slow swimming mutants, there was a significant increase

in the speed of anterograde trains. Many also had significantly increased retrograde IFT train speed. PF16 deletion caused the most significant and striking increase in IFT train speeds in both directions.

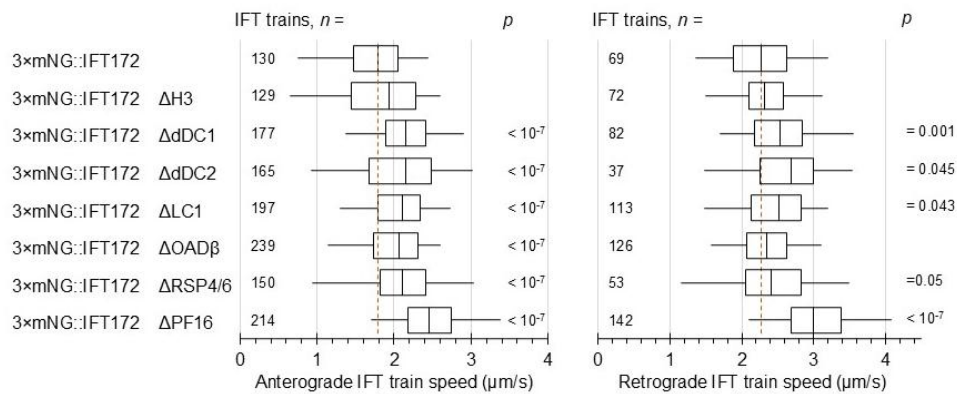


Figure 4-9, Adapted from Gray et al., 2024 | In deletion mutants with reduced flagellar movement, the speed of anterograde IFT trains is higher. Boxplots displaying the speeds of anterograde and retrograde IFT trains in the parental and slower-swimming mutant cell lines. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. The number of IFT trains (n) is presented. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented.

4.2.8 Increased frequency beating flagella have slower retrograde IFT trains

So far, we have seen that IFT train speed had a broadly inverse correlation with reduced cell swimming speed and propensity of flagella to beat. To test if this phenomenon was bidirectional, I investigated the speed of IFT trains in an LC4-like deletion mutant which has previously been shown to have higher frequency beating and faster swimming (Edwards et al., 2018). After being unable to generate this deletion in a 3xmNG::IFT172 cell line using puro and neo drug selection markers, possibly because LC4-like is highly GC-rich; an existing LC4-like deletion mutant cell line (generated using blast) was defrosted from liquid nitrogen (Edwards et al., 2018), and IFT172 tagged with mNG using drug selectable markers (puro and neo) to generate two cell lines. It was not possible to tag this defrosted cell line with 3xmNG, as the plasmid encoding 3xmNG uses the blast drug selectable marker for integration. In 5.2.6, we will witness the challenges of tagging with blast on a deletion mutant already possessing an integrated blasticidin drug selectable marker.

The cell path maps (Figure 4-10A) and swimming speed (Figure 4-10B) of mNG::IFT172, mNG::IFT172 (puro) Δ LC4 and mNG::IFT172 (neo) Δ LC4 were largely similar, though mNG::IFT172 (neo) Δ LC4-like had a significantly increased mean swimming speed. Both Δ LC4-like mutants had longer median flagella, though this was only significant in mNG::IFT172 (puro) Δ LC4-like (Figure 4-10C).

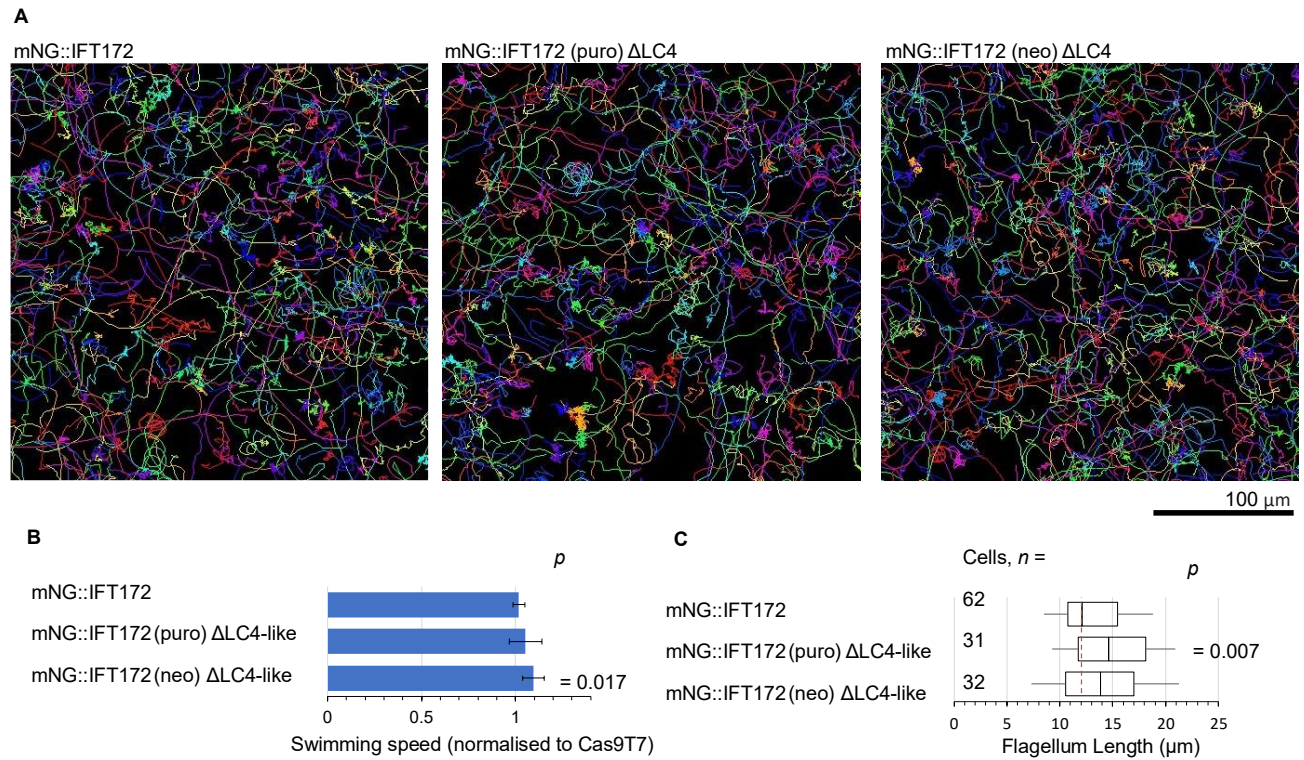


Figure 4-10 | mNG::IFT172, mNG::IFT172 (puro) Δ LC4 and mNG::IFT172 (neo) Δ LC4 have similar patterns of swimming. A. Cell path maps of mNG::IFT172 and deletion mutants showing traced cells for a portion of the full FOV for the full duration of the 10 $\times$ dark-field 5 Hz image sequences. Each trace, presented in different colours, represents a single cell. The scale is indicated by a scale bar. B. Adapted from Gray et al., 2024. Bar chart depicting the average swimming speed of mNG::IFT172 and the two LC4-like deletion mutants. The error bars show the standard deviation of the six replicates. Each replicate comprises the average swimming speed of over 1000 cells analysed, normalized against a sample from the Cas9T7 cell line (the (grand)parental line) taken on the same day. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented. C. Boxplots displaying the length of the flagellum the parental and mutant cell lines. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. The number of cells (n) is presented. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented.

Phase contrast stills (Figure 4-11A) and tangent angle kymographs (Figure 4-11B) showed that the flagella of mNG::IFT172 (puro) Δ LC4-like and mNG::IFT172 (neo) Δ LC4-like retained largely normal flagella waveforms, comparative to mNG::IFT172. Both Δ LC4-like cell lines had a significant difference in proportion of time spent in different wavelengths (Figure 4-11C), however this may be due to the

sample size, variability and high sensitivity of the chi-squared test rather than a biological difference.

As expected, in both Δ LC4-like cell lines, the frequency of symmetric tip-to-base beat was increased, at 33.3 ± 7.3 Hz ($n=23$, mean $\pm$ standard deviation, $p = 0.02$, two-tailed t-test) and 31.6 ± 7.7 Hz ($n=27$, $p = 0.02$, two-tailed t-test), compared to 27.0 ± 8.4 Hz for mNG::IFT172 ($n=39$).

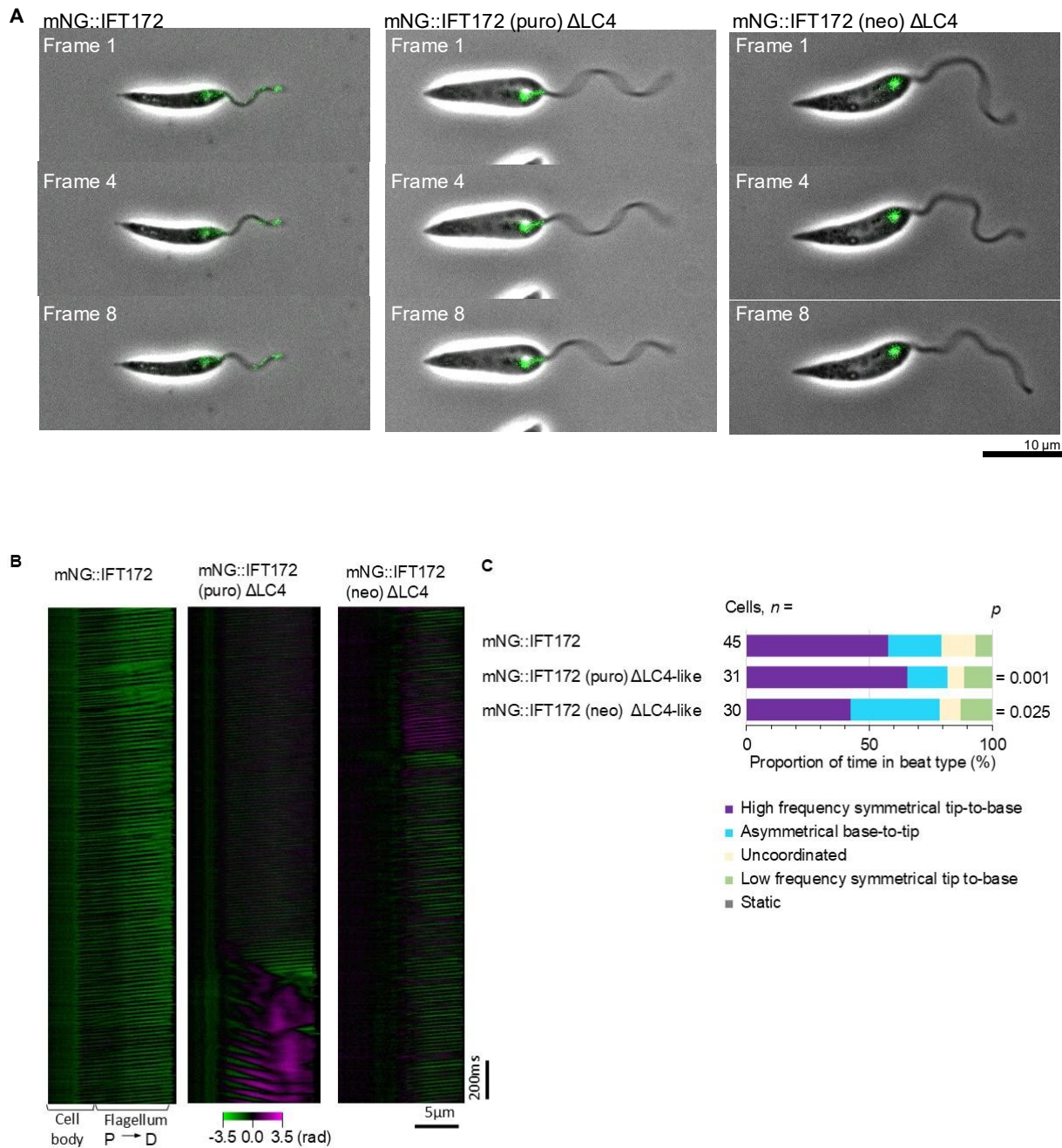


Figure 4-11 | Δ LC4-like have higher frequency flagellar beating. A. Three frames taken from high-frame rate 63 $\times$ phase contrast videos of example cells of each of the cell lines to show one beat cycle of a symmetric tip-to-base beat. The number of the frame is displayed on each still. The scale is indicated by a scale bar. B. Cell lines are labelled above their tangent angle kymographs. A tangent angle in radian of each pixel along the entire length of the cell relative to the flagella base, over the length of the video is represented by its colour. For the first kymograph, the cell body and flagellum are labelled. The cell body does not bend a lot, whereas the flagellum has high curvature. The proximal (P) and distal (D) ends of the flagellum are labelled. Time and distance scale bars are available. C. Adapted from Gray et al., 2024. A stacked bar graph representing the proportion of time spent in the different beat types for cell line. The number of cells (*n*) is presented. For significant differences (chi-squared test, *P* < 0.05) *P*-values are provided.

As in immobilised flagella, in free flagella mNG::IFT172 had higher speed of IFT trains compared to 3×mNG::IFT172 (Figure 4-12 vs Figure 3-9). In both cell lines, LC4-like deletion mutants had a statistically significant decrease in anterograde and retrograde IFT train speed compared to mNG::IFT172 control, with retrograde being most markedly reduced (Figure 4-12).

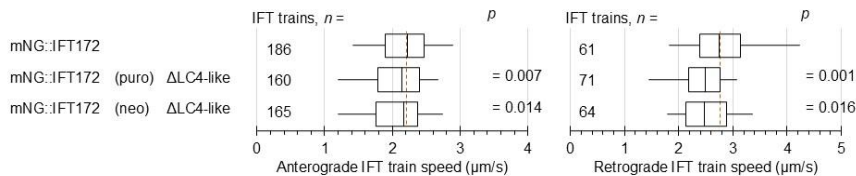


Figure 4-12 Adapted from Gray et al., 2024 | In deletion mutants with increased flagellar movement, the speed of anterograde and retrograde IFT trains is slower. Boxplots displaying the speeds of anterograde and retrograde IFT trains in the parental and faster-swimming mutant cell lines. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. The number of IFT trains (n) is presented. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented.

4.2.9 Investigating if proximal distal axonemal asymmetry in the flagellum alters IFT train speed

To investigate the existence of proximal-distal differences in IFT train speed and if proximal-distal structural asymmetry impacts this, we set out to quantify IFT train speeds in 3×mNG::IFT172 and 3×mNG::IFT172 ΔdDC2. In 3×mNG::IFT172, the distribution of pDCs and dDCs is characterised as being 20% and 80% of the axoneme respectively, so these percentages were used to designate the proximal and distal sections of the axoneme respectively.

With dDC2 deletion, it has been shown that pDCs extend further along the axoneme towards the distal end, putatively due to the loss of the distal ODAs causing a shift in the proximal-distal boundary which is normally maintained by a concentration gradient of the pDCs generated through IFT (Edwards et al., 2018). To quantify this pDC1 was tagged with fluorescent marker mCh in the 3×mNG::IFT172 ΔdDC2 cell line. The distribution of red fluorescence showed that in 3×mNG::IFT172 ΔdDC2 pDC1::mCh, the pDCs covered the proximal $36 \pm 10.6\%$ (n=13) of the flagellum. As there was significant variation, we

used the presence or absence of red fluorescence signal to define the proximal and distal regions of the flagellum.

Live cells were adhered to glass and visualised at 63× in phase contrast and green fluorescence channels, and red fluorescence (for 3×mNG::IFT172 ΔdDC2 pDC1::mCh) (Figure 4-13A). In adhered cells, a large proportion of the proximal axoneme resides inside the cell body. The section of the axoneme inside the cell body is not itself adhered to the glass slip, and therefore is able to move in a way that the adhered flagellum is unable to (Figure 4-13B). An additional challenge we found with visualising differences in IFT asymmetries is that the positioning of the pDCs overlaps with the transition zone at the proximal end of the flagellum where IFT trains are loaded onto the microtubule doublets. In the IFT kymograph this is represented as an area of constantly high fluorescence and is partially within the flagellar pocket, thus suffers from cytoplasmic background signal too. Any IFT train movement in this part of the proximal flagellum may be more difficult to observe.

In both 3×mNG::IFT172 and 3×mNG::IFT172 ΔdDC2 pDC1::mCh, anterograde trains were significantly slower in the proximal flagellum than in the distal flagella (Figure 4-13C). This could be due to there being less space in the proximal region where the dynein arms are present. Alternatively, trains may not have reached peak speed after loading or it could support the hand over from slower to a faster and more processive kinesin as occurs in *C. elegans*. In 3×mNG::IFT172 retrograde trains were significantly faster in the distal flagellum than the proximal flagellum, but there was no significant difference in 3×mNG::IFT172 ΔdDC2 pDC1::mCh. If the proximal-distal asymmetry in retrograde transport is related not to the presence or absence of ODAs but to reduced speed in the transition zone, then proximal-distal asymmetry may be hidden in 3×mNG::IFT172 ΔdDC2 pDC1::mCh.

For free swimming cells, in order to visualise both the proximal/distal docking complex boundary and the IFT trains, cells were visualised using green and red fluorescence. This meant that there was no phase contrast image sequence to be used for tracing. Attempts to develop a novel method of tracing using the combined fluorescence of the two channels and developing a string and dot mechanism

yielded a success rate of tracing that was too low and too biased i.e. in a cell undergoing asymmetric base-to-tip beating where the flagella circles round the identification of the closest 'dot' of fluorescence could be signal from an IFT particle on the other end of the flagellum as seen in Figure 4-13D. Each image sequence is 950 frames long, therefore manually tracing the flagellum in each frame in the number of videos required to have a large enough sample size would not be feasible.

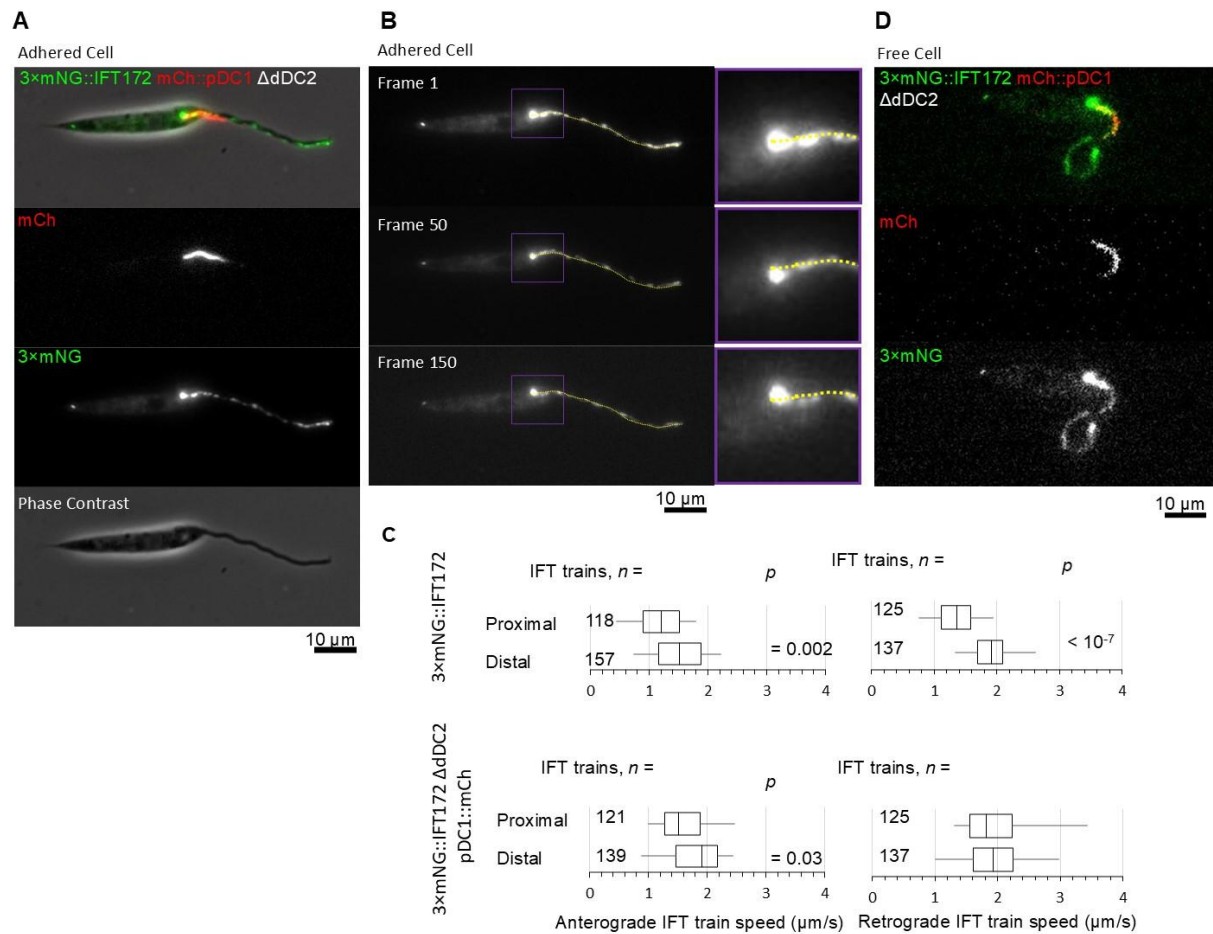


Figure 4-13 | Proximal-distal asymmetry in IFT train speed. A. Phase contrast, red fluorescence and green fluorescence stills taken of adhered 3xmNG::IFT172 ΔdDC2 pDC1::mCh. B. Green fluorescence stills taken of adhered 3xmNG::IFT172 ΔdDC2 pDC1::mCh at frames 1, 50 and 150, with an enlarged view of the purple boxed area highlighting movement of axis of green fluorescence within the cell body. C. Boxplots displaying the speeds of anterograde and retrograde IFT trains in the proximal and distal flagella of adhered cells. The boxes demarcate the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. The number of IFT trains (n) is presented. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented. D. A composite red and green fluorescence, red fluorescence and green fluorescence still from a high-frame rate dual-colour video of free swimming 3xmNG::IFT172 ΔdDC2 pDC1::mCh cell.

4.3 Discussion

This chapter explored the impact of permanent genetically-induced changes to flagellar beating on IFT train speed. We found that slow-swimming mutants with disrupted flagellar beating had increased anterograde IFT train speed, whereas faster-swimming mutants with increased beat frequency had reduced anterograde and retrograde train speed. Notably, the most severe motility defect, paralysis through PF16 deletion, caused the largest increase in IFT train speed. As IFT train speed was shown to not be strongly affected by beat type with naturally occurring switches from symmetric to asymmetric beat potentially resulting in a small decrease (Figure 3-8); the increase in IFT train speed in Δ dDC1 and 2 is unlikely due to alterations in spatial geometry surrounding IFT trains during the characteristic tight radius bends for asymmetric base-to-tip waveforms.

We showed that in *L. mexicana*, OAD β deletion mutants had structural and functional deficits, namely being unable to correctly assemble ODAs or undergo normal beating, whilst LC1 deletion did not result in significant structural changes in the axoneme. Previous work had shown that the deletion of dDC1 or dDC2 cause the loss of ODAs in the distal axoneme (Edwards et al., 2018). However, all four of these deletion cell lines with significantly reduced swimming speed and altered flagellar beating had increased anterograde IFT train speeds. This suggests that IFT train speed increase is an effect of altered flagellar motility, rather than ODA loss.

Moreover, if the increase in IFT train speed was predominantly driven by spatial phenomena, deletion of LC4-like or LC1 which are both small ODA-associated proteins not necessary for ODA assembly, would be expected to have a similar IFT train speed phenotype. Instead, they gave inverse phenotypes, consistent with increased axonemal ATP consumption for the higher LC4-like deletion mutant beat frequency.

This is supported by results from the Δ RSP4/6 and Δ PF16 cell lines. As expected from RSP4 and RSP6 structural contributions in other species (Wei et al., 2010), RSP4/6 deletion led to loss of radial spoke heads (Figure 4-1). PF16 has previously been shown to cause loss of either one or both of the central

pair singlets or the central pair orientation. The mechanism by which these changes disrupt beating is not fully understood, but likely involves disruption of a charge-based interaction between the radial spoke head and central pair complex which detects axonemal mechanical strain as well as the loss of structural integrity in the axoneme (Grossman-Haham et al., 2021). Both RSP4/6 and PF16 deletion had a significant and severe effect on swimming speed and flagellar movement (Figure 4-3 - Figure 4-8), and caused an increased anterograde IFT train speed (Figure 4-9) without altering in any way the structure of the ODAs or outer microtubule doublet decoration (Figure 4-1) in areas near to where the IFT trains move.

Collectively, this indicates an indirect mechanism of beating/IFT train speed interaction, like competition for ATP. Previous research has shown that damaged or otherwise movement-inhibited flagella have a movement-dependent reduction in ATP consumption, reducing to almost zero when otherwise functional flagella are immobilised by high viscosity (Brokaw and Benedict, 1968; Chen et al., 2015; Gibbons and Gibbons, 1972). This shows that even if ODAs are present and functional in a flagellum, they use very little ATP when not driving flagellar beating, thus meaning an increased availability of ATP for IFT motor proteins. Therefore, it is to be expected that the deletion of proteins resulting in flagella that are unable to beat (Δ PF16, Δ RSP4/6), have reduced activity (Δ LC1) or are missing all (Δ OAD β) or a significant proportion (Δ dDC1, Δ dDC2) of their ODAs will use dramatically less ATP for flagellar motility. Whilst flagella with increased beating frequency (Δ LC4-like) would use more ATP for driving flagellar movement. Anterograde and retrograde IFT were differentially affected by changes in flagellar beating, and predicted changes in ATP availability suggesting the effect may be nuanced for kinesin-2 and dynein. Kinesin is highly processive and can move several microns without dissociating, carrying up to several piconewtons (Howard et al., 1989; Block et al., 1990; Meyhöfer and Howard, 1995). Though single dyneins have stochastic stepping and enter unproductive states, they are able to achieve higher processivity and long-range transport through cooperative action (Mallik et al., 2004; Reck-Peterson et al., 2006). Given the different copy number per train and

mechanical properties of their respective motor proteins, it is unsurprising that changes in ATP availability would alter anterograde and retrograde train speed differently.

In *Chlamydomonas*, some glycolytic enzymes localise to the flagellum and may represent a further ATP source within the flagellum (Mitchell et al., 2005). Trypanosomatid flagella similarly contain orthologs of glycolytic enzymes, however they appear to be catalytically inactive (Brown et al., 2014). Glycolysis and oxidative phosphorylation occur in the glycosomes and mitochondrion respectively thus diffusion from the flagellar base is the only plausible ATP source. However, a similar interplay of beating and IFT velocity is implied in *Chlamydomonas* from mutants with temperature sensitive flagella paralysis (Iomini et al., 2001). Therefore, while this phenomenon might not be universal, the interaction of genetically-induced changes to flagellar motility and IFT train speed changes may be conserved in other flagellated species.

Interestingly, many of the slower swimming mutants had reduced flagellum length, namely Δ dDC1, Δ PF16, Δ RSP4/6 and most severely Δ OAD β (Figure 4-2). This could be due to the effect of the loss of the protein on subunit trafficking to the flagellar tip. For example in *T. brucei*, deletion of LRRC56, a protein required for trafficking of dDCs led to shorter flagella (Bonnefoy et al., 2024).

Alternatively, flagellum shortening could also be due to changes in axonemal stability. In ciliated epithelial cells, exposure to shear stress caused PKC mediated MPK phosphorylation, independent of potassium or potassium ion signalling, with PKC or MPK inhibition decreasing cilia length, suggesting shear stress may be important for PKC-MPK driven flagellum length maintenance (Davies, 1995; Abdul-Majeed et al., 2011). In neurons, PKC activation increased tubulin deacetylation which altered microtubule stability (Cho and Cavalli, 2012). Given that most severe shortening is seen in the least motile cell lines (Δ PF16, Δ RSP4/6, Δ OAD β), reduced exposure to shear stress i.e. the flagellum not beating or moving through media, may lead to reduced microtubule stability and shorter flagella. Whereas, for the higher frequency beating LC4-like deletion mutants - the median flagellum length was increased for both, though this was only a statistically significant increase in one of the cell lines.

In *Chlamydomonas*, DC2 deletion caused loss of the ODAs but did not reduce flagella length, however the deletion of IDA components *ida4*, *ida6* caused slight reduction and the deletion of *pf22* (and resultant loss of IDAs and ODAs) caused dramatic flagella shortening (Yanagisawa and Kamiya, 2004). Whether it is the loss of the dynein arm structures themselves or the resultant alteration to flagella beating that causes flagella shortening it is unknown. However it does collectively suggest there is a complex relationship between dynein arm presence, flagellar beating and flagellum length.

Proximal anterograde IFT train speed was slower than distal anterograde IFT train speed for both 3×mNG::IFT172 and 3×mNG::IFT172 ΔdDC2 pDC1::mCh. Given that this pattern was consistent in both cell lines, it is likely not an effect of increased space due to missing ODAs. However, it could point to an alteration in local energetic environment created by proximal and distal ODAs, or a lack of ODAs. Alternatively it could point to a hand over between kinesins with different processivity or recently loaded trains not having reached their typical speed. In *C. elegans* it has been shown that kinesin-II pauses close to the ciliary base, putatively docking to assembling trains, before becoming mobile and accelerating after passing the transition zone (Mitra et al., 2024). Unexpectedly, 3×mNG::IFT172 had proximal-distal asymmetry in its retrograde IFT train speed whilst 3×mNG::IFT172 ΔdDC2 pDC1::mCh did not. This again highlights that loss of ODAs is not a prominent factor in increased IFT train speed – given that retrograde IFT train speed did not increase distally with ΔdDC2. It also points to a more complex regulatory relationship between the ODAs and the dyneins powering retrograde IFT trains that should be further explored.

5 Novel IFT components, tracks and Regulators

5.1 Introduction

If we think about any train system, there are several elements that come to mind with the potential to affect speed – the train, its tracks and its regulators. We can tell how essential each part is and the role it has by removing it from the system and observing the effect on the ability of the train to function. Does the train run faster or slower, does it run at all? In this chapter I investigate a cohort of putative particle, track and regulator proteins of the IFT system using tagging and/or deletion mutants.

5.1.1 The IFT Particle

Firstly, the train itself and its constituent parts – for example the engine, the wheels, the carriages. In the IFT system this is the IFT particle. Previously, genome-wide subcellular protein maps were made using TrypTag, covering 89% of the *T. brucei* proteome, including the IFT particle (Sunter et al., 2023; Dean et al., 2017). Further work, revealed a previously unidentified, WD40 repeat-containing IFT dynein associated protein in *T. brucei*: [Tb927.10.12590](#) (Figure 5-1). Investigating the localisation of the *L. mexicana* orthologue of this previously unidentified dynein associated protein will further elucidate the composition of the IFT particle.

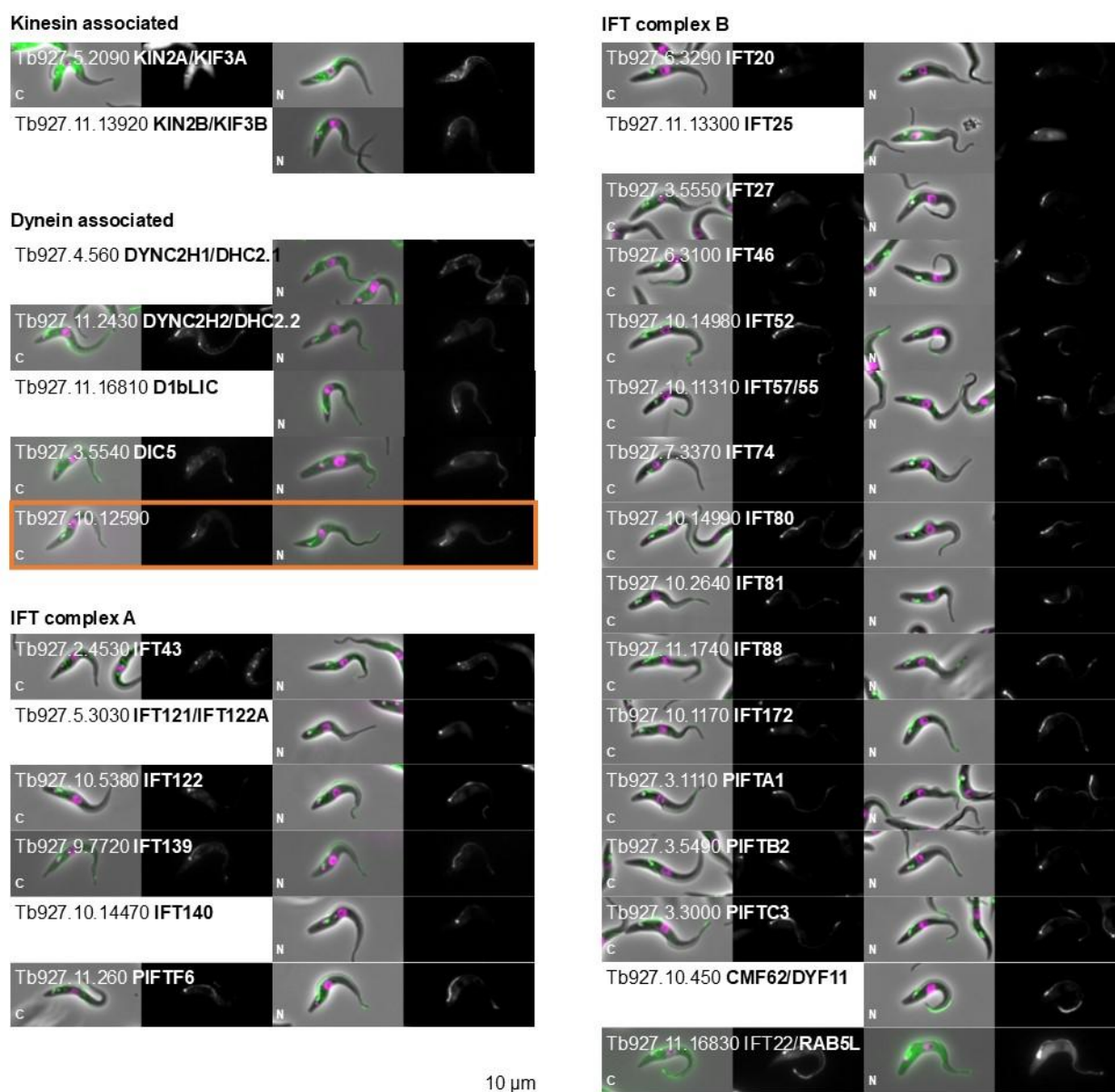


Figure 5-1 | IFT particle proteome has uncharacterised protein. Example stills for C- (C) and N-terminally (N) tagged (if successful) IFT associated proteins from TrypTag. For each gene, ID and name (if existing) is written and an overlay of mNG (green) Hoescht (magenta) and phase contrast and greyscale mNG signal image are presented. Genes are organised by the complex with which they are associated. Orange box surrounds unannotated IFT dynein associated protein.

5.1.2 IFT Tracks

The next essential element is the tracks the train runs on. A train itself is not enough to transport cargo – movement of the train relies on a surface which it interacts with. The foundation of this track in the IFT system is the microtubule doublets, however it is likely other components are important in coordinating the double-track railway (Stepanek and Pigino, 2016). A- and B-microtubules of each

doublet have the same B-lattice tubulin arrangements, could restrictions to IFT localisation be a result of tubulin post-translational modifications? Or is it possible microtubule doublet decorations interact with IFT motor proteins to regulate train routes? Such systems must be necessary for facilitating bi-directional separation and restriction of trains to certain doublets. Long or repeating external MAPs that localise along the length of the flagellum, are expressed during flagellum formation, or are known to be involved in flagellum maintenance, are strong candidate track proteins. A recent paper which used cryo-EM to reconstruct the 48 nm microtubule repeat in *Leishmania tarentolae* revealed that CFAP97, an external MAP, which when tagged with mNG has a signal starting $\sim 0.6 \mu\text{m}$ from the kinetoplast, corresponding to the transitional zone and basal plate and to the ciliary gate that regulates IFT entry (Doran et al., 2025). CFAP97 has a conserved periodicity (24 nm) and localisation at the junction between the A and B microtubules (A10 and B01) on the outer face of the microtubule doublets in mammalian sperm and trypanosomatids (Leung et al., 2023; Doran et al., 2025). The Pro-Gly-Pro-Gly-X-Tyr (PGPGXY) motif (SHIPPO-domain) in ODF3 or ODF3-like proteins— has been shown to mediate binding to the outer wedge of the axonemal doublet microtubules in sea urchin and bovine sperm (Leung et al., 2023). In particular the ciliary MAP1 (CIMAP1) family of proteins has PGP repeat motifs around every 40 residues, giving it an 8 nm repeat, corresponding roughly to kinesin step distance and distance between tubulin binding sites (Coy et al., 1999; Leung et al., 2023; Egydio de Carvalho et al., 2002). In *T. brucei*, SHIPPO-domain containing protein TZIP250 (Tb927.7.7200), an ODF3 orthologue, is located along the length of the transition zone and putatively acts as a “molecular ruler” for the transition zone complex, and deletion leads to severe morphology and cell cycle defects (Dean, 2016). Personal correspondence with Alan Brown indicated that CFAP97 and SHIPPO-domain containing proteins may be important in *Leishmania* flagella construction and maintenance – whether they do this through interaction with IFT was previously unknown.

5.1.3 IFT regulators

The final element of a functional train network is the regulators, without which there is the potential for chaos. Regulators can be on board the train itself, such as a driver, or they can interact with the train transiently – think a speed limit or a red signal. In mammalian cells, kinases have been shown to be involved with various aspects of the IFT system function, from mediating kinesin handover (DYF-5) and train turn around (calcium-dependent protein kinase 1) to associating directly with the IFT particle or motor proteins driving transport (CILK1, MAK and MOK) to influence velocity (Broekhuis et al., 2014).

As previously discussed in the introduction, *L. mexicana* MPKs are known to be crucial regulators of flagellum growth (MPK3) and length maintenance (MPK9). A BLASTP for CILK1, MAK and MOK in *L. mexicana* yielded many significant results, the top three of which were: MPK9 (LmxM.19.0180), MPK14 (LmxM.27.0100) and MPK13 (LmxM.34.5010). A recent systematic analysis of protein kinase functionality in *Leishmania* revealed MPK13 (LmxM.34.5010) and MPK14 (LmxM. 27.0100) are localised to the basal body and flagellum, respectively (Baker et al., 2021). One study in *L. mexicana* found a putative protein target of MPK13 to be an OSM-3 like kinesin (Emmerson, 2014). The Baker et al., 2021 study revealed that deletion of a kinase encoded by LmxM.29.600 caused severe flagellum length shortening. Collectively this suggests that MPKs may play a crucial role in IFT regulation in *L. mexicana* and therefore should be investigated.

In this chapter, I explore the roles of putative constituent, track and regulator proteins in the IFT system. Beginning with characterising a novel IFT dynein intermediate chain, that may be essential for survival. Followed by testing the localisation and interaction of a CFAP7 orthologue and two SHIPPO-domain containing proteins with IFT to begin to unravel the mechanisms directing IFT routes along the microtubule doublets. Finally identifying MPKs which modulate flagellum length via regulating IFT train speed in *L. mexicana* which are orthologous to those in mammalian cells, implying their role is fundamental to flagellar function across diverse eukaryotes.

5.2 Results

5.2.1 A novel kinetoplastid IFT dynein intermediate chain

In *L. mexicana*, the orthologous gene to Tb927.10.12590 is LmxM.18.1460. A BLASTP was completed in *H. sapiens* and *Chlamydomonas*. In *H. sapiens* the reciprocal best hit was cytoplasmic dynein 2 intermediate chain 1 (WDR60). In humans, the cytoplasmic dynein 2 intermediate chain 1 (WDR60) forms a heterodimer with cytoplasmic dynein 2 intermediate chain 2 – also known as WD34 or DIC5 (Asante et al., 2014). The reciprocal best BLASTP for the human Cytoplasmic dynein 2 intermediate chain 2 in *L. mexicana* was the intermediate chain DIC5 (LmxM.08_29.0100).

In *C. reinhardtii*, the best hit for LmxM.18.1460 was CHLRE_02g076100v5 an unannotated protein, however, a literature search yielded that in *Chlamydomonas* a WD60 protein exists which is the orthologue of the human WDR60 protein (Pazour et al., 2005; Patel-King et al., 2013). A BLASTP using the linked sequence for WD60 in the Patel-King et al., 2013 , revealed LmxM.18.1460 was the best hit for *L. mexicana* and Tb927.10.12590 for *T. brucei*.

This suggests that LmxM.18.1460 encodes an IFT dynein intermediate chain that forms a heterodimer with DIC5, with functional parallels in *T. brucei*, *H. sapiens*, and *C. reinhardtii*.

5.2.2 IFT dynein intermediate chain moves with IFT particle

To investigate if localisation of LmxM.18.1460 was conserved, the protein was N-terminally tagged with mNG. PBS-washed live mNG::LmxM.18.1460 cells were adhered to a glass slide before being visualised by epifluorescence microscopy at 5 Hz for 512 frames. The flagella were manually traced and digitally straightened to generate IFT kymographs. This revealed that the mNG::LmxM.18.1460 signal was localised to the flagellum and moved along the axoneme with the canonical behaviour of IFT at the same speed as mNG::IFT172 (Figure 5-2A). This strongly suggests that LmxM.18.1460 is part of the IFT particle and supports it encoding a novel IFT dynein intermediate chain.

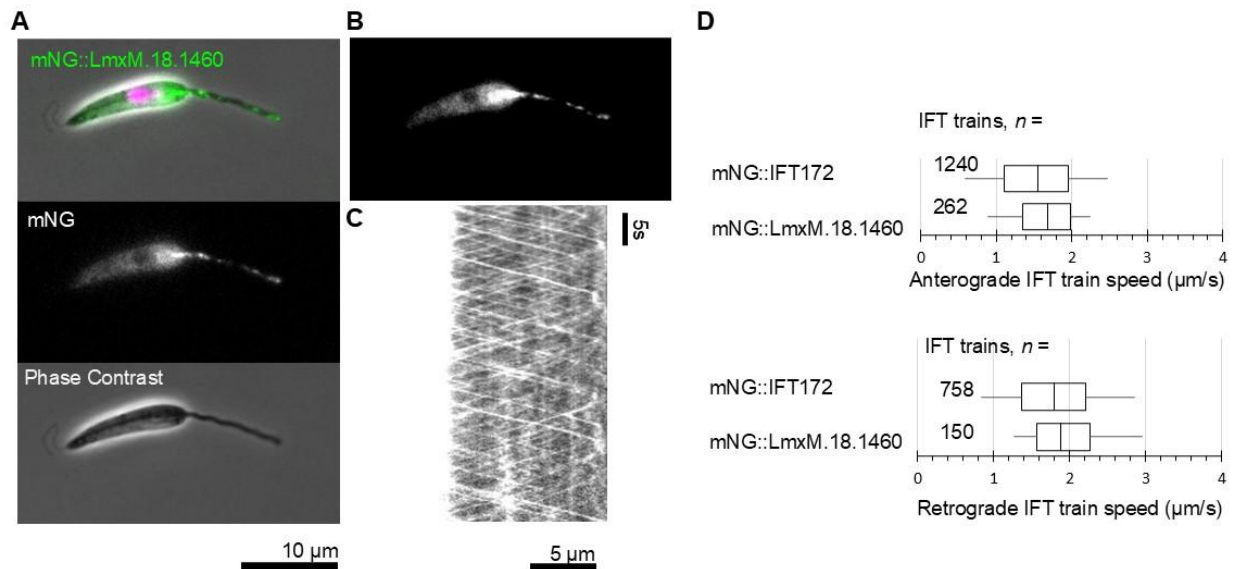


Figure 5-2 | mNG::LmxM.18.1460 has a pattern of fluorescence signal like IFT, and the speed of the movement was not significantly different than mNG::IFT172. A. Reference phase contrast and green fluorescence images of mNG::LmxM.18.1460. Images are laid out in the following format: first image, an overlay of the mNG fluorescence (green) over phase contrast (grey), second image, mNG fluorescence in greyscale, third image, the phase contrast. B. Repeated mNG fluorescence image for reference with kymograph below. C. Green fluorescence kymograph showing the location of mNG::LmxM.18.1460 along the length of the flagellum (x-axis), over time (y-axis). D. The speed of the green fluorescence of mNG::LmxM.18.1460 and of mNG::IFT172 are not significantly different.

It was not possible to generate a homologous deletion of LmxM.18.1460. The LeishGem project, a collaboration between multiple laboratories also failed to generate a deletion cell line for this gene. Is it possible that this putative dynein intermediate chain LmxM.18.1460, similar to dynein-2 heavy chain DHC2.1 (Adhiambo et al., 2005), is essential for *L. mexicana* survival? This is discussed further in 5.3.

5.2.3 LmxM.05.0690 has conserved localisation

The orthologous protein to TZIP250 in *L. mexicana* is LmxM.05.0690, a 2573 amino acid long protein which possesses 28 PGPXY repeats. Given that an alpha helix has roughly 3.6 aa per turn and 0.15 nm distance given per turn, we could imagine the maximum predicted length of the alpha helix to be around 0.39 μm. N- (mNG::LmxM.05.0690) and C-terminally (LmxM.05.0690::mNG) tagged cell lines were stained with Hoechst 33342 and imaged live using an Axio Observer A1 (Zeiss) at 63× (Figure 5-3). Depending on the angle of the cell, and potentially the cell cycle phase, the fluorescence signal of the N- and the C-terminally tagged LmxM.05.0690 can be seen as two distinct puncta. The N-

terminal fluorescence is directly distal to the kinetoplast with localisation similar to the TAC or basal body, with C-terminal location similar to transition zone (Halliday et al., 2019). The average distance between the peak of fluorescence signal for the kinetoplast (as stained by Hoechst 33342) and the N-terminal tag was $0.56 \pm 0.18 \mu\text{m}$ (n=13) and for C-terminal was $0.74 \pm 0.15 \mu\text{m}$ (n=32). This may suggest that like in *T. brucei* (Dean, 2016), TZP250 acts as a molecular ruler for the TZ complex. Alternatively, its localisation is equivalent to the region that kinesin-II operates in *C. elegans*, so it could also be involved in kinesin-II distribution, however confirmation of this would require further investigation (Snow et al., 2004; Pan et al., 2006; Prevo et al., 2015).

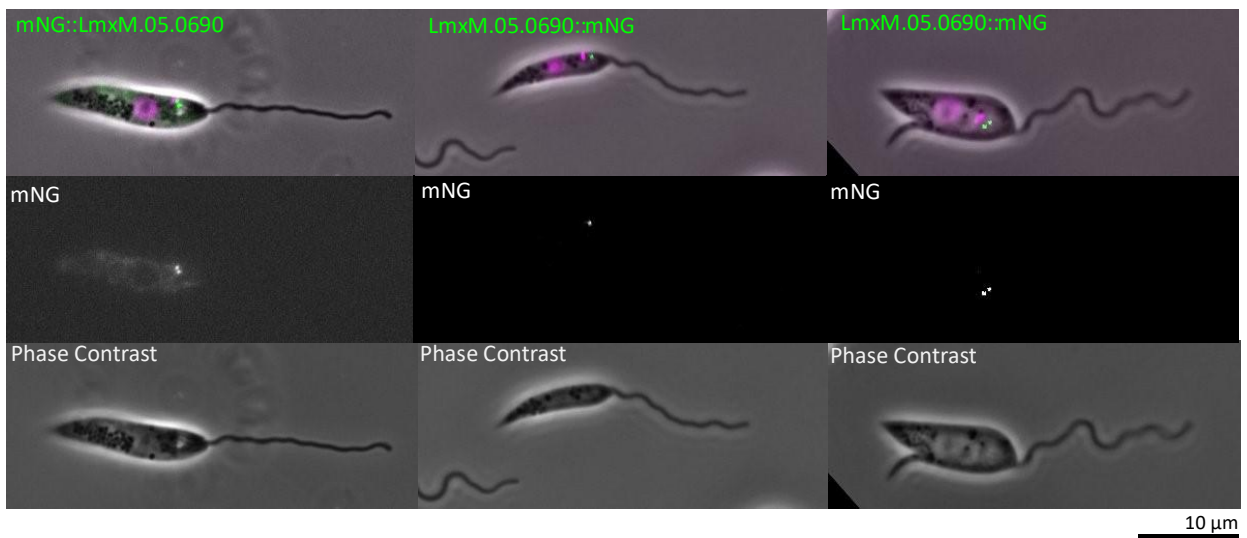


Figure 5-3 | Localisation of LmxM.05.0690 is conserved. Reference phase contrast and green fluorescence images of mNG::LmxM.05.0690 and LmxM.05.0690::mNG cell lines. For each cell line the protein name and gene fusion are shown in the top left. Images are laid out in the following format: first row, an overlay of the mNG fluorescence (green) over phase contrast (grey), second row, mNG fluorescence in greyscale, third row, the phase contrast image.

5.2.4 PGPXY rich protein LmxM.34.3925 is localised along the length of flagellum

LmxM.34.3925 is another PGPXY motif rich protein, which contains seven repeats, spread throughout the protein with 36 to 107aa between the repeats. Cells with mNG N-terminally tagged LmxM.34.3925 (mNG::LmxM.34.3925) were adhered to a glass cover slip and imaged live using an Axio Observer A1 (Zeiss) at 63× (Figure 5-4). LmxM.34.3925 was localised along the length of the flagellum.

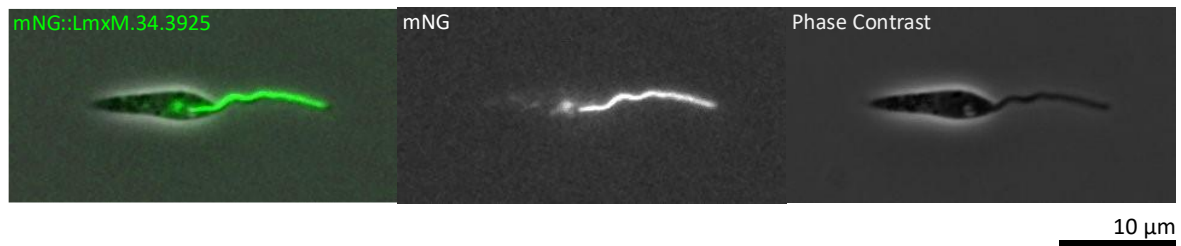


Figure 5-4 LmxM.34.3925 is localised along the flagellum. Reference phase contrast and green fluorescence images of mNG::LmxM.34.3925. The protein name and gene fusion are shown in the top left. Images are laid out in the following format: left image, an overlay of the mNG fluorescence (green) over phase contrast (grey), middle image, mNG fluorescence in greyscale, right image, the phase contrast image.

5.2.5 LmxM.34.3925 deletion reduces IFT train speed without affecting swimming speed or axoneme structure

A cell line in which both alleles of LmxM.34.3925 were deleted (Δ LmxM.34.3925) was generated using 3xmNG::IFT172 as a parental cell line. 3xmNG::IFT172 Δ LmxM.34.3925 and their parental cell line were analysed using 10 $\times$ dark-field to study swimming behaviour. The pattern of swimming in the cell path maps of 3xmNG::IFT172 and 3xmNG::IFT172 Δ LmxM.34.3925 are largely similar (Figure 5-5A), and LmxM.34.3925 deletion caused no significant difference in swimming speed (Figure 5-5B).

Adhered PBS-washed cells and free-swimming cells were visualised by phase contrast and green fluorescence, using the methodology for adhered and free cells previously described. The deletion of LmxM.34.3925 caused no significant change in flagellum length (Figure 5-5C) or severe morphological changes (Figure 5-5D) and when free to swim, Δ LmxM.34.3925 flagella were able to generate a normal symmetric tip-to-base pattern of beating (Figure 5-5E). TEM completed by Cecile Fort reveal that LmxM.34.3925 deletion caused no major alterations to axonemal structure (Figure 5-5F).

Consistent with previous results the speed of IFT trains in swimming cells was significantly faster than in adhered cells (Figure 5-5G). Interestingly, in both adhered and swimming cells, the deletion of LmxM.34.3925 resulted in decreased anterograde train speed (Figure 5-5G).

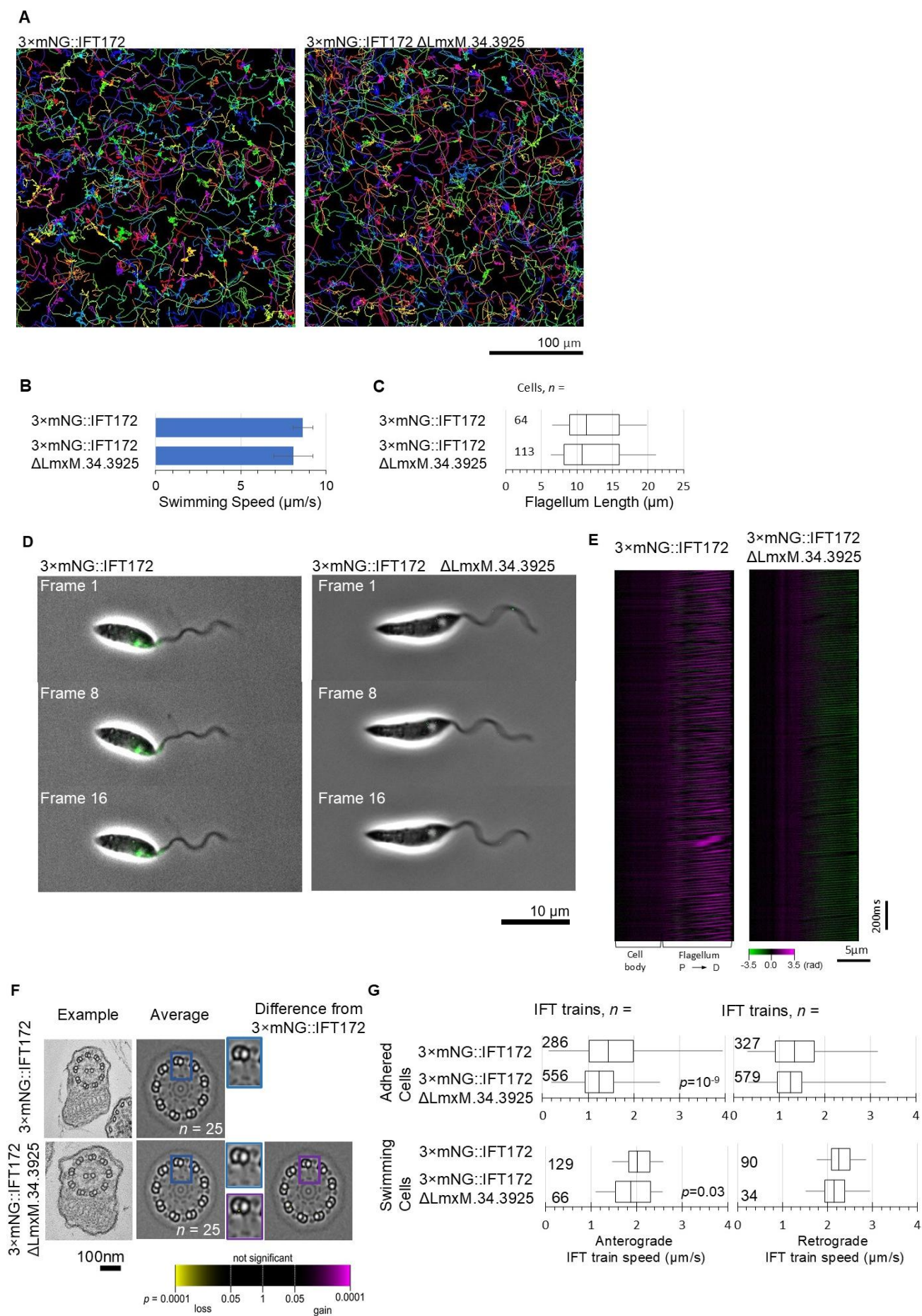


Figure 5-5 | IFT train speed decreases with LmxM.34.3925 deletion. A. Cell path maps of parental 3×mNG::IFT172 and deletion mutant 3×mNG::IFT172 ΔLmxM.34.3925 showing traced cells for a portion of the full FOV for the full duration of the 10× dark-field 5 Hz image sequences. Each trace, presented in different colours, represents a single cell. The scale is indicated by a scale bar. B. Bar chart depicting the average swimming speed of 3×mNG::IFT172 and 3×mNG::IFT172 ΔLmxM.34.3925. The error bars show the standard deviation of the six replicates. Each replicate comprises the average swimming speed of over 1000 cells analysed. C. Box plot displaying flagellum length of parental 3×mNG::IFT172 and 3×mNG::IFT172 ΔLmxM.34.3925. The number of cells *n* is written. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented. D. Three frames taken from high-frame rate 63× phase contrast videos of example 3×mNG::IFT172 and 3×mNG::IFT172 ΔLmxM.34.3925 cells showing one beat cycle of a symmetric tip-to-base beat. The number of the frame is displayed on each still. Scale is indicated by a scale bar. E. Cell lines are labelled above their tangent angle kymographs. A tangent angle in radian of each pixel along the entire length of the cell relative to the flagella base, over the length of the video is represented by its colour. For the first kymograph, the cell body and flagellum are labelled. The cell body does not bend a lot, whereas the flagellum has high curvature. The proximal (P) and distal (D) ends of the flagellum are labelled. Time and distance scale bars are available. F. Transmission electron micrographs of axonemal cross sections of 3×mNG::IFT172 and 3×mNG::IFT172 ΔLmxM.34.3925. Electron microscopy completed and averaged by Cecile Fort. The images are as described: the first column displays an example transverse section of the flagellum, showing both the axoneme and paraflagellar rod. The second column displays an average of 25 nine-fold rotationally averaged axonemes, with an enlarged view of the area highlighted in blue shows a single microtubule doublet and its associated complexes. The third column compares the electron density to that of 3×mNG::IFT172 using a per-pixel Mann–Whitney U test, with an enlarged view of the purple boxed area highlighting a single microtubule doublet. G. Boxplots displaying the speeds of anterograde and retrograde IFT trains in 3×mNG::IFT172 and deletion mutant 3×mNG::IFT172 ΔLmxM.34.3925 in adhered and in swimming cell samples. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented.

5.2.6 LmxM.30.0890 (CFAP97) is localised along the flagellum

CFAP97 ([Tb927.10.6490](#)) in *T. brucei* is localised along the length of the axoneme in a cell cycle dependent manner. Its orthologue, LmxM.30.0890 was N-terminally tagged with mNG and visualised in phase contrast and green fluorescence (Figure 5-6). The LmxM.30.0890 protein localised along the length of the flagellum.

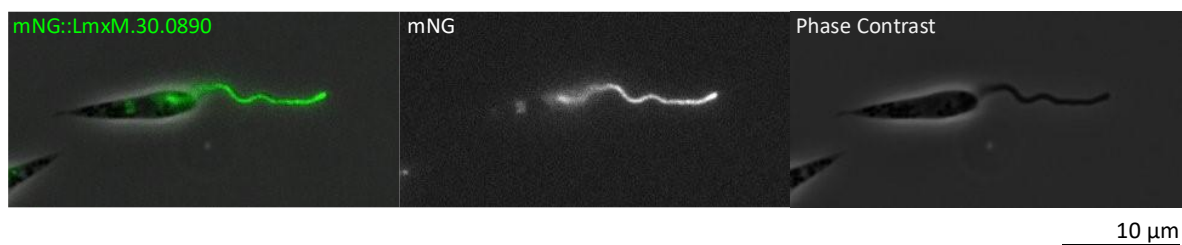


Figure 5-6 | Reference phase contrast and green fluorescence images of mNG::LmxM.30.0890. The protein name and gene fusion are shown in the top left. Images are laid out in the following format: left image, an overlay of the mNG fluorescence (green) over phase contrast (grey), middle image, mNG fluorescence in greyscale, right image, the phase contrast image.

Chromosome 30 on which LmxM.30.0890 is located is tetraploid (Fiebig et al., 2015). pT Neo, pT Puro, pT, Phleo and pT Blast were used to delete all four alleles of LmxM.30.0890 (Δ LmxM.30.0890). This mutant shows no significant change in swimming behaviour (Figure 5-7A), swimming speed (Figure 5-7B), flagellum length (Figure 5-7C) or axonemal structure (Figure 5-7D).

Generating a cell line with LmxM.30.0890 deletion and 3mNG::IFT172, which is required for imaging IFT, was extremely difficult as there are only six useable drug selection markers, all of which were already being used in Δ LmxM.30.0890 – Hyg & NTC to maintain Cas 9 and T7 respectively, and Neo Puro Phleo and Blast drug selectable markers to delete all LmxM.30.0890 alleles. LmxM.30.0890 was transformed using a 3mNG::IFT172 tag which was blasticidin selectable; a concentration of 20 μ g/ml blasticidin, ten times the normal selection concentration, was required for fluorescent cells to be detectable, even then there was fluorescence in only around 1 in 10 cells. The fluorescence was lost if cells were no longer under drug selection, so drug selection was maintained.

PBS-washed cells were adhered to slides and low frequency green fluorescence videos taken at 63 $\times$. As 3mNG::IFT172 Δ LmxM.30.0890 was maintained under blasticidin drug selection, 3mNG::IFT172 under blasticidin drug selection of 5 μ g/ml was used as an additional control for IFT train speed. 3mNG::IFT172 Δ LmxM.30.0890 had decreased anterograde IFT train speed compared to both controls and decreased retrograde IFT train speed compared to the control under drug selection (Figure 5-7E).

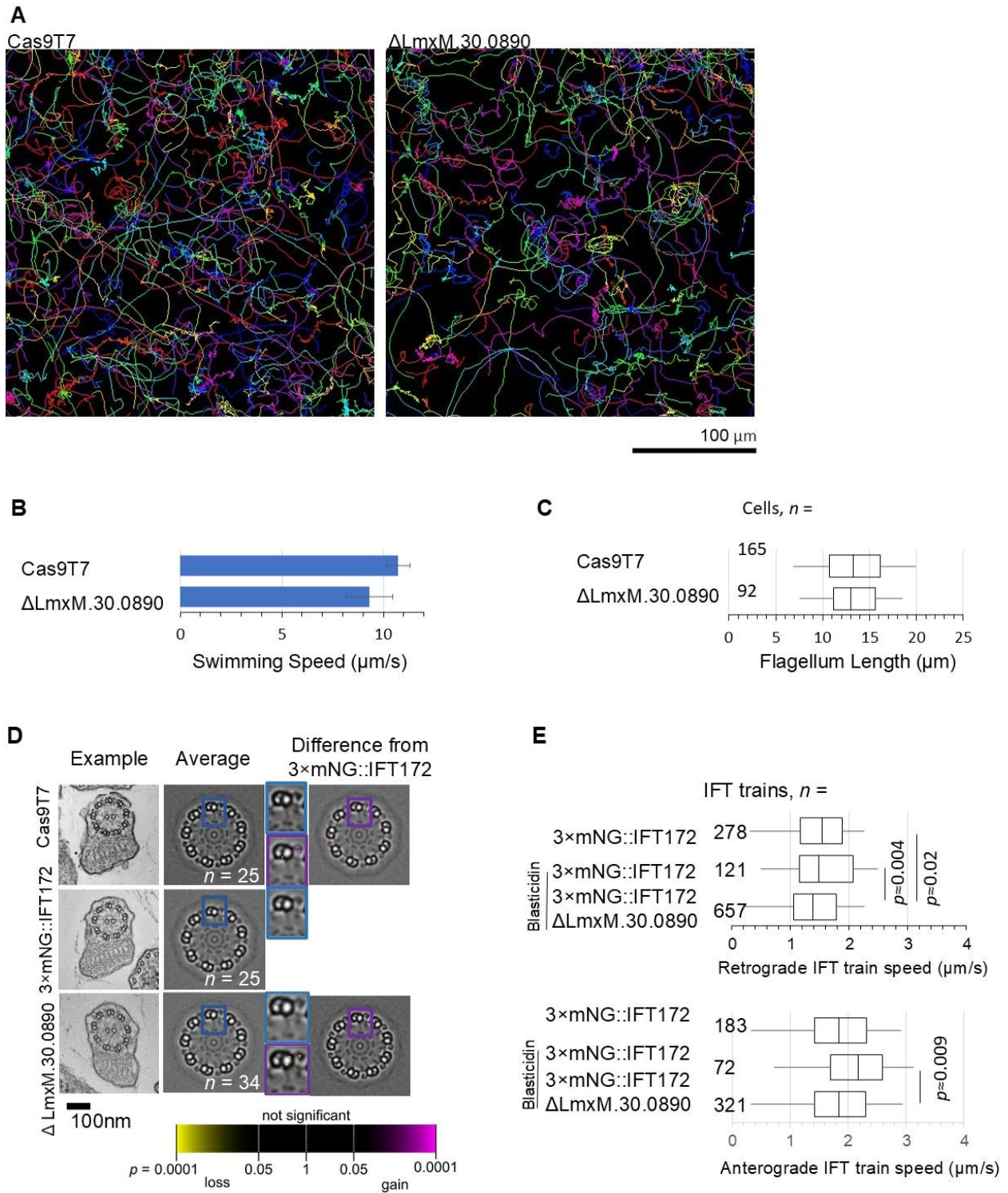


Figure 5-7 | LmxM.30.0890 deletion reduced anterograde IFT train speed. A. Cell path maps of parental Cas9T7 and deletion mutant Δ LmxM.30.0890 showing traced cells for a portion of the full FOV for the full duration of the 10× dark-field 5 Hz image sequences. Each trace, presented in different colours, represents a single cell. The scale is indicated by a scale bar. B. Bar chart depicting the average swimming speed of parental 3×mNG::IFT172 and deletion mutant 3×mNG::IFT172 Δ LmxM.30.0890. The error bars show the standard deviation of the six replicates. Each replicate comprises the average swimming speed of over 1000 cells analysed. C. Box plots displaying flagellum length of parental 3×mNG::IFT172 and 3×mNG::IFT172 Δ LmxM.30.0890. The number of cells

n is written. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented. D. The images are as described: the first column displays an example transverse section of the flagellum, showing both the axoneme and paraflagellar rod. The second column displays an average of 25 nine-fold rotationally averaged axonemes, with an enlarged view of the area highlighted in blue shows a single microtubule doublet and its associated complexes. The third column compares the electron density to that of 3xmNG::IFT172 using a per-pixel Mann–Whitney U test, with an enlarged view of the purple boxed area highlighting a single microtubule doublet. E. Boxplots displaying the speeds of anterograde and retrograde IFT trains in the parental and mutant cell line in adhered cell samples. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented.

The image analysis pipeline for visualising IFT in swimming cells has quite a significant attrition rate, due to the requirement for freely moving cells to be traceable in high frame rate videos. Therefore, even if every cell has a fluorescently tagged IFT172 signal, it is challenging and time consuming to generate enough videos to give an adequate sample size for analysis of IFT train speed downstream. Given that this cell line had no significant changes in motility, fluorescent IFT172 signal was found in as little as 1 in 10 Δ LmxM.30.0890 cells and the requirement to capture an additional blasticidin-treated 3xmNG::IFT172 control, IFT train speed analysis was not completed in swimming cells for this cell line.

5.2.7 Candidate MPKs have flagellar localisation

Candidate kinases for IFT interaction were chosen due to their known effect on flagellum length or flagellar localisation: MPK3, MPK9, MPK13, MPK14 and LmxM.29.0600. Cell lines with mNG N-terminal tagging of these MPKs were generated. PBS-washed cells were adhered to glass and visualised live in phase contrast and green fluorescence at 63 $\times$ (Figure 5-8). MPK3 and MPK9 signals localised to the cell body and flagellum. MPK13, MPK14 and LmxM.29.0600 had weaker signal and were localised to the flagellum and some compartments of the cell body.

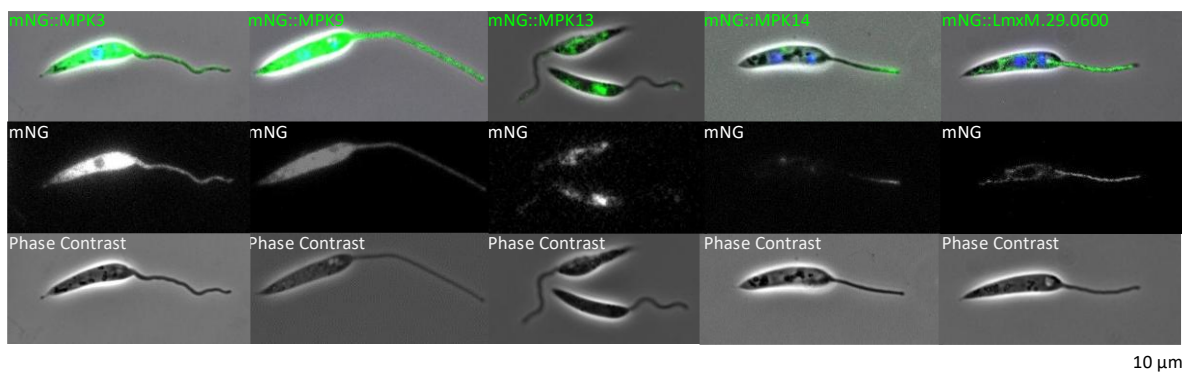


Figure 5-8 | Candidate MPKs have flagellar localisation. Reference phase contrast and green fluorescence images of mNG-tagged MPK cell lines. For each cell line the protein name and gene fusion are shown in the top left with the gene ID in brackets. Images are laid out in the following format: first row, an overlay of the mNG fluorescence (green) over phase contrast (grey), second row, mNG fluorescence in greyscale, third row, the phase contrast images. Scale is indicated by a scale bar.

5.2.8 MPK13 and MPK14 have IFT-associated patterns of movement

To gain insight into dynamic localisation of MPKs and detect those likely to be interacting with IFT, 5 Hz green fluorescence videos were taken of live cells adhered to a glass slide (Figure 5-9A). Flagella of the cells were manually traced and digitally straightened. The fluorescence signal along the length of the flagella for each frame was stacked to create a green fluorescence kymograph (Figure 5-9B). In this green fluorescence kymograph, the pattern of mNG signal along the length of the flagellum (x-axis) can be seen over time (y-axis). The patterns of signal localisation for MPK3 and MPK9 suggest diffusion in the flagellum. LmxM.29.0600 localisation appears in vertical stripes along the flagellum, this could indicate periodicity of localisation, or static localisation which causes photobleached areas to remain present throughout imaging. MPK13 and MPK14 signal localisations are similar to that of IFT train movement, however weaker, with MPK14 fluorescence signal brightest at the flagellar tip. This may suggest that MPK13 and MPK14 interact with IFT trains.

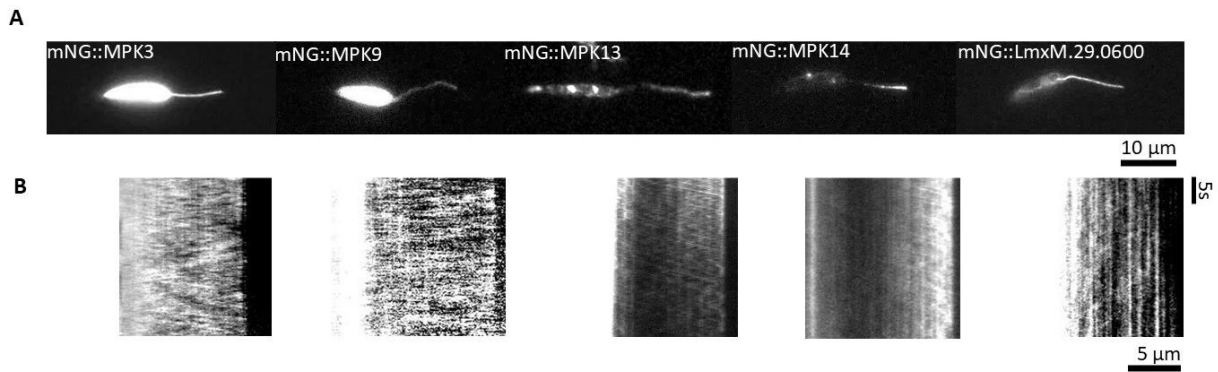


Figure 5-9 | MPK13 and MPK14 have similar movement over time to IFT trains. A. Images showing green fluorescence signal in a still frame from 5 Hz green fluorescence video. B. Green fluorescence kymograph showing the location of mNG tagged MPKs along the length of the flagellum (x-axis), over time (y-axis).

5.2.9 MPK9, MPK13 and MPK14 deletion mutants have increased anterograde IFT train speed

Given that MPK13 and MPK14 had patterns of signal similar to IFT train movement, these were of most interest in studying MPK interaction with IFT trains. TriTrypDB CLUSTAL O(1.2.3) multiple sequence alignment function revealed that MPK9 and MPK14 have a high degree of similarity, particularly in their N-terminal regions, suggesting they may be paralogues. Therefore, MPK9, MPK13 and MPK14 were selected for further investigation.

Deletion mutants of MPK9 (Δ MPK9), MPK13 (Δ MPK13) and MPK14 (Δ MPK14) were generated using 3xmNG::IFT172 as a parental cell line. The cell lines were visualised using 10 $\times$ dark-field microscopy to study swimming behaviour. The cell path maps of the deletion mutants were largely similar to their parental cell line (Figure 5-10A), however 3xmNG::IFT172 Δ MPK13 had significantly increased swimming speed (Figure 5-10B). MPK9 and MPK13 deletion cell lines both had increased flagellum length (Figure 5-10C). None of the MPK deletion mutants had further severe morphological changes (Figure 5-10D) and all were able to generate a symmetric tip-to-base beat (Figure 5-10E).

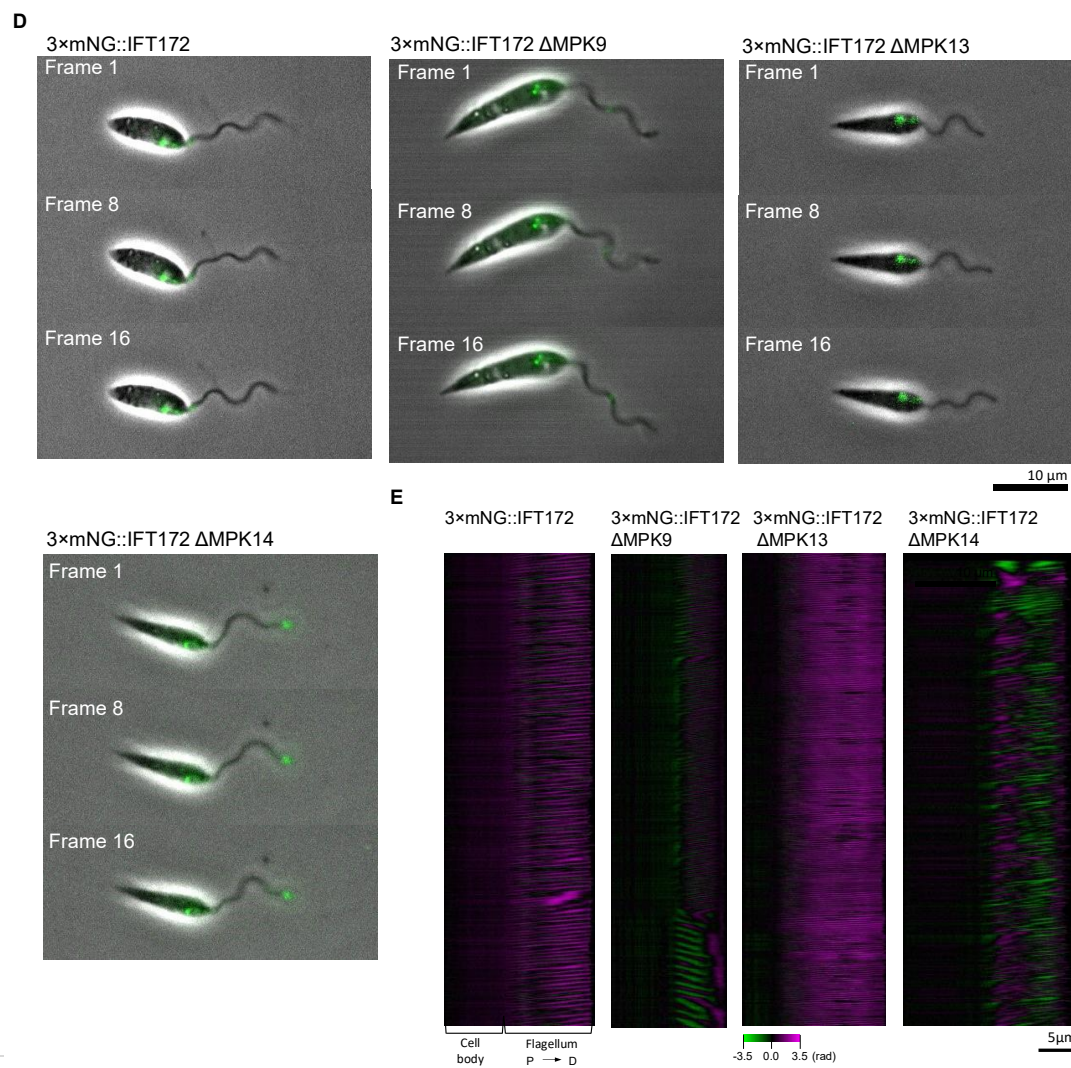
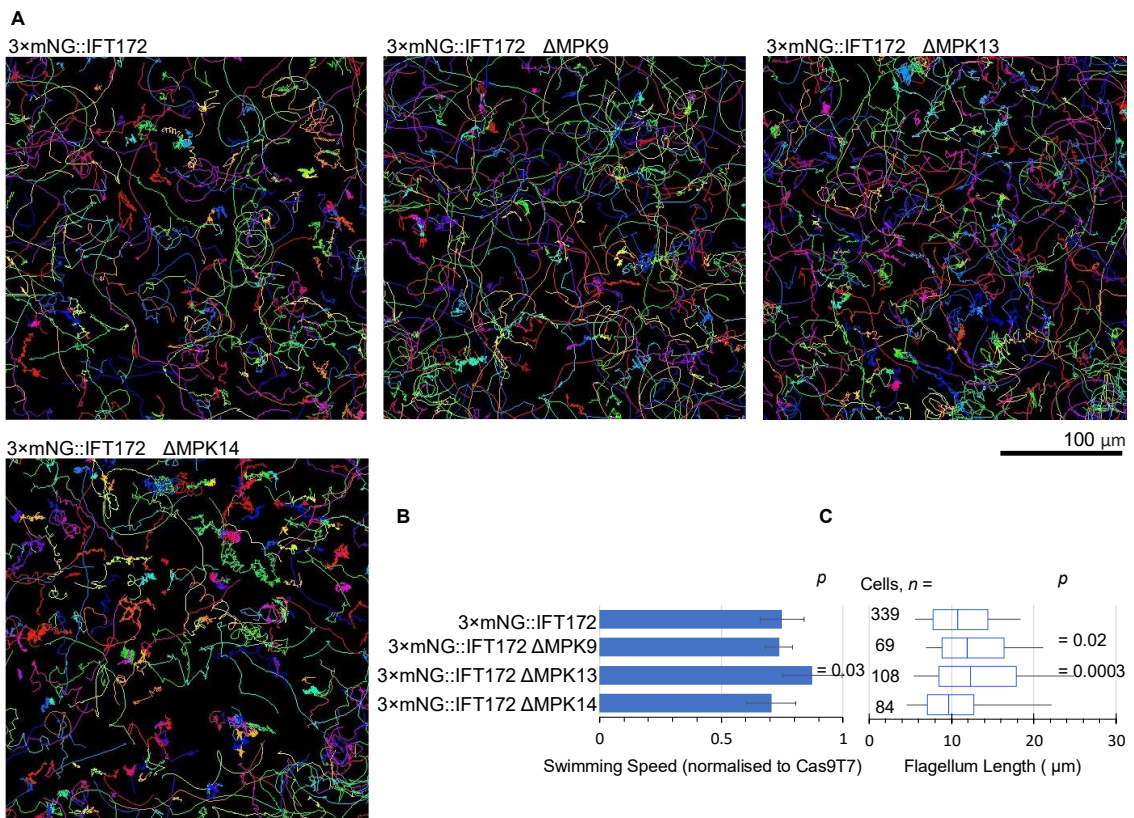


Figure 5-10 | MPK deletion mutants are able to generate wild type beats. A. Cell path maps of parental 3×mNG::IFT172 and MPK deletion mutants showing traced cells for a portion of the full FOV for the full duration of the 10× dark-field 5 Hz image sequences. Each trace, presented in different colours, represents a single cell. The scale is indicated by a scale bar. B. Bar chart depicting the average swimming speed of MPK deletion mutants, derived from 3×mNG::IFT172. The error bars show the standard deviation of six replicates. Each replicate comprises the average swimming speed of over 1000 cells analysed, normalized against a sample from the Cas9T7 cell line (the (grand)parental line) taken on the same day. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented. C. Box plots displaying flagellum length of parental 3×mNG::IFT172 and MPK deletion mutants. The number of cells n is written. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented. D. Three frames taken from high-frame rate 63× phase contrast videos of example 3×mNG::IFT172 and MPK deletion mutant cells showing one beat cycle of a symmetric tip-to-base beat. The number of the frame is displayed on each still. Scale is indicated by a scale bar. E. Cell lines are labelled above their tangent angle kymographs. A tangent angle in radian of each pixel along the entire length of the cell relative to the flagella base, over the length of the video is represented by its colour. For the first kymograph, the cell body and flagellum are labelled. The cell body does not bend a lot, whereas the flagellum has high curvature. The proximal (P) and distal (D) ends of the flagellum are labelled. Time and distance scale bars are available.

IFT train speed was quantified in adhered and free swimming cells for each of the MPK deletion mutants (Figure 5-11). In adhered cells, only MPK13 deletion mutants had a significantly increased anterograde and retrograde IFT train speed. However, when free to swim, all three MPK deletion mutants had significantly increased anterograde and retrograde IFT train speed.

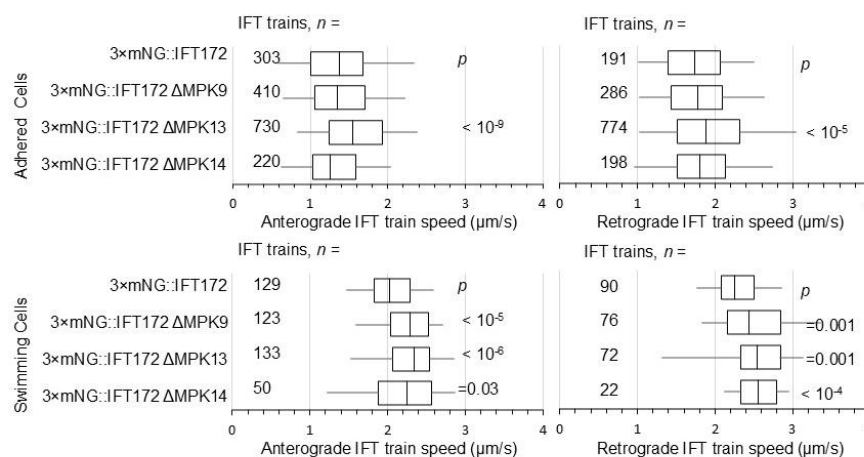


Figure 5-11 | Anterograde train speed is increased in beating flagella of MPK deletion mutants cohort. Boxplots displaying the speeds of anterograde and retrograde IFT trains in the parental and MPK deletion cell lines in adhered and swimming cell samples. The boxes demark the lower quartile, median, and upper quartile, and the whiskers denote the 5th and 95th percentiles. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented.

5.3 Discussion

This chapter investigated a range of proteins that may be involved with IFT functioning: a putative dynein intermediate chain, IFT track proteins and regulator MPKs. We found LmxM.18.1460 moved in an IFT-associated pattern and is a putative dynein intermediate chain, for which we were unable to generate a deletion mutant suggesting that it may be essential for cell survival. In several species, cytoplasmic dynein-1 and IFT motor protein dynein-2 share subunits (Asante et al., 2014) – so it could be possible that the intermediate chain encoded by LmxM.18.1460 is involved in the functioning of more than one dynein complex, as there is a weak cytoplasmic fluorescence signal in mNG::LmxM.18.1460, however this is unknown in *Leishmania*. In *Chlamydomonas* WD60 knockdown resulted in ciliary assembly defects, and in ciliated human cells WDR60 knockdown cause defects in retrograde trafficking and mutations in both WDR34 and WDR60 are known to cause disease (Hamada et al., 2018; Asante et al., 2014). Therefore, LmxM.18.1460 could be required for the correct functioning and assembly of the dynein-2 complex. If generating a homozygous deletion of LmxM.18.1460 continues to be unsuccessful, this could be investigated in the future by using knockdown systems such as RNAi-mediated gene silencing.

We found that the SHIPPO-domain containing proteins had disparate localisations in *L. mexicana*. LmxM.05690 was localised to the transition zone and appears to have a conserved function from its orthologue in *T. brucei* acting as what Dean et al 2016 described as a ‘molecular ruler’. Whereas LmxM.34.3925 was localised along the length of the flagellum. The deletion of LmxM.34.3925 did not alter swimming speed (Figure 5-5A), flagellum length (Figure 5-5B) or axonemal structure (Figure 5-5C); however, it did result in a significant decrease in anterograde IFT train speed in adhered cells (Figure 5-5D). This deletion mutant showed that it is possible to impact IFT train speed without altering flagellar motility or macromolecular axonemal structure – suggesting that LmxM.34.3925 functionality is specifically related to IFT train movement. The PGPGXY motif has been shown to mediate

microtubule binding (Dean, 2016), it is possible that through binding to the face of the microtubules along which IFT trains walk, LmxM.34.3925 mediates IFT train progression?

CFAP97 is evolutionarily conserved from *Chlamydomonas* to mammals, and in mammalian sperm its deletion compromises flagellar axoneme maintenance, and lead to the loss of the fourth microtubule doublet in over half of the cells (Merlino et al., 1991; Sampson et al., 2001; Oura et al., 2020). In *L. tarentolae*, CFAP97 is distributed on the outer face of the microtubule doublets at the junction between the A and B microtubules (A10 and B01) from the transition zone to the flagellar tip (Leung et al., 2023; Doran et al., 2025). In *L. mexicana*, the deletion of CFAP97 orthologue (LmxM.30.0890) did not cause microtubule doublet loss (Figure 5-7D), significant changes in flagellum length (Figure 5-7C) or cause reductions in swimming speed (Figure 5-7B). However, it did reduce anterograde IFT train speed (Figure 5-7E). Given its localisation, it is possible that it forms part of the separation mechanism for anterograde and retrograde transport onto separate microtubules of the doublet. The effect on IFT train speed following the deletion of LmxM.34.3925 and LmxM.30.0890 is moderate rather than severe suggesting they are part of a collection of proteins involved in forming the tracks that direct IFT trafficking.

We identified three MPKs that negatively regulate IFT train speed in *L. mexicana*. Localisation data suggests that MPK13 and MPK14 do so through interaction with substrates on component of or associated with the IFT particle itself for instance the motor proteins driving movement, whilst MPK9 diffuses along the flagellum and interacts more transiently with either the train or the microtubules to influence IFT train progression. Overall, our data suggests that MPK13 is a negative regulator of flagellum length, IFT train speed and swimming speed. MPK9 acts as a negative regulator for flagellum length and IFT train speed and MPK14 IFT train speed only. MPK9 and MPK13 deletion caused an increase in flagellum length, however MPK14 did not.

Interestingly, MPK13 deletion caused an increase in anterograde and retrograde train speed in adhered and free-swimming cells. Whereas, MPK9 and MPK14 deletion increased IFT train speed in a

similar magnitude to MPK13 in free swimming cells but not in adhered cells. One explanation is that the IFT regulatory activity of MPK9 and MPK14 is modulated in response to flagellar beating activity. As discussed in Chapter 4, there is evidence from epithelial cells that shear stress can induce PKC mediated MPK phosphorylation, independent of potassium or potassium ion signalling, with PKC or MPK inhibition decreasing cilia length (Davies, 1995; Abdul-Majeed et al., 2011). This suggests mechanical force induced MPK activation could be involved in flagellum length control. A recent *in vitro* study showed that MPK9 was important for sand fly colonisation, therefore the role it has in controlling IFT train speed and flagellum length are biologically important (Baker et al., 2021). One possibility is that MPK9 is key to optimising flagellar motility and thus migration during the changing environments of the *L. mexicana* life cycle by regulating flagellum length regulation in response to changes in shear force.

Interestingly, Δ MPK13 cells also had increased swimming speed. Increased flagellum length can create stronger propulsive force but also additional drag, therefore there is an optimum flagellum length for motility. Metacyclic promastigotes have longer flagellar than procyclic promastigotes, and *in vitro* also swim faster and straighter (Findlay, 2018; Findlay et al., 2021), therefore it could be that the increased flagellum length with MPK13 deletion directly lead to increased swimming speed. Alternatively, MPK13 could phosphorylate targets on the motor proteins driving both IFT and flagellum beating. The differing combination and severity of effects on swimming speed, flagellum length and IFT train speed with the deletion of the MPKs implied that each has unique mechanisms of action which remains to be elucidated.

6 Light, redox, calcium and swimming

6.1 Introduction

Regulation of flagellar beating is a complex process that is not fully understood. While completing imaging of swimming cells at 63× using both transmitted light and epi-illumination source for visualising flagellar beating and IFT movement, we observed that cells that began in a symmetric tip-to-base beat frequently switched to an asymmetric base-to-tip beat during the video. Switching from asymmetric base-to-tip to symmetric tip-to-base beating was more rarely observed. Is this a light-mediated motility response or an indirect effect of light exposure on cell physiology?

Many species of flagellated organisms have motility responses to light. Helical or spiral swimming trajectories, known as ‘phototurns’ are common to phototactic, and chemotactic micro-organisms, with the photosensor and steering mechanisms having evolved convergently across species (Jennings, 1901; Jékely, 2009). *Chlamydomonas*, and other *Volvocine* algae, have a photosensitive ‘eyespot’ organelle and exhibit phototactic responses dependent on light levels and directions (Kreimer, 2009; Foster and Smyth, 1980). Ion influxes at the eyespot in response to light modulate the pattern of flagellar beating as well as directionality of swimming (Witman, 1993). Notably, the adhesion of *Chlamydomonas* to surfaces is light-switchable (Kreis et al., 2018). Another set of organisms exhibiting photo movement is kinetoplastid *Euglena*, who have a light responsive paraflagellar body comprised of chromoprotein, and photoactivated adenylyl cyclase (PAC) which is localised along the length of the flagellum (Ntefidou and Häder, 2005; Iseki et al., 2002). A PAC molecule comprises dual α - and β -subunits, which harvest light with their blue light receptor using BLUF 1 and 2 domains and convert cAMP to ATP with their adenylyl cyclases domains (Häder and Iseki, 2017). A phylogenetic study showed the PAC catalytic domain was not shared in a lineage with trypanosomes, however, that *E. gracilis* had two trypanosome-type adenylyl cyclases whose *T. brucei* and *L. donovani* orthologues putatively have N-terminal receptor function (Koumura et al., 2004; Alexandre et al., 1990; Sanchez

et al., 1995). Light and dark conditions are thought to be involved in trypanosomatid post-translational protein abundance regulation and trypanosomatids are known to affect insect host behaviour, including modifying phototactic responses (Piel et al., 2022; Ebenezer et al., 2019; Marlière et al., 2015). However, it is not well documented whether the swimming behaviour of trypanosomatid parasites is regulated by light.

The excitation of macromolecules within the cell during live fluorescence microscopy can influence cell physiology, an effect that can be exacerbated by the presence of fluorescent markers (Icha et al., 2017; Kiepas et al., 2020). Phototoxicity can be reduced by decreasing the total time of exposure to light i.e. having short video lengths, which we have done for our high-frame rate dual-colour videos. However, it is still possible that light-induced redox changes could occur in a cell during this time, especially the generation of ROS.

At high concentrations intracellular ROS can be deadly. Antileishmanial therapeutic compounds Quercetin and Carajurin work by inducing ROS generation and disturbing mitochondrial function in *L. amazonensis* promastigotes (Fonseca-Silva et al., 2011; Silva-Silva et al., 2022). In *L. amazonensis*, treatment with 2 mM hydrogen peroxide (H_2O_2) for an hour induced DNA damage, cell cycle arrest and for some cell death – though surviving cells developed oxidative stress resistance indicating the importance of redox-metabolism pathways for survival (da Silva et al., 2017). In *L. donovani*, H_2O_2 -induced oxidative stress led to increased cytosolic calcium ion concentrations, mitochondrial membrane potential loss, ATP level reduction and at high doses cell death (Mukherjee et al., 2002). However, this oxidative stress response was highly time and dose dependent, with lower concentrations (0.1 mM) of H_2O_2 not causing any significant decrease in mitochondrial membrane potential, whilst exposure to moderate concentrations (1.0mM) only caused significant change in mitochondrial membrane potential and ATP after 240 minutes (Mukherjee et al., 2002).

Importantly, ROS are also mediators of cellular and flagellar function. In trypanosomatids, the trypanothione reductase system pathway is key for redox metabolism. The number of TXNs vary

between trypanosomatid protozoa species, for example *C. fasciculata* only has a single TXN whilst *Trypanosoma* and *Leishmania* possess several (Beltrame-Botelho et al., 2016; Alpey et al., 2003). TrypTag localisation of TXNs in *T. brucei*, shows TXN1a and TXN1b have cytoplasmic localisation with flagellar enrichment, which is unusual and distinct compared to ‘normal’ cytoplasm localisation without enrichment (Jeilani et al., 2022) (Figure 6-1). Is it possible that in some trypanosomatids TXNs are involved in redox-based regulation of flagellar motility?



Figure 6-1 | In *T. brucei* TXNs have flagellar enrichment. Phase contrast, green fluorescence and Hoechst (DNA stain) overlaid images obtained from TrypTag of example cells from mNG tagged TXN cell lines in *T. brucei*. For each gene, ID and name are presented. Scale is indicated by a scale bar.

Ca^{2+} is known to be an important regulator of flagellar motility and IFT, with calcium sensitive domains discovered in many axonemal components and calcium-dependent signalling pathways involved in correct flagellar beating. Genetically encoded calcium sensors have previously been used to study calcium flux *in vitro* in flagella in the context of IFT train injection (Ishikawa et al., 2023), however to our knowledge Ca^{2+} has not been well studied in beating flagella.

This chapter is the most speculative of the thesis, though it does highlight some interesting avenues for future investigation at the nexus between light, redox and flagellar motility and for calcium sensing. We find that changes in light intensity alter swimming behaviour but that changes in wavelength do not. Given that swimming behaviour is also affected by H_2O_2 -induced ROS generation, it is possible that this response is due to internal redox-dependent pathway that regulates flagellar motility. We putatively suggest that TXNs with flagellar localisation may be involved with redox-poised mediated changes in cell swimming, however, more experiments would be required to further

investigate this. We also complete an initial investigation into the use of GCaMP3 for visualising Ca^{2+} in beating flagella.

6.2 Results

6.2.1 Light intensity alters beat type switching

We wished to investigate if the pattern of beat type switching observed is a response to light intensity. Light intensity for the transillumination (100W halogen bulb) light source with a red filter and epi-illumination (120 V metal halide light source) with dichroic filter cube, were measured as 20.2 mW and 156 mW respectively. Therefore, samples subject to trans- and epi-illumination are exposed to almost nine-fold light intensity than those under trans-illumination only. Additionally trans-illumination light source provides 615 -730 nm (red), whilst epi-illumination provides 450–490 nm (blue) and 550–590 nm (yellow/green) wavelengths.

To test if this was a result of ROS generated under transmitted light and epifluorescence, an SMP1::KillerRed cell line was generated. SMP1 is crucial for flagellar membrane stabilisation and is required for flagella elongation and function (Tull et al., 2010). Killer red is phototoxic protein which generates ROS upon irradiation with light with peak irradiation at 561 nm, it has an $\text{O}_2^{\bullet-}$ quantum yield ($\Phi_{\text{O}_2^{\bullet-}}$) of 0.97×10^{-3} (Onukwufor et al., 2020; Bulina et al., 2006). High frame rate videos were taken of live Cas9T7 and SMP1::KillerRed cells in exponential growth phase at 40× under transmitted light and under transmitted and epifluorescence light. The beating behaviour of each cell in each field of view was categorised as symmetric tip-to-base, asymmetric base-to-tip, uncoordinated or switch from symmetric tip-to-base to asymmetric base-to-tip.

In Cas9T7, there was a significant difference in beat behaviour observed under transillumination and trans- and epi-illumination (χ^2 (3, N = 279) = 244.72, $P \approx 10^{-15}$). With higher intensity light less cells maintained an symmetric tip-to-base and a higher percentage switched from symmetric tip-to-base to asymmetric base-to-tip (Figure 6-2). Interestingly, in SMP1:KillerRed cells there was no significant

difference between the two imaging conditions, and the vast majority of cells underwent symmetric tip-to-base beating.

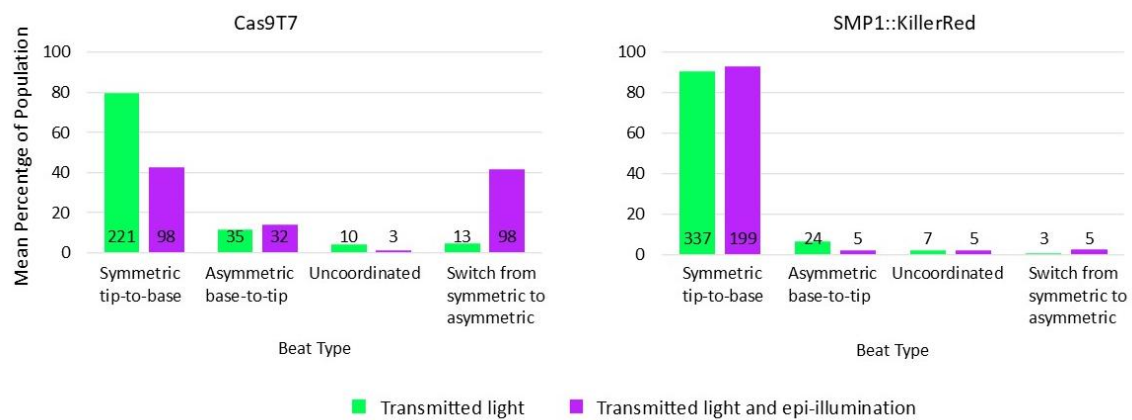


Figure 6-2 | Cas9T7 beat switching is increased with increased light intensity whereas SMP1::KillerRed is not. Bar chart showing mean percentage of cell population for Cas9T7 and SMP1::KillerRed undergoing each beating behaviour for two light conditions. Number of cells visualised and categorised is described for each bar.

6.2.2 There were no obvious light sensitive BLUF1 or BLUF2 in *L. mexicana*

A BlastP search for the protein sequence of the light sensitive BLUF1 and BLUF2 regions of the PAC α -subunit in *Leishmania* and *Trypanosoma* species yielded no significant hits, with all E-values greater than 1. BlastP for the entire PAC α -subunit, revealed many significant proteins, including a cysteine peptidase, a CHAT domain containing domain and a protein localised to the PFR (Figure 6-3). There is no evidence these proteins are regulated by light, however they may be of more general interest in signal transduction pathways involved in flagellar beating. BlastP for the PAC β -subunit yielded no significant results.

Gene ID	Gene Name or Description	E-value
LmxM.29.2040	cysteine peptidase, Clan CA, family C2, putative, calpain-like cysteine peptidase,	0.000003
LmxM.21.0180	CHAT domain containing protein, putative	0.000006
LmxM.04.1200	hypothetical protein, conserved	0.000007
LmxM.36.4230	paraflagellar rod component, putative	0.0006
LmxM.20.0170	leucine-rich repeat-containing protein	0.001
LmxM.07.0320	hypothetical protein, conserved	0.001
LmxM.34.2400	Flagellar Member 5	0.01

Figure 6-3 | Table showing best BLASTP hits in *L. mexicana* to PAC α -subunit sequence (query sequence). Gene ID, gene name – or if unavailable the description is listed, along with an E-value. E-value represents the probability that the match between the query sequence and the stated protein sequence is a result of chance. Hits with an E-value of <0.01 are considered significant.

6.2.3 *Leishmania* swimming is not altered by exposure to different light wavelength

Given that the addition of epi-illumination source alters both light intensity and the spectrum of wavelengths – it was important to decode the effect of these on swimming behaviour. Beat type and swimming speed are closely related. Cells undergoing symmetric tip-to-base forward swimming will have a higher speed than those undergoing uncoordinated or ciliary beating.

To test if wavelength of light exposure affects beating and therefore swimming speed, 10× low frame rate darkfield videos of Cas9T7 cells free to swim with filters on the transmitted light source of different wavelengths compared to an unfiltered darkfield (400-700 nm) control were taken. These filters were as follows: 49 DAPI (365±5 nm), 38 Green Fluorescent Protein (470±40 nm), 20HE Rhodamine (546±12 nm), 63HE Red Fluorescent protein (572±25 nm) and 50 Cy5 (640±30 nm) (Figure 6-4). There is an internal control present, as cells were taken from the same flask for visualisation under different conditions. Initial results suggested a change in swimming speed with a shift in wavelength of the excitation light, however filters have differing comparative optical powers and adding a line representing light intensity for each filter suggested this might be a confounding variable.

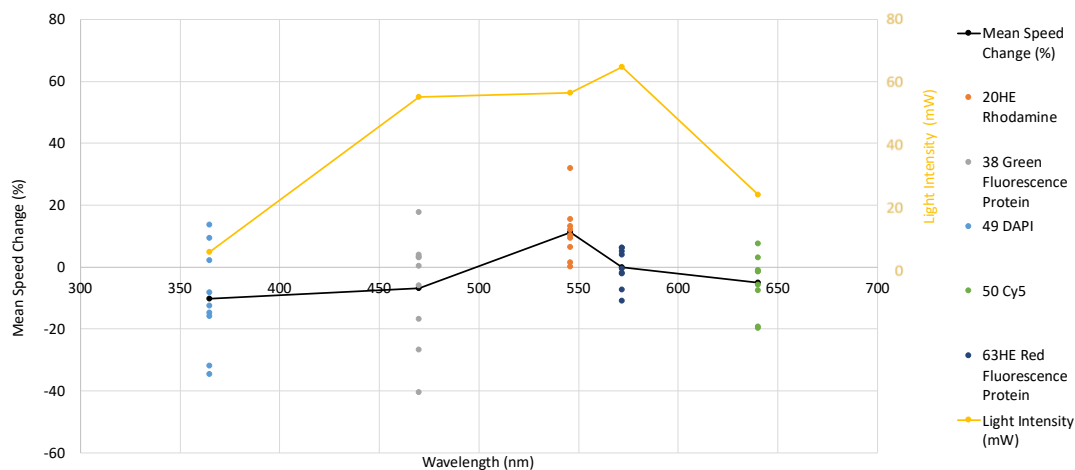


Figure 6-4 | Mean swimming speed changes with use of filters. Line graph showing mean swimming speed change of cells in low magnification videos with exposure to differentially filtered light compared to unfiltered light. The swimming speed change for each of the replicates relative to the unfiltered control for each wavelength is shown with a dot with a black line connecting the mean of each condition. The light intensity of the filters in mW is displayed in yellow.

The light intensity for 49 DAPI and 50 Cy5 filters was much lower than the other three. Therefore, it was decided to complete the motility assay for the three remaining filters compared to darkfield, standardising the light intensity of each to 50 mW. This showed that when light intensity was controlled for wavelength of light exposure had no impact on swimming speed (Figure 6-5).

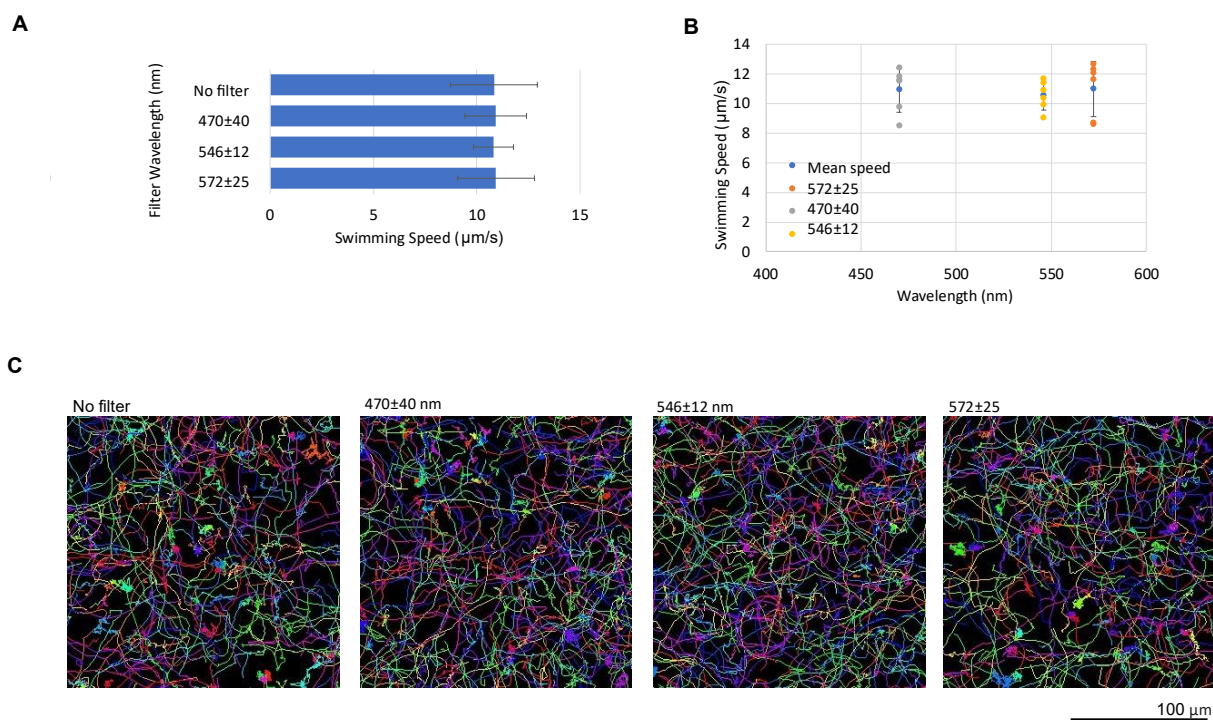


Figure 6-5 | swimming speed is not affected by changes in wavelength of light source. A. Bar chart depicting the average swimming speed of Cas9T7 exposed to different light wavelengths. The error bars show the standard deviation of the six replicates. Each replicate comprises the average swimming speed of over 1000 cells analysed. B. Graph showing mean swimming speed of each of the replicates for under filtered light conditions, with mean of the means presented with a blue dot. C. Cell path maps of Cas9T7 exposed to different light wavelengths showing traced cells for a portion of the full FOV for the full duration of the 10× dark-field 5 Hz image sequences. Each trace, presented in different colours, represents a single cell. The scale is indicated by a scale bar.

6.2.4 In *L. mexicana*, oxidative stress altered swimming speed

H₂O₂ has been used in many studies as a method to initiate ROS generation in *Leishmania* (Fonseca-Silva et al., 2011; Silva-Silva et al., 2022). The following concentrations of H₂O₂ were selected for the initial motility assay: 0 mM, 0.1 mM, 0.5 mM, 1 mM and 2 mM. For each concentration of H₂O₂, 10× low frame rate darkfield videos of Cas9T7 cells free to swim after 0, 15, 30 and 60 minutes of exposure were captured (Figure 6-6). This showed that concentrations of H₂O₂ above 0.5 mM resulted in cells reaching a baseline of low to no movement (equivalent speed to PF16 deletion).

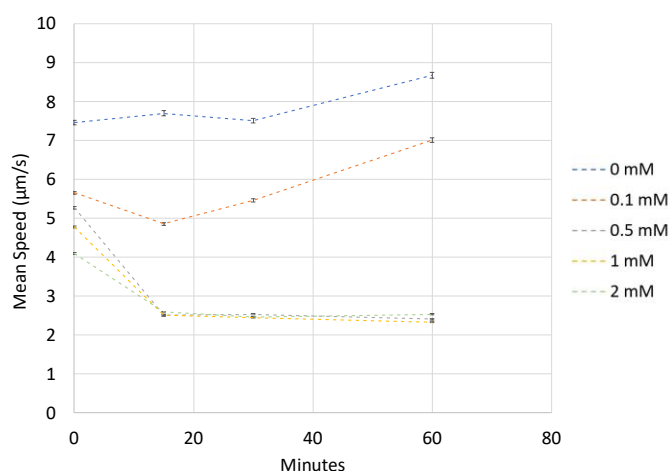


Figure 6-6 | H₂O₂-induced oxidative stress alters swimming speed. A. Line graph showing mean swimming speed of cells at varying H₂O₂ concentrations over time. Standard error of the mean is presented in vertical error bars that act as the markers for the sample; all standard errors are below 0.07.

For 0.1 mM the increase in mean speed over time (Figure 6-6) and the traces in cell path maps (Figure 6-7) suggest that following an initial reduction in movement, cells partially recovered the ability to undergo forward swimming after sustained exposure. Though, it should be noted that there is a slight

increase in the speed of the control sample, this could be due to the focal depth at which the video was taken or an environmental factor such as a change in temperature in the incubation chamber.

This assay was only completed once, though it is a matrix of conditions on cells from the same population meaning it is internally controlled and the time points act in part as replicates, showing largely consistent trends across time points and H_2O_2 concentration. For each time point of each H_2O_2 concentration, over 6000 cells were traced and the standard error of the mean is presented (Figure 6-6).

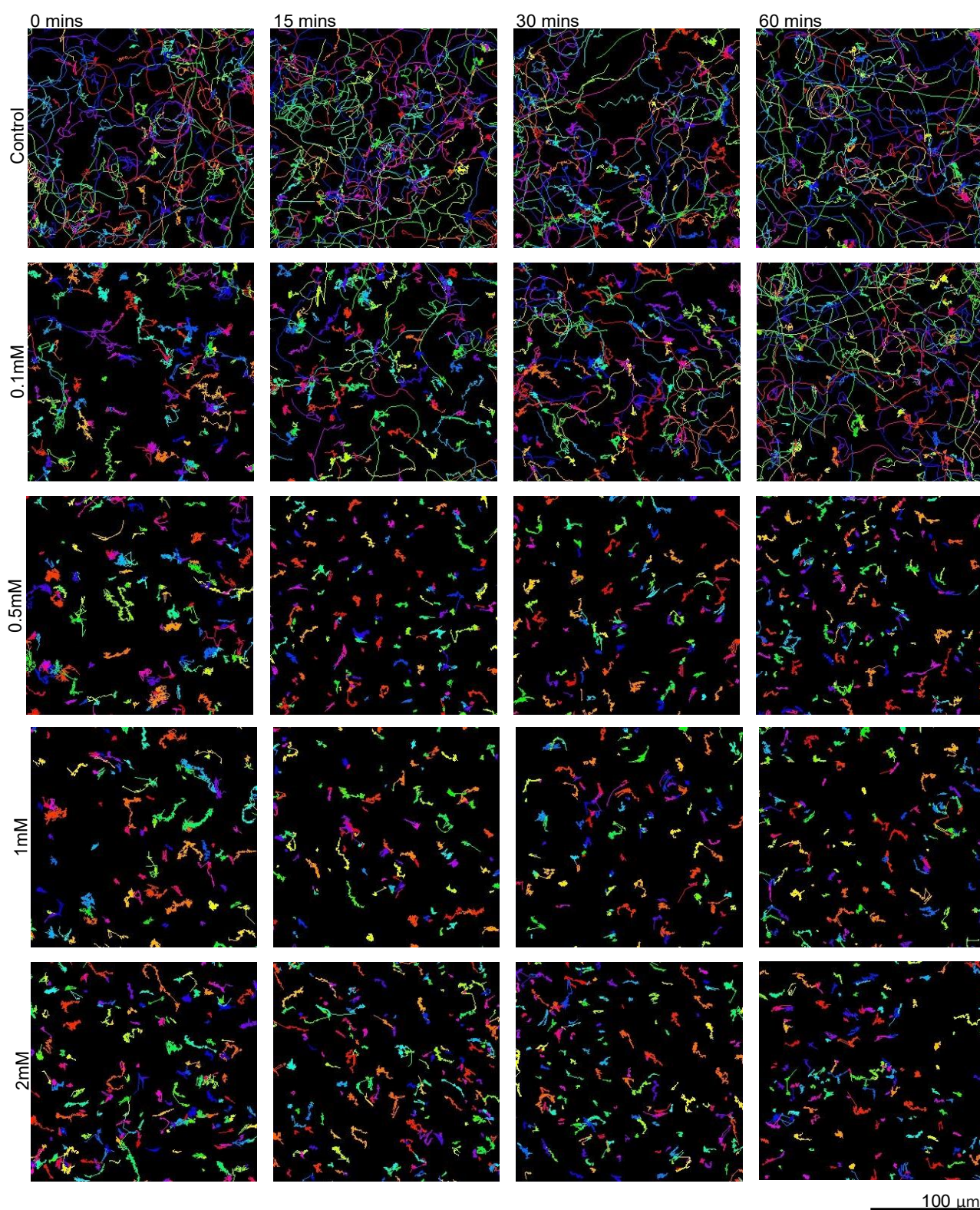


Figure 6-7 | Cell swimming is altered by H_2O_2 exposure. Cell path maps of Cas9T7 showing traced cells at varying H_2O_2 concentrations over time for a portion of the FOV for the full duration of the 10 $\times$ dark-field 5 Hz image sequences. Each trace, presented in different colours, represents a single cell. The scale is indicated by a scale bar.

6.2.5 TXNs are encoded by gene arrays across trypanosomatids

TXNs are an important part of the redox metabolism pathway, therefore we wanted to investigate their role in redox recovery and whether they vary across trypanosomatids. A phylogenetic tree of TXNs in *L. mexicana*, *T. brucei*, *L. braziliensis* (MHOM/BR/75/M2904), *T. cruzi* (DM28 strain) and *C. fasciculata* was constructed (Figure 6-8). Both species of *Leishmania* have more TXN encoding genes than the *Trypanosoma* species. The single TXN of *C. fasciculata* is more closely related to the TXN1 of the various species, suggesting this is the original gene that duplicated in the trypanosomatid lineage, and later mutated and diverged in function to create expanded gene arrays.

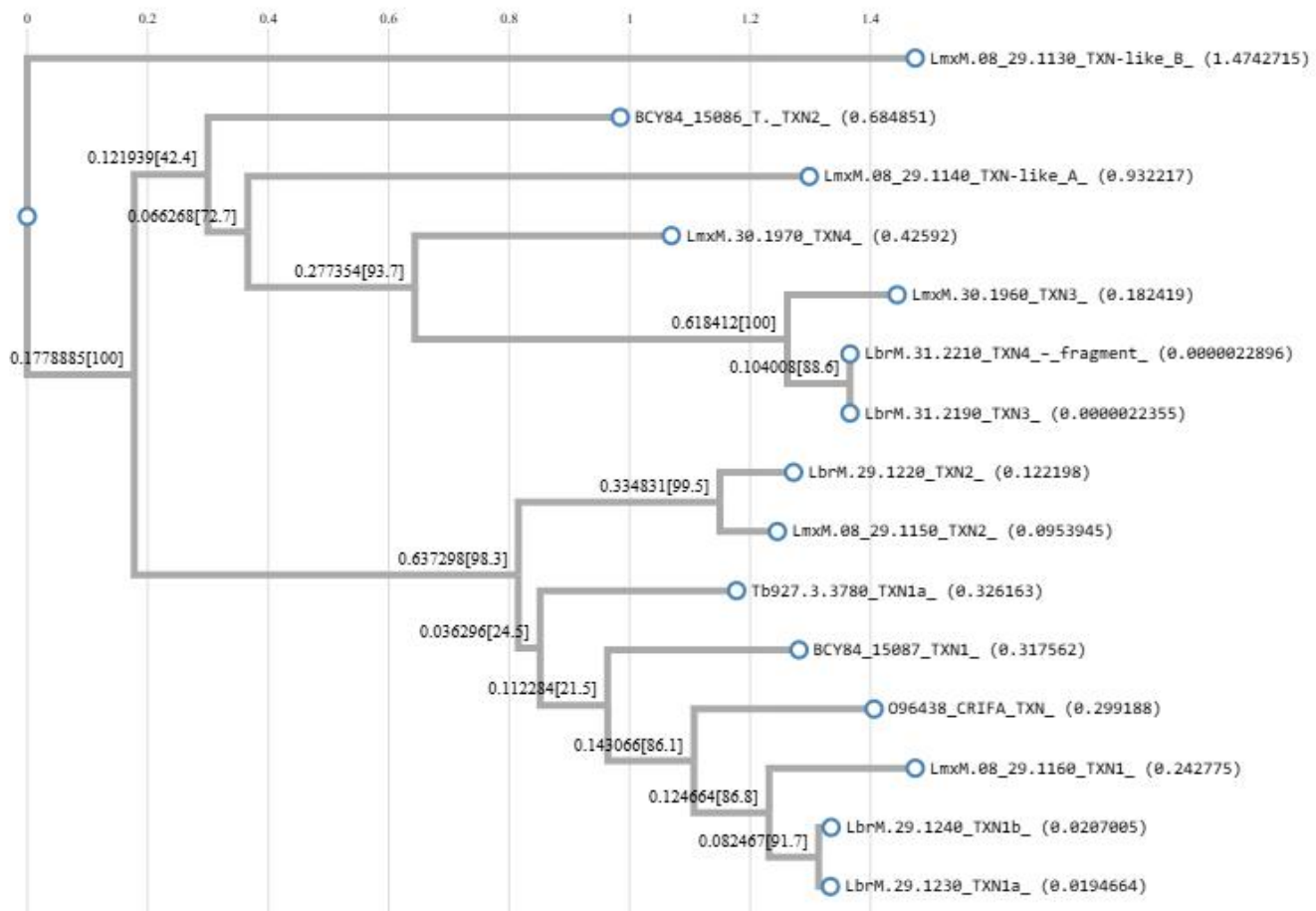


Figure 6-8 | TXNs have duplicated and evolved across kinetoplasts. The horizontal axis represents evolutionary distance as pertained by changes in sequence i.e. the substitution rate. The branch lengths are provided before nodes and in round brackets after gene ID and names. Bootstrap support values are presented in square brackets as a measure of confidence in the location of branching nodes representing the divergence of genes.

6.2.6 *Leishmania* possess TXNs with flagellar localisations

TXNs with flagellar localisation may be involved in redox-poise based regulation of motility. To test if *L. mexicana* possessed TXNs with flagellar localisation, TXN and TXN-like proteins were N-terminally tagged with mNG (Figure 6-9). The cellular landmark data from Halliday et al., 2019 was used to aid localisation interpretation. TXN1 and TXN2 had cytoplasmic and flagellar localisation, as seen in TXN1a and TXN1b of *T. brucei*. The pattern of signal putatively shows TXN-like A is localised to the mitochondrion, TXN3 and TXN-like B appear lysosomal and TXN4 is cytoplasmic with some vesicular enrichment.



Figure 6-9 | TXNs have diverse localisations. For each cell line the protein name and gene fusion are shown in the top left. Images are laid out in the following format: first column, an overlay of the mNG fluorescence (green) and Hoescht (magenta) over phase contrast (grey), second column, mNG fluorescence in greyscale.

6.2.7 TXN deletion does not significantly impact swimming speed under normal conditions

Given their flagellar localisation TXN1 and TXN2 deletions were generated. A TXN-like B deletion mutant was also engineered as this was the most evolutionarily divergent gene, which may indicate a specific purpose in *L. mexicana*. Swimming speed or behaviour were not altered significantly in these deletion mutants compared to controls under normal conditions (Figure 6-10).

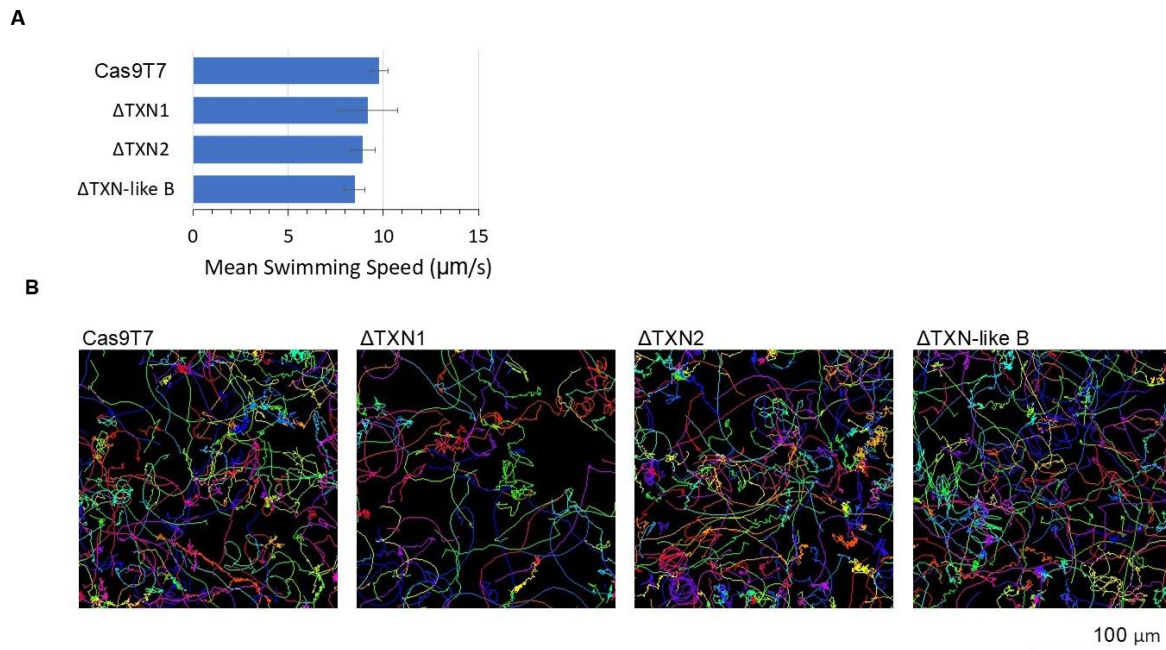
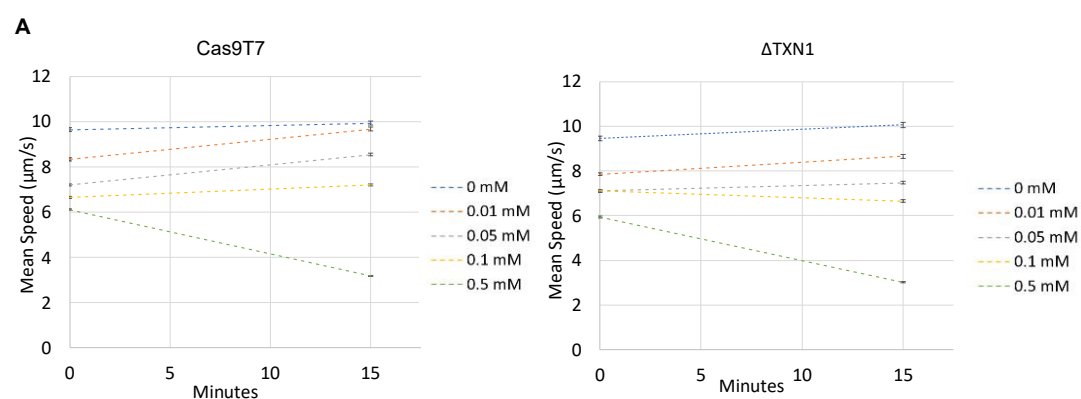


Figure 6-10 | TXN deletion does not affect swimming behaviour in normal conditions. A. Bar chart depicting the average swimming speed of Cas9T7 and the TXN deletion mutants. The error bars show the standard deviation of the six replicates. Each replicate comprises the average swimming speed of over 1000 cells analysed. For statistically significant differences (two-tailed T-test compared to the parental line, with $P < 0.05$) P-values are presented. B. Cell path maps of Cas9T7 and the TXN deletion mutants showing traced cells for a portion of the full FOV for the full duration of the 10× dark-field 5 Hz image sequences. Each trace, presented in different colours, represents a single cell. The scale is indicated by a scale bar.

6.2.8 TXN1 deletion may reduce recovery from oxidative stress-induced changes in swimming

To investigate if TXNs are involved in flagellar motility response to oxidative stress, a motility assay was completed on Cas9T7 and ΔTXN1 at a range of H_2O_2 concentrations up to 0.5 mM, as this concentration was previously shown to halt cell swimming, at 0 and 15 minutes after exposure (Figure 6-11). ΔTXN1 had reduced recovery of swimming speed at 0.01 mM, 0.05 mM and 0.1 mM compared to Cas9T7.



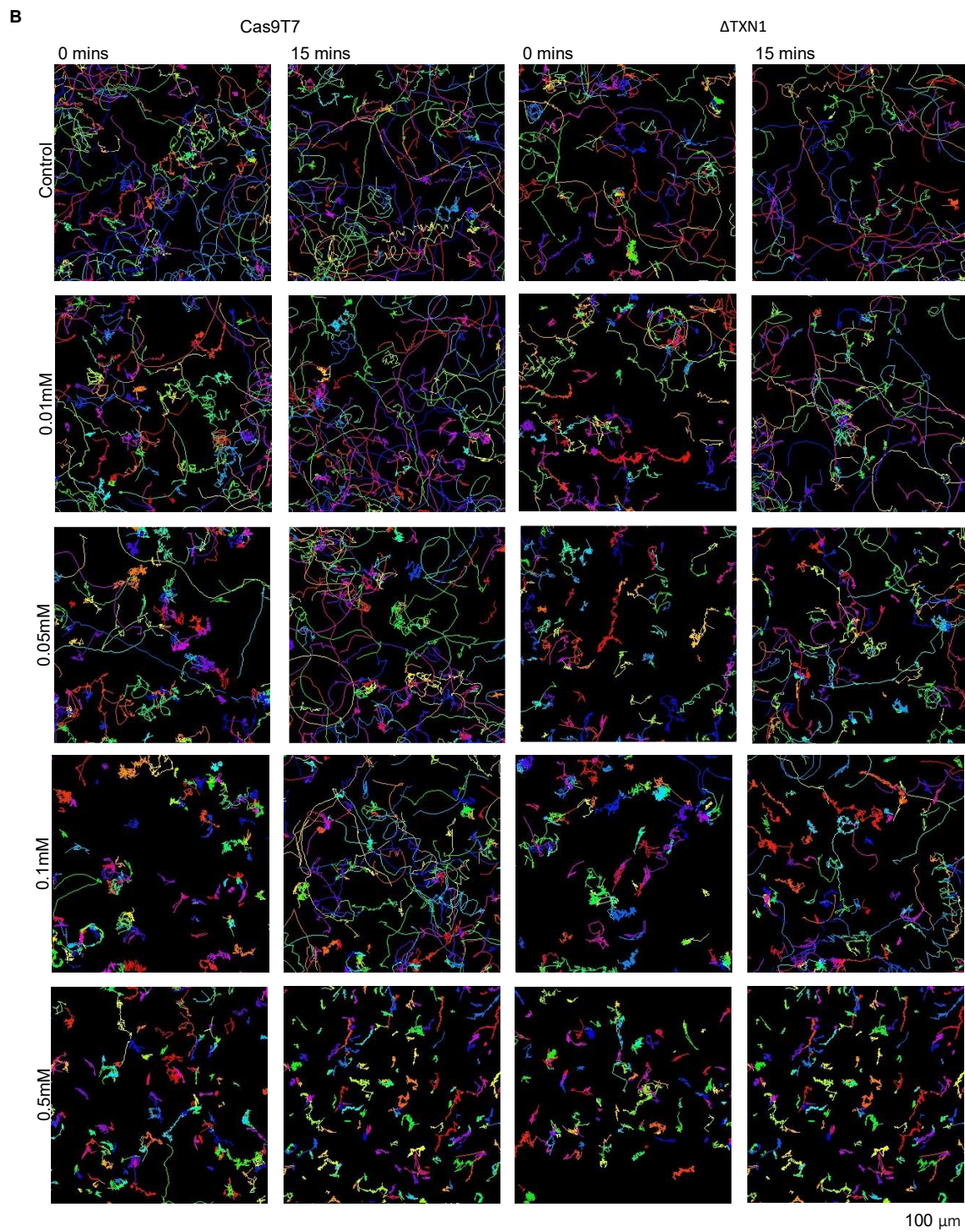


Figure 6-11 | TXN1 deletion may alter oxidative-stress induced changes in motility. A. Line graph showing mean swimming speed of cells at varying H_2O_2 concentrations over time in Cas9T7 and Δ TXN1 cell lines. Standard error of the mean is presented in vertical error bars that act as the markers for the sample; all standard errors are below 0.03. B. Cell path maps of Cas9T7 and Δ TXN1 showing traced cells at varying H_2O_2 concentrations over time for a portion of the FOV for the full duration of the 10 $\times$ dark-field 5 Hz image sequences. Each trace, presented in different colours, represents a single cell. The scale is indicated by a scale bar.

6.2.9 Genetically encoded calcium sensor visible at low frame rates in beating flagella

Understanding calcium ion activity in beating flagella is important in decoding its activity in beat regulation in response to environmental changes including redox. The ODAs are thought to be involved in calcium-based beat regulation so ODA component LC4-like was chosen for tagging with GCaMP3.

LC4-like::GCaMP3 cells were PBS-washed and adhered to glass slides for 63× epifluorescence imaging. The genetically encoded calcium responsive sensor was brightest in incompletely adhered cells and in cells with disrupted calcium regulation (such as the damaged cell pictured in the second row of Figure 6-12A) . Short, low frame rate videos were captured of LC4-like::GCaMP3 cells with free flagella, with a reference phase contrast image. From these, fluorescence can be seen in the flagella but it is subject to motion blur. When LC4-like::GCaMP3 were visualised at high frame rates the short exposure rates meant fluorescence emitted by GCaMP3 was too weak and not significantly detectable compared to the background.

Figure 6-12 | LC4-like::GCaMP3 signal is visible at low frame rates in beating flagella but not at high frame rates. A. Stills of LC4-like GCaMP3. Images are laid out in the following format: first column, an overlay of the mNG fluorescence (green) and phase contrast (grey), second column, phase contrast, third column mNG fluorescence in greyscale. B. Phase contrast reference photo of cell and frames 1-4 from a green fluorescence low frame rate video. C. Stills from high frame rate video of LC4-like::GCaMP3. From left to right: composite phase contrast and green fluorescence, green fluorescence, tangent angle kymograph showing successful tracing, fluorescence kymograph of straightened LC4-like::GCaMP3 signal which is not visible above the background.

6.3 Discussion

This chapter explored the interaction between light, redox and swimming and investigated the use of genetically encoded calcium sensors in beating flagella. Increasing light intensity during imaging caused an increased tendency for symmetric base-to-tip to asymmetric tip-to-base beat type switching. Given that changes in wavelength did not alter swimming behaviour and no obvious light sensitive domains were found in *L. mexicana*, this points to ROS generation as a byproduct of light exposure being the underlying mechanism. Therefore, suggesting that in *L. mexicana*, modulating redox-poise of the flagella cytosol could be a key mechanism utilised to control beat type switching and flagellar motility.

The effect of high intensity light exposure on beat type was ablated in an SMP1::KillerRed cell line. One explanation could be that KillerRed was causing low levels of ROS generation under ambient lab light which the cell acclimatised to or there could be a threshold concentration or timescale over which ROS generation must occur for beat type switching which KillerRed disrupted. An alternative explanation is that the generation of ROS by KillerRed is highly localised to where the protein is activated by light, with ROS being short lived and remaining close to their site of generation. In SMP1::KillerRed, KillerRed is localised to the membrane, therefore it absorbs light and locally produces ROS at the flagellar membrane, potentially reducing light exposure and/or ROS production specifically in other areas which would ordinarily be involved in redox-based regulation of flagellar motility. In the future, generation of cell lines with KillerRed tags localised on axonemal skeleton structures, and with alternative tags known to have lower cytotoxicity i.e. mCh could further decode this result.

H₂O₂ induced oxidative stress caused swimming speed to be reduced in a time and dose dependent manner. Previous research in *L. donovani*, has shown that at 1.0 mM, H₂O₂ only significantly affected mitochondrial membrane potential and ATP production after 240 minutes exposure. If this is the case in *L. mexicana*, it suggests that changes in motility during the first 60 minutes at concentrations of H₂O₂ at 1.0 mM or lower are independent of ATP production. Instead an alternative mechanism of flagellar motility regulation in response to ROS exist.

This could be directly due to ROS-mediated oxidative modifications to proteins. ROS can react with biomolecules and generate acidic byproducts; the consequent drop in intraflagellar pH could influence the pronation state of acidic residues of proteins on the radial spoke head and alter its interaction with the central pair. In *Chlamydomonas*, mutations to acidic residues at the centre of the negatively charged acidic head disrupted swimming, showing their function is essential to correct flagellar beating (Grossman-Haham et al., 2021). There are a multitude of other possible ROS-mediated pathways that could be involved in controlling cell swimming, including ROS-mediated calcium signalling or protein activation, however decoding them all will require a volume of investigation outside the scope of this project.

Previous studies have shown that upregulation of trypanothione peroxidase increased H₂O₂ metabolism and conferred resistance to antimonial therapeutics (Wyllie et al., 2008). The results from our study putatively suggest that TXN1 could be involved in mediating recovery of swimming phenotype in the context of H₂O₂ induced ROS generation, through maintaining a reduced environment. TXN1 and TXN2 are part of a gene array and both have flagellar localisation, if they have overlapping antioxidant function then TXN1 deletion would cause an insufficiency phenotype, not deletion phenotype. Therefore, repeating this assay on a cell line with deletion of both alleles of TXN1 and TXN2 may yield a stronger phenotype and give further insight into redox-based regulation of flagellar beating.

The sandfly midgut epithelial cells produce ROS during some parasitic infection however not to *L. mexicana*, therefore reduced swimming speeds in response to ROS production is unlikely to be a major

mechanism for adhesion of the *L. mexicana* to the epithelial lining and allow colonisation (Diaz-Albiter et al., 2012). However, reduced swimming on encountering increased oxidative-stress may guide cells away from ROS-rich areas. Additionally, by reducing metabolic activity related to flagellar movement *L. mexicana* cells might reduce intracellular ROS production to avoid damage until their homeostatic mechanisms overcome accumulation. In response to oxidative-stress, such as that induced by host macrophages, *L. donovani* elevates cAMP, which binds to PKA to regulate expression of antioxidant genes (Kumar et al., 2021b). PKA activity has also been shown to directly alter flagellar beating (Vassella et al., 1997; van den Abbeele et al., 1995). It is possible that the recovery in swimming deficits seen in this project after exposure at the lower H₂O₂ concentration is due to cells overcoming ROS accumulation using a combination of redox metabolism pathways.

Visualisation of calcium flux in a beating flagellum would give insight into the role of Ca²⁺ in flagellar motility. We began initial investigations into the feasibility of this by tagging ODA component LC4-like with genetically encoded calcium sensor GCaMP3. We found that the signal brightness was visible in slow frame rate videos but not at higher frame rates. Additionally, although GCaMP3 has a relatively fast response to calcium, it has relatively slow dissociation kinetics (Tian et al., 2009). More sensitive for visualising calcium ion activity on the timescale of a single beat cycle a faster more sensitive GCaMP calcium indicator will be required (Zhang et al., 2023).

7 Perspectives and conclusions

7.1 Introduction

Cilia and flagella are nearly ubiquitous across eukaryotes, with IFT being indispensable for their assembly and maintenance in most organisms. *L. mexicana* is a trypanosomatid parasite whose single motile flagellum, present in promastigote form, is essential for infectivity, both during migration through the sandfly and in the human host. This project used advanced microscopy and novel image analysis pipelines to explore key aspects of IFT and flagellar motility. Notably, high-frame-rate, dual-colour microscopy enabled the visualisation of IFT trains in the beating flagellum of *L. mexicana*, opening new avenues for exploring dynamic interactions within the flagellum that could not be addressed by prior microscopy methods dependent on mechanically or genetically immobilising flagella. The findings in this thesis provide novel insights into the complex relationship between the structure and function of these two motor protein driven systems which underpin flagellar functioning.

7.2 Considerations of experimental methodology

The traditional methodologies of visualising IFT rely heavily on immobilised or genetically paralysed flagella. Immobilisation via PBS washing and adherence to a glass slide significantly reduced IFT train speed in a time-dependent manner, with retrograde trains most severely affected. Adhesion also caused stop-start stalling of IFT trains that we did not observe in free flagella (Figure 3-6). These effects are likely due to substrate interaction involving the adhesion of flagellar membrane proteins to the glass slide, combined with the metabolic effects of being in nutrient-poor PBS. Conversely, genetic paralysis of flagella, via PF16 deletion, resulted in increased IFT speeds compared to its 3×mNG::IFT172 parental control (Figure 3-2); however, IFT train speed still decreased over the window of observation (Figure 3-3). These findings underscore key experimental considerations, including the need to

minimise immobilisation time in the case of mechanical immobilisation and to acknowledge the impact of genetic paralysis on IFT and interpret IFT train speed data accordingly.

Any methodology or critical tool in experimental biology has challenges, limitations and biases that need to be understood and considered to make meaningful biological conclusions. For high-frame rate dual-colour imaging these arose largely from the steps taken to increase fluorescence signal enough to visualise IFT at high frame rates required to capture an unblurred flagellum during beating. Firstly the use of brighter fluorescent markers (mNG and 3xmNG). In order to maximise brightness we utilised 3xmNG where possible, however this large endogenous tag did cause a significant decrease in flagellum length and IFT train speed. This highlights the importance of consistency in the use of fluorescent marker tagging to ensure valid controls.

In addition, exposure to light is a necessary evil for visualising IFT using genetically encoded fluorescent markers, both in adhered and in beating flagella – however the intensity of this was increased when visualising IFT in swimming cells to allow increased fluorescence signal generation. In Chapter 4, we quantify that the increased light intensity used in dual-colour high-frame rate epifluorescence microscopy causes altered flagellar motility compared to lower intensity transillumination used for phase contrast microscopy. As quantified in Cas9T7, this was an increased frequency of symmetric tip-to-base to asymmetric base-to-tip beat type switching – the biological ramifications of this phenomenon are discussed later in this chapter. Unexpectedly tagging membrane protein SMP1 with KillerRed ablated the switch from symmetric tip-to-base to asymmetric base-to-tip. This could be due to localising ROS generation to the membrane, a threshold above which ROS generation causes faster swimming rather than slower or cell adaptation to higher ROS levels. Nevertheless it suggests that the effect of high intensity light may be different across a cohort of differently tagged cell lines.

On an experimental level, this is important to consider as a possible inducer of bias into our reporting of time spent in beat types and likely underlies any discrepancies between changes in beat type and changes in swimming speed and behaviour observed with low magnification darkfield microscopy.

We found cell swimming behaviour, especially in mutants is nuanced and was understood through engagement with cell path maps, swimming speed and in the case of complex motility disruptions through the proportion of time spent in each beat type. For example, Δ dDC2 and Δ LC1 both have reduced swimming speed (Figure 4-3, Figure 4-4) however the speed changes are a result of biologically different processes. Δ dDC2 cells more frequently switch to asymmetric beating, while Δ LC1 cells have slower symmetric beating (Figure 4-6 - Figure 4-8). In addition, these assays are important for understanding diversity of cell behaviour within the sample, such as for H₂O₂ motility assays where cells have differing severity of swimming deficit or recovery (Figure 6-7, Figure 6-11). Largely the behaviours of cell lines that have been previously characterised are as expected i.e. Δ PF16 were paralysed and Δ LC4-like had higher symmetric base-to-tip beat frequencies though we did note a different phenotype for LC1 deletion than observed in *T. brucei* (Beneke et al., 2019; Gadelha et al., 2007; Wheeler, 2017).

In this research, both immobilised and swimming IFT train speed analysis was used. Obtaining large sample sizes (>100) of IFT trains, especially in the retrograde direction, can be time and labour intensive in swimming cells – with restriction points on sample size during both capture and analysis, whilst doing so in immobilised cells is much less challenging. As scientists, we have limited time and resources, even more so during a DPhil, therefore it is important to allocate these in a meaningful way. Completing swimming assays to detect changes in swimming behaviour and visualising IFT in adhered flagella can be used to identify the cell lines in which visualising IFT in swimming cells will be biologically meaningful.

The interactions between flagellar beating, cell swimming and IFT train speed is undoubtedly a complex and nuanced system. This study showed that though some cells showed increased IFT train

speed compared to controls of a similar magnitude in adhered and beating flagella, others did not. For instance, deletion of putative track protein LmxM.34.3925 led to a small but significant decrease in anterograde train speed in adhered and swimming cells (Figure 5-5). Whilst the results for MPK-based IFT regulation were more nuanced, with differences in reported IFT train speed changes in adhered and free flagella MPK9 and MPK14 suggesting their regulation of IFT is beat driven (Figure 5-11). This highlights the importance of visualising IFT in flagella which are free to move if it is likely to obtain more biologically meaningful results.

7.3 Biological findings: IFT train speed, flagellar motility and their interaction

We demonstrated that analysing IFT in flagella free to undergo their natural beating behaviour is important in answering decades old questions on IFT behaviour. For the first time we were able to define the nature of IFT train movement in beating flagella which was largely similar to that of adhered flagella but faster and with an altered pattern of train stalling. Additionally, quantifying IFT in motile flagella enabled the investigation of the interaction between IFT train speeds with beat type switching allowing the exclusion of several hypotheses about beat-type-IFT co-regulation. It also permitted further investigation of indirect interactions between the sets of motor proteins driving IFT and beating, the nature of IFT track and IFT regulators. Overall we have discovered a complex relationship between flagellar motility and IFT train speed (Figure 7-1), highlighting the importance of visualising IFT in free flagella to more accurately recapitulate the dynamic system of motile flagella.

Mutant Cell Line	Change compared to parental cell line		
	Flagellar structure	Flagellar beating & cell swimming	IFT train speed in swimming cells
mNG::IFT172	None	None	None
3×mNG::IFT172	Shortened flagellum	Slower swimming	Decreased anterograde and retrograde train speed
3×mNG::IFT172 ΔPF16	Altered presence and orientation of central pair microtubules, shortened flagellum	Paralysed flagellum, low to no swimming	Large increase in anterograde and retrograde train speed
3×mNG:IFT172 ΔH3	None	None	None
3×mNG:IFT172 ΔdDC1	Missing ODAs in distal flagellum, shortened flagellum	Less time spent in symmetric base-to-tip beating, slower swimming	Increased anterograde and retrograde train speed
3×mNG:IFT172 ΔdDC2	Missing ODAs in distal flagellum	Less time spent in symmetric base-to-tip beating, slower swimming	Increased anterograde and retrograde train speed
3×mNG:IFT172 ΔLC1	None	Low frequency tip-to-base as dominant beat type, slower swimming	Increased anterograde and retrograde train speed
3×mNG:IFT172 ΔOADβ	Missing ODAs, severely shortened flagellum	Largely uncoordinated flagellar movement, slower swimming	Increased anterograde train speed
3×mNG:IFT172 ΔRSP4/6	Missing radial spoke head, shortened flagellum	Largely uncoordinated flagellar movement, slower swimming	Increased anterograde and retrograde train speed
mNG:IFT172 (puro) ΔLC4-like	Longer flagellum	Higher frequency symmetric tip-to-base beat	Decreased anterograde and retrograde train speed
mNG:IFT172 (neo) ΔLC4-like	None	Higher frequency symmetric tip-to-base beat, faster swimming	Decreased anterograde and retrograde train speed
3×mNG::IFT172 ΔLmxM.34.3925	None	None	Small decrease in anterograde train speed
3×mNG::IFT172 ΔLmxM.30.0890	None	None	N/A
3×mNG::IFT172 ΔMPK9	Longer flagellum	None	Increased anterograde and retrograde train speed
3×mNG::IFT172 ΔMPK13	Longer flagellum	Increased swimming speed	Increased anterograde and retrograde train speed
3×mNG::IFT172 ΔMPK14	None	None	Increased anterograde and retrograde train speed

Figure 7-1 | Summary table of changes in flagella structure, swimming behaviour and IFT train speed in *L. mexicana* deletion mutants.

7.3.1 IFT system

IFT trains stalling has been observed in adhered flagella and paralysed flagella, and in *Chlamydomonas* plays a role in gliding motility through substrate interaction, however, if train stalling occurs in beating

flagella was unknown. In adhered flagella of *L. mexicana* we observed stalled trains, however no gliding motility. We found that IFT trains can stall in beating flagella without contact with a substrate, however that the stalling tends to be for longer periods of time (>4.75s) and occurs half as frequently as in adhered flagella (Figure 3-6). Motor proteins hydrolyse ATP and convert it into movement producing a force to step along the microtubule, if cargo load or resistance exceed this force then the motor protein stops and eventually dissociates, *in vitro* this was 6-7 piconewtons for one kinesin (Svoboda and Block, 1994). Therefore, it may be possible that interaction of the IFT train with surrounding structures, for example the IFT-A subunit in anterograde trains which is pressed against the membrane with transmembrane proteins or excessive cargo could result in stalling and eventual dissociation in a free flagellum.

Our data suggests proximal-distal asymmetry in anterograde IFT train speed (Figure 4-13). If true, this would support the existence of two kinesin proteins with different speeds and processivities as in *C. reinhardtii*. Further experiments should be completed to confirm this – namely the tagging of TbKin2a and TbKin2b orthologues in *L. mexicana* with different fluorescent markers to measure speed and observe if a ‘handover’ is visible.

Previous research has found IFT train routes along microtubules to be organised, including through the separation of anterograde and retrograde transport onto the B-microtubules and A-microtubules in *Chlamydomonas* and the restriction of IFT to two sets (3 to 4 and 7 to 8) of microtubule doublets in *T. brucei* (Stepanek and Pigino, 2016; Bertiaux et al., 2018). However, the mechanisms underlying this have not been characterised. In this project, we explored the possibility that a set of microtubule binding proteins could contribute to forming IFT train tracks.

In humans and *L. tarentolae*, external MAP CFAP97 is distributed on the outer face of the microtubule doublets at the junction between the A and B microtubules (A10 and B01) from the transition zone to the flagellar tip (Leung et al., 2023; Doran et al., 2025). We showed that its deletion reduces anterograde train speed without significantly affecting flagellum length (Figure 5-7). Its conserved

localisation suggests it has a key role in microtubule stability – could it be important for defining the bi-directional railway tracks of IFT?

Some SHIPPO-domain containing proteins for example the CMAP1 family (Coy et al., 1999; Leung et al., 2023; Egydio de Carvalho et al., 2002), have PGP motifs approximately every 40 residues creating an 8 nm repeats which corresponds both to the average kinesin and dynein step size and to the tubulin dimer repeat, making it possible for them to be involved with IFT track formation. We found 28 PGPXY repeat containing LmxM.05.0690 was localised to the basal body or transition zone (Figure 5-3). This could be as a “molecular ruler” as suggested for *T. brucei* orthologue TZP250 (Dean, 2016). Given that, IFT trains dock along the transition zone for assembly prior to flagellar entry, it could also have a role in IFT motor protein binding. Visualising co-localisation in a dual tagged 3xmNG::IFT172 and mCh::LmxM.05.0690, and investigating the impact of LmxM.05.0690 deletion on cell morphology and on IFT movement with 3xmNG::IFT172 tagging could further elucidate this. The PGP-domain containing protein, LmxM.34.3925, was found to localise along the length of the flagellum, with its deletion causing a small but significant decrease in anterograde IFT train speed (Figure 5-5). Is it possible that the proximal-distal asymmetry in anterograde IFT train speeds is driven by two kinesins with differing processivity, with their handover being guided by the asymmetric distribution of MAPs, such as those containing the SHIPPO domain, that influence microtubule binding?

Though it leaves us with many questions, our data suggest that the binding of MAPs to the outer face of the microtubule doublets are important for correct IFT function. This could be through enhancing motor protein binding to tubulin binding sites or to selectively block specific binding sites to organise IFT train movement (Leung et al., 2023). Given that the effect size of deletion of each MAP on IFT train speed is small - it is likely that if these microtubule binding proteins are responsible for directing IFT train traffic by designating a route of specific binding sites for motor proteins - they do so in concert. Investigating the distribution of IFT and putative tracks within the flagellum through dual tagging of

IFT or track proteins and a protein with known asymmetric distribution such as SPEF1 or PRF1 with mCh (Edwards et al., 2018), could yield further insights into their specific roles.

In humans and *Chlamydomonas*, IFT dyneins have intermediate chains with distinct functions however to our knowledge only one intermediate dynein chain has been annotated in trypanosomatids (Hamada et al., 2018; Asante et al., 2014). Our data suggests that LmxM.18.1460 is a novel IFT dynein intermediate chain (Figure 5-2). We did not find it possible to delete this protein putatively suggesting it could be required for survival, for example due to being a subunit required for the functioning of other dynein proteins— however, it could also just be a challenging protein to delete. The effect of LmxM.18.1460 loss could be investigated using knockdown models to further characterise its role in IFT to investigate if dynein intermediate chains have distinct functions in *L. mexicana*.

Several kinases are known to alter IFT train speed in mammalian cells and MPKs have been shown to influence flagellum length in *Leishmania*. However whether any MPKs interact directly with IFT in *Leishmania* was unclear. We found three MPKs, MPK9, MPK14 and MPK13 which act as negative regulators of IFT train speed (Figure 5-11). Their disparate localisation, effect swimming speed and flagellum length suggest they likely have distinct mechanisms of action and phosphorylation targets (Figure 5-9 - Figure 5-11). For example, data suggests that MPK13 is a negative co-regulator, either directly or indirectly, of IFT train speed, flagellum length and swimming speed, as all increased when the protein was deleted. Whilst MPK9 deletion has no effect on swimming speed, it did increase flagellum length and IFT train speed, which may underlie its importance in sandfly colonisation (Baker et al., 2021). MPK14 deletion had no effect on swimming speed or flagellum length, only on IFT train speed. The conserved function and structure of mammalian MAK, MOK and CILK with MPK9, MPK13 and MPK14 suggests that MPKs are key players in regulatory pathways which are fundamental for eukaryotic cilia/flagella. In addition, conserved kinase structure raises the possibility of conserved kinase target sequences. Completing targeted mutagenesis of conserved serine/threonine phosphorylation sequences from known MAK, MOK and CILK targets in the *L. mexicana* IFT particle or

IFT associated protein and measuring the effect on IFT train speed may be a way to investigate this further.

7.3.2 IFT speed and flagellar motility

Although flagellar beating and IFT occur over vastly different timescales, namely one beat cycle over tens of milliseconds (Friedrich and Riedel-Kruse, 2014) compared to several seconds taken for a train to travel the axoneme (Santi-Rocca et al., 2015), both arise from the action of motor proteins using ATP hydrolysis to move along the axonemal microtubule doublets (Webb et al., 2020). However, their interaction had not yet been investigated.

Given their spatial proximity and shared environment it is plausible that the motor proteins driving IFT and flagellar beating would experience co-regulation. We found that IFT train speed was not strongly dependent on natural switches in flagellar beat (Figure 3-8), suggesting beat type switching does not underlie flagellum length changes. However, genetic disruption of normal flagellum beating did result in significant changes in the speed of IFT trains (Figure 4-9C, Figure 4-12C). In slower-swimming mutants anterograde IFT train speed was increased, independent of ODA presence or absence, whilst in faster swimming mutants retrograde train speed was decreased. Overall it suggested that IFT train speed changes were driven by effects on flagellar motility rather than the severity of structural changes to the axoneme, though the two are clearly linked. Previous studies in sea urchin sperm have supported that ATP dephosphorylation in motile flagella is coupled to flagellar movement, with ATP consumption having a linear relationship with beat frequency (Brokaw and Benedict, 1968; Chen et al., 2015; Gibbons and Gibbons, 1972). Given catalytic inactivity of flagellar glycolytic enzymes in trypanosomatids, which putatively act as an intraflagellar ATP source in *Chlamydomonas*, diffusion of ATP into the flagellum is likely the only ATP source. Collectively this indicates competition for ATP as an energy source as the dominant effect behind the broadly inverse relationship between IFT train speed and the rate of flagellar movement. With kinesin and dynein motor proteins driving anterograde and retrograde trains respectively having nuanced responses to ATP availability.

One exception to this inverse relationship was seen with MPK13 deletion wherein a faster swimming cell had faster IFT train speed (Figure 5-9 - Figure 5-11), though this was likely a result of the loss of phosphorylation-based negative regulation on IFT trains having a stronger impact than the relative decrease in ATP availability. MPK13 may provide weak negative co-regulation of IFT and flagella beat frequency— though not in the context of beat type switching. There is also a possibility that MPK9 and MPK14 are flagellar motility-dependent regulators of IFT train speed – given that their deletion caused an increase in IFT speed in swimming cells but not in adhered cells. IFT is required for maintaining asymmetric distribution of ODAs (Fort et al., 2024; Edwards et al., 2018). Could MPK-driven changes in IFT speed be responsible for dynamic axoneme restructuring underlying beat regulation in the context of environmental adaptation? Further research into the up- and down-stream components of their signalling pathways would be required to investigate this hypothesis.

7.3.3 Flagellum length, IFT and swimming

Interaction between flagellum length, IFT and swimming is complex and not fully understood. In this project we observed that despite possessing faster IFT train speeds, the flagella of Δ dDC1, Δ OAD β , Δ RSP4/6 and most notably Δ OAD β were significantly shorter (Figure 4-2). Both LC4-like and MPK13 deletion caused an increase in flagellum length and swimming speed, however they had opposite effects on IFT speed (Figure 4-12, Figure 5-11). Across the cohort of deletion mutants in this study there was no meaningful relationship between the mean anterograde ($r_s=-0.09$, $n=15$, $p>0.05$) or retrograde ($r_s=0.1$, $n=15$, $p>0.05$) train speed and mean flagellum length, however both display large variability. This agrees largely with previous literature. Though one study in *Chlamydomonas* found, IFT train size, frequency and speed inversely proportional to flagellum length, another study found that flagellum length was not coupled to IFT (Bauer et al., 2021; Engel et al., 2009). In *T. brucei* after flagellum assembly arresting IFT did not alter flagellum length and largely it is the amount of IFT proteins that correlated with flagellum length rather than number of trains (Bertiaux et al., 2020). Given that the flagella we are visualising are free and therefore fluorescence may be captured from

slightly varying focal depths with different amounts of motion blur depending on velocity of movement of that section of the flagellum, our methodology is not appropriate for accurately measuring the amount of IFT train proteins. However, we can say that IFT train speed was not the dominant force driving flagellum length, and is more likely to be microtubule stability.

There was a moderate positive correlation between flagellum length and swimming speed ($r_s=0.49$, $n=15$, $p<0.05$) across the deletion mutant cohort. Metacyclic promastigotes have longer flagella than procyclic promastigotes and have faster swimming speeds, so the swimming speed is potentially a result of a longer flagellum length. Longer flagella have increased Ca^{2+} channels which have been suggested as mechanism for kinase-mediated signalling pathways which interact with beat frequency (Beck and Uhl, 1994; Rosenbaum, 2003). Alternatively, higher frequency swimming and increased shear stress on the flagellar membrane could influence microtubule stability and alter the flagellum balance point. During their life cycle *Leishmania* are required to swim through fluids of varying viscosities, including the extremely viscous biofilm in the fly mid-gut. Flagellum length modulation via shear stress or changes in medium viscosity is seen in various ciliated or flagellated mammalian cells in the form of membrane-receptor mechanosignalling transduction through adenylate cyclase-cAMP-PKA and/or PKC-MPK mediated pathways (Davies, 1995; Abdul-Majeed et al., 2011). Either way, the investigation of kinase signalling, flagellum beating and flagellum length may be an interesting area for future study given the importance of parasite motility for infectivity and transmission.

7.3.4 Flagella beat and swimming regulation

Despite its popularity as a research subject, the exactly mechanisms driving and regulating flagellar beating remain largely unknown. A wide range of mediators have been proposed, including calcium signalling, kinases and phosphatases, mechanical feedback between axonemal components, cellular stress, and responses to environmental stimuli. It is likely that the true mechanism is a complex system integrating many of these factors.

In phototactic organisms, motile flagellar beating can be modulated by light. In *L. mexicana*, we found no obvious light sensitive molecules in *L. mexicana*, and light wavelength did not affect swimming behaviour. Therefore, the effect of high intensity light exposure on cell swimming behaviour is likely a result of ROS generation as a by-product. Exposure to H_2O_2 , influenced swimming in a time and concentration dependent manner. Above 0.5 mM H_2O_2 cells stopped swimming and remained largely immobile with no forward swimming (Figure 6-6). Previous research in *Leishmania* has shown that mitochondrial membrane potential and ATP production was only affected after 240 minutes exposure at 1.0 mM or higher (Mukherjee et al., 2002). This suggests in our experiments ROS-mediated regulation of motility occurs independently of ATP production, possibly through oxidative modifications to flagellar proteins. At very low concentrations of H_2O_2 (0.01 mM to 0.1 mM), cells initially experienced a reduction in swimming speed, but recovered partially within 15 minutes suggesting that cells are able to adapt to ROS inducing environments putatively through upregulating proteins involved in maintaining a reducing environment. The deletion of TXN1, a trypanothione with flagellar localisation, caused a small reduction in swimming recovery, although this is just an initial assay (Figure 6-11). Given that TXN1 is part of a gene array with TXN2 that has similar localisation, it may be an insufficiency phenotype and the assay should be repeated on a mutant with deletion of TXN1 and TXN2 to assess if these proteins are working in concert to manage flagella redox poise. Overall, this suggested that the effect on swimming from strong light intensities are due to be ROS generation as a byproduct of light exposure and that redox poise may be important in modulating flagellar motility in *L. mexicana*.

Given the importance of Ca^{2+} and calcium dependent signalling in IFT and flagellar beating, visualising calcium in a beating flagellum would be an important step towards decoding flagellar motility regulation. Several components of the ODA have been shown to interact with calcium, therefore we tagged ODA light chain LC4-like with genetically encoded calcium sensor GCaMP3. We found that fluorescence was visible in partially adhered flagella, and flagella of cells whose calcium regulation was disrupted. At low frame rates (2 Hz) calcium signal was visible in beating flagella, however at

higher frame rates (>100 Hz) the signal from GCaMP3 was too low to be detectable above background in beating flagella. This raises the possibility of using GCaMP3 at low frame rates for deciphering any overall differences in signal brightness, equating to calcium levels, during different beat types however excludes the possibility of use to detect calcium flux over the course of a beat cycle. For this a brighter, faster responding fluorescent sensor will be required.

7.4 Avenues for future research

7.4.1 Microscopy optimisation and multi-focal plane imaging

During our research we observed that high-intensity light may interact with flagellar motility. Therefore, the use of an alternative light source, such as an LED to reduce cellular stress and heat generation with possible wavelength selection and pulse modulation for targeted excitation of fluorophores could reduce light-induced effects on cell motility at high magnifications and decrease photobleaching. Although the impact of this on the short 4.5s videos used for IFT visualisation in beating flagella might be incremental, it could be combined with a higher sensitivity camera sensor to allow for better IFT visibility even with mNG, a fluorophore potentially less disruptive to IFT train movement. In the future, high-frame rate dual-colour, multifocal plane imaging, made possible by replacing fixed focal length lenses in the optical pathway with electrotunable lenses to allow rapid refocusing between frames, could provide the opportunity to visualise fluorescently tagged proteins in live beating flagella in three dimensions.

7.4.2 Genetically encoded fluorescent sensors

Many important findings in this project related to the structural and functional roles played by proteins in the flagellum and collectively pointed to the importance of small signalling molecules including ATP, cAMP, Ca^{2+} and ROS in modulating IFT and flagellar beating function. Therefore, utilising high-frame rate dual-colour microscopy to elucidate the localisation and abundance of such molecules in a flagella undergoing different beat types or in various deletion mutants could provide

unprecedented insight into the nature of regulatory networks in the flagellum in real time during beating. Initial investigations into the use of calcium sensor GCaMP3 (Figure 6-12) suggest doing this will require sensors with brighter signal and faster on-off kinetics. For example GCaMP3_{fast} has a 40-fold faster decay of 3.3 ms which would theoretically be fast enough to capture calcium flux in flagellar beating at 25 Hz (40 ms). However, faster decay also means less time for photon emission per calcium flux so this needs to be balanced with camera sensor sensitivity to ensure signal will be above background. Therefore, although a wide range of ultrasensitive biosensors exist for small cations and anions (Chen et al., 2013; Subach et al., 2020; Barykina et al., 2017; Subach et al., 2023; Shen et al., 2019; Torres Cabán et al., 2022), ROS (Marchetti et al., 2023), ATP (Lobas et al., 2019) and cAMP (Massengill et al., 2022), finding fluorescent sensors that are bright enough and have fast enough on-off kinetics to produce a signal that can be visualised in a beating flagellum will likely require much optimisation.

7.5 Conclusion

This thesis explored the nature of IFT and its interaction with flagellar motility using unflagellate parasite *L. mexicana* as a model of flagellar motility. Through the use of advanced microscopy techniques, including high-frame-rate dual-colour imaging, the project overcame limitations of traditional immobilisation methods and provided novel insights into IFT dynamics in freely beating flagella. The findings showcase the importance of studying IFT in its natural context to uncover the nuanced interplay between motility and IFT train speed. We observed that IFT train speed was largely independent of beat type, that genetic perturbations to flagellar motility influence IFT and that IFT trains can stall in freely beating flagella, albeit less frequently than in adhered flagella. We identified key proteins that play a role in IFT system architecture and regulation – some of which appear to be highly conserved from trypanosomatids to mammalian cells. Finally we began initial investigations into the action of light, redox and calcium in the context of flagellar motility and cell swimming. Future

studies involving multi-focal plane imaging and real-time visualisation of signalling molecules using biosensors could further elucidate the regulatory networks governing IFT and flagellar function.

8 References

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