Motility of microorganisms in microfluidic geometries

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December 2024

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

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Abstract (English)

Biological microswimmers, both eukaryotic and prokaryotic, exhibit a variety of swimming patterns in response to external cues to thrive in their natural habitats. In confined spaces or near boundaries, they respond with altered behavioural patterns, underscoring their adaptability for survival. Microbial responses to environmental stress are shaped by factors such as resource foraging, competition with other microorganisms, and ecological dynamics. Among these microorganisms, Euglena species are a suitable model due to their diverse motility modes, such as flagellar swimming and bodily deformations (metaboly or euglenoid movement). Euglena exhibits versatile responses under stress, such as transitioning from helical swimming to polygonal spinning under sudden increases in light intensity or effective crawling under severe confinement. These traits make euglenids ideal candidates for investigating motility and behavioural responses. Understanding their adaptations aids in gaining deeper insights into their evolutionary dynamics and behavioural response to external stressors, with implications for the development of applications, including biocomputation, soft robots, and biomedical applications. To progress the understanding of space searching in confined geometries, we studied their behaviour in microfluidic structures presenting varying confinements and geometries.

The first part of the work aims to probe motility patterns and behavioural responses of two euglena species, *E. gracilis* and *E acus,* in microfluidic-based structures with varying confinements and geometries. In highly confined environments, both species altered their motility patterns, switching from helical swimming to either back-and-forth motility or cell deformations, also known as metaboly. Additionally, this study examines the correlation between different confinements and swimming efficiency and factors that influence behavioural responses. We also report the structure that the cells were previously subjected to has a temporary impact on the swimming patterns of the cell, indicating prior structural exposure was found to transiently influence subsequent swimming patterns.

The second part of the work analyzes flagellar beat patterns and behavioural responses such as metaboly initiation, escape strategies, and avoidance responses under varying confinements. We observed drastic modulations in flagellar beat patterns and distinct changes in the behaviour of the cell when confined. The flagellum undergoes a series of

shape modulations, enabling the cell to regulate the flagellum beating and consequently select the most efficient responses reflecting on the changes in behaviour to free itself from tight spaces. The findings from this study indicate that metaboly is associated with the compromised flagellum, providing insights into cellular response, metaboly initiation, and the role of flagellar flexibility and behavioural adjustments. How unicellular microbes such as euglena use the flexibility of their flagellum and body to select an optimal response to escape and navigate through confined space shows the intricacies of their morphological plasticity.

The findings from motility and behavioural studies advance the understanding of underlying characteristics of flagellar motility and how euglena leverages flagellar and body flexibility to optimize navigation and escape responses. The study provides a foundation for future exploration, critical for developing templates for network-based biocomputation, soft robotics inspired by bio-adaptability, and biomedical applications.

Abstract (French)

Les micro-nageurs biologiques, qu'ils soient eucaryotes ou procaryotes, présentent une variété de modèles de nage en réponse à des signaux externes afin de prospérer dans leurs habitats naturels. Dans les espaces confinés ou à proximité de frontières, ils réagissent en modifiant leur comportement, ce qui souligne leur capacité d'adaptation pour survivre. Les réponses microbiennes au stress environnemental sont déterminées par des facteurs tels que la recherche de ressources, la compétition avec d'autres microorganismes et la dynamique écologique. Parmi ces micro-organismes, les espèces d'Euglena constituent un modèle approprié en raison de leurs divers modes de motilité, tels que la nage flagellaire et les déformations corporelles (métabolie ou mouvement euglénoïde). Euglena présente des réponses polyvalentes en cas de stress, comme la transition de la nage hélicoïdale à la rotation polygonale en cas d'augmentation soudaine de l'intensité lumineuse ou la reptation efficace en cas de confinement sévère. Ces caractéristiques font des euglènes des candidats idéaux pour l'étude de la motilité et des réponses comportementales. La compréhension de leurs adaptations permet de mieux comprendre leur dynamique évolutive et leur réponse comportementale aux facteurs de stress externes, avec des implications pour le développement d'applications, y compris la biocomputation, les robots mous et les applications biomédicales. Pour mieux comprendre la recherche d'espace dans des géométries confinées, nous avons étudié leur comportement dans des structures microfluidiques présentant des confinements et des géométries variables.

La première partie du travail vise à sonder les modèles de motilité et les réponses comportementales de deux espèces d'euglènes, E. gracilis et E acus, dans des structures microfluidiques présentant des confinements et des géométries variables. Dans des environnements très confinés, les deux espèces ont modifié leurs schémas de mobilité, passant d'une nage hélicoïdale à une mobilité de va-et-vient ou à des déformations cellulaires, également connues sous le nom de métabolie. En outre, cette étude examine la corrélation entre les différents confinements et l'efficacité de la nage, ainsi que les facteurs qui influencent les réponses comportementales. Nous rapportons également que la structure à laquelle les cellules ont été soumises précédemment a un impact temporaire sur les schémas

de nage de la cellule, indiquant qu'une exposition structurelle antérieure a influencé de manière transitoire les schémas de nage ultérieurs.

La deuxième partie du travail analyse les schémas de battements flagellaires et les réponses comportementales telles que l'initiation du métabole, les stratégies de fuite et les réponses d'évitement dans des conditions de confinement variables. Nous avons observé des modulations drastiques dans les rythmes flagellaires et des changements distincts dans le comportement de la cellule. Le flagelle a subi une série de modulations de forme, permettant à la cellule de réguler le battement du flagelle et par conséquent de sélectionner les réponses les plus efficaces reflétant les changements de comportement pour se libérer des espaces restreints. Les résultats de cette étude indiquent que la métabolie est associée au flagelle compromis, ce qui permet de mieux comprendre la réponse cellulaire, le déclenchement de la métabolie et le rôle de la flexibilité flagellaire et des ajustements comportementaux. La façon dont les microbes unicellulaires tels que l'euglena utilisent la flexibilité de leur flagelle et de leur corps pour sélectionner une réponse optimale afin de s'échapper et de naviguer dans un espace confiné montre les subtilités de leur plasticité morphologique.

Les résultats des études sur la motilité et le comportement permettent de mieux comprendre les caractéristiques sous-jacentes de la motilité flagellaire et la manière dont euglena tire parti de la flexibilité de son flagelle et de son corps pour optimiser ses réactions de navigation et de fuite. L'étude jette les bases d'une exploration future, essentielle au développement de modèles pour la biocomputation en réseau, la robotique douce inspirée par la bio-adaptabilité et les applications biomédicales.

Acknowledgements

Completing a Ph.D. is a monumental achievement, and I still find it hard to believe that I have reached this milestone. I want to raise a toast to everyone who has been a part of this incredible journey with me.

Firstly, I would like to express my sincere gratitude and humbly thank my Ph.D. supervisor, Professor Dan V. Nicolau, for giving me the opportunity to join his research group as a master's and Ph.D. student. His exceptional academic expertise, invaluable guidance, dynamic vision, and dedication have inspired me to develop analytical skills, learn new techniques, and carry out my research explicitly.

I would also like to sincerely thank my Ph.D. Advisory committee members, Prof. Danilo Bzdok, Prof. Andrew Kirk, and Prof. Adam Hendricks for their valuable guidance and feedback. Thank you for your time, effort, and thought-provoking questions which have improved the quality of my work. I would like to extend my thanks to Dr. Ayyappasamy, for his guidance and support. I would like to thank my teammates and peers for their support and encouragement. Many thanks to the administrative team in the Biological and Biomedical engineering department; especially Pina Sorrini and Sabrina Teoli for all the support and help. I would like to thank the Bioengineering Department; Melissa Vitaro, Nicholas Milette, Rola Chbaklo, Horia Roman, Xavier Elisseeff, for all the wonderful support and guidance. I would like to thank my friend Monalisha for her constant encouragement, phone calls, and endless conversations over numerous cups of coffee. She has been there for me since day one as a support system and a great friend.

I would like to extend my deepest gratitude to McGill University and the Department of Bioengineering for providing me with the opportunity to pursue my academic aspirations and for the invaluable support and resources throughout my journey. I thank the Faculty of Engineering for awarding the McGill Engineering Doctoral Award (MEDA) and many recognitions as fellowships such as George Stinnes Fellowship in Engineering, Grad Excellence Award in Engineering, J W McConnell Memorial Fellowship, in support of my graduate studies. I would like to thank TISED for Mike Zahn fellowship. This has helped me lighten my financial burden and provided encouragement to focus on my academic pursuits.

I extend heartfelt appreciation to the University for their dedication to fostering academic

excellence and empowering students worldwide by providing opportunities to achieve their academic goals. My heartfelt gratitude and many thanks to the Graphos 3MAYT team.

I would like to take a moment to express my deep gratitude and appreciation to my mother, Punitha, father, Rajendran, and my sisters Bhavs and Pals (lovingly referred to as thangachi papas) for being my pillars of support throughout my academic journey. Their unwavering love, encouragement, and sacrifices have been instrumental to my success. I would like to thank my beautiful baby girl Hridha, whom I was pregnant with during my defence and the last few months of my Ph.D. journey, she has been my sweet little cheerleader. I am also extremely grateful to my husband, Venkatesh, for being my source of strength and motivation. He has provided me with constant encouragement during the successful and stressful moments of this journey. I would like to thank everyone whose name isn't mentioned here but has supported me throughout and made this a smooth experience, I want you to know that your support has been invaluable to me. I am grateful to all the people and experiences that have inspired and motivated me to pursue science. Finally, I would like to thank myself for believing in my abilities, never giving up, and discovering my inner strength along this journey. Thank you all from the bottom of my heart and I hope to continue to grow and contribute to the scientific community.

Contribution to original knowledge

Motility patterns of Euglena species in confining microfluidic geometries: The present work encompasses several elements that can be regarded as distinct contributions to knowledge and original scholarship. The novelty of this work lies in advancing the understanding of how various microfluidic-based geometrical confinements trigger distinct species-specific motility patterns and behavioural responses in euglenids. The motility studies on euglenids not only showed distinctive motility patterns in response to confined geometries but for the very first time, we report that the structure design and complexity impacted the trajectory of the cell. This work describes the ability of cells to develop modulations between flagellar swimming, back-and-forth motility, and metaboly showcasing an instance of behavioural adjustments in euglenids. We report a correlation between flagellar length and biological behaviours including scattering dynamics. While the majority of investigations concerning euglenids focus on *E. gracilis*, our study incorporates *E. acus* along with *E. gracilis* as model organisms. By examining the capacity of this species to change motility patterns and exhibit behavioural responses, our research broadens the scope of inquiry and identifies opportunities for the exploration of additional species such as *E. acus* in various research applications. Insights from this study will provide a template to develop and fabricate microfluidic structures for applications critical to developing biocomputation and further extendable to bio-inspired soft-robots, and biomedical applications.

Flagellar shape modulations and behavioural changes to confinement in Euglena gracilis: This study advances in understanding how motility responses triggered by confinement impact the flagellar beat patterns and how this was integrated into the overall behaviour and biological responses of the organism. We report that the flagellum undergoes a series of shape modulations. By undergoing drastic shape changes, the cell may choose an optimal response to free itself from physical barriers or tight spaces, enabling it to resume a free-swimming state (helical, flagellar swimming). These modulations reflect a complex interplay between the physiology of the cell, its surrounding milieu, and the need for efficient motility. Additionally, we also report that metaboly (movement coupled with bodily deformations) is a response associated with an impaired/compromised flagellum. This

finding brings us one step closer to comprehending metaboly initiation and represents a significant advancement in our understanding of cellular responses to external stressors. The behavioural responses enhance our understanding of metaboly initiation and cellular responses with a new perspective on their role in motility. The findings of this study can contribute to our broader understanding of the underlying characteristics and strategies of flagellar motility, with potential implications to stimulate further research in this field.

Most biological response or motility studies in euglenids are often limited to studying them between two glass plates or under simple geometrical constraints, typically focusing on the effects of light, chemicals, or gravity as external stressors. While these studies provide valuable insights, they fall short of examining motility and behavioural changes at the organismal scale. In this work, we employ a microfluidics-based design to evaluate how varying degrees of confinements govern motility and behaviour in euglenids offering a reproducible method to study their responses. This thesis encompasses the body of work I have contributed to throughout my Ph.D. journey.

Contribution to Authors

Thesis contribution to the current state of knowledge

Kavya Rajendran, Gala Montiel Rubies, Ayyappasamy Sudalaiyadum Perumal, Karine Baassiri, Dan V. Nicolau, *Motility patterns of Euglena species in confining microfluidic geometries***. First author**, in preparation to be published. D.V.N. designed the research and contributed to writing the manuscript; K.R. planned and optimized the experiments, carried out the device fabrication, carried out the experiments, performed the data analysis and result interpretation, and wrote the manuscript. G.M.R performed the statistical analysis, A.S.P. provided the device designs, and contributed to SEM imaging, K.B. contributed to scaling up the designs. D.V.N. provided resources.

Kavya Rajendran, Ayyappasamy Sudalaiyadum Perumal, Dan V. Nicolau, *Flagellar shape modulations and behavioural changes to confinement in Euglena gracilis* **First author**, in preparation to be published. D.V.N. designed the research and contributed to writing the manuscript; K.R. planned and optimized the experiments, carried out the device fabrication, carried out the experiments, performed the data analysis and result interpretation, and wrote the manuscript. A.S.P. provided the device designs, D.V.N. provided resources.

Kavya Rajendran, Ayyappasamy Sudalaiyadum Perumal, Dan V. Nicolau, *Proof-of-concept: Photosynthesis-driven biological computation*. D.V.N. and team designed the research; A.S.P. fabricated initial devices and guided the initial experiments, K.R. fabricated the master's and replicated structures for experiments, optimized device setup, modification of designs, carried out the preliminary experiments, and performed data analysis. D.V.N. provided feedback to shape the research. This is in the preliminary phase of experimentation. The research is focused on establishing foundational methodologies and validating hypothesis. While promising results have been observed, additional experiments are required to advance towards a fully developed manuscript.

Viola Tokárová, Ayyappasamy Sudalaiyadum Perumal, Monalisha Nayak, Henry Shum, Ondrej Kašpar, **Kavya Rajendran**, Mahmood Mohammadi, Charles Tremblay, Eamonn A.

Gaffney, Sylvain Martel, Dan V. Nicolau Jr, and Dan V. Nicolau, Proceedings of the National Academy of Sciences. (2021*). Patterns of bacterial motility in microfluidics-confining environments 118* (17).

D.V.N. designed the research; V.T., A.S.P., M.N., H.S., O.K., M.M., C.T., and D.V.N. Jr. performed research; S.M. contributed new reagents/analytic tools; V.T., A.S.P., M.N., H.S., O.K., K.R., E.A.G., D.V.N. Jr., and D.V.N. analyzed data; and V.T., A.S.P., M.N., H.S., O.K., and D.V.N. wrote the paper.

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SUPPLEMENTARY INFORMATION

Chapter I

1. Introduction

Microorganisms are remarkable in their ability to survive and thrive in a wide range of environments. One of the most important traits is their motility, which enables them to carry out a variety of essential biological processes, from moving towards resources and colonizing, to the dispersal of progeny and escaping hostile environments, motility is key to their survival. Microorganisms are in general sub-ten microns in size and their swimming is fundamentally governed by fluid dynamics and influenced by their surroundings. Swimming in low Reynolds numbers has allowed them to develop sophisticated self-propulsion mechanisms to navigate and thrive in their natural environment. Most microorganisms use thin hair-like appendages such as flagella, cilia, or pili as primary structures for motility. These structures have been extensively studied in both prokaryotes and eukaryotes to uncover the underlying principles of microbial physiology, biophysical mechanisms, and navigation strategies for applications in, biosensing, biocomputation, biomimetics, disease control, selective breeding, and cell sorting. In nature, microorganisms are capable of performing versatile feedback control strategies by changing their motility behaviour in response to environmental stimuli. For instance, bacteria navigate to chemical gradient cues [1] by alternating between running and tumbling states [2]. *Chlamydomonas reinhardtii* uses its flagella to transition between positive and negative phototaxis [3-7]. Euglenids respond to a number of external stimuli such as phototaxis [8-10], gravitaxis [11-13], chemotaxis [13], and mechanical responses [14] to orient and survive in their natural habitat. Apart from light or chemical gradients, boundaries and confined spaces trigger intriguing behavioural responses among microswimmers [15-19]. In the presence of a wall or a physical boundary, the motility behaviour of microswimmers is highly influenced by several factors including hydrodynamics, steric effects, mechanical forces, and biological responses. Based on these factors a microswimmer (depending on the swimming type) either swims in closer vicinity to the wall (wall accumulator) or away from the wall (wall escaper) [20]. Alternatively, several studies reported this type of swimming behaviour in sperm cells [15, 21-23], bacteria [20, 24-26], and ciliates [27, 28]. *How the*

microswimmers develop their feedback mechanism to stimuli and control their motility accordingly is still poorly understood.

Euglenids are a diversified family of photosynthetic, unicellular, flagellates that mainly habitat aquatic ecosystems, brackish water, and damp soil. They are one of the earliest reported microorganisms [29] and occupy a center stage in research among eukaryotes due to their wide range of applications in multiple disciplines including biophysics [30-32], biochemistry [30, 33-35], nutraceuticals [36], biotechnology [37], biocomputation [38, 39]. Despite their potential and established applications, the general behaviour of euglenids is largely unknown. Euglenids are eukaryotic microorganisms that exhibit complex and diverse swimming behaviour, the flagellar swimming, propelled by the flagellum and metaboly, elegantly coordinated bodily deformation also called euglenoid movement [40-42]. The earliest observations of euglenids and metaboly date back to the first microscopists [29, 43], and it has been an intriguing phenomenon ever since. Despite numerous research studies, the functional retention and precise mechanism of this phenomenon remain elusive. These complex biological response or motility studies in euglenids are limited to studying them between two glass plates or simple geometrical constraints [14]. Moreover, understanding motility and behavioural changes associated with the biology of the organism at the organismal scale is not only challenging but also demands reproducible methods to obtain accurate results. Thanks to the state-of-the-art microfluidic technologies which provide an excellent test bed to design and develop new methodologies to study behavioural patterns of biological microswimmers. This work is focused on studying the motility patterns, the effect of confinements on the swimming efficiency of the cell, and behavioural responses of photosynthetic *Euglena species*. The goal is to understand the motility patterns and behavioural changes of euglenids in confined spaces preliminarily for the design and development of microfluidic-based biocomputational devices employing euglenids as biological agents and further extendable to biologically inspired soft robotics and biomedical applications. Sequentially operating electronic computers can efficiently solve several tractable problems in polynomial time, however, are unsuitable for solving combinatorial (particularly NP-complete) problems. These problems have an exponential number of solutions to be explored by brute force, thus demanding unreasonable computation time for conventional electronic computers that can only operate sequentially. Additionally, the

complexity of combinatorial problems translates to ever-increasing energy consumption by electronic computers, making the computing process highly unsustainable energy-wise. Consequently, the development of efficient, parallel computation strategies that employ scalable processors/agents to solve these combinatorial problems is necessary. Previously proposed parallel computation techniques using biological agents such as Physarum [44], DNA [45-47], and molecular motor proteins [48], suffered from critical limitations such as scalability issues, demand for impractical, large amounts of computing agents, and experimental difficulties in handling [49]. Employing self-propelled, replicating (scalable), microorganisms as biological agents, particularly *Euglena species,* to explore physical microfluidic network encoding instances of NP-complete mathematical problems is a potential alternative. The features of Euglena based biocomputation such as replication, Floating-point Operation Per second (FLOPS), and error rate are discussed in the biocomputation chapter. To employ euglenids as biological agents for biocomputational applications it is critical to first understand their intricate motility patterns and the complexity of their behavioural responses in microfluidic structures. Aiming at the objective of the study the following primary aims are designed. The first work is focused on studying the motility patterns of euglenids in microfluidic-based geometrical confinements with varying complexities. The motility studies will aid in advancing our understanding on, species-specific motility patterns in confinements, spatial distribution in channels, swimming efficiency in terms of velocity, rate of success, and time, and characteristics of swimming patterns. It is important to consider how the motility responses of euglenids impacted the flagellar beat and how this was integrated into the overall behaviour of the cell. Therefore, the second work is focused on investigating the flagellar beats, behavioural responses and, escape strategies adapted by cells under complex and simple confinements. Modulation of flagellar beat patterns according to the type of confinement, flagellum and body shape modulations, and factors triggering metaboly are studied to obtain critical insights into the underlying characteristics of flagellar motility. The findings from motility and behavioural studies will aid in domains such as bio-inspired soft robotics, developing a methodological template critical to the design and development of biocomputational devices and further extendable to domains such as bio-inspired soft robotics, cell sorting, biomedical applications.

2. Background

2.1 Motility in microorganisms

Motility enables movement towards resources, colonization, dispersal of progeny, pathogenicity escape from hostile environments, and is key for survival in most microorganisms. Most microorganisms use thin hair-like appendages such as flagella, cilia, or pili for motility.

"No more pleasant sight has ever yet come before my eye than these many thousands of living creatures, seen all alive in a little drop of water, moving among one another, each several creatures having its own proper motion." He goes on to say "…I can make out the paws too, which are also pleasant to behold, because of their swift motions. We see those little living animals whose paws we can distinguish, and estimate that they are more than ten thousand times thinner than a hair of our beard… but I see… animalcules which are yet more than a hundred times less, and on which I can make out no paws… I am persuaded that they too are furnished with paws withal. [29]"

(From Antonie van Leeuwenhoek's 18th letter to the Royal Society on observations made on 6 August 1676 of "little eels" from a flask of water containing pepper, as translated by Clifford Dobell)[29]

The "paw" in this context is the flagellum, the little living animals whose paws were distinguishable were protozoans and the ones whose paws were not distinguishable were bacteria.

Besides the thinness and length of the flagellum, there are more striking features such as wave propagation, rotation, structure, composition, and energy source that remained an open question. Although the flagellum was identified in the 1600s the answer to these questions, at least "broadly outlined" were obtained only in the late 1900s [50-53] with great advances

in bacterial motility witnessed in the past decade [54-57], however, to a vast degree remains unclear.

2.2 Motility in bacteria

2.2.1 Bacterial flagella architecture

Flagellum (plural – flagella) is a hair-like appendage present in a wide range of both prokaryotes and eukaryotes cells, it is a molecular machine that is used for locomotion. The flagellum significantly differs both structurally and mechanically between eukaryotes and prokaryotes. Bacterial flagella are long, rigid, helical structures composed of flagellin protein. The basic structure (Figure 1) consists of a basal body, hook (universal joint), and filament (propeller) [58, 59]. The basal body is a series of rings threaded by a rod and divided into two major parts, stator (remains stationary) and rotor (rotates). The hook is a short, highly curved, and flexible segment that connects the basal body and the filament. The filament is a rigid, helical macromolecular structure composed of the flagellin protein. The basal body is a rotary motor that enables the flagellum to rotate and propel. The rotation or movement of the rotary motor is powered by pumping proton – or – sodium ions creating electrochemical gradients across the cell membrane, the rotation of the flagellum is dictated by the movement of the rotor [60-62].

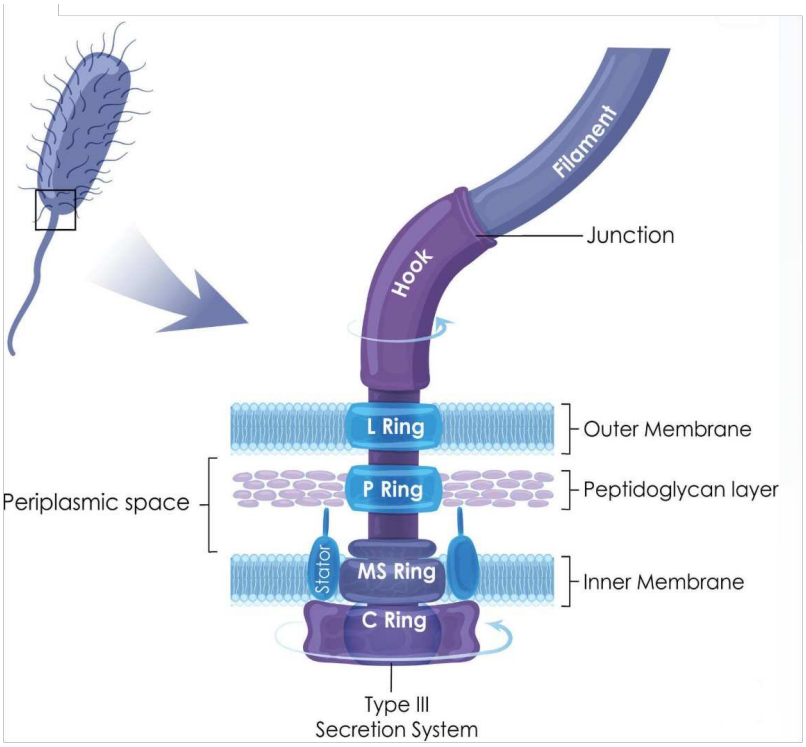

Figure 1: Illustration of the bacterial flagellum. The figure depicts the structural components of a bacterial flagellum, a helical propeller used for motility. It showcases, the basal body embedded in the cell membrane, the hook that connects the basal body to the filament, and the filament that enables locomotion. The flagellum operates via a rotary motor. Reproduced from vector art [63].

2.2.2 Flagellar arrangements

The flagellar arrangements differ between each bacterial species and are divided into six types (Figure 2): (i). Atrichous – no flagella, (ii). Monotrichous – a single flagellum at one polar end of the cell [64], (iii). Amphitrichous – one flagellum on each polar end of the cell [65], (iv). Lophotrichous – multiple flagella (tuft) present at one polar end of the cell [66], (v). Peritrichous – multiple flagella present all over the cell body [67], (vi). Cephalotrichous – multiple flagella present on both polar ends of the cell [68]. Bacteria swim by rotating their flagella, this movement can be broadly classified into two types, run; from counterclockwise rotation of the flagella and tumble; from clockwise rotation of the flagella. Run results in

pushing the bacteria forward and tumble results in erratic movement which mainly serves to reorient the direction for the next run. Bacteria can change their swimming direction simply but rotating their flagella counterclockwise or clockwise. Run-and-tumble series is a hallmark motility mechanism employed by bacteria [69] which dictates their spread and transport. As motility is directly related to virulence in virulent strains [70-72], there are studies particularly interested in understanding the run-and-tumble dynamics [69]. In addition to run-and-tumble, bacteria also adapts other motility patterns such as run-reverse and run-reverse-flick [73].

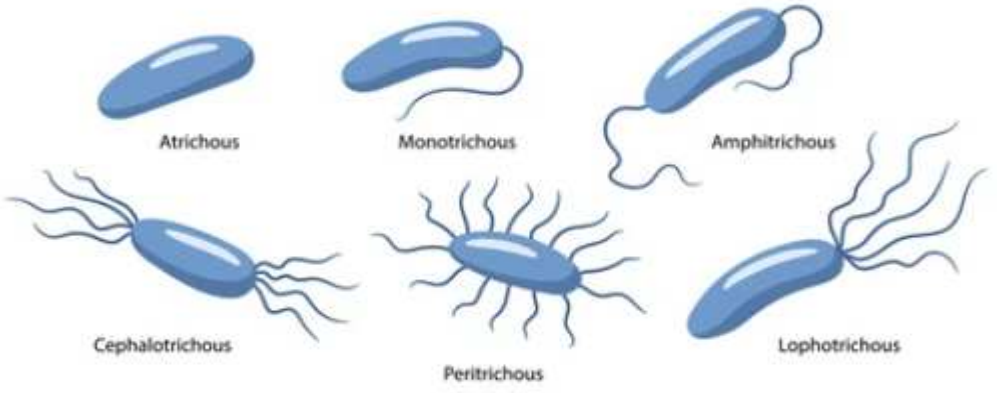

Figure 2: Flagellar arrangements in bacteria. This image illustrates the diversity of flagellar arrangements in bacteria, including monotrichous (single flagellum), lophotrichous (multiple flagella at one end), amphitrichous (flagella at both ends), and peritrichous (flagella distributed all around the cell). These arrangements support various modes of bacterial motility and adapt to diverse environmental conditions. The illustration highlights the structural and functional variations across bacterial species. Reproduced from Shutterstock[74].

Motility patterns in bacteria, although not mutually exclusive can be divided into several categories including, swimming, swarming, twitching, gliding, and sliding [75, 76]. Swimming is movement propelled by flagella typically in a liquid environment [77], swarming is a coordinated flagella-mediated surface migration of bacteria. Swarming involves intercellular interactions, surfactant secretions, and cellular differentiation into

elongation and hyper-flagellated phenotype [75, 78]. Twitching can be described as an uneven, surface-bound motility, occurring in both flagellated and non-flagellated bacteria, independent of flagella [75, 79]. The movement is unorganized and intermittent, cells predominantly move singly with occasional collective movement. Gliding is also a surface translocation occurring mainly in the non-flagellated bacteria, the movement is organized and smooth with whirls and bands by unknown mechanisms that involve adhesins to attach to a substratum [75, 80]. Sliding is more of a passive movement and occurs in a growing colony by expansive forces and bacteria's surfactants. The surfactants reduce the surface tension resulting in reduced friction between the cells and the substratum. The morphology appears like a uniform sheet of closely packed cells which moves as a unit, generally in a single-layer [75, 81].

2.3 Motility in Euglena

2.3.1 General structure

Euglenids or euglenoids are a diversified family of photosynthetic, unicellular, flagellates that mainly habitat aquatic ecosystems, brackish water, and damp soil. Euglenids are one of the earliest reported microorganisms [29] and occupy a center stage in research among eukaryotes due to their wide range of applications in multiple disciplines including biophysics [30-32], biochemistry [30, 33-35], nutraceuticals [36], biotechnology [37], biocomputation [38, 39], bio-regenerative life support in space [82]. They are typically cylindrical or spindle-shaped with rounded or tapered ends, the size varying between 5 to 150 microns in length, although some can be several hundred microns or longer.

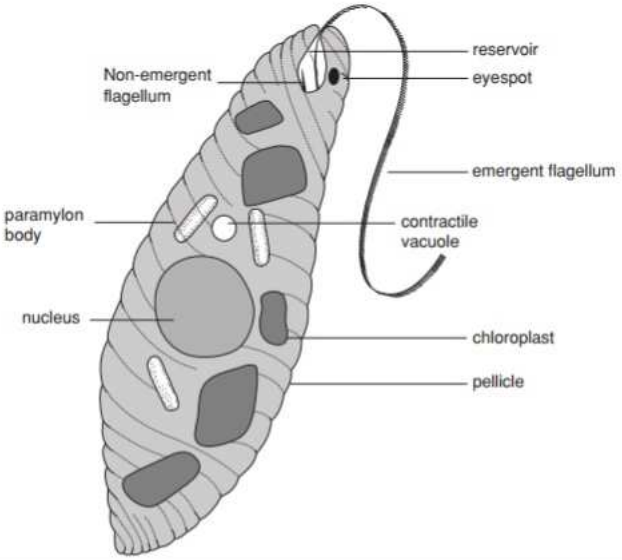

Figure 3: General structure of Euglena. Key features include two flagella, emergent and non-emergent, eyespot (stigma), which functions in phototaxis, chloroplasts, essential for photosynthesis, indicating autotrophic capabilities; pellicle highlights its ability to sustain dynamic shape changing. Additional organelles, such as the nucleus contractile vacuole, reflect the organism's complexity and ability to thrive in diverse environments. Reproduced from Akash education [83].

They are generally biflagellates, both the flagella originate from a small reservoir located at the anterior end, with one emergent and one non-emergent based on extrusion and retention respectively from the flagellar reservoir (Figure 3).

2.3.2 Euglenoid flagella architecture

A large variety of eukaryotic microswimmers are propelled by active flagellar structures, the beat patterns of the flagella (essentially helical and planar) are widely studied in eukaryotes including protists, ciliates, and sperm cells. The structure of the euglenoid flagellum comprises an evolutionarily conserved organelle, the axoneme as a central core. The axoneme comprises of "9+2" axonemal structure (Figure 4), nine cylindrically arranged microtubule doublets (peripheral) and an internal central singlet pair of microtubules [84, 85]. The internal pair is connected by radial spokes to the nine peripheral microtubule doublets, the nine doublets are linked by an inter-doublet link protein called nexin. The

dynein motor proteins form transient bridges between adjacent periphery microtubules causing microtubule sliding. Dynein hydrolyzes adenosine triphosphate (ATP) to generate forces and because sliding is constrained by the link protein nexin and the basal body the generated force is converted into the flagellar bending [86]. This complex, yet brilliantly orchestrated microtubule movement and motor protein activity are thought to self-regulate in euglenids based on the external mechanical feedback [87].

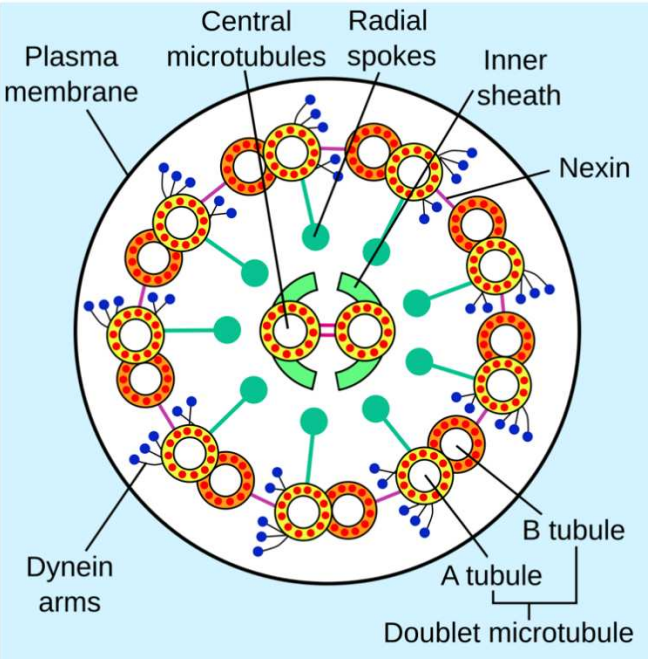

Figure 4: The figure illustrates the ultrastructure of a eukaryotic flagellum in cross-section, highlighting its characteristic "9+2" microtubule arrangement. The flagellum comprises nine doublet microtubules arranged in a circular pattern around two central singlet microtubules. Nexin links interconnect the doublet microtubules. Reproduced from smartse [88].

2.3.3 Flagellar swimming algorithm and response to stimuli

The flagellar swimming in euglenids is propelled by the lateral beating of the emergent flagellum. Bending waves emerge from the base of the flagellum rooted in the reservoir, through the length of the flagellum and fade at the tip. The flagellum bends while swinging the cell body around, entailing spiralling trajectories coupled to body rotations, thereby, the motility pattern is a characteristic sinusoidal path or a periodic swinging motion. The

swimming propulsion mechanism of the flagellum can be summarized as a complex sequence of non-planar waveforms with a characteristic movement mimicking a spinning lasso or figure “8” [31]. Microorganisms respond to environmental cues and actively seek most favorable conditions for growth, optimal metabolic activity, and survival. The ability to respond and navigate to various physicochemical gradients is called taxis.

Taxis are innate behavioural responses to external stimuli, largely observed in microorganisms and animals, some examples include moving in response to light – phototaxis, temperature – thermotaxis, chemicals – chemotaxis as well as magnetic and electric fields – magnetotaxis and galvanotaxis. Euglenids respond to a number of external stimuli to orient and survive in their natural habitat, namely phototaxis [8-10], gravitaxis [11-13], chemotaxis [13], and some mechanical responses [14]. Interestingly, they are unique in that they are both heterotrophic (derive nutrients and energy by consuming other organisms) and autotrophic (they contain chloroplasts and can produce their own food via photosynthesis). But being predominantly photosynthetic; to survive, in their natural habitat euglena exploit both phototaxis and gravitaxis to optimally position themselves to efficiently perform photosynthesis and avoid photobleaching. Their motility is often affected by these external cues or stimuli, several studies exploit this behaviour to understand biological algorithms in euglenids and for applications in multiple fields [11, 12, 33, 89, 90]. In our work, we exploit the innate ability of Euglena to swim without interference from such external cues to understand their motility patterns and behavioural responses in confined geometries which can be directly translated to employing them for biocomputational studies and also has potential applications in diverse fields.

2.3.4 Metaboly or euglenoid movement

Apart from flagellar swimming, euglenids are also capable of performing highly coordinated bodily deformations called metaboly or euglenoid movement [40-42]. The earliest observations of metaboly date back to the first microscopist’s [29, 43] and it has been an intriguing phenomenon ever since. Euglenoid movement or 'metaboly' is a unique, elegantly coordinated deformation supported by an active striated cell envelope, called pellicle (Figure 3,5) or pellicle strips [32, 91-93]. These epiplasmic pellicle strips serve as an elastic motor system to control metaboly. The active sliding of adjacent pellicle strips and self-regulation

of this elastic motor system is the operational principle of the peristaltic deformations [14, 92]. Depending on the species and the pellicle strip arrangements/features metaboly can range from gentle bending, and rounding, to large amplitude of highly coordinated peristaltic waves [93, 94]. Despite numerous research studies, the functional retention and precise mechanism of this phenomenon remain elusive. Additionally, although the morphogenesis of pellicle strips is highly studied, the correlation between pellicle strip's origin and metaboly is still intangible.

2.3.5 Pellicle – the machinery

Pellicles in euglenids are longitudinal proteinaceous, epiplasmic strips that extend along the length of the cell (Figure 5), this complex structure is underlain by microtubules and molecular motor proteins. Depending on the species, some have 12 or fewer strips making their pellicular structure rigid and others with 14-80 strips making them more flexible [95]. The pellicle is an active striated envelope which controls the cell shape and metaboly, however, the precise mechanism, function, and what triggers metaboly is still an open question.

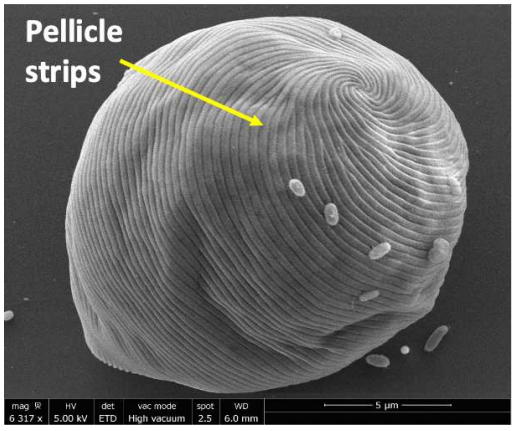

Figure 5: The figure showcases the pellicle strips of *Euglena gracilis* visualized through Scanning Electron Microscope (SEM) from our experimental sample. The pellicle, composed of interlocking proteinaceous strips, provides structural support and enables the organism's characteristic flexibility facilitating its unique mode of locomotion known as metaboly.

The pellicle strip sliding model has gained widespread acceptance in the field [96, 97], and other notable developments include the observation that cell volume does not change during metaboly [98]. Additionally, cells that were subjected to high confinement crawled by exploiting frictional propulsion showing that the pellicle is an elastic and extended motor system that, once biologically activated cells can use the pellicle deformations to self-adjust to the degree of confinement to produce an effective gait in order to escape. The adaptable mode of locomotion by mechanical self-regulation of the pellicle strips was observed in *Euglena gracilis* subjected to confinement [14].

2.4 Microfluidic technologies for research

Microfluidics is a multidisciplinary path that manipulates fluids typically in a sub-millimetre scale, ideally to rescale conventional laboratory analysis onto centimetre-level chip; lab-on-a-chip (LoC) for higher throughput, rapid detection time, and reduced sample volumes. This technology has demonstrated great potential in several research fields including point-of-care (PoC) diagnostics [99], microbiology [100], [101], cell biological analysis [102], food [103], chemical [104, 105] and mineral processing [106, 107] due to its size-matching effect with samples (biological, chemical, small organisms) and unique flow characteristics (laminar, enhanced surface properties and effects). Microfluidic technologies find a unique place in microbiome-relevant research and engineered systems, particularly because, they have great versatility in terms of design, material, and physical and chemical conditions [108]. As a result, microfluidic devices can reproduce different scenarios, including physical, chemical or biological variations that exist in natural environments. For example, simple linear channels [109-111], complex chips with mixing channels [112] [113], porous devices with topographic modifications to mimic obstacles or natural habitats like soil [114] [115, 116], droplet microfluidics [117-119], etc.

2.4.1 Motility and behavioural studies

Microfluidics allows the recreation of 3D microenvironments, which otherwise allows a more realistic study of certain bacterial phenotypes than traditional methods. The main

advantage is the ability to recreate biological environments more realistically than conventional macroscopic cultures in flasks, petri dishes, and well plates [120]. For instance, an extracellular matrix can be simulated by using three-dimensional cultures of microorganisms embedded in hydrogels or by creating a flow to mimic the interstitial fluid flow that carries nutrients and microbes. In order to examine adaptations to the changing environment, it is also possible to change the physical (rigidity, pressure forces, fluid flows, etc.) and chemical (pH, attraction and repulsion factors, etc.) circumstances [102, 121]. For example, the simplest devices consisting of linear channels have been used to track bacterial migration in response to tactic stimuli [122-126], analyze bacterial sensitivity to drugs or study biofilm formation [127]. More complex chips include mixing channels or those with more than one floor that allow greater control of conditions within the channels and/or the co-culture of various species of microbes [128]. In addition, the surfaces of these devices can be modified to fabricate non-flat channels, resulting in porous devices or topographic devices that recreate natural environments, such as soil, or obstacles with specific shapes[115, 116, 129-131]. Finally, droplet-based microfluidic devices isolate microbes on microdroplets, allowing single-cell studies mainly focused on testing drug resistance and biofilm formation. Together, microfluidics offers versatile approaches to studying multiple microbiological scenarios.

2.4.2 Biocomputation

Biocomputation is an interdisciplinary study of computer science, engineering and biology, the intersection of biological agents and computation offers an exciting avenue for innovation. The inherent and unique biochemical properties showcased by biological materials including bacteria, fungi, protists, and cell components such as DNA, and molecular motor proteins can be leveraged for computational tasks, propelling the field of biological agents-based computation forward. Traditional silicon-based computing, while tremendously successful, has encountered obstacles in addressing certain complex problems that can be elegantly tackled through the inherent properties of living organisms. Microorganisms, as living entities, exhibit dynamic responses to their environment and possess inherent biochemical processes such as space partitioning or searching, replication, and autonomous motility that can be harnessed to perform specific tasks. A deeper

understanding of these organisms' capabilities and limitations will pave the way for transformative applications across various sectors, leading us into an era where living organisms contribute to computation in unprecedented ways. Microfluidic based biocomputational devices have gained a lot of attention due to the versatility they offer. They allow control and ease in replication, dynamic flow regimes, ability to develop structures in the microns scale suitable for microorganisms.

2.5 Microorganisms as computational agents

The ability of microorganisms to space partition and optimization, pattern recognition, and decision-making demonstrates their unparallel efficiency to adapt to constantly changing microenvironments and solve intricate existential problems both temporally and spatially. This innate ability and additional qualities like fast replication, autonomous motility, varied motility rates, etc., make them ideal candidates to serve as biological computational agents. Several types of microorganisms including bacteria, protists, and fungi have been successfully used as computational agents to solve combinatorial problems. Studies report the efficiency of fungal mycelium and hyphae in exploring confined spaces and solving a range of computational geometrical problems [132-137]. Slime mold *Physarum polycephallum* is another classic example of biological computation capable of sensing and solving computational geometry problems and has aided in the development of unconventional computing devices. *Physarum* solves problems by developing an optimal network by decentralized propulsion of the protoplasmic tubes, doing this, it is capable of distributed sensing and parallel information processing/computing [138]. The ability of slime mold to efficiently space explore has been exploited to find the shortest path and solve computational problems including, simple labyrinth [139-142], spatially extended geometrical problems such as Delaunay triangulation and Voronoi diagram [143], and to approximate travelling salesman problem [144]. Similarly, unconventional computation using bacteria [145-148] and protists [38, 89, 149, 150] as biological agents shows that the symbiotic integration of biological systems with computational tasks paves the way for unparalleled efficiency, adaptability, and sustainability in solving intricate problems that extend beyond the reach of conventional computers.

2.6 Rationale and Innovation

2.6.1 Motility studies

Euglenids are a diversified family of photosynthetic, unicellular, flagellates that mainly habitat aquatic ecosystems, brackish water, and damp soil. They are one of the earliest reported microorganisms [29] and occupy a center stage in research among eukaryotes due to their wide range of applications in multiple disciplines. Euglenids are shown to exhibit versatile swimming mechanisms - the flagellar swimming, propelled by a single, emergent flagellum located at the anterior end of its body with a characteristic movement mostly dubbed to a "spinning lasso" or figure "8" [31]. Apart from flagellar swimming, they are also capable of performing highly coordinated bodily deformations called metaboly or euglenoid movement [40-42]. Though the purpose of the latter is still being researched, some report that culture crowding and confinement trigger metaboly [14]. and most of their functional strategies remain elusive. It is intriguing to think about the complexities of these organisms and how much more we are yet to discover about them. A plethora of studies report motility behaviours in biological microswimmers, in response to light [10, 30, 33, 151], fluid [152], chemicals [153-155], and more but relatively limited to examining responses to physical boundaries or complex geometry [20, 23, 27, 28, 156]. It is important to note that the studies involving external stimuli such as light, chemical, gravity may lead to a partially biased understanding of cell behaviour as these responses are typically in response to stimuli and may not entirely be of their natural instinctive behaviour. And this research gap is larger in euglenids, although these studies provide valuable insights into understanding the hydrodynamics and mechanisms that govern motility, many are limited to studying them between two glass plates or simple geometrical constraints [14]. An alternative approach would be to see how euglenids navigate around complex structures/physical barriers, exhibit changes in behaviour, and adapt their strategies to achieve successful navigation. Therefore, developing a robust and comprehensive design framework is essential to probe the motility patterns and understand the physical mechanisms that govern the cell behaviour of euglenids in complex geometries. Additionally, understanding motility and behavioural changes associated with the biology of the organism at the organismal scale is not only challenging but also demands reproducible methods to obtain accurate results. Thanks to the state-of-the-art microfluidic technologies which provide a versatile test bed to design and develop new

methodologies to study behavioural patterns of biological microswimmers. The motility patterns and behavioural responses were studied in microfluidic-based complex geometries with varying confinements. The novelty of this work lies in understanding species-dependent, intricate behaviours and responses adapted by euglenids to achieve navigational tasks in response to various geometrical confinements.

2.6.2 Biocomputation

Conventional electronic computers, while tremendously successful, due to their sequential operation are inefficient, energy-consuming, thermodynamically unsustainable, and mostly intractable even for medium-sized combinatorial mathematical problems. These problems have an exponential number of solutions to be explored by brute force, thus demanding unreasonable computation time for conventional electronic computers that can only operate sequentially. Additionally, the complexity of combinatorial problems translates to ever-increasing energy consumption by electronic computers, making the computing process highly unsustainable energy-wise. Consequently, the development of efficient, parallel computation strategies that employ scalable processors/agents to solve these combinatorial problems is necessary. Parallel computing in biology is not a new phenomenon, most living beings process signals presented to them in analog forms that help them make decisions to either react or flee, comparable to zero's and one's used in the binary form of electronic computing. Such parallel computing has been demonstrated in an *in vitro* set-up to approximate solutions for computation using several agents such as *Physarum* [44], nucleic acid-based DNA [45-47] computing and recently by our team using molecular motor proteins [48], and bacteria (currently explored by our team). The earliest form of biological computing using DNA was demonstrated to solve an instance of Hamilton path problem [157], although an excellent and convincing parallel computing approach, it has hardly scratched the surface of the complexity of these NP-complete problems due to their key scalability issues meaning demanding incredibly large resources for solving larger size problem [45-47]. It has been shown that even the recent high-end, state-of-the-art quantum computing (though not biological computing) is limited in scale to solve such intractable, exponentially growing NP-complete problems [158]. To address this problem Prof. Nicolau and his team proposed a new computational paradigm, for solving the combinatorial problem

by allowing a very large number of motile biological agents, here molecular motors to explore the microfluidics network [48]. These self-propelled agents explore the network independently and parallelly through channels by brute force to solve the graphically represented mathematical problem. This approach was successful, and the experiment-based computer-aided simulations revealed the proof-of-concept that parallel computation using the biological agents has the capability to efficiently solve the NP-complete problem of medium to higher sample sizes. However, the approach employing molecular motor proteins faced challenges like detachment of molecular motor proteins from one another, handling of molecular motor proteins, and scalability associated with readout for computing. Microorganisms, though memory-less, exhibit dynamic responses to their environment and appear to have recognition and decision-making abilities in receiving and processing signals like chemical, light, etc., as either an independent or collective entity resulting in efficient space-searching, adaptation, and colonization. Using photosynthesis microbes (euglenids) as biological agents offers several advantages such as self-propelled, easy to handle, low-cost maintenance, replication, and the most appealing factor about euglenids is that they are photosynthetic and require minimal nutrients to survive. We propose the use of microorganisms, in particular, photosynthetic euglenids as biological agents for network-based parallel computing. Euglenids are photosynthetic, unicellular flagellated protists abundantly found in aquatic ecosystems. They are unique in that they are both heterotrophic (derive nutrients and energy by consuming other organisms) and autotrophic (can produce their own food via photosynthesis - the most appealing factor), making them easily culturable microorganisms that live stably without stringent culture conditions. Using microbes as biological agents offers several advantages such as a plethora of microbial species to choose from, different sizes, flagellar arrangements, varied motility rates and patterns, and various replication times – replication, being a critical factor in the parallel exploration of solutions to an exponentially growing problem. Employing self-propelled, replicating (scalable), **microorganisms as biological agents, particularly *Euglena species,*** to explore physical microfluidic network encoding instances of NP-complete mathematical problems. The goal is to develop an energy-efficient, low-cost-driven computation system with autonomous, self-propelled biological agents that require minimum maintenance.

3. Approach

3.1 Motility and behavioural studies in Euglenids

3.1.1 Culture of cells and maintenance

The model organisms used in this study, *Euglena gracilis* and *Euglena acus* were obtained from Carolina biological supply. The organisms were cultured in Cramer Meyer's media and maintained under a wide spectrum illumination of 40W, white light at a light:dark cycle of 12:12 hour. 7-day-old culture was used for the experimental study in the microfluidic structures as the growth rate of the microorganisms was at the peak of the log phase.

3.1.2 Device design and fabrication - Motility studies and behavioural

The microfluidic network to probe the motility behaviour of euglenids comprises a 3.5 mm x 3.5 mm layout of uniquely structured grids arranged parallelly in six columns with five rows of identical geometrical structures separated by quasi-open space. Each column of the parallelly arranged reservoirs is a total length of 3.5 mm, connected through a 1 mm x 1 mm-wide 'bus' channel. A total of seven different types of structures were used namely, W1 – W6, wavy and zig-zag channel systems and O, quasi-open space (plaza). The channel systems in W1 – W6 were as follows, C0 common in all structures is a 14 μm wide linear channel, C1-C3, channels with varying confinement and turning complexities namely, number of turn angle, turn angle variety, and channel member variety. All the channels (C0-C3) in the structures were a uniform length of 350 μm. The patterns were designed using AutoCAD software and printed on Mylar or Polyethylene terephthalate (PET) masks, the printed patterns were translated on a silica wafer using negative photoresist-based SU-8 fabrication and optical mask aligner set-up (UV settings at Z-40 and 55 s exposure time) in the Nano-UQAM facility located at Université du Québec à Montréal. The wafers after fabrication were subjected to salinization using methyltrichlorosilane. A mixture of Polydimethylsiloxane (PDMS), Sylgard 184, Dow Corning and cross-linker (weight ratio 10:1) was poured onto the silicon master, degassed inside the vacuum chamber to remove air bubbles, and cured at 65°C overnight to ensure complete cross-linking. After cutting and peeling, the PDMS replica was treated with air plasma for 45 seconds to render the surface

hydrophilic before irreversibly bonding it onto the glass coverslip (also plasma-activated for 45 seconds). Further, the fabricated microfluidic devices were flooded with Cramer Meyers (CM) media and stored at 4°C overnight to facilitate sufficient wetting. All experiments were carried out under uniform light. For motility studies, the W1, W2, and W3 wavy structures with plazas (Figure 6.D) were utilized, while the complete structure, incorporating both wavy and zig-zag channels, was employed for behavioural studies (Figure 12).

3.1.3 Experiments and analysis

The microfluidic devices were removed from 4°C and brought to room temperature before introducing the euglenids. Separately, a log-phase suspension of euglenids was added around the device and allowed to explore the microstructures through the open ends of the device. The motility behaviour of euglenids was imaged using Olympus IX83, an inverted - Spinning Disk Confocal Olympus IX83 microscope, with MetaMorph® (from Molecular Devices) Microscopy Automation & Image Analysis Software, 10x (NA 0.4), 20x (NA 0.75) and 40x (NA 0.95). Data analysis was performed using ImageJ, a public domain software platform [159]. The analysis included tracking and plotting trajectories of motility patterns, velocity, density maps, and quantification of the residence time. The trajectories of single euglenids were tracked manually with point-and-click tracking using Manual tracking plugin in ImageJ and the x-y-time coordinates from the trajectories were used for quantification of the velocity. Replotting the tracks and graphs were represented using ‘Origin’ software.

Chapter II

Motility studies

Euglenids are photosynthetic, unicellular, that are shown to exhibit versatile swimming mechanisms - the flagellar swimming, propelled by the flagella and highly coordinated bodily deformations called metaboly or euglenoid movement. A plethora of studies report motility behaviours in biological microswimmers, in response to external stimuli such as light, fluid, chemicals, gravity, and more but are relatively limited to examining responses to physical boundaries or complex. Most motility studies involving euglenids are phototactic, chemotactic or gravitactic based. Additionally, many studies that provide valuable insights into the hydrodynamics and mechanisms that govern motility under physical constraints are limited to studying them between two glass plates or simple geometrical structures. An alternative approach would be to see how euglenids navigate around complex structures/physical barriers, exhibit changes in behaviour, and adapt their strategies to achieve successful navigation without the influence of light or chemicals. The primary focus of this work is to investigate the motility behaviours of two euglena species namely, *Euglena gracilis* and *Euglena acus*, in microfluidics-based complex geometries with varying degrees of confinement. This work offers advances in understanding of characteristics of spatial distribution, species-specific motility patterns or swimming trajectories, the impact of confinement on swimming efficiency and various behaviours including avoidance maneuvers for escaping physical barriers and wall corners. The findings not only reveal motility and behavioural patterns adapted by euglenids in response to confined environments but also highlight the influence of structure design on cellular motility and behaviour.

This chapter encompasses the first study to which I have contributed. The data obtained from this study is of publishable quality and is currently being prepared for publication

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4. Motility patterns of Euglena species in confining microfluidic geometries.

Abstract

The motility patterns of two euglena species with varied flagellar lengths and slight morphological differences were extensively studied in microfluidic-based simple and complex geometries with varying confinements. We report under complex confinements cells change their motility patterns, swimming trajectories, swimming efficiency, and exhibit various behavioural responses. The cells switch from helical swimming to back-and-forth motility or metaboly in highly confined channels. Furthermore, cells exhibit gyrating behaviour when confined in the channel corners and exploit their innate ability of forward inclination to escape from wall-corner trapping. These intricate behaviours and responses by cells were species-specific highlighting the adaptability of each species to navigate through different geometrical confinements. The results of this research provide advancements in understanding of the correlation between complex confinements and swimming efficiency, and factors that conceivably influence biological behaviours such as metaboly. The ability of cells to develop modulations between flagellar swimming, back-and-forth motility, and metaboly provides a instance of self-organized, behavioural adjustments in euglenids. This study finds its relevance in the design and control of soft, self-adaptive, bio-inspired robots, developing motility-based cell separation, and further applicable to biocomputation.

4.1 Introduction

Euglenids are a diversified family of photosynthetic, unicellular, flagellates that mainly habitat aquatic ecosystems, brackish water, and damp soil. They are one among the earliest reported microorganisms [29] and occupy a center stage in research among eukaryotes due to their wide range of applications in multiple disciplines including biophysics [30-32], biochemistry [30, 33-35], nutraceuticals [36], biotechnology [37], biocomputation [38, 39], bio-regenerative life support in space [82]. Despite their potential and established applications, the general behaviour of euglenids is largely unknown. Biological microswimmers exhibit a variety of swimming behaviours to thrive in the environment under various complex conditions, understanding their motility patterns and behavioural responses is critical for several applications including biosensing, biocomputation, disease control, single-cell genomics, selective breeding, and cell sorting. Euglenids are shown to exhibit versatile swimming mechanisms - the flagellar swimming, propelled by a single, emergent flagellum located at the anterior end of its body with a characteristic movement mostly dubbed to a “spinning lasso” or figure “8” [31]. Apart from the flagellar swimming they are also capable of performing highly coordinated bodily deformations called metaboly or euglenoid movement [40-42]. Though the purpose of the latter is still being researched, some report that culture crowding and confinement triggers metaboly [14]. Several studies report motility behaviours in biological microswimers, in response to complex geometry [20, 23, 27, 28, 156], light [10, 30, 33, 151], fluid [152], chemicals [153-155], and more. Our previous work, studying the motility of bacterial species in confining microfluidic environments showed that bacterial motility patterns are the result of a “tug-of-war” between hydrodynamics and local nano mechanics [20]. However, the swimming strategies of eukaryotes differ substantially from the prokaryotes, albeit the flagella are used for propulsion, their propulsion mechanism is largely distinct. Although these studies provide valuable insights on understanding the hydrodynamics and mechanisms that govern motility, the general hydrodynamic arguments neglect the scattering dynamics of the cell swimming near boundaries. Furthermore, the complex biological response or motility studies in euglenids are limited to studying them between two glass plates or simple geometrical constraints [14]. We employ microfluidic-based technologies to study behavioural patterns in euglenids. The present work reports on the motility behaviour of two photosynthetic

Euglena species, namely, *Euglena gracilis* and *Euglena acus*, which present different cell morphologies, in microfluidics structures with various geometries and confinement levels. The understanding of the species-specific relationship between cell morphology and motility patterns in confining environments can provide insights into the design and fabrication of microfluidics devices to areas as diverse as biocomputation, soft robotics, cell sorting, and biomedical applications.

4.2 Methods

4.2.1 Culture of cells and maintenance

The detailed methodology mentioned in Chapter 1, section 3.1.1

4.2.2 Device design and fabrication

The detailed methodology mentioned in Chapter 1, section 3.1.2

4.2.3 Motility experiments

The microfluidic devices were removed from 4°C and brought to room temperature before introducing the euglenids. Separately, a log-phase suspension of euglenids was added around the device and allowed to explore the microstructures through the open ends of the device. To ensure the motility behaviour in the microstructures were in response to physical geometries alone and to avoid any chemotaxis or phototaxis-driven motility, the working fluids had an excess of nutrients, and the experimental time of recorded motility was shorter at 5 min and 20 sec to ensure that the level of nutrients remains practically constant, furthermore, uniform illumination of the microstructures were maintained throughout the experimental duration.

4.2.4 Image acquisition and analysis

The detailed methodology mentioned in Chapter 1, section 3.1.3

4.3 Results and Discussion

The motility patterns of euglenids were extensively studied in the microfluidic channels with varying geometrical confinements namely, linear channel, quasi-open space (plaza), wavy, and zig-zag channel systems (W1-W3) with three complexity dimensions, 1. turn angle complexity, 2. number of turn angles, and 3. channel member variety (Figure 6.D), the channel complexity is defined by variations in width, tortuosity, and branching structures. This study provided insights on modulation and adaptation of swimming patterns in response to the nature of confinement, species-specific motility patterns, velocity, residence time, and spatial position as an effect of channel confinement and geometric complexity. Additionally, the behavioural responses (species-specific) such as avoiding responses, escape strategies at physical barrier, metaboly, and back-and-forth motility was explored.

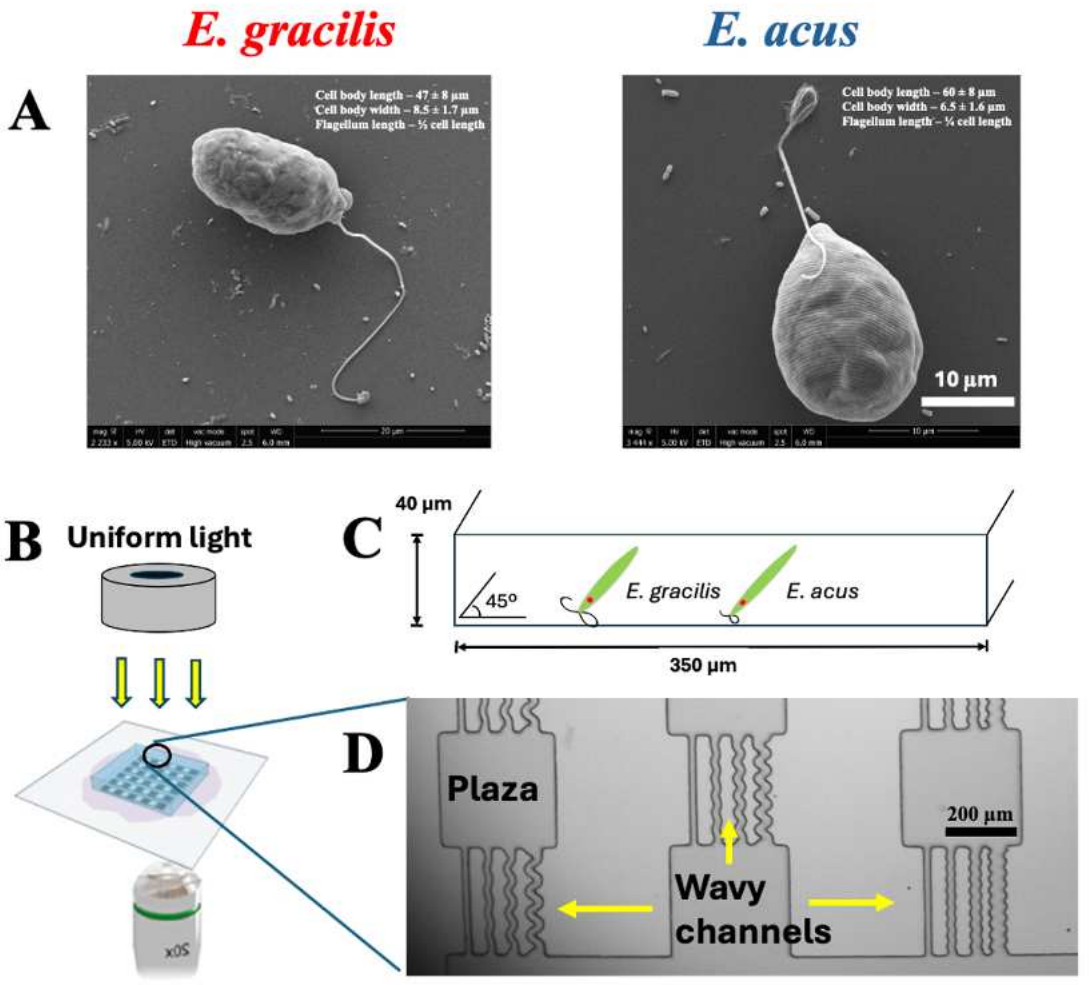

Figure 6: Device design and setup to probe the motility behaviour of euglenids. A. Scanning Electron Microscopy images of the two euglena species B. Schematic representation of microfluidic device flooded with euglenids culture imaged under uniform light. C. Schematic of device dimension and the spatial orientation of the tested Euglena species within microfluidic structures. D. The overall architecture of the chip showing a sequence of wavy channels separated by plazas

4.3.1 Motility and spatial distribution in the Plaza

The spatial distribution of cells within the plaza as they transitioned from confined structures into the plaza was obtained using density maps and 2D projection of individual euglenids trajectories. Specifically, O1 corresponds to W1 as cells exit W1 they enter O1, O2 denoted the transition from structure W2, and so forth respectively. Density maps represent a relative density scale of individual euglenids trajectories across the microstructures over a period of time, produced as a fluorescence micrograph. The distribution of *E. gracilis* in the open space O1-O3 (Figure 7.A, B), showed a rather uneven distribution throughout the structure. The density near the walls was short and broken, additionally, cells exhibited ping-pong-like collisions and reflections at the wall (Movie S1). Furthermore, cells were trapped in the wall corners (Movie S1) of the open plaza which translated as saturated spots at wall corners in the density maps and dense trajectories at the channel corners in the 2D visualization of the individual trajectories (Figure 2B). In contrast, the distribution of *E. acus* in the open spaces (O1-O3) exhibited some intriguing motility patterns. The density maps (Figure 7.C) in O1 and O2 showed higher density near the walls. The motility patterns were long trajectories with corner trappings, very short movements in close vicinity to the wall, and wall bouncing (ping-pong-like) motility (Movie S2) similar to *E. gracilis*. Several cells took multiple laps, traversed as repetitive paths and scattered off the wall at relatively the same points forming a characteristic “square” shaped trajectory pattern in the structure (Figure 7.D - O2). Conversely, in O3, the trajectories were predominantly linear, with null to minimum interaction with the wall. Furthermore, we observed that the cell interaction with the microfluidic channel walls notably diminished with the decrease in the degree of confinement and difficulty level of the structure design which the cells were previously subjected. This is delineated in Figure 7.C and D, underscoring the interplay between confinement-induced alterations in motility patterns exhibited by *E. acus*.

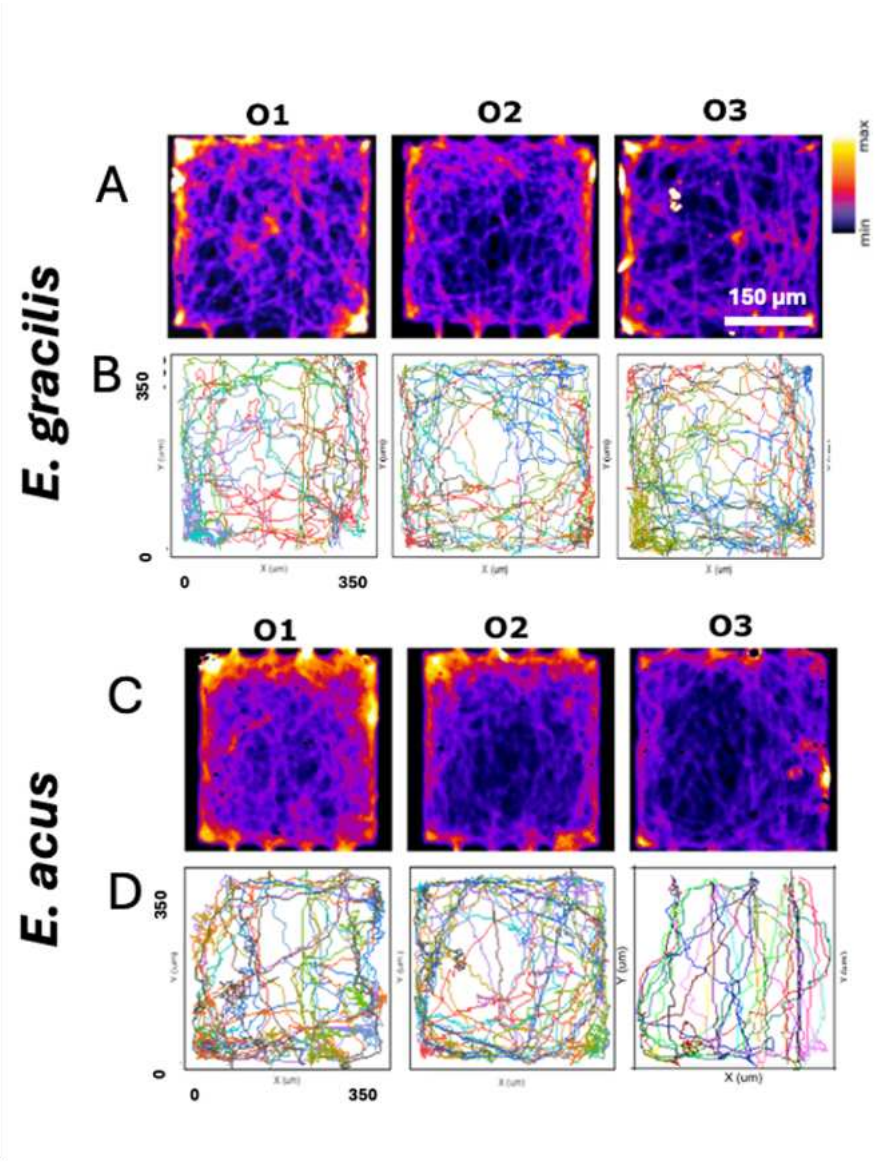

Figure 7: Characteristics of spatial distribution and swimming trajectories of euglenids in geometrical structures. **A.** Density maps of *E. gracilis* highlighting the presence of cells in the test structures, bar showing color code reference, far-right on the figure, "min" and "max" represents absence and highest presence of cells, respectively. **B.** 2D projection of swimming trajectories of *E. gracilis* in structures (O1-O3). **C.** Density maps of *E. acus* highlighting presence of cells in the quasi-open space corresponding to the structure W1-W3 **D.** 2D projection of swimming trajectories of *E. acuis* in the open space O1-O3. Structure O1

corresponds to structure W1 and similarly O2-O3 corresponds to structures W2-W3 respectively.

Euglenids are typically ellipsoid (cigar-shaped) bodies that swim rotating counterclockwise along their long axis, the flagellar swimming in euglenids is propelled by the lateral beating of a single emergent flagellum located at the anterior end of its body. Swimming under these "ideal" (flagellum-driven, ellipsoid shape) conditions, the motility pattern is a characteristic sinusoidal path or a periodic swinging motion [31]. The swimming propulsion mechanism can be summarized as a complex sequence of non-planar flagellum beats with a characteristic movement mostly dubbed to figure "8" [31]. In the presence of a wall or a physical boundary, the motility behaviour of microswimmers swimming in low Reynold's number is highly influenced by several factors including hydrodynamics, steric effects, mechanical forces, and biological responses. Based on these factors a microswimmer (depending on the swimming type) either swims in closer vicinity to the wall (wall accumulator) or away from the wall (wall escaper) [20]. To illustrate further, when thrust is generated behind the cell body otherwise classified as a pusher, the cell tends to swim closer to the wall showing wall accumulating behaviour, and when the thrust is generated in front of the cell body (puller), the cell swim away from the wall showing wall escaping behaviour [24]. Our previous work explicated such behaviours in five bacterial species [20]. Alternatively, several studies reported this type of swimming behaviour in sperm cells [15, 21-23], bacteria [20, 24-26], and ciliates [27, 28]. In quasi-open structures (O1-O3), the simplest geometric constraint bound by vertical and horizontal walls *E. gracilis* presented an uneven or broken near wall density and exhibit abrupt wall-bouncing reflections like ping-pong ball, however, cells swam in closer vicinity to the wall for short distances (wall accumulator) before abruptly scattered by the wall (wall escaper). Conclusively, in the quasi-open space, under the "ideal" swimming conditions *E. gracilis* oscillates between a wall accumulator and a wall escaper. A report on the flow field analysis of swimming behaviour reported *E. gracilis* exhibit mixed puller-pusher behaviour during a stroke [160]. The motility behaviour of *E. acus* showed distinct trajectory patterns, in O1 and O2, long trajectories with wall bouncing

(ping-pong ball like) motility. Several cells that collided head-on with the wall, retracted their anterior end as an initial mechanical response triggered by the collision and scattered off by taking a sharp turn from the wall showing a wall escaper behaviour. However, some cells swam parallel to wall for very short distances until scattered off. Although these structures showed higher density localized near the wall, this was due to a cumulative effect of frequent scattering of cells upon collision. In O3, predominantly short, linear trajectories with null to minimum interaction with the wall featuring a wall escaping behaviour highly dominated by the hydrodynamics was observed. Based on this behaviour *E. acus* can be classified as a swimmer that marginally oscillates between wall accumulator and wall escaper, then again dictated largely as a wall escaper. Although the two species are morphologically similar with slight differences, their flagellar length varies significantly by ½ the cell length in *E. gracilis* and ¼ the cell length in *E. acus* (Figure S 1.A, B), this variation could be a contributing factor for the difference in these swimming patterns and are discussed in the subsequent sections. Based on the observed behaviour, the impact of the length of the emergent flagellum and how it affected the motility behaviour in confinement remains elusive and needs further study. Mutational studies on the flagellar length with the two species and further elucidation by motility experiments may answer this scenario in objective facts. However, the first-hand study described here showed that flagella length impacted the behaviour of Euglena in confined geometry during the transition from confined geometries to plazas. Moreover, the elastic motor system – pellicle strips which differ species to species is critical for mechanical responses. The variation in these could be the two contributing factors for the difference in these swimming patterns and are discussed in the subsequent sections.

4.3.2 Characteristics of swimming trajectories:

When cells exit relatively complex and confining W1 and W2 (Figure 8.B) structures they opted for long, circular trajectories with longer residence time in the open plaza, when they exit the least complex and confining W3 (Figure 8.B) structure their trajectory patterns were predominantly linear in the corresponding open space, with a shorter residence time and quick transition to the next structure. This was distinctly observed in

E. acus, to understand the novelty of this behaviour the radius of curvature (Figure 8.A). of the trajectories and percentage frequency of cells that adapted circular trajectories and straight trajectories were quantified (Figure 8.C, D). The radius of curvature of the trajectories of *E. gracilis* in the plazas of W1, W2, and W3 networks was 142.6 µm, 143.5 µm, and 102.6 µm, respectively. However, the average radius of curvature for *E. acus* was 136.5 µm, 72.4 µm, and 25.8 µm, respectively, suggesting a significant difference between species on their exiting behaviours when decreasing the channel confinement.

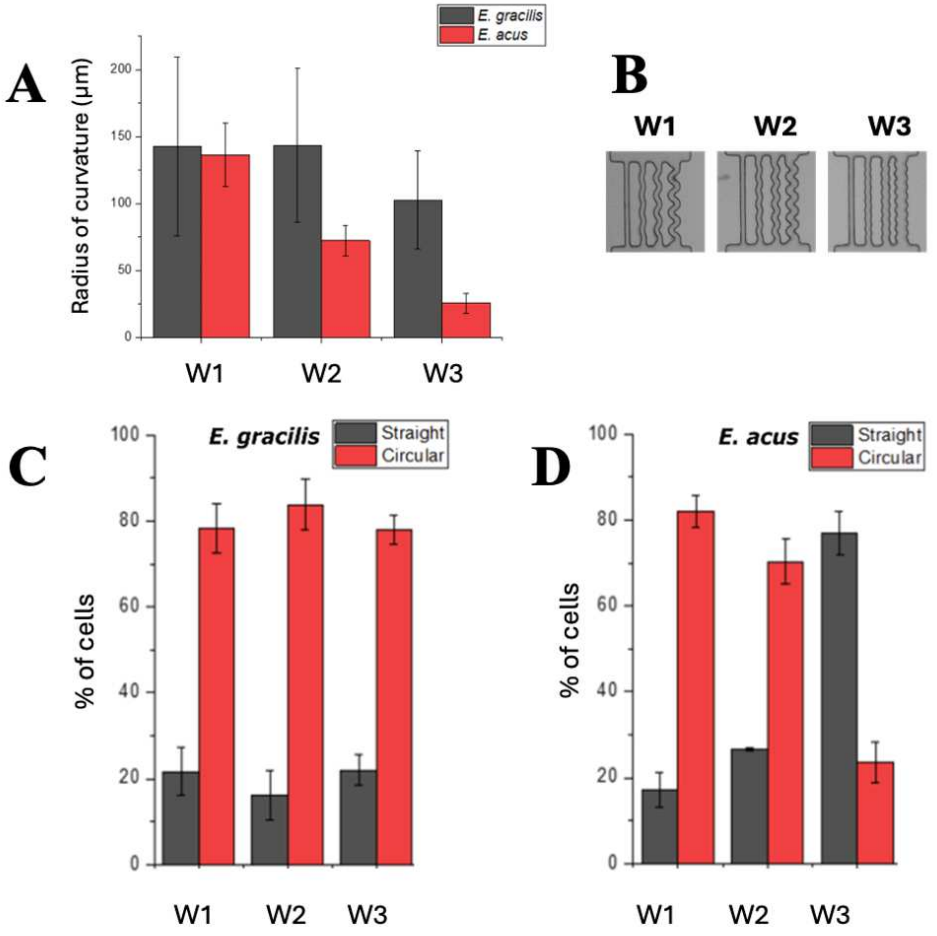

Figure 8: Quantification of swimming trajectories comparing the two species. A. Radius of curvature in the open structures as cells exit the channels (n=50) indicating effects of channel confinement on swimming trajectories, B. Optical images of corresponding

structures which cells exit from. Percentage of cells exhibiting straight and circular trajectories in C. *E. gracilis* and D. *E. acus* (n=400) with respect to each structure.

Here, straight/linear trajectories that transitioned from one structure (W) to the next (W) without interaction with the wall in the open plaza (O) were classified as straight trajectories, this was irrespective of the channel they exit from or enter. Long trajectories where cells loop and circle with wall collisions or interactions, in the open space was classified as circular trajectories. About 82.1% and 70.4%, of cells that exit from W1 and W2, respectively adapted circular trajectories. In cells that exit from W3 the percentage of cells that adapted circular trajectories were only about 23.5% in *E. acus*. It appeared that the structures they were previously subjected to had a temporary impact on their motility pattern in the corresponding open space. Euglenids contain epiplasmic pellicle strips that serves as an elastic motor system to adapt highly coordinated bodily deformations or metaboly. Some studies showed an adaptable mode of locomotion in Euglena by mechanical self-regulation of the pellicle strips to confinement [14]. The channel design in the structures W1and W2 imposed agitation, severely restricted forward motion that prompted cells to adapt various biological responses mainly metaboly in *E. acus*. The mechanical adaptability of the active pellicle (mechanical machinery) to the dynamics of constraining microenvironment could presumably contribute to this distinctive behaviour. Although, this behaviour was not distinctively observed in *E. gracilis*, the percentage of cells that opted for straight trajectories was slightly higher when they exit W3 compared to the other four structures. This could be explained by the number of pellicle strips, which differ in both species 36-46 strips with a strip width of 0.58 ± 0,04 µm in *E. gracilis* and 45-52 strips with a strip width of 0.79 ± 0.03 µm in *E. acus* (Figure S1). The higher number and larger width of pellicle strips, which is directly related to cell flexibility, likely provide enhanced mechanical flexibility, enabling *E. acus* to more effectively adapt metaboly in complex microenvironments. Conclusively, the confinement-induced collisions and geometric constraints imposed by the channels impacted the motility of both species, with mechanical stress on both the flagellum and the cell body triggering adaptive responses like metaboly. Another factor to consider is the potential influence of memory. For instance, *Tetrahymena*, when confined within a small droplet, swam in small circular trajectories. This ciliate continued to reproduce circular trajectories for extending

durations from minutes to an hour, even after being released from the droplet [161]. Similarly, for the algae *Paramecium,* when confined within a narrow-ended long capillary tube, the cells altered the swimming to short-term backward swimming as an avoidance response [162]. This backward swimming persisted for five to ten times longer. These findings showed that microorganisms have the ability to temporarily retain the memory of their surrounding environment. Cells in the structures with many turn angles continued to reorient their swimming direction to navigate through the channel, while cells in relatively less confined channels swam freely with minimum interaction with the channel walls. Cells that exited the complex confined channels, tended to continue reorienting their swimming direction in the open space, likely due to 'memory' of multiple turns they experienced within the channels. This residual behaviour, where cells swim in long, circular trajectories with several reorientations, suggests that the cells 'remember' and continue to adapt as though they are still navigating the confined channel. In contrast, cells in the wider channels, rarely reorient their swimming direction in the channel. As a result, their trajectory remains linear both within the channel and in the open space, highlighting the impact of confinement-induced memory on subsequent motility patterns. Additionally, Euglenids are shown to modulate their flagellum beat patterns to change distinctive trajectory patterns in response to light stimuli [10]. Similarly, modulation of the flagellar beats to change swimming direction in the channels, retained after exiting the channel causing cells to swim in circular trajectories could be another factor to consider in understanding post-confinement behaviour. One of the observations was the ability of cells to adapt and self-adjust their motility pattern according to the type and degree of confinement. Some cells that exit the relatively complex channels show erratic behaviour immediately after exiting the complex channels.

4.3.3 The dynamics of scattering mechanism in quasi-open channel

Cells in quasi-open channels exhibited a wall bouncing, ping-pong like trajectories, showed the primary scattering dynamics was governed by mechanical responses followed by interactions with the wall (Movie S 4.A and S 4.B) for *E. gracilis* and *E. acus* respectively). Cells swimming near the wall largely showed two phenomena, 1. impact/collision with the wall followed by scattering and 2. escape from the wall without contact. Figure 9.A showed the scattering dynamics of *E. gracilis*, when cells swam near the wall three trajectory patterns were observed, a. green trajectories, cells approaching the wall collide, scatter, swim parallel to the wall for short distances (≈ 160 ± 70µm) and swim away by escaping the wall. The incidence angle is mostly symmetrical to the exit angle, additionally cells collided with the wall two or more times before escaping showing a characteristic w-type trajectory pattern. In this case, the scattering is initiated by short flagellar interactions (steric effects) with the wall, the turn is smooth and cells swim in close proximity to the wall. b. blue trajectories, the incidence angle is asymmetrical to the exit angle, cells are trapped in closer proximity to the wall for short distances (≈ 75 ± 25µm) and swiftly escape from the steric effects of the wall. This type trajectories are usually initiated when cells collided head-on and the initial response was a shock mechanical response. c. red trajectories (dashed), cells change swimming direction and escape from the wall without contact, the type of swimming behaviour dominated by hydrodynamic forces. In type a and b, the swimming behaviour was a result of short steric interactions with the wall until hydrodynamic forces dominated. The impact with the wall instigated mechanical responses where cells immediately scatter, yet remain trapped in close proximity to the wall showing a brief hydrodynamic escape, the phenomenon of hydrodynamic escape was also observed in ciliates [28]. The initial mechanical responses when scattered adds as a critical factor in memory of incidence angle and symmetry of the exit angle, the blue trajectories showed a sharp turn/scatter upon collision resulted in asymmetrical exit angle, when the scattering was smooth (green trajectory) the exit angle was symmetrical, and swimming was dominated by the steric effects until cells escape the wall.

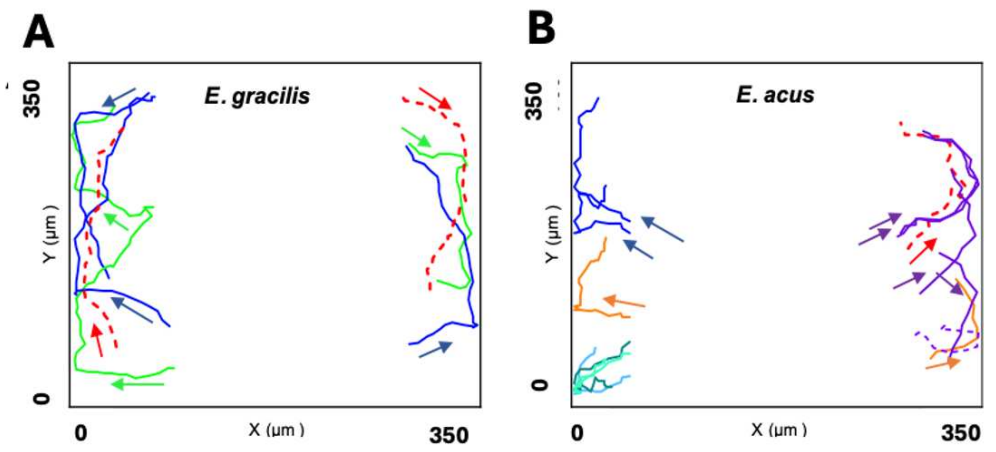

Figure 9: Shows the scattering mechanism between the two species and how cells escape from the wall in the open plaza with A. *E. gracilis* and B. *E. acus*. Colored trajectories depict the different type of escape the cells perform before scattering from the wall. Arrows indicate the swimming direction before cell-wall interaction.

Figure 9.B showed the scattering dynamics of *E. acus*, the following patterns were observed, a. purple trajectories, the angle of incidence was symmetrical with the exit angle indicated the memory of incidence angle is not lost upon scattering, cells scattered off quickly with a sharp turn upon collision without being trapped near the wall, the scattering was predominantly by mechanical shock responses from collision. b. orange trajectories, exit angle was asymmetrical to the incidence angle, the memory of incidence angle is lost upon collision, c. blue trajectories, cells swim in close proximity to the wall for shorter distances parallel to the wall for d. red trajectories (dash), cells escape from the wall without impact. Furthermore, cyan trajectories, close to the channel corner showed symmetrical incidence and exit angles, color variations indicated the memory of incidence angle not lost irrelevant of the entry/incidence angles. However, in all cases cells course corrects the trajectory to parallel to the wall maintaining at least 100 µm distance from the wall and continue to swim after course correction. *E. acus* is predominantly a wall escaper, the cells scattering was

primarily dominated by mechanical forces upon collision, resulting sharp turns when scattered by the wall.

The scattering angles upon collision with the wall varied between the two species with 90 ± 30º and 118 ± 30 º in *E. gracilis* (Figure S 3) and *E. acus* (Figure S 4) respectively. Both species have a single flagellum and do not vary significantly in the body morphology, the flagellum length however varies significantly. The scattering angle decreased with flagellum length, showed the scattering angle predominantly depends on the flagellum length. Additionally, cells collide head-on with the wall and scattering was initially dominated by the mechano-elastic response of the cell and later by the steric or hydrodynamic forces. We hypothesize that the dynamics of scattering mechanism depends on the mechanical responses and flagellum length of the cells as observed in this study.

4.3.4 Entrapment and reconfiguration

When cells come in contact with the structure's corners they were trapped and tend to escape by deflecting from the wall. As the wall corner is bound by two walls, cells began to gyrate in the channel corner for several seconds and this continued until the cells can negotiate a way around the obstruction (Movie S 5). The gyration time when trapped is 560 ± 100 ms in *E. gracilis* and 240 ± 94 ms in *E. acus* respectively. In *E. gracilis* the cells trapped in the wall corner gyrated for longer times, as the angle of deflection is low. Failure to negotiate the way leads to a series of responses dominated by biological behaviours as an escape mechanism, cells adapt metaboly, spins, switches orientation while swimming, recovers or escapes from the trap (Movie S 5.A). Additionally, cells also escaped by tilting the anterior end in negative horizontal angle, the length of the flagellum is longer in this species which added an additional challenge for the lateral beating flagellum, the hindered flagellum when trapped in the wall corners, negotiates the trap by attempting to incline multiple time resulting in longer trapped duration. *E. acus* escaped the trap in the wall corners by inclining horizontally at a negative angle (Movie S 5.B), moreover, the entrapment time was short, almost half the trap time in this species, the shorter flagellum of the cells facilitated a fast escape strategy. Euglena species innately exhibits gravitaxis [163] to position itself for optimal photosynthesis this feature enabled cells to escape from the trap by efficiently by

inclining their anterior end forward or Z-plane without having to adapt the complex series of metaboly.

4.3.5 Structure design triggers distinctive motility patterns and behaviour.

In channels C2, C3 (W1 andW2,) the distance between each turn was approximately 50-70 μm while the length of a swimming cell was 45-65 μm (ellipsoid/cigar shape), obligatorily, cells collided with the wall and were trapped end-to-end in the channel bend before switching their orientation. Several cells innately traverse in soft sinusoidal motion resulting in frequent collisions with the wall, when end-to-end entrapment and frequent collisions occur cells adapted various biological avoiding responses such as back and forth motility, reorientation, and metaboly. In the most laterally confined (C0) or relatively laterally confined (C1) channels, *E. gracilis* predominantly adapted back and forth motility. The cell's orientation appeared slant in contact with and sliding against the wall. The lateral confinement stimulated disinclination and cells negotiate the space by moving back and forth without switching their orientation. They position themselves at an angle where the posterior end and/or anterior end are in contact with one or both walls and thrust forward and backward (Movie S 3). The long-range hydrodynamics and short-range steric interactions with the wall could credit for this behaviour, in other words, an interplay between hydrodynamics and frictional forces contributes to the back-and-forth motility. Under confinement, euglenid are shown to generate propulsive forces against the wall which is balanced by the resistive hydrodynamic force [14]. The flagellar beats are lateral in euglenids, thereby the lateral confinement in this channel type hinder flagellar beats and triggers an avoiding response in the cells. In this state, the cells exploit the frictional forces to pull backward that cause the sliding or dragging of cells against the wall resulting in backward motility as an avoiding response. This phenomenon continues until the cell exits the channel traversing backwards or reorients the swimming direction (Movie S 3). Back and forth motility was observed but not highly pronounced in *E. acus*, instead cells adapted metaboly.

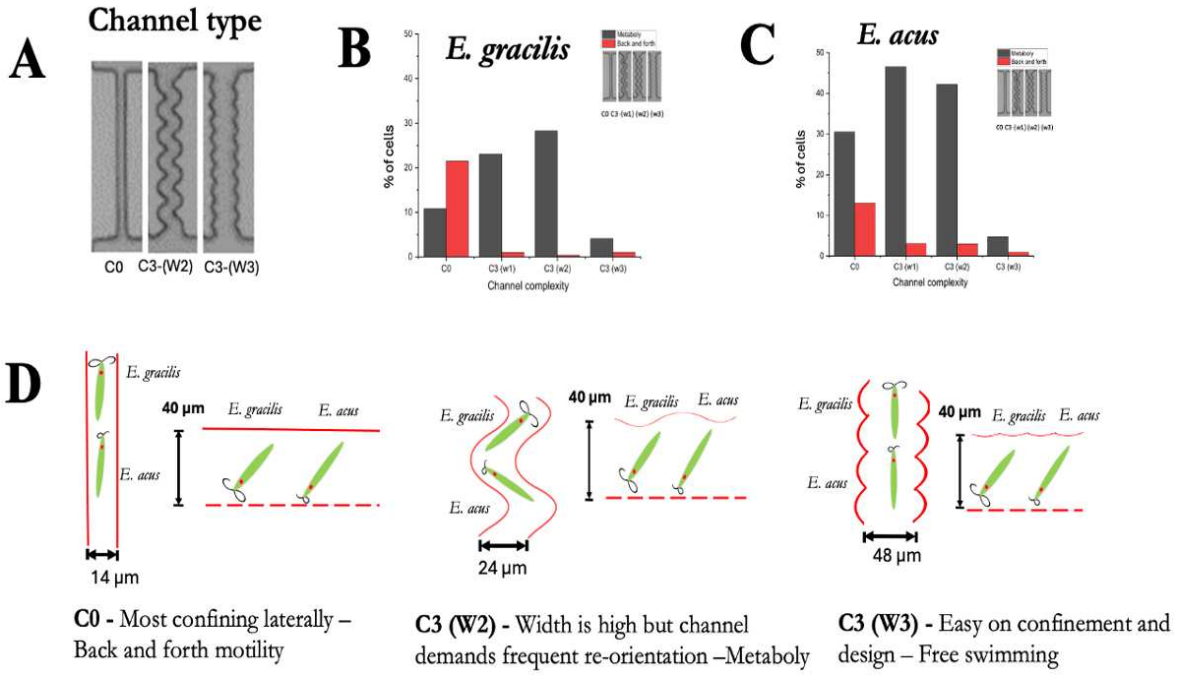

Figure 10: Channel structure triggers distinctive avoidance responses in both species. A. Optical image of channel structure with varying confinement and design. Percentage of cells that adapted avoidance response with respect to each channel in B *E. gracilis* and C. *E. acus* (n=300), showing biological behaviours distinctive to each channel type. D. Schematic of cell orientation in each channel showing types of swimming triggered by each channel.

When end-to-end entrapment and frequent collisions occurred, cells adapted various swimming maneuvers to avoid wall-accumulating responses such as back-and-forth motility, reorientation, and bodily deformations i.e., metaboly. The percentage of cells that exhibited any of these behaviours across various channel configurations was quantified (Figure 10.B, C). Linear channel C0 triggered a higher percentage of cells to adapt back and forth motility, The more complex wavy channel C3 (W2) cells mainly metaboly with significantly higher percentage of *E. acus*. C3(W3) was easy confinement, in the channel most cells displayed free flagellar swimming, all these represented as schematics in Figure 10.D.

4.3.6 Effect of confinement on swimming efficiency

Motility in the structures: The distribution and motility patterns of euglenids in channels of different confinement and complexity were obtained using density maps (collective behaviour and 2D projections, Figure 11) of individual trajectories (single-cell behaviour,). The density maps from motility in structures W1 to W3 indicated that *E. gracilis* (Figure 11A) predominantly moved along the central axis of the channels rather than along the walls. In the linear channels (C0), saturated areas were observed as a result of the back-and-forth motility along the channel triggered by impact with the walls, which translated as short, overlapped trajectories in the 2D projection. This saturation in the density maps also suggests that *E. gracilis* struggled more to successfully navigate tightly confined channels than *E. acus*. The channels (C1, C2, and C3) in the W1 and W2 networks presented an additional challenge of longitudinal confinement at each bend or turn of the wavy channels. The frequency of pausing and restricted motility was high at these points as cells were required to reorient their swimming direction at every turn. The reorientation was not smooth; the cells collided with the wall at each turn, resulting in gyration behaviours and temporary clogging of the channel, which was principally observed in channels C2 and C3. The density maps derived from the motility patterns in the wavy channels suggested that the bends and turns in these channels did not induce corner preference in *E. gracilis*. The channel design (C1, C2, and C3) in structures W3 presented a relatively "linear" structure (easy on confinement and geometry), and cells largely traversed straight in the middle of the channel. Fewer cells were trapped end-to-end in the $C3_3$ channel of the W3 network, and when this occurred, cells tried to re-orient or take a U-turn in the channel, leading to entrapments for short periods of time.

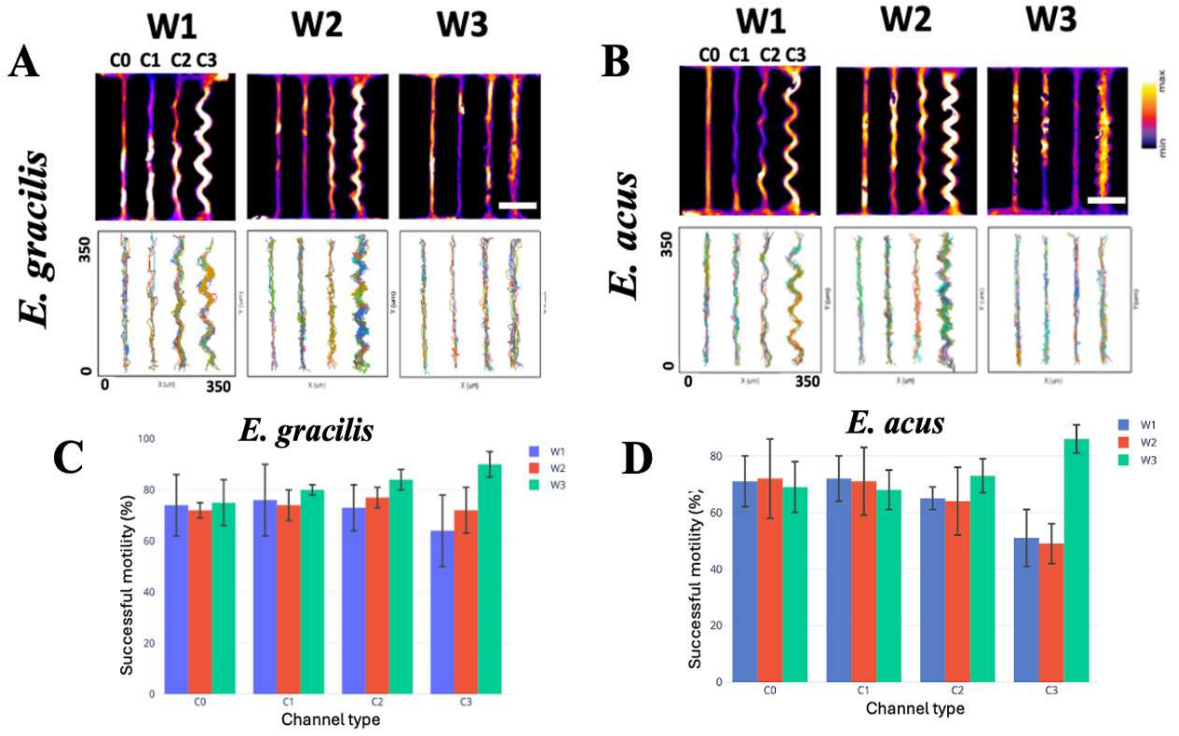

Figure 11. Spatial distribution of cells in the wavy structures. Density map (top grid) and 2D motility trajectories (bottom grid) showing the presence of A. *E. gracilis* and B. *E. acus* in the confined geometries. Quantification of swimming efficiency, successful motility percentages of C. *E. gracilis* and D. *E. acus* in different wavy structures (n=100/channel), C0-C3 represents channel type.

The swimming efficiency of *Euglena species* in complex channels was characterized by quantifying the velocity, average time (residence time) spent in the channel, and rate of success against the channel complexity. The rate of success (Figure 11.C, D was quantified to determine the swimming efficiency (n=100 per channel and n=400 per structure), here a complete trajectory is when a cell enters the channel through one end and exits the channel through the other end, any other type of trajectory namely, U-turn, trapped in the channel, or exit without changing orientation, simply put, exit from the entry side was classified as an incomplete or failed trajectory. The percentage of success in *E. gracilis* (Figure 11.C), shows

an average of 74.4% successful trajectories in C0. In structure W1, the rate of success declines with channel complexity with 75.7 %, 73.3%, and 63.9% in C1, C2, and C3 respectively. In W2, the rate of success was 73.9%, 76.9%, and 72.3% in C1, C2, and C3 respectively. In W3 the rate of success increases from C1 through C3 with 80%, 83.8%, and 89.7%, the highest number of successful trajectories was observed in the C3 channel in the W3 structure. *E. acus*, on the other hand (Figure 11.D), had an average success rate of 71% in C0 in all the structures, In W1, the percentile of successful trajectories dropped steeply from C1 through C3 with a 72%, 65%, and 52% in C1, C2, and C3 respectively. In W2, the rate of success was the least with 71%, 64%, and 48.7% in C1, C2, and C3 respectively Rate of success was the least in W1(C3) - 63.9% and W2(C3) - 48.7% in *E. gracilis* and *E. acus* respectively, this corroborated with the least velocity and highest residence time observed in these channels.

Conclusively, the channel complexity had a greater impact on swimming efficiency in both the species, specifically structure W1 (C3) for *E. gracilis* and structure W2 (C3) for *E. acus*. The turn angle variations such as turn angle depth, turn frequency instigated a significant difference in the time spent and swimming velocity in these channels. The extreme geometric confinement and complexity stimulated the cells to switch from flagellar swimming to a robust and efficient mode of motility – the euglenoid movement or metaboly. Although both species adapted metaboly, a higher percentage of *E. acus* showed this trend compared to *E. gracilis*, resulting is an overall lower success rate. Furthermore, cells largely adapted metaboly when their anterior end was agitated, a higher percentage of cells in the C2 and C3 channels of structures W1 and W2 adapted metaboly, although C0 was the laterally confining, fewer cells adapted metaboly in this channel confirming anterior-end agitation predominantly triggers this response. Factors such as end-to-end entrapment at turns/physical barrier and re-orientation by spinning contributed to the difference in residence time and velocity, biological responses such as gyration, backward motility, metaboly also play a critical role in the swimming efficiency of cells.

E. gracilis (Figure S 2.A): in structure W1 as the complexity of the channel increased the velocity decreased, the cells swam with a velocity of 38.4 μms^{-1}, 37.9 μms^{-1}, and 29.1 μms^{-}

[1] in C1, C2, and C3 respectively. The residence time showed an opposite trend with 6.9 s, 7.9 s, and 21.8 s in C1, C2, and C3 respectively. In the structure W2, the velocity decreased with the channel complexity from a 44. 3 μms^{-1}, 37.5 μms^{-1}, and 34. 3 μms^{1} in C1, C2, and C3 respectively, the residence time was 7.1 s, 8.9 s, and 15.9 s in C1, C2, and C3 respectively. In structure W3, the velocity linearly increased from C1 through C3, with 35.4 μms^{1}, 38.2 μms^{1}, and 43.5 μms^{1} and the residence time in these channels were 8 s, 10.5 s, and 7.5 s in C1, C2, and C3 respectively evidently, due to the ease in channel confinement and complexity. Overall, the swimming efficiency of *E. gracilis* was the lowest in W1(C3), the velocity was the lowest and the residence time in this channel was the highest. The turn frequency in the channel (W1-C3) was lower (8 turns) compared to W2-C3 (12 turns), but the turn angle was higher and cells constantly collided head-on with the walls to switch their swimming orientation. The velocity of *E. acus* (Figure S 2.B) was 35.4 μms^{-1}, 30.4 μms^{-1}, and 29.2 μms^{-1} and the residence time being, 5.8 s, 8.4 s, and 15.4 s in C1, C2, and C3 respectively. In W2, the velocity decreased with the channel complexity and the time spent was the highest in the C3 channel. In structure W3, the velocity increased linearly with 29.5, 33.6, and 35.4 μms^{-1} in C1, C2, and C3 respectively, the residence time was 4.1, 2.1, and 1.6 s in C1, C2, and C3 respectively, this trend was probable as the channel confinement and complexity was low. The swimming efficiency was the least in W2-C3 channel, the velocity was the least with longest residence time in this channel. Furthermore, the residence time was higher in the C3 channels of structure W1 and W2 because a higher percentage of cells adapted metaboly due to the complexity of the channel.

The swimming velocity decreased in the channel with the highest lateral confinement (C0) in both species however, very steeply (twice) in *E. acus* (Figure S 2.B). The flagellar beats are lateral, non-planar waveforms in euglenids [31], the lateral confinement reduces the adequacy of flagellar beating in both the species rendering them to swim at a lower velocity, this mechanism was also observed in ciliates when confined between glass plates [28]. Additionally, the characteristic motility in euglenids is a wiggly motion (soft sinusoidal waves) with helical rotation [31], this wiggly motion leads to increased collisions with the wall. Alternatively, the decrease in velocity as an effect of confinement could presumably be

due to decrease in lubrication forces as also reported in ciliates [28] or disturbance in flagellar beat frequency.

4.4 Outlook

The motility patterns of two euglena species with flagellar length and slight morphological differences provided a comprehensive understanding of species-dependent, intricate behaviours and strategies adapted to achieve navigation in response to various geometrical confinements. The experimental observations provide valuable insights on the surface scattering dynamics of cells swimming in close proximity to the wall, how cells exploit their ability of vertically incline to escape from a wall-corner trap by inclining in tdirection, and the correlation between complex confinements and swimming efficiency. The ability of cells to switch between flagellar swimming, back and forth motility, and metaboly provides insights into flexibility and adaptations in euglenids. However, how they benefit from these modulations and the precise mechanism in their natural habitats where cells swim in water remains elusive. This study finds its relevance in design and control of soft, self-adaptive, bio-inspired robots, and develop motility-based cell separation, biomimetics, and biocomputation. Future work will illustrate various biological behaviours, correlation between properties of elastic motor system – pellicle strips and mechanical responses of the cell, the factors that trigger metaboly, and why cells have retained this ability still adapt metaboly.

4.5 Supplementary Information

4.5.1 Supplementary figures

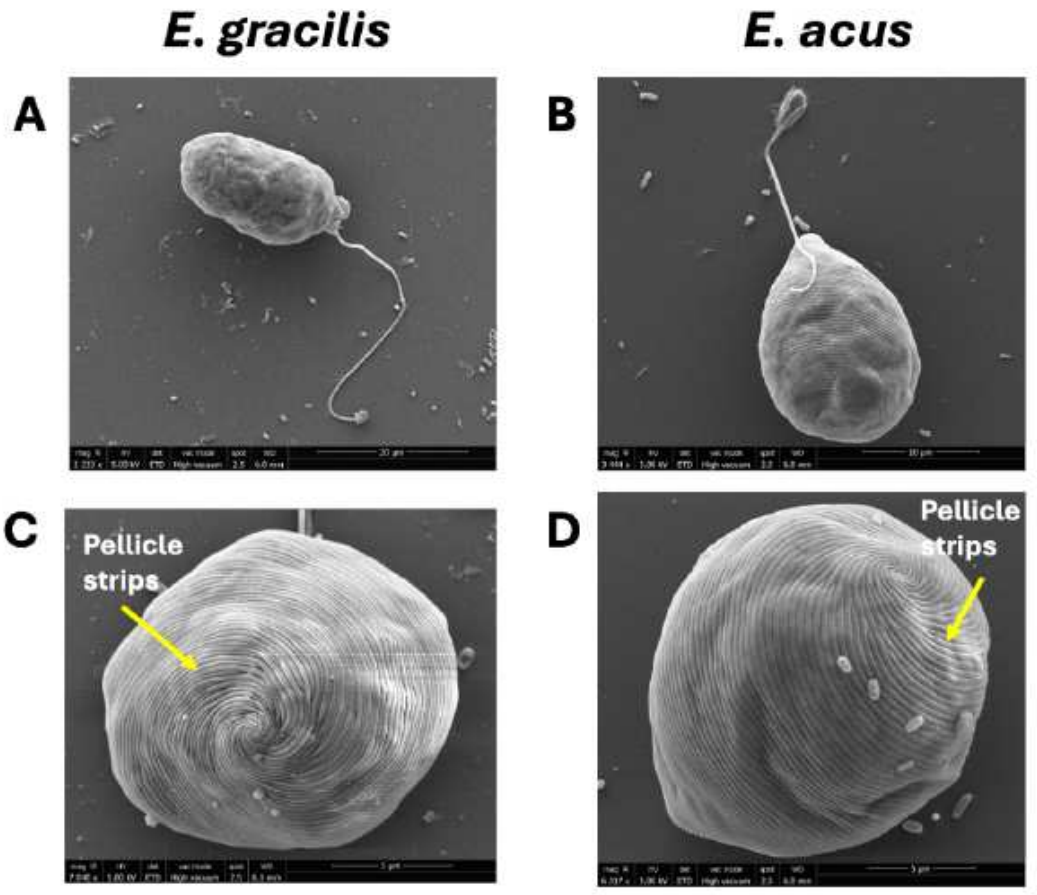

Figure S 1: SEM images of highlighting cell body and flagellum (A. *E. gracilis*, B. *E. acus*) and pellicle strips (machinery for bodily deformations) in C. *E. gracili* and D. *E. acus*.

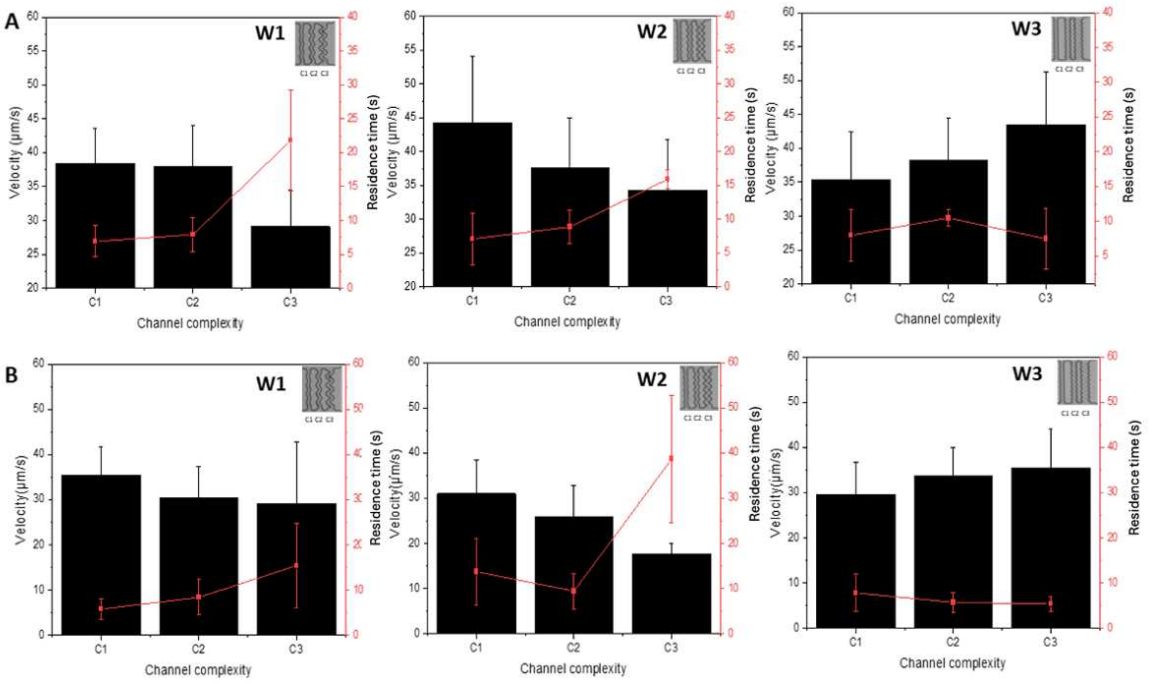

Figure S 2: Effect of channel complexity on swimming efficiency quantified byvelocity and residence time against channel type in A. *E gracilis* and B. *E. acus* n =100 per channel, that is 300 per structure

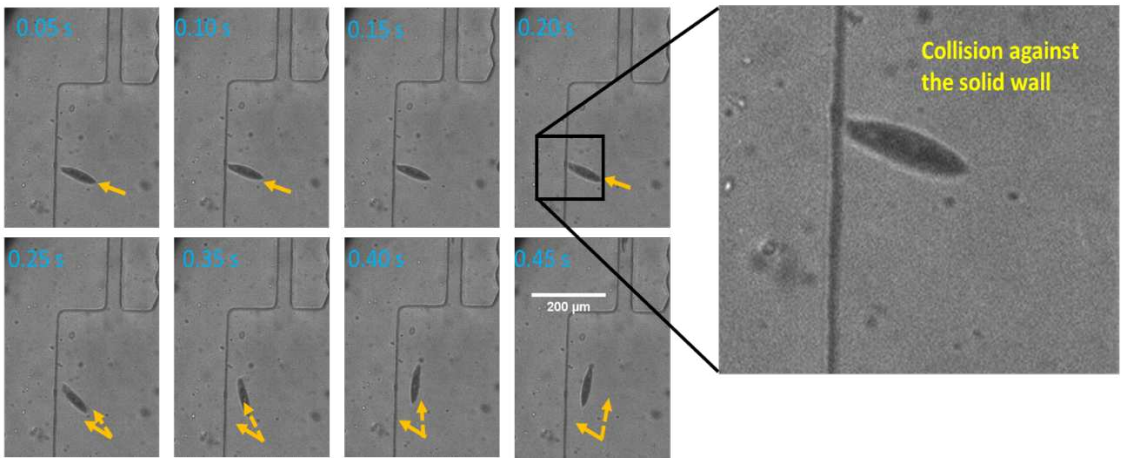

Figure S 3: Montage of optical images of *E. gracilis*, deflecting from wall (scattering) upon anterior-end collision with the wall.

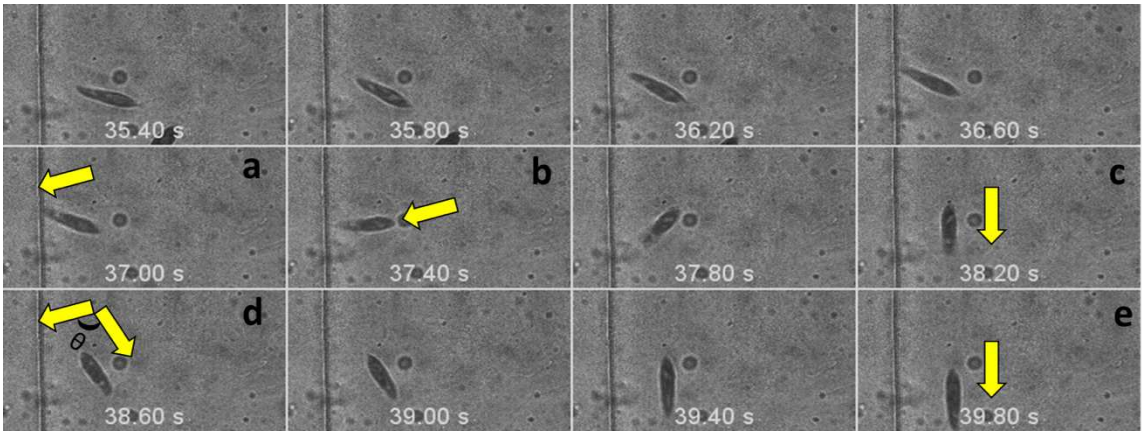

Figure S 4: Montage of optical images of *E. acus* performing a sharp turn (scattering) upon anterior-end collision with the wall. A. Point and angle of collision, b. retraction from the original position, c-d. sharp turn with an exit angle θ = 119º, e. trajectory course correction.

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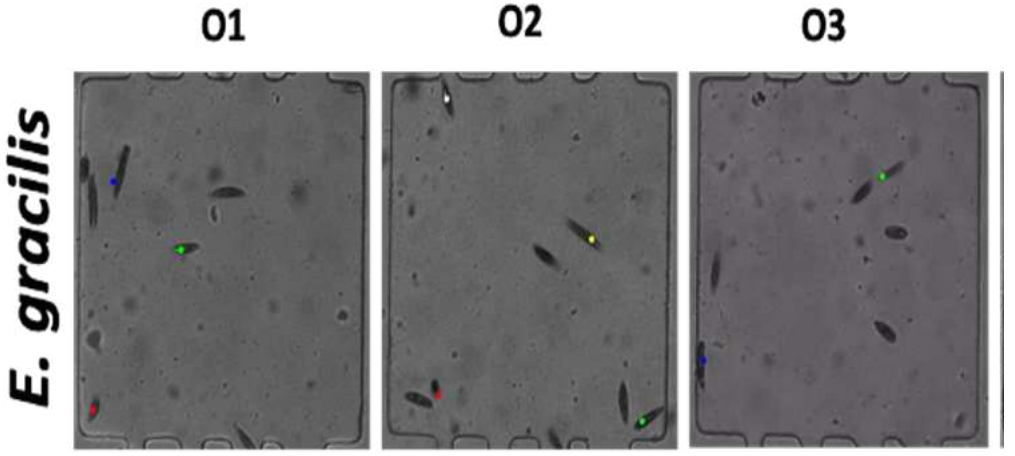
4.5.2 Supporting movies

Supplementary movies related to this study are available at the Zenodo repository and available as PowerPoint file.

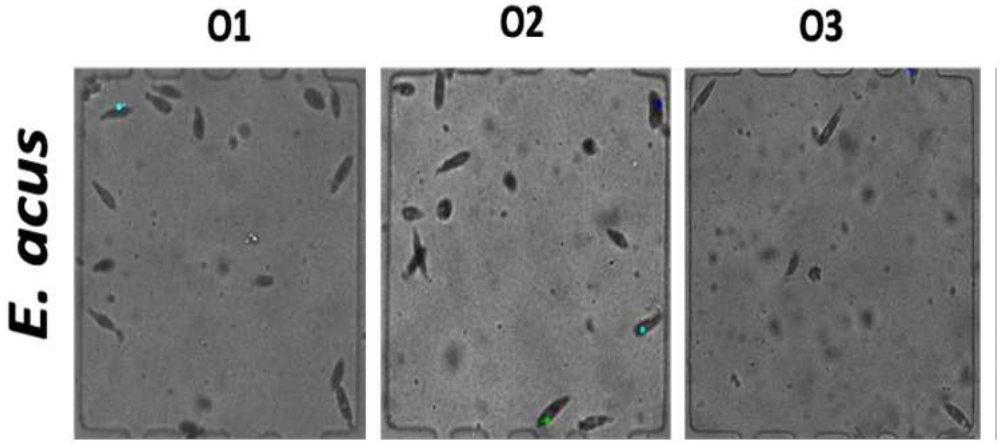
https://zenodo.org/records/14579594?token=eyJhbGciOiJIUzUxMiIsImlhdCI6MTczNTU5NzQxMiwiZXhwIjoxNzM4MTk1MTk5fQ.eyJpZCI6ImFiMmJiYjViLTEyZDEtNGFiMy1iNTUzLWNjYWUwNzQ2YTI3NSIsImRhdGEiOnt9LCJyYW5kb20iOiJjZGRiOWYxNjkxNDBkZWNjMzYyMjRmZmMxMWEzNmZlOCJ9.pKP6_gMVQfJqVuP2TxR26itD4dh8BwQkdXYn2eGZlZU_Y32Q3ZSxPMtpwAGye2nYsMWwk2vHodf02Xuw_e8I6w

1. Motility of *E. gracilis* in open channels.



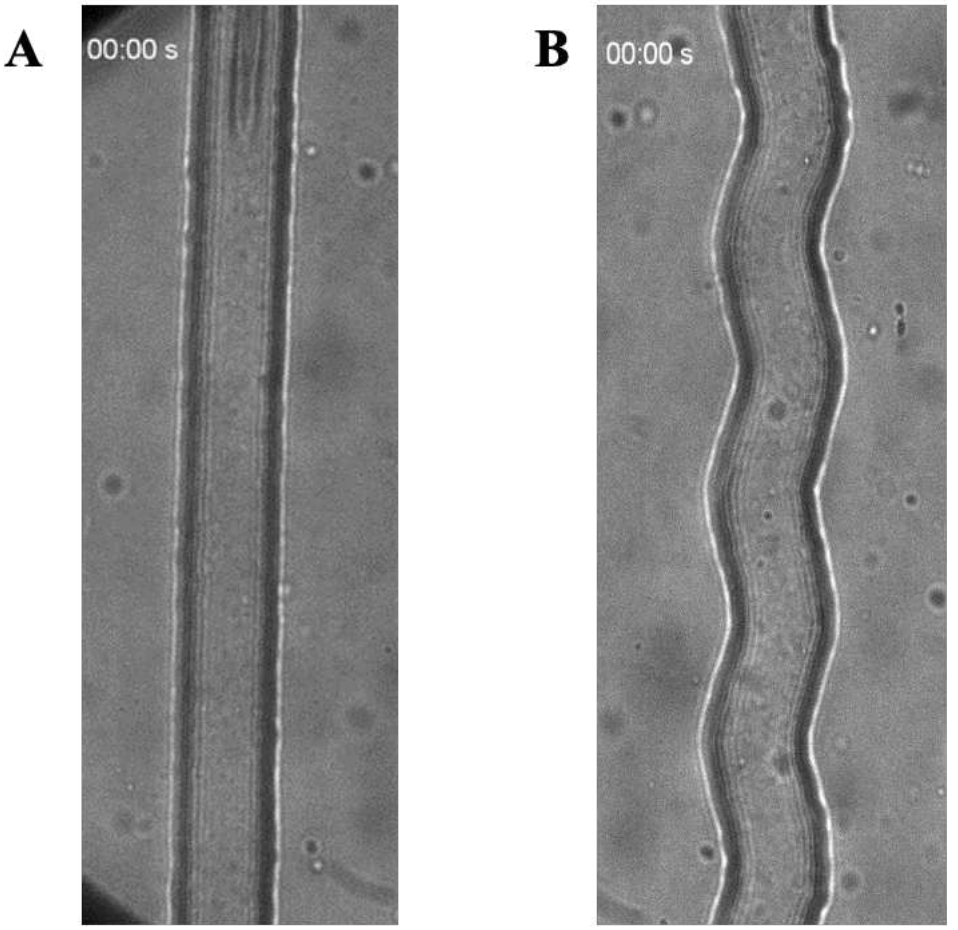
Movie 1: Motility in quasi-open spaces in *E. gracilis*: Random motility patterns, frequent change of directions with high deflection angles, small circular motions. Broken or un-even near-wall density, wall bouncing (ping-pong like) motility and corner trappings observed.

2. Motility of *E. acus* in open channels.



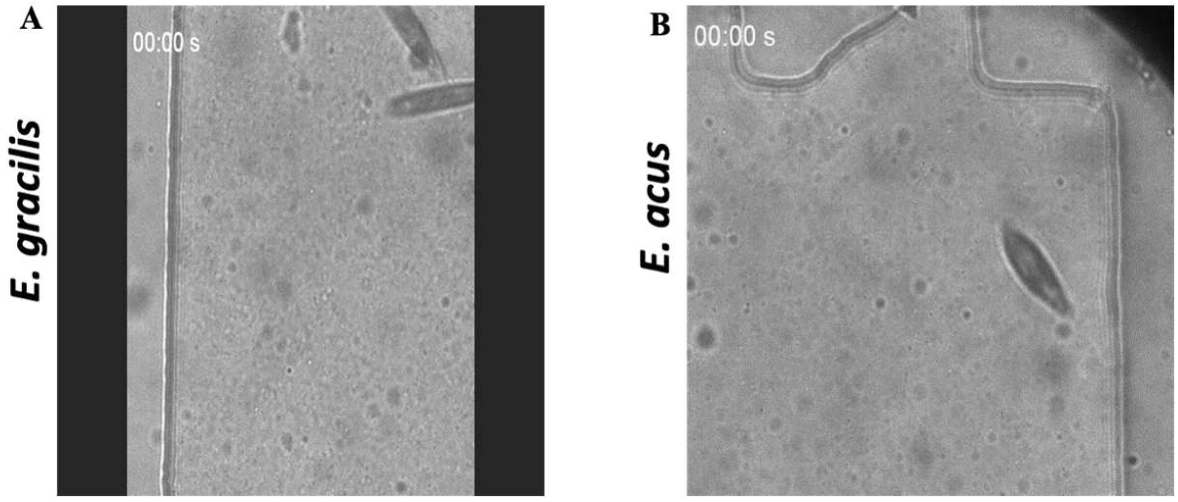
Movie 2: Motility in quasi-open spaces in *E. acus*: Distinctive motility patterns observed with each structure, O1, O2– long trajectories with wall bouncing (ping-pong like) motility and corner trappings observed. Short movement parallel to vertical walls until scattered off. O3– Predominantly short, linear trajectories with minimum to null interaction with the wall.

3. Analysis of altered motility patterns in the confined channels.



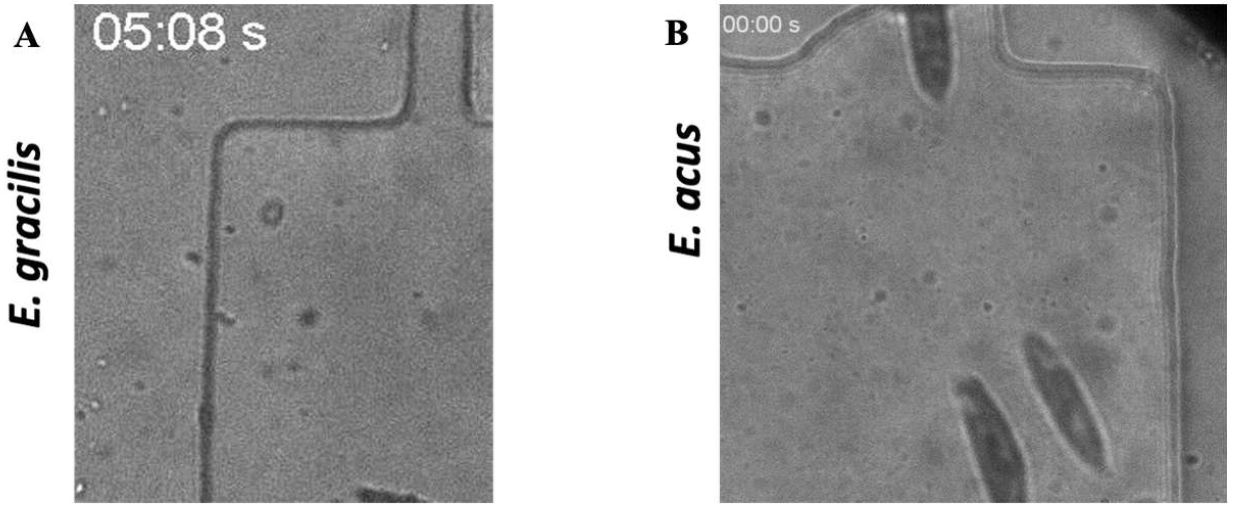
Movie 3: In movie 3A, *E. gracilis* exhibited various behavioural responses, the linear channel (C0), provides lateral confinement physically, and several cells adapted back-and-forth swimming patterns in this type of channel, a similar trend was observed in relatively linear movie 3B (C1) channel type as well.

4. Scattering in quasi-open channel.



Movie 4: Cells perform sharp turns upon collision at a physical barrier. The cells in quasi-open channels exhibited wall bouncing or ping-pong-like trajectories, here the scattering was initiated by the mechanical responses followed by interactions with the wall in 4 A. *E. gracilis* and 4 B. for *E. acus*.

5. Analysis of swimming responses in situations of wall corner entrapment.



Movie 5 A. *E. gracilis,* some cells exhibit a series of responses as an escape mechanism, anterior end of the cell recedes or retracts, pulling the flagellum from the entrapment, this increases the distance between the wall corner and the cell body, and the cell body exhibits metaboly and spins. Cells switch orientation during swimming and eventually recover their original swimming pattern. S 5 B. *E. acus,* recedes and tilts the cell body in the z-direction until the trapped cell restores its ability to swim.

Chapter III

Behavioural studies

The following work is focused on using microfluidics-based varying confined structures to probe the flagellar beat patterns and behavioural responses of *Euglena gracilis*. We investigated the flagellar beats, behavioural responses and, escape strategies adapted by cells under complex and simple confinements. This work focuses on how the flagellum undergoes a series of shape excursions, allowing the cell to consequently select the most efficient responses reflecting on changes in behaviour to free itself from tight spaces. Furthermore, this study sheds new light on the process of metaboly, which has remained inadequately understood. Our findings reveal a correlation between metaboly and compromised flagellar, thereby advancing our understanding of this intriguing phenomenon.

This chapter encompasses the second study to which I have contributed. The data obtained from this study is of publishable quality and is currently being prepared for publication.

5. Flagellar shape modulations and behavioural changes to confinement in *Euglena gracilis*

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Abstract

The flagellum is an essential component that powers the movement of microorganisms. Understanding the intricate mechanisms behind the interaction between the flagellum and its surrounding environment is paramount to gaining valuable insights into the physical principles that govern the motility and behaviour of microorganisms. The focus of this work is to examine the behavioural responses of *Euglena gracilis* under microfluidics-based confined geometries in the absence of external stimuli such as light or chemical gradients. We investigate the changes in flagellar beat patterns, cell behaviour, and escape strategies adopted by cells in both complex and simple confinements. We observed significant differences in flagellar beat patterns and distinct characteristic changes in the behaviour of the cell. The flagellum undergoes a series of shape excursions, allowing the cell to regulate the flagellum beating and consequently select the most efficient responses reflecting on changes in behaviour to free itself from tight spaces. In less confined spaces the cell chose alternative swimming modes to escape from physical barriers or obstacles. The observed patterns reflect the complex interplay between the cell's physiology, external cues, and the need for efficient motility or escape. The findings from this work also suggest that metaboly, is highly related to the compromised flagellum, which brings us closer to a better understanding of metaboly adaptation. This research sheds light on how unicellular microbes can use the flexibility of their flagellum and body to select an optimal response to escape and navigate through confined spaces. The insights from this study have potential implications for fields such as soft robotics, biomimetics, and bioengineering, by providing critical insights into the underlying characteristics and strategies of flagellar motility.

5.1 Introduction

Motility in microorganisms facilitates a range of biological processes essential for survival, including movement towards resources, colonization, dispersal of progeny, pathogenicity and, escape from hostile environments. Most microorganisms use thin hair-like appendages such as flagella, cilia, or pili as primary structures for motility. The role of these appendages in motility has been widely studied to understand the fundamental aspect of microbial physiology, biophysical mechanisms, and their navigational strategies in both prokaryotes and eukaryotes. Microorganisms in nature are capable of performing versatile feedback control strategies by changing their motility behaviour in response to environmental stimuli. Bacteria navigate to chemical gradient cues by alternating between running and tumbling states [164-167], the motility of bacteria is the result of a "tug-of-war" between hydrodynamics and local nanomechanics influenced by confining microenvironments [168]. *Chlamydomonas reinhardtii* uses its flagella to transition between positive and negative phototaxis [3-7]. Euglenids respond to a number of external stimuli such as phototaxis [8-10], gravitaxis [11-13], chemotaxis [13], and mechanical responses [14] to orient and survive in their natural habitat. Apart from light or chemical gradients, boundaries and confined spaces trigger intriguing behavioural responses among microswimmers [15-19]. How the microswimmers develop their feedback mechanism to stimuli and control their motility accordingly is still poorly understood.

The flagellar beat patterns (essentially helical and planar) of eukaryotic flagellates including several protists, and sperm cells have been studied to understand the underlying mechanisms that govern their motility and beat patterns [169-174]. Euglenids are unicellular eukaryotic microorganisms that exhibit complex and diverse swimming behaviours. Motility in euglenids is propelled by the lateral beating of the emergent flagellum. Bending waves emerge from the base of the flagellum rooted in the reservoir, through the length of the flagellum and fade at the tip. The flagellum bends while swinging the cell body around, entailing spiralling trajectories coupled to body rotations, thereby, the motility pattern is a characteristic sinusoidal path or a periodic swinging motion. The swimming propulsion mechanism of the flagellum can be summarized as a complex sequence of non-planar waveforms with a characteristic movement mimicking a spinning lasso or figure "8" [169].

Apart from flagellar swimming, euglenids are also capable of performing highly coordinated bodily deformations called metaboly or euglenoid movement [40-42]. The earliest observations of metaboly date back to the first microscopist's [29] and it has been an intriguing phenomenon ever since. Euglenoid movement or metaboly is a unique, elegantly coordinated deformation supported by an active striated cell envelope, called pellicle or pellicle strips [32, 91-93]. In spite of numerous research studies, the functional retention and precise mechanism of this phenomenon remain elusive.

The previous work with motility studies on euglenids showed motility patterns in response to confined geometries, we also observed that the structure design and complexity impacted the cell's motility and behaviour. This work is focused on using microfluidics-based varying confined structures to probe the flagellar beat patterns and behavioural responses of *Euglena gracilis*. Microfluidics is a start-of-the-art technology that enables the recreation of simple and complex microenvironments, which otherwise allows a more realistic representation to study microswimmers than traditional methods. We investigated the flagellar beats, behavioural responses and, escape strategies adapted by cells under complex and simple confinements.

The study proposes that the flagellum undergoes a series of shape excursions, allowing the cell to select an optimal response to navigate physical barriers and confined spaces to restore normal motility. By exploring these patterns, we aim to better understand the adaptive mechanisms that underlie cellular motility in response to mechanical constraints. The proper functioning of flagella is fundamental to the motility of microorganisms, and the intricate interplay between the flagellum and its surrounding milieu will aid gain a better understanding of physical principles that govern the behaviour of microorganisms, particularly applicable to the field of biomechanics. Additionally, we also report that metaboly is a complex response related to the compromised flagellum. This finding brings us one step closer to comprehending metaboly adaptation and represents an advancement in our understanding of intriguing cellular responses to external stressors. The key mechanisms in the way these simple unicellular organisms, process periodic signals to optimal light, optimal position, and confinement, by exhibiting complex behaviour coupled with bodily rotations/alignment and altering flagellar beating implies the intricacies of their

morphological flexibility. The findings of this study can contribute significantly to our understanding of underlying characteristics and strategies of flagellar motility, with potential implications for fields such as microbiology, biomimetics, and bioengineering.

5.2. Methods

5.2.1 Culture of cells and maintenance

The detailed methodology mentioned in Chapter 1, section 3.1.1

5.2.2 Device design and fabrication

The detailed methodology mentioned in Chapter 1, section 3.1.2

5.2.3 Motility experiments

The detailed methodology mentioned in Chapter 1, section 3.1.3

5.2.4 Image acquisition and analysis

The motility behaviour of euglenids was imaged using Olympus IX83, an inverted - Spinning Disk Confocal Olympus IX83 microscope, with MetaMorph® (from Molecular Devices) Microscopy Automation & Image Analysis Software, 10x (NA 0.4), 20x (NA 0.75) and 40x (NA 0.95). Overall, the experiments were carried out at a magnification of 100X and an acquisition speed of 100 and 142 frames per second, at this rate we could capture details with minimal loss of information. Data analysis was performed using ImageJ [175], a public domain software platform. The analysis included tracking, flagellum mapping, curvature analysis. Curvature analysis was done using Kappa analysis [176] a plugin compatible with Image J.

5.3. Results

Molecular motors play a crucial role in driving the movement of eukaryotic flagella, these motors aid in driving flagellum movement by facilitating bending, [177] while the flagellar elasticity counters the bending. Other than the bending and opposing forces, the fluid forces of the surrounding liquid act on the flagellum, the coordinated action and balance between the three forces governs the flagellar motility and swimming of the cell. In addition to the above, the flagellum-body mechanical interaction with the surroundings plays a critical role in the movement of the flagellum. This study highlights the pivotal role played by the mechanical interaction between the flagellum and the surrounding environment in governing the dynamics of flagellum beating and the development of escape strategies.

The motility patterns of euglenids were studied in the microfluidic channels (schematic of device set up in Figure 12.A) with varying geometrical confinements (Figure 12.B(i)) namely, linear channel, quasi-open space (plaza), wavy (W1-W3), and zig-zag channel systems (W4-W6) with three complexity dimensions, 1. turn angle complexity, 2. number of turn angles, and 3. channel member variety (Figure 12.B(ii)). This study provided interesting insights into the adaptation of an optimized algorithm or search strategy to escape from confinement, modulation of various flagellar beat patterns as a feedback mechanism, and a deeper understanding of factors that trigger metaboly.

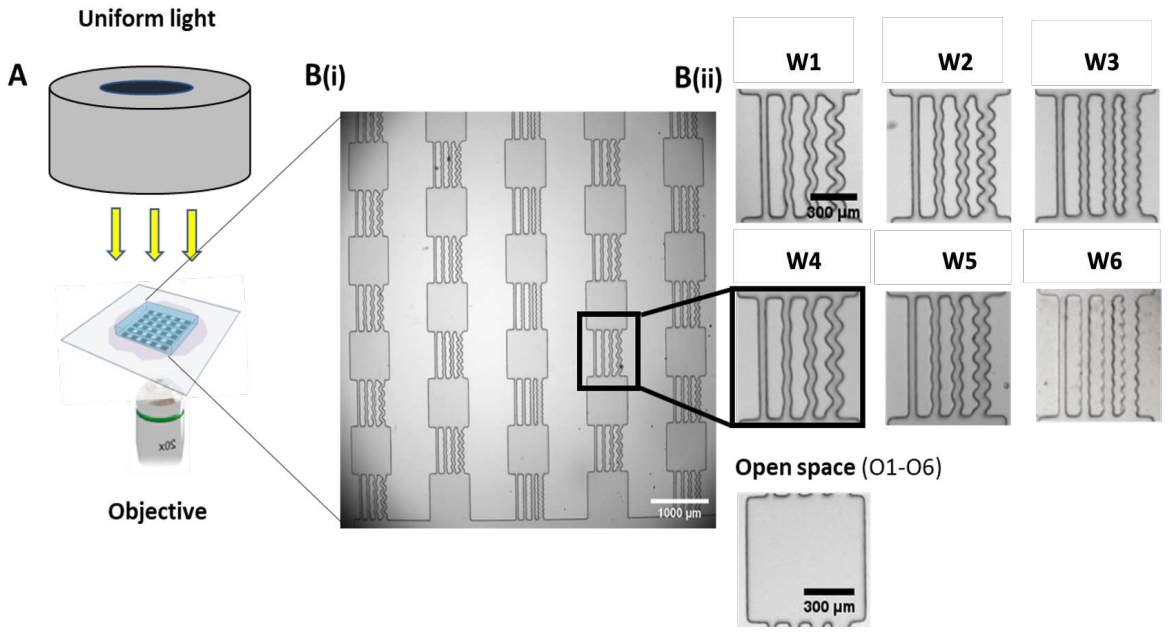


Figure 12: Device design and setup to probe the motility patterns of *Euglena gracilis*. **A.** Schematic representation of microfluidic device flooded with euglenids culture imaged under uniform light. **B.** Sequential, from left to right, (i) the overall architecture of the chip showing a sequence of wavy and zig-zag channels separated by open plazas; (ii) zoom-in of one lane of the experimental structure; detailed image of the experimental structures used in this study, structure W1-W3 comprised of linear and wavy channels with different levels of confinement (top row), structure W4-W6 comprised of linear and zig-zag channels with different levels of confinement (middle row), quasi-open space (bottom row, structure O1 – O6 corresponding to each W1-W6 channel respectively, as shown in **B**(i)).

5.3.1 Euglena switches flagellar beat patterns to achieve states of helical swimming.

To investigate the interplay between swimming motion and flagellar beat patterns, we conducted high-resolution imaging of cells at a higher magnification and frame rate. Cells swimming in the channels adapted various flagellar beat pattern modulations as a result of mechanical shock from the collision with the wall followed by malfunction of flagellar beating. Figure 13 shows that the free-swimming cell, characterized by helical motion (Figure 13.A) switches from the conventional flagellar beating to various beating modulations (Figure 13.B) upon collision with the wall in a complex, confined space. The

series of events includes helical swimming, collision with the wall, malfunction or incompetence of conventional flagellar beat patterns due to confinement, retraction of the anterior end, and significant modifications in flagellar beat pattern (Movie S 6). The flagellum undergoes a series of shape changes to resume conventional beating, additionally, the cell retracts the anterior end to create additional space to address the former. During the retraction, the cell tilts vertically almost entirely in the z-direction before lifting back to the swimming plane. Interestingly, the retraction of the body was highly controlled, and the retraction was successful in that the anterior end had enough space for the flagellum to modulate and resume the flagellar beating. It was also observed that the posterior end of the cell exploits the channel wall to support itself during the retraction and beating modulations. In free-swimming cells, the conventional flagellum beating can be summarized as nonplanar, asymmetrical waveforms mimicking the number 8 (Figure 13.A) or a swinging lasso [169]. When the cell free swimming was hindered due to confinement, we observed that controlled regressive behavioural responses were triggered. Microswimmers are known to respond to physical stimuli using mechanosensitive channels, these channels function as molecular detectors and are found in most living organisms ranging from microbes to humans, they play a critical role in multiple functions including photo, sound, gravity perception, taste, touch and pain sensation, heat sensation, fluid flow, etc., *Paramecium* showed to reverse ciliary beating as an avoidance response upon collision with the wall. [178]. Recent studies on mammalian cells have identified mechanosensitive channels in endothelial and vascular muscle cells [179], as well as immotile cilia or crown cells in the nodal cavity that respond to mechanical stimuli and sense the magnitude and direction of flow [180]. These channels play an important role in protecting bacteria against osmotic shock and varying membrane tension [181]. Mechanosensitive channels in *Euglena gracilis* aid in changes in the movement and course correction of the flagellum during gravitaxis, and it is assumed that these channels are located at the front end of the cell underneath the flagellum as the modulated signals occur when the flagellum points downwards [182]. More on this continued in the later section. Considering the immediate yet controlled avoidance reaction after the collision, and instead of a full body deformation the cell attempts to create additional space from the wall to resume conventional beating, it is safe to hypothesize that this feedback response could be due to the mechanosensitive channels present in the anterior end of the

cell. The cell used the posterior end to attach itself and used one side of the channel wall as a support to efficiently execute the flagellar beat modulations.

Euglenids are capable of propelling themselves using frictional forces [98], here, the ridges of the pellicle strips likely interact with the channel wall to generate frictional forces to support itself against the channel wall.

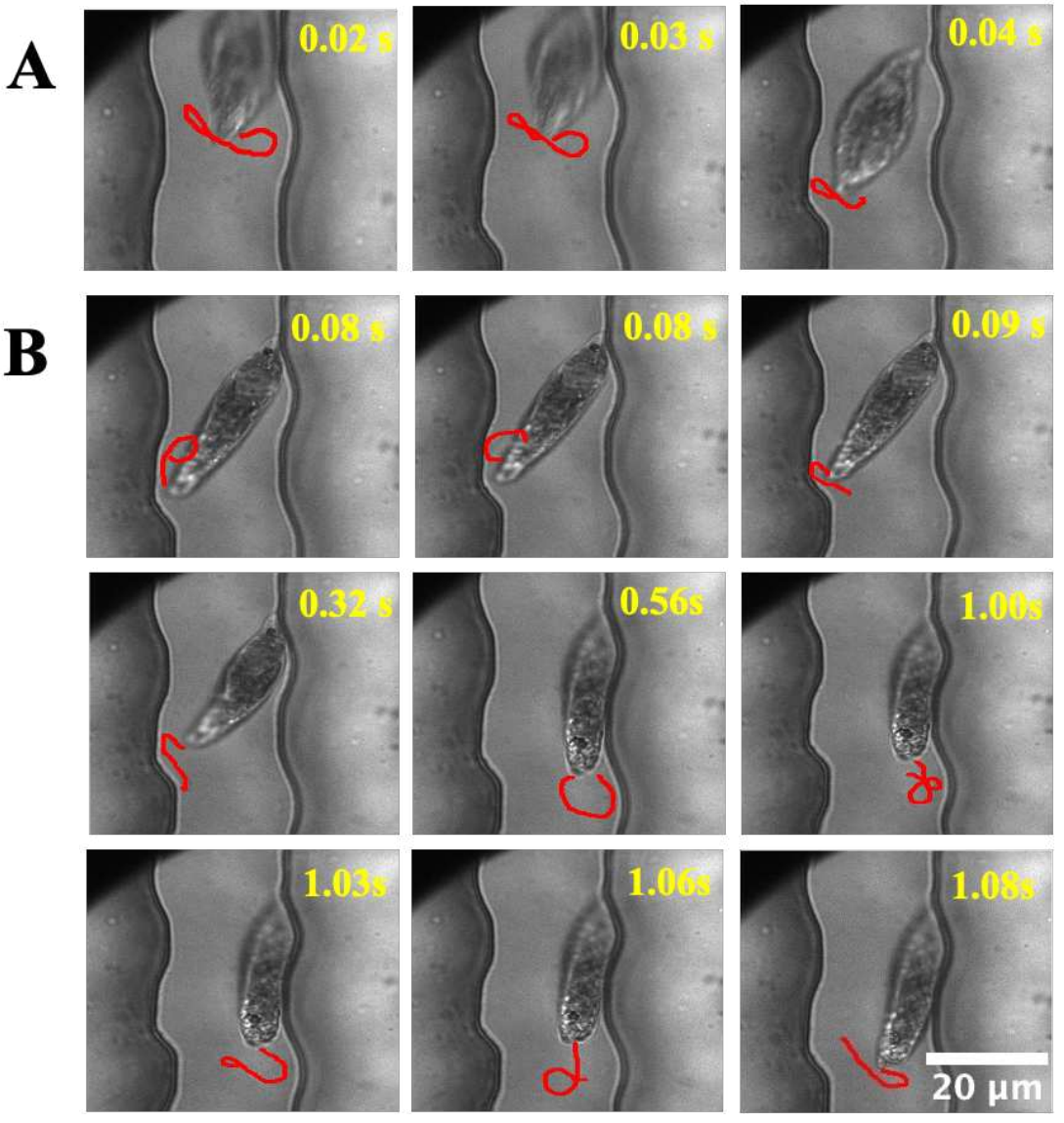


Figure 13: Image sequences showing mapped flagellum obtained from high-speed videos. **A.** Conventional flagellar beat patterns of helical swimming *Euglena gracilis*. **B.** Flagellar beat pattern modulations adapted by *Euglena gracilis* followed by collision with the wall. The flagellum is mapped in red colour.

5.3.2 Flagellum shape excursions - The change is regulatory, not merely haphazard.

To obtain a comprehensive analysis of the complex dynamics of flagellar beat pattern modulations, we studied the shape excursions by frame-by-frame mapping of the flagellum from high-resolution movies (Figure 14). The mapping was done when the entire flagellum

was in the plane, therefore the difference in timings. In this channel type in addition to confinement, the channel presents itself with continuous bends, each bend is almost the length of the cell. To effectively navigate this channel the cell needs to continuously make sharp turns. With the flagellum present at the anterior end steering the body, with each sharp bend, the flagellum as well as the body is obligated to collide with the wall. In most cases, this collision results in hindered free-swimming resulting in behavioural changes. We observed that the modulations exhibit several repeated patterns, despite their seemingly haphazard appearance. It is apparent that the changes in the flagellar beating are commissioned to regulate the conventional figure-eight beat pattern characterized by double loops on either side of the cell. The cell continuously attempts to unwind and re-loop the flagellum (Movie S 7). In this process, the shapes undertaken are, a. Open (Figure 14 at 1.39, 1.47, 1.50, 1.53, 2.09 s), b. Single loop (1.36, 1.49, 4.05 – 4.07 s), c. Twisted loop (1.40, 1.44 (i, ii), 1.46 (ii), 1.58, 2.08 s), d. Double loop (1.37, 1.38, 2.12 s). The observed complex dynamics of flagellar beat pattern modulations appeared to involve multiple attempts of reconfiguration, characterized by systematic changes with repeated shapes. Remarkably, the flagellum is capable of entirely unwinding itself in order to restore optimal beating and facilitate efficient locomotion to escape confinement.

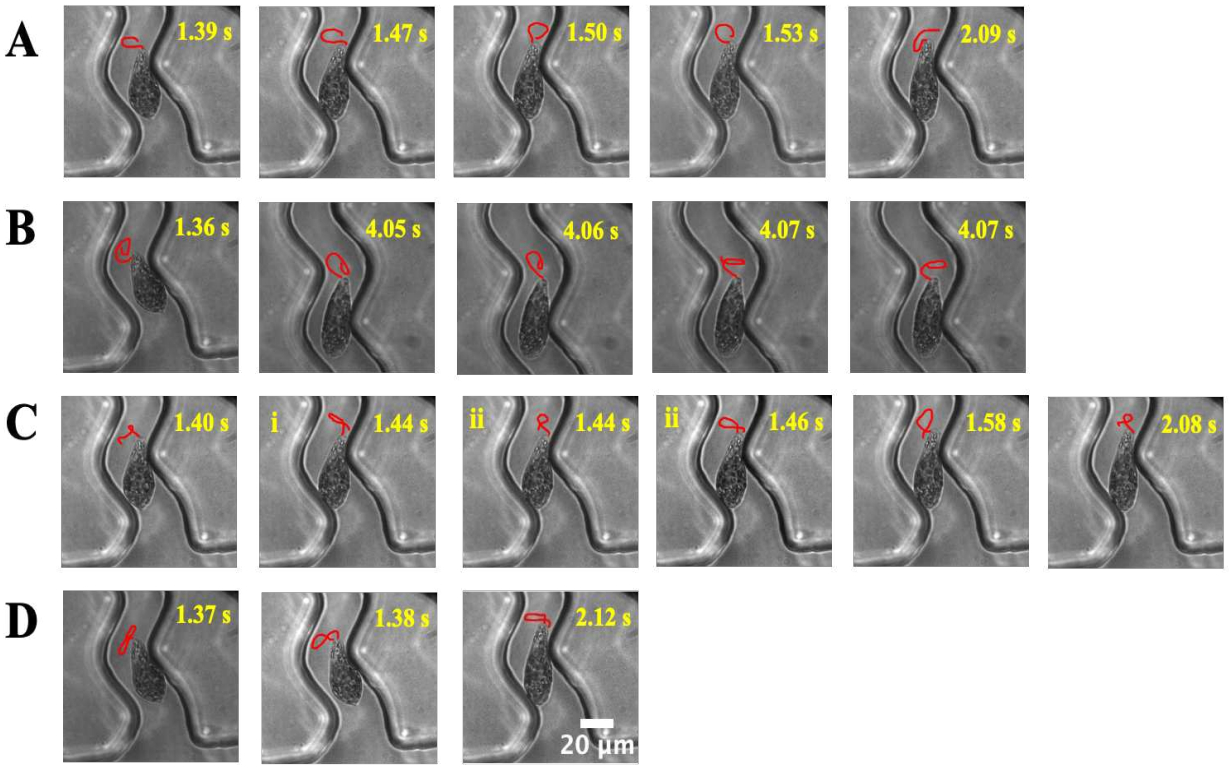


Figure 14: Image sequence demonstrates the systematic and dynamic changes in the flagellar beat patterns of *Euglena gracilis* as it attempts to regulate its conventional beating in a confined environment. The flagellum, highlighted in red, exhibits remarkable flexibility and reconfiguration capabilities to optimize locomotion. Throughout the sequence, the flagellum undergoes several distinct configurations: A. Open, B. Single loop, C. Twisted loop, and D. Double loop.

Eukaryotic ciliates and flagellates use sensory cues to navigate in their habitat, when cells need to change the swimming direction, it becomes challenging as unsynchronized movements are required to produce the appropriate torque for the changes in swimming direction. Reports on sensory reception in eukaryotic organisms date back 100 years, this sensory reception includes mechanosensation, phototactic, chemotactic sensation etc., The avoiding reaction was first reported in *Paramecium* [19, 183], when the cell collides with a solid object, it briefly reverses swimming direction and orientation, then resumes forward motion, this continues until the organism successfully avoids the obstacle. It was later discovered that the underlying mechanism for this avoiding reaction was the initial contact with the obstacle triggered a Ca^{2+}-based depolarizing mechanoreceptor potential a temporary rise in intraciliary Ca^{2+} concentration [184-187]. This change in the Ca^{2+} action potential initiates a temporary reversal of axonemal beating, resulting reversal in the swimming direction. A similar mechanoreceptor-mediated Ca^{2+} influx was also observed in *Chlamydomonas* [188, 189], furthermore, it has also been reported that *Chlamydomonas* is equipped with other flagellum-mediated sensory roles [190-192]. The sensory function of the motile flagellum in protozoan *Heteromastix* showed the flagellum movements constantly feeling for obstructions, and upon encounter seizes the hold of it [193]. It's important to note that the specific flagellar-based functions can vary among different eukaryotic cells and organisms. The observed complex dynamics of flagellar beat pattern modulations in our study suggest that the flagellum's intricate movements are not random but follow a purposeful and regulated process aimed at optimizing the cell's locomotion. These repeated patterns, particularly, the attempt to resume the conventional figure-eight pattern characterized by double loops on either side of the cell, indicate a deliberate effort to reconfigure the flagellum's shape during the beating. Furthermore, the observed capability of the flagellum to entirely unwind itself, extend, and sense the confined space around suggests an adaptive mechanism employed by the cell to overcome confinement and facilitate escape. Different shapes may provide varying propulsive forces and directional control, allowing the cell to navigate and understand the space effectively. As iterated earlier, perhaps the changes in the flagellum shape could be a result of such mechanoreceptors present at the anterior end of the cell. However, a pivotal question that warrants consideration is whether the eukaryotic flagellum is an organelle exclusively used for motility/locomotion

or does it possess many additional functions such as serving as an active sensory organelle. As observed in this study, the controlled regressive behaviour to retract the anterior end just to make more space to beat the flagellum, or the continued effort to entirely unwind and rewind the flagellum to regulate the beating while continuously sensing the space indicates a complex layer of characteristics to the flagellum. This implies that the flagellar beat pattern modulations are not arbitrary but rather purposeful adjustments aimed at ensuring the cell's effective movement in response to surrounding challenges. Further research and experimentation would be necessary to provide more detailed insights into the underlying mechanisms and functional significance of flagellar shape modulations in specific euglenids.

5.3.3 Correlation between flagellar malfunction and metaboly.

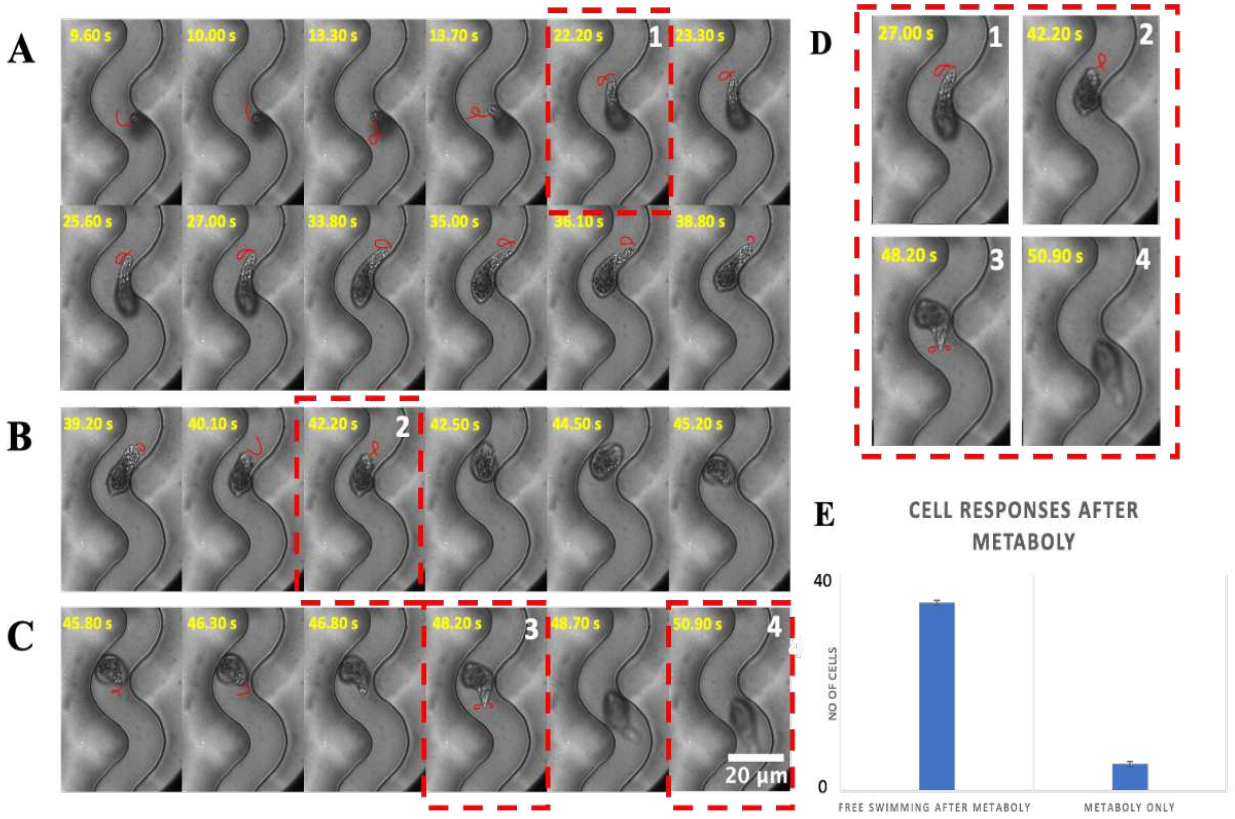


Figure 15: Image sequences showing the correlation between flagellum malfunction and metaboly. The figure depicts the transition from flagellar swimming to bodily deformation after a flagellum malfunction. A. Flagellar beat modulations to resume conventional beat pattern after collision with the wall, B. Failure to resume flagellar beating and metaboly/bodily deformation initiation as an alternative swimming, C. Metaboly swimming, reorientation, flagellum resume to conventional beating, cell resume shape and free swimming, D. Highlight of the events to resume free swimming 1. flagellum beat pattern modulation, 2. metaboly initiation, 3. re-oreint and resume flagellum beating, 4, free swimming. E. Graph showing flagellum regulation and metaboly-based swimming outcomes (n=40). The flagellum is mapped in red color serves as a critical marker in this sequence revealing cell response to physical constraints.

We observed cells adapted metaboly after multiple attempts to regulate their flagellar beats as an ultimate resort to escape from the complex confinement. Metaboly, also known as

euglenoid movement refers to highly coordinated bodily deformations exhibited by euglenids. Despite extensive research, the exact function of this interesting phenomenon is rather unclear. However, we may have an interesting theory from our experimental observations to obtain a better understanding of the purpose. Figure 15 shows that the cell in confinement begins to regulate the flagellum beats by undergoing modulations (Figure 15.A), at 38.80 s, the cell then initiates retraction at the anterior end ultimately leading to bodily deformations almost entirely (Figure 15.B) at 44.50 s (Movie S 8.A). Following the deformation, the cell undergoes a small spin or rotation to reorient itself, while still endeavouring to regulate the aforementioned flagellum beats. Once the cell was able to restore the flagellar beating (48.70 s), the cell quickly resumed its elongated cell shape to escape or swim away from confinement (Figure 15.C). In certain instances, it has been observed that cells are unable to resume their flagellum beating activity after adapting metaboly. As a result, these cells continue to tumble incessantly attempting to exit the channel (Movie S 8.B), whereupon they revert to their conventional mode of swimming. When we quantified the outcomes of metaboly-mediated free swimming (Figure 15.E), the majority of cells (87.5%) successfully utilized metaboly to reorient and subsequently resumed conventional flagellar beating for free swimming, 12.5 % of cells failed to regulate free swimming and traversed the channel with metaboly swimming or incessant tumbling. This indicates metaboly is a successful alternate swimming mode or escape response in both cases, with an endpoint to navigate confinements.

The flagellar motion hindrances caused by confinement result in changes in the cell orientation and position in 3D space ultimately when the flagellum fails to resume conventional beating, metaboly takes over as an alternative swimming strategy. The complex feedback between the flagellum beat patterns and the accompanying space sensing (stimulated by flagellum unwinding and modulating) enables the selection of an optimal response and facilitates strategic decision-making for effective navigation through complex confinements/environments. We observed here was although metaboly is a quick and faster escape strategy this does not seem to be an immediate/first response to confinement. Rather, the cells first attempt to unwind the flagellum and modulate its beating, and perform various trials to regulate the beating, in this process, sensing the availability of space or level of confinement to resume free helical swimming, when unsuccessful ultimately adapt metaboly.

As understood in previous studies, metaboly was not due to culture crowding or just confinement [98], metaboly appears to be largely associated with the agitation at the anterior end and the inability to beat the flagellum conventionally for free swimming. In other words, metaboly is not a first response to confinement, rather a series of alternative methods are adopted by the cell which when failed leads to metaboly. Additionally, the escape doesn't just stop with metaboly, when unsuccessful to resume free swimming, the cell undergoes tumbling-type motility to escape the confined channel as witnessed in Movie S 8.B.

5.3.4 Backward motility.

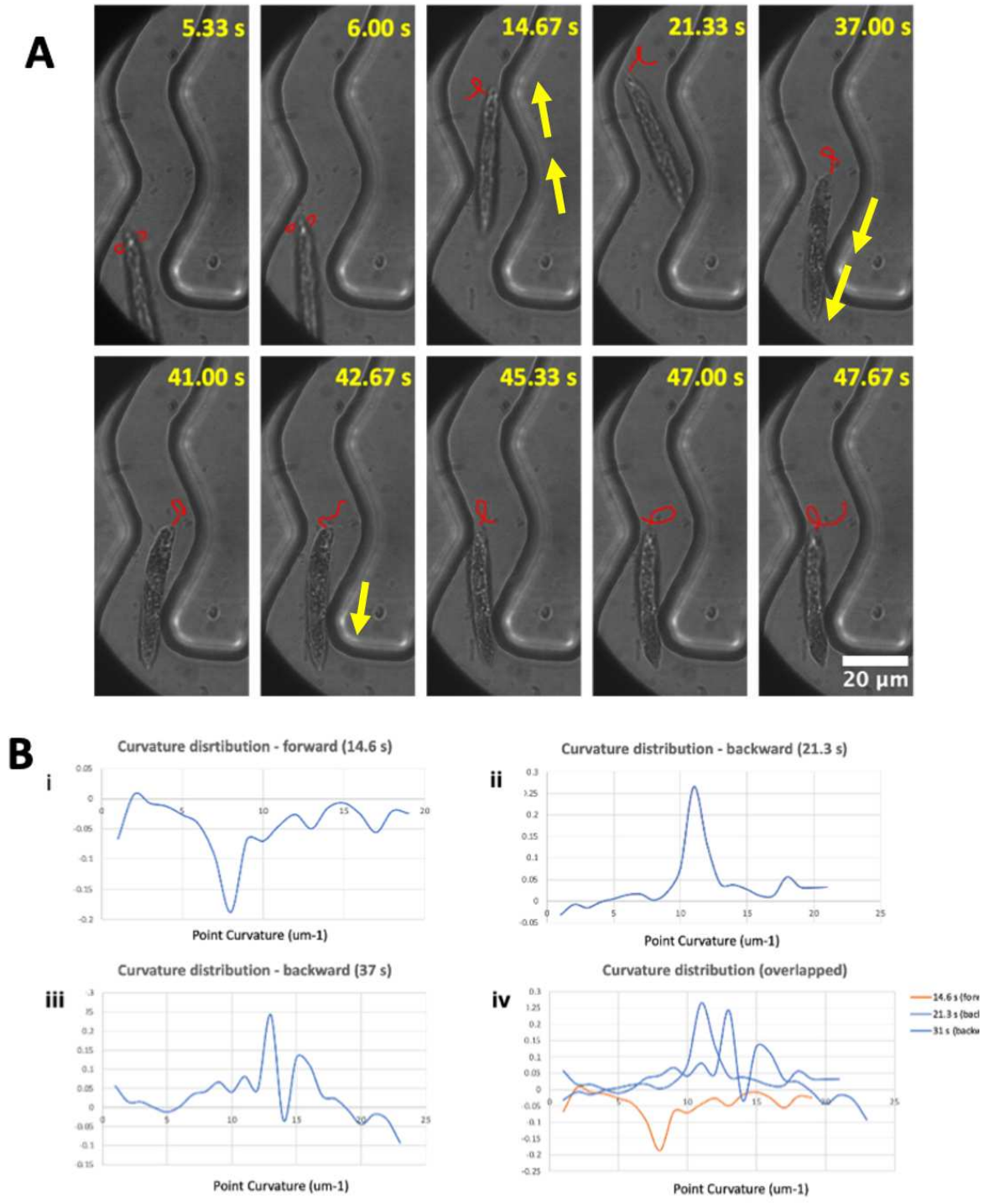


Figure 16: Figure A. Presents a time-lapse sequence illustrating the transition of *Euglena gracilis* from conventional forward swimming to backward swimming within a confined

microchannel. Arrows indicate the swimming direction. B. Point curvature analysis of the flagellum during forward and backward swimming using Kappa curvature with B-spline curve fit. The flagellum, mapped in red, undergoes distinct modulations in its beating pattern as the cell navigates its environment. The flagellum is mapped in red colour.

Fewer cells were observed to spontaneously adapt a reverse or backward swimming as an avoidance response to the confined channel. Figure 16. A illustrates that cells swimming through the channel first adapted modulations in flagellar beating and very quickly transitioned to brief backward or reverse swimming. The principal observation here was that the cell unwinds the flagellum, however, the cell itself held its original shape (ellipsoid) while swimming in the backward direction, the flagellum beat patterns continued to vary as the cells descended the channel. Eventually, the cell retracted its anterior end and adapted metaboly to change the swimming direction, as evidenced in Movie 9. Although elusive, it appeared that when the cell swam backward there was a directional switch from clockwise rotation to anticlockwise rotation, based on the movement of the eyespot position (Movie 9). To investigate if there is any difference in the flagellar beating, we measured the curvature of the flagellum during the forward and backward swimming phases. For a more accurate comparison, the conventional flagellum beating during forward swimming was excluded, and the curvature was analyzed when the cell was swimming with the untwisted flagellum both forward and backward. The curvature was measured on various locations on the flagellum with a B-spline type curve fit. The resulting curvature analysis showed altered curvature when the cell swimming forward compared to when it was swimming backward (Figure 16.B). Specifically, Figure 16.B(i). shows the curvature distribution when the cell is swimming forward at 14.6 s, while 16.B(ii). and (iii). show the curvature distribution of the cell swimming in the reverse direction at 21.3 s and 37 s, respectively. Figure 16.B (iv). is an overlapped graph of the above three time points which indicates the difference in the curvature of the flagellum. The curvature was measured from the base of the flagellum to the tip of the flagellum, it can be observed that there was an offset in average curvature in the backward swimming compared to forward swimming. It can be hypothesized that there was an altered wave propagation in cells swimming backward. The propulsion in euglenids can be summarized as non-planar waveforms resulting in helical trajectories coupled to body

rotations [169]. These waves are generated from the root and extend to the tip of the flagellum with each beating stroke thus pushing the swimming cell forward. It is noteworthy that backward swimming is not a predominant trait in euglenids, however, some studies report a reversed flagellar motion with a shift in curvature and wave propagation in euglena as a photoinduced response [194]. Microswimmers show interesting responses to confined spaces, such as, bacteria showing circular swimming near boundaries due to hydrodynamics [195], accumulation near solid surfaces [196], reverse the swimming direction by flagellar reorientation at an obstacle [16], cell-surface scattering and long residence time on surfaces [197]. Eukaryotes like sperm cells also exhibit such circular swimming due to hydrodynamics [198], accumulation near flat surfaces [15], and preferential swimming such as boundary following navigation [17]. *Paramecium* also exhibits avoidance [19], meandering and bent or circulating navigation in confined spaces [18]. Our study did not involve an external stimulation such as phototaxis, however, it is evident that confined spaces act as a physical cue that triggers very intriguing self-organized responses in euglenids which can be exploited for the design and control of biomimetic soft robots.

5.3.5 Other intricate behaviours and strategies to achieve navigational tasks.

5.3.5.1 Spinning and vertical inclination

In the quasi-open channel where the confinement is very limited few interesting motility patterns were observed. The free-swimming cells transition from helical swimming to spinning to avoid an obstacle. The reference to spinning here is adapted from [90], during spinning-type motility the flagellum is twisted into a single loop in comparison to two loops while helical swimming. Two types of flagellar shape effecting can be observed, a. single loop extended towards the front of the cell (Figure S 6.A), b. single loop extended opposite to the turning direction (Figure S 6.B). Free-swimming cells as soon as they encounter an obstacle, switch from helical swimming to spinning, during this the cell initially twisted the flagellum into one loop extended on the side opposite to the swimming direction, steered almost a 180 turn from the obstacle (obstacle here was another euglena stuck to the wall of the channel) to change the swimming direction as observed in Movie 10.A. Spinning as a behavioural change was observed in *Euglena gracilis* as a phototactic response to changes in light intensity [90]. In our study, this response was a result of behavioural changes or mechanical responses to obstacles, particularly in relatively unconstrained spaces. Decisively, the flexibility of the flagellum coupled with prompt stimulus to physical cues trigger an active change in switching of swimming behaviour resulting in avoidance reaction without exertion. In addition to spinning, we also observed cells exploiting their innate gravitactic ability [199] to escape or switch swimming direction. This was observed in both confined channels and in quasi-open spaces. Movie 10. B demonstrates a cell using spinning behaviour to navigate and change swimming direction, it can be observed that although the cell was swimming in open space, the flagellum is hindered by the proximity of the wall. The cell swiftly vertically tilts the anterior end at 180 in the Z-direction changing the swimming direction to swim away and back in the horizontal plane.

5.3.5.2 Gliding motility

Gliding motility in this context is defined as substrate-dependant, whole-cell motility with or without instigation of bodily deformations when trapped in the substrate interface. We observed two types of gliding behaviour associated with the flagellum, stiff flagellum, and motile flagellum without cell propulsion. The flagellum appears to be moderately stiff lacking significant bends, but a slight wiggle and occasional undulations produced from the base through the tip. The cell glides on the substrate, which is the channel floor, slowly glides while undergoing bodily deformations (Movie 11.A). It appeared that the trapped cell starts gliding to free itself from the trap, additionally, with the inability to beat the flagellum, it starts undergoing bodily deformations while gliding. In the second type of sliding (Movie 11.B) the flagellum is free and motile with the ability to perform conventional beating, nonetheless as the cell is stuck, this hinders the ability of the cell to propel forward. In this situation, the flagellum continues to beat and the cell glides sideways along the channel. It was observed that the posterior end of the cell was trapped in the channel floor hindering the cell rotation. The cell attempts to rotate itself as it glides along the substrate to resume helical swimming. In both cases, there was a lack of linear forward motion. However, the cell ultimately frees itself from the trapped substrate and continues free helical swimming, not shown here. Gliding motility was reported in *Euglena mutabilis*, this species lacks the emergent flagella [200]. As the flagellum does not play a role in motility the cell resorts to an alternative mode of movement which is, gliding. This type of motility is facilitated by the secretion of localized mucus while the cell attaches itself to the substrate. In context to our study, although the cell has an emergent flagellum, being trapped at the substrate interface and inability to beat the flagellum or propel forward, the cell switches to an alternative mode to facilitate movement. This observation underscores the remarkable plasticity and versatility of euglenids in adopting alternative modes of movement as a survival strategy. It reflects the cell's capacity to adapt and respond to external challenges on an as-needed basis, thus enabling it to thrive in diverse and fluctuating conditions.

5.4 Future work and Outlook

The in-depth investigation into the complex interplay between swimming motion and flagellar beat patterns of *E. gracilis* revealed intricate behavioural responses to confinement including flagellar beat pattern modulations, controlled regressive behaviour, metaboly, spinning, and gliding. These findings shed light on the purposeful adjustments made by the cell to navigate through complex confinements indicating a deliberate effort to optimize motility and escape. The modulations in the flagellar beat patterns revealed systematic changes with repeated patterns suggesting a regulated process to restore helical swimming. Furthermore, the correlation between flagellar beating malfunction and metaboly suggests that metaboly is an alternative swimming strategy when conventional beating fails to resume. Alternate motility such as back and forth motility, spinning, and gliding highlights the remarkable adaptability underscoring the versatility and plasticity of *E. gracilis* in response to external stressors. Building upon the findings from this study, further research could delve into understanding the underlying mechanisms driving these behavioural responses, further investigating the role of mechanosensitive channels in mediating these responses and elucidating the signalling pathways could provide valuable insights into cellular mechanisms governing the flagellar beat modulations. Understanding the regulatory mechanisms governing metaboly adaptation could enhance our knowledge of euglenoid physiology and the functional significance of this behaviour. These findings enhance our understanding of diverse strategies employed by euglenids to thrive in dynamic environments. Further investigation into the motility and behaviour of euglenids holds promise for advancing our understanding of cellular biomechanics, dynamic adaptability, and design and development of bioinspired robots, opening up new avenues for interdisciplinary research and technological innovation.

5.5 Supplementary figures

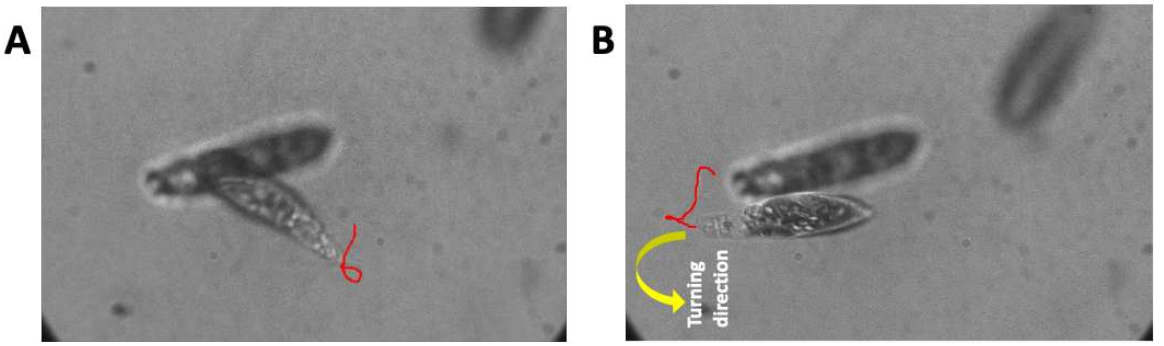


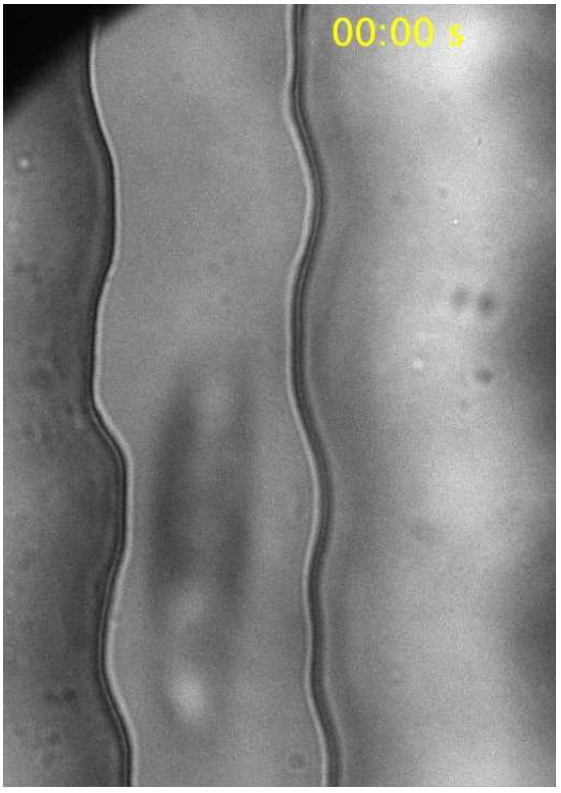
Figure S 5: Images of cells exhibiting spinning-type motility, the flagellum is mapped in red colour.

5.6 Supporting movies

Supplementary movies related to this study are available at the Zenodo repository and available as PowerPoint file.

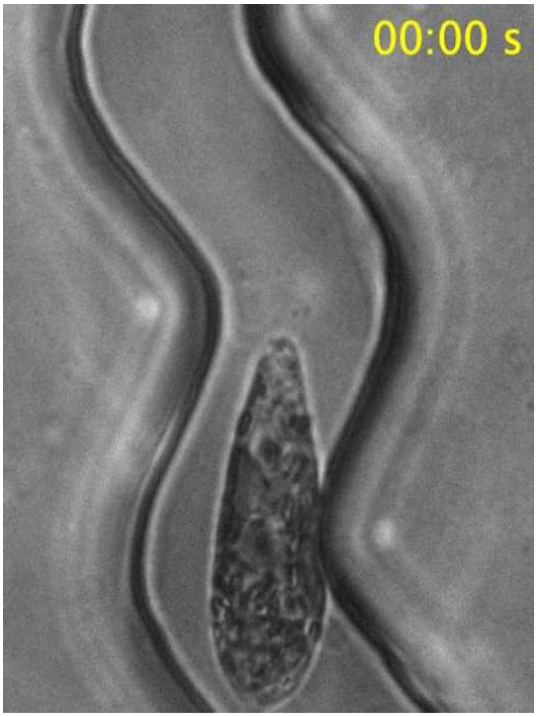
https://zenodo.org/records/14579594?token=eyJhbGciOiJIUzUxMiIsImlhdCI6MTczNTU5NzQxMiwiZXhwIjoxNzM4MTk1MTk5fQ.eyJpZCI6ImFiMmJiYjViLTEyZDEtNGFiMy1iNTUzLWNjYWUwNzQ2YTI3NSIsImRhdGEiOnt9LCJyYW5kb20iOiJjZGRiOWYxNjkxNDBkZWNjMzYyMjRmZmMxMWEzNmZlOCJ9.pKP6_gMVQfJqVuP2TxR26itD4dh8BwQkdXYn2eGZlZU_Y32Q3ZSxPMtpwAGye2nYsMWwk2vHodf02Xuw_e8I6w

6. Euglena switches flagellar beating patterns to achieve states of helical swimming



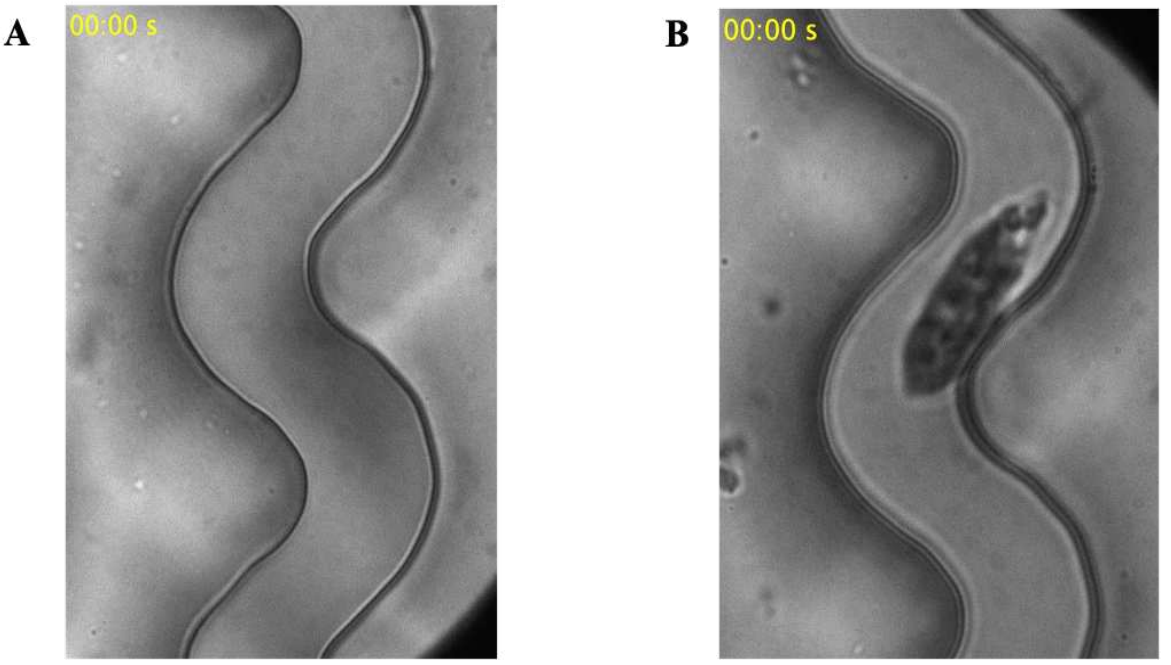
Movie 6 showcases free helical swimming motion of *Euglena gracilis* before the collision with the wall resulting in modulation of the flagellar beat patterns after the collision.

7. Flagellum shape excursions - The change is regulatory, not merely haphazard



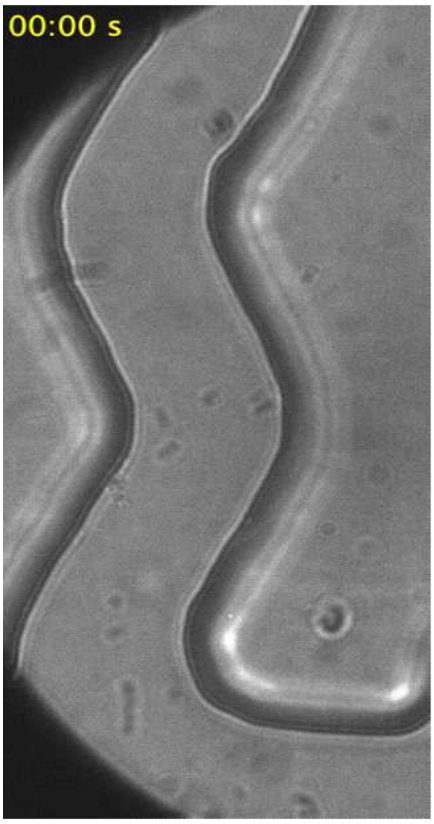
Movie 7 shows the cell's multiple attempts to unwind and re-loop the flagellum. Despite the seemingly haphazard appearance, the changes are commissioned to regulate the conventional figure-eight beat pattern characterized by double loops on either side of the cell. The dynamics of flagellar beat pattern modulations appeared to involve multiple attempts of reconfiguration, characterized by systematic changes with repeated shapes.

8. Correlation between flagellar malfunction and metaboly



Movie 8 A shows that the cell in confinement begins to regulate the flagellum beats, when unsuccessful, the cell then initiates retraction at the anterior end at 38.80 s ultimately leading to deformation almost entirely at 44.50 s showing metaboly adaptation. Cells finally resume conventional flagellar beating and exit the channel. Movie 8 B shows that when cells are unable to resume their flagellum beating even after adapting metaboly, they continue to tumble incessantly attempting to exit the channel.

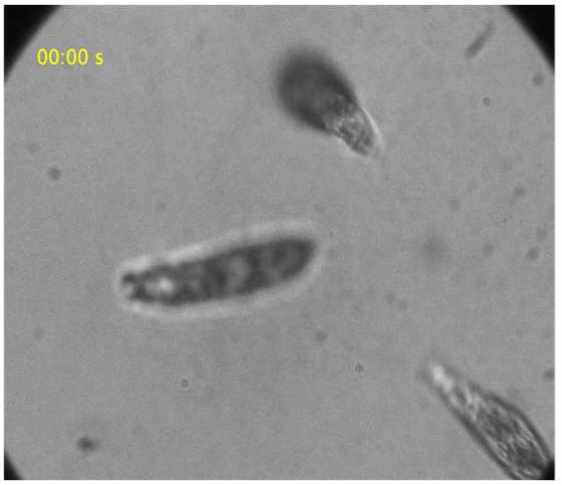
9. Backward motility



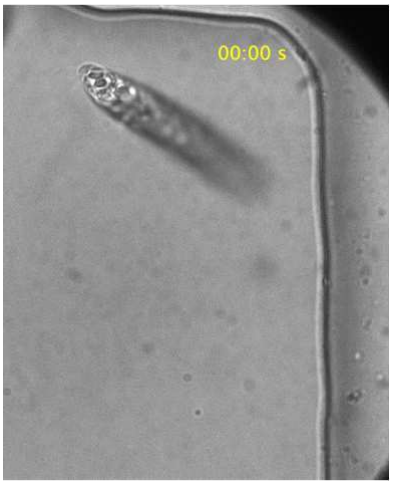
Movie 9 shows that cells swimming through the channel first adapted modulations in flagellar beating and quickly transitioned to brief backward or reverse swimming. However, the cell itself held its original ellipsoid shape while swimming in the backward direction, the flagellum beat patterns continued to vary as the cells descended the channel.

10. Spinning

A

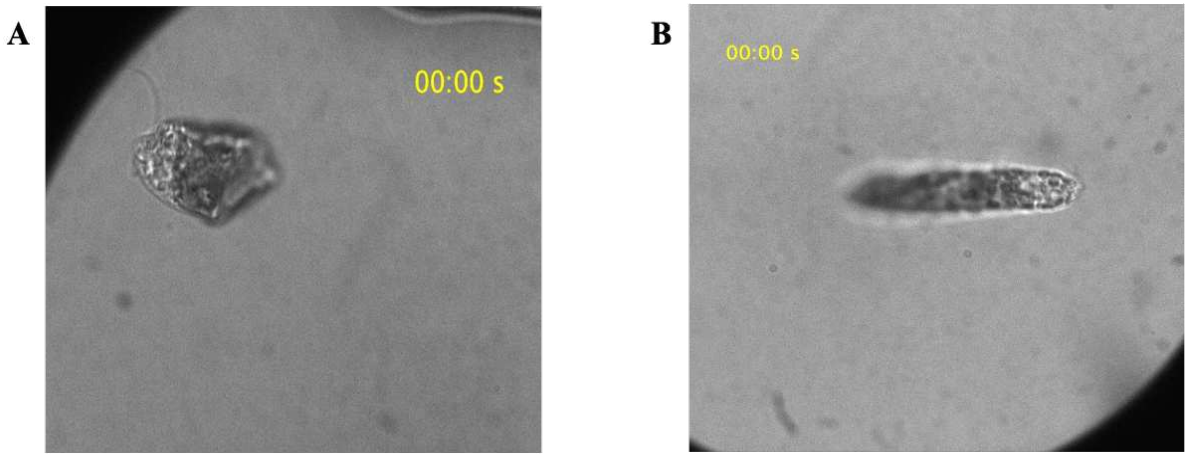


B



Movie 10 A shows that in the plaza, free-swimming cells switch from helical swimming to spinning when they encounter an obstacle, during this the cell initially twisted the flagellum into one loop extended on the side opposite to the swimming direction, steered almost a 180 turn from the obstacle to change the swimming direction. Movie 10 B shows that a cell using spinning behaviour to navigate and change swimming direction, when the flagellum is hindered by the proximity of the wall, the cell swiftly vertically tilts the anterior end in the Z-direction changing the swimming direction to swim away and back in the horizontal plane.

11. Gliding motility



Movie 11 A shows gliding motility with stiff flagellum, the cell glides on the substrate, which is the channel floor while undergoing bodily deformations. Movie 11 B shows gliding with motile flagellum but without cell propulsion, the flagellum is free and motile with the ability to perform conventional beating, nonetheless as the cell is stuck, this hinders the ability of the cell to propel forward. In this situation, the flagellum continues to beat and the cell glides sideways along the channel. It was observed that the posterior end of the cell was trapped in the channel floor hindering the cell rotation. The cell attempts to rotate itself as it glides along the substrate to resume helical swimming.

Chapter IV

Biocomputation

This work aims to design and develop the operation of a computational device driven by photosynthesis, employing euglenids as biological agents – photosynthesis-driven biocomputation. A graph-theory based microfluidic network encoding mathematical problem for biocomputation was designed and developed by Prof. Nicolau's team. Preliminary experiments started with experimental set-up and trials for solution exploration of euglenids for biocomputation and investigating if the euglenids follow the rules in the network. These self-propelled agents explore the network independently and parallelly through channels by brute force to solve the graphically represented mathematical problem. The end goal is to achieve a biocomputational device that works on light and water employing euglenids as biological agent to solve a network-based computation problem.

The research findings presented in this chapter stem from preliminary experiments aimed for employing euglenids as biological agents for photosynthesis-driven biocomputation.

6. Proof-of-concept: photosynthesis-driven biological computation

6.1 Introduction

Computers have proven to be an efficient interface for solving numerous research questions and has revolutionized human life in solving combinatorial problems during drug discovery, sequencing and alignment, online management of queue system, object tracking, optimal network routing, scheduling, travel planning, and other astronomical feats like black hole mergers. However, many mathematically relevant combinatorial problems demand brute force computing to explore solutions. To solve a problem of input size C, 2^C solutions must be visited, the exponentially increasing workload needs to be distributed in space, time, and per computing agent. Moreover, the complexity of combinatorial problems translates to ever-increasing energy consumption by electronic computers, making the computing process highly unsustainable energy-wise. Consequently, efficient, both computational and energy-wise parallel computation strategies are needed to solve these combinatorial problems. The previously proposed parallel computation techniques using biological agents suffered from critical limitations such as scalability, demand for impractical large amounts of computing agent, energy inefficiency due to the rapidly increasing physical size of the network [47, 48, 157]. The new computational paradigm, in solving the combinatorial problem by allowing a very large number of motile biological agents, the molecular motors to explore the microfluidics network [201],[98] was successful and the experiment-based computer aided simulations revealed the proof-of-concept that parallel computation using the biological agents. However, the approach employing molecular motor proteins faced challenges like detachment of molecular motor proteins from one another, handling of molecular motor proteins, and scalability associated with readout for computing. Motility of microbes in microfluidic geometries agents has the capability to efficiently solve the NP-complete problem of medium to higher sample sizes. Using microbes as biological agents offer several advantages such as, a plethora of microbial species to choose from, different sizes, flagellar arrangements, varied motility rates and patterns, and varied replication rates - a critical factor in the parallel exploration of solutions to an exponentially growing combinatorial problem.

Furthermore, photosynthetic microbes (euglenids) as biological agents offer several advantages such as self-propelled, easy to handle, low-cost maintenance, replication, and the most appealing factor about euglenids are that they are photosynthetic and require minimal nutrients to survive. The goal is to develop an energy-efficient, low-cost driven computation system with autonomous, self-propelled biological agents that require minimum maintenance. Our work is focused on the state-of-the-art network-based computation employing motile euglenids biological agents in microfluidic channels. Here, the microfluidic device encoding the combinatorial problem of interest is the hardware and the motile autonomous agents that explore the physical network in brute force to solve the solution act as processors. The operational functionality of our proposed work is that the motile biological agents are allowed to autonomously explore the microfluidic network encoded with the combinatorial problem, agents pass through a sequence of junctions in order to report for solutions at exit. The key to addressing the exponentially increasing solutions with problem size is employing growing resources – establishing massively parallel computation. This physical network encoding the mathematical problem serves as a rigid master for fabricating and replicating microfluidic devices, particularly by the soft lithography process. This work aims to design and develop the operation of a computational device driven by photosynthesis, employing euglenids as biological agents – photosynthesis-driven biocomputation. Each euglenoid is an autonomous, independent computing agent that explores the microfluidic network encoding instances of a benchmark combinatorial problem and essentially, multiply, translating to growing resources for an exponentially growing problem size – establishing a potential for parallel computation. The proof-of-concept of a photosynthesis-driven biological computation holds the potential to develop a computing device that is independent with longer run times and hold the potential to successfully scale up.

6.2 Method

A graph-theory based microfluidic network encoding mathematical problem for biocomputation was designed and developed by Prof. Nicolau's team. This physical network encoding the mathematical problem serves as a rigid master for fabricating and replicating

microfluidic devices, particularly by the soft lithography process. The model organism used in this study, *Euglena gracilis* was obtained from Carolina Biological Supply. The cells were cultured in Cramer Meyer's media and maintained under a wide spectrum illumination of 40W, white light at a light:dark cycle of 12:12 hours. The 7-day-old culture was used for the experimental study in the microfluidic structures as the growth rate of the microorganisms was at the peak of the log phase. The design of the network encoding the mathematical problem was translated to silicon wafer from master design using negative photoresist-based SU-8 fabrication and optical mask aligner set-up (UV settings at Z-40 and 55 s exposure time) in the Nano-UQAM facility located at Université du Québec à Montréal. The wafers after fabrication were subjected to salinization using methyltrichlorosilane. A mixture of Polydimethylsiloxane (PDMS) and cross-linker (weight ratio 10:1) was poured onto the silicon master, degassed inside the vacuum chamber to remove air bubbles, and cured at 65°C overnight to ensure complete cross-linking. After cutting and peeling, the PDMS replica was treated with air plasma for 45 seconds to render the surface hydrophilic before irreversibly bonding it onto the glass coverslip for short experiments and glass Petri plates for longer experiments (also plasma-activated for 45 seconds). Further, the fabricated microfluidic devices were flooded with sterile water and stored at 4°C overnight to facilitate sufficient wetting. The experiments were carried out under uniform light.

The microfluidic device was removed from 4°C and brought to room temperature before introducing the euglenids. Separately, a log-phase suspension of euglenids was added around the device and allowed to explore the network from the entry point, furthermore, uniform illumination of the microstructures was maintained throughout the experimental duration. The motility of agents was imaged using Olympus IX83, an inverted - Spinning Disk Confocal Olympus IX83 microscope, with MetaMorph® (from Molecular Devices) Microscopy Automation & Image Analysis Software, 10x (NA 0.4), 20x (NA 0.75) and 40x (NA 0.95). Data analysis was performed using ImageJ [175], a public domain software platform.

6.3 Research findings.

The preliminary experiments started with experimental set-up and trials for solution exploration of euglenids for biocomputation and investigating if the euglenids follow the rules in the network. These self-propelled agents explore the network independently and parallelly through channels by brute force to solve the graphically represented mathematical problem. One of the key factors necessary for the proposed work to be of practical relevance is to have low error rates particularly due to the stochastic movement of biological agents in the network. Therefore, the preliminary analysis of this study began with studying motility, replication, and quantification of error rates in the channels and junctions of the SSP network. Based on the above the optimization of the experimental setup and network is in progress. The process of computation using microfluidic devices involves a series of stages, 1. Graphically encode network with mathematical problems of interest. 2. Translate the graph to a physical network using lithography techniques, 3. Fabricating a microfluidic network comprising entry and exit ports, channels, and junctions encodes the problem of interest, 4. Allow biological agents in parallel to solve through stochastic exploration. The microfluidic structure comprising entry, exits, nodes, junctions, channels are the computing network that encodes the combinatorial problem of interest. To make this device photosynthesis based, sterile waster is used as a working fluid to wet the microfluidic device encoding mathematical problems and euglenids are allowed to explore under uniform light. The agents are flooded at the entry port of the device and the movement of the agents is optically recorded along the network at high precision.

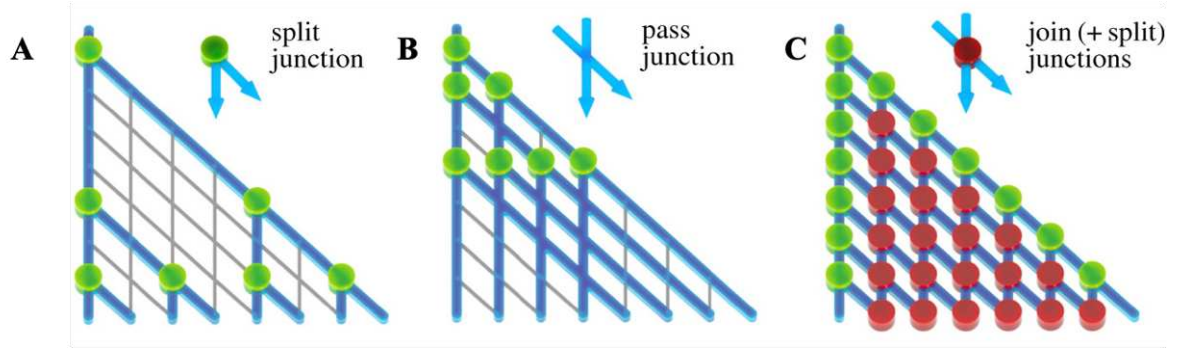


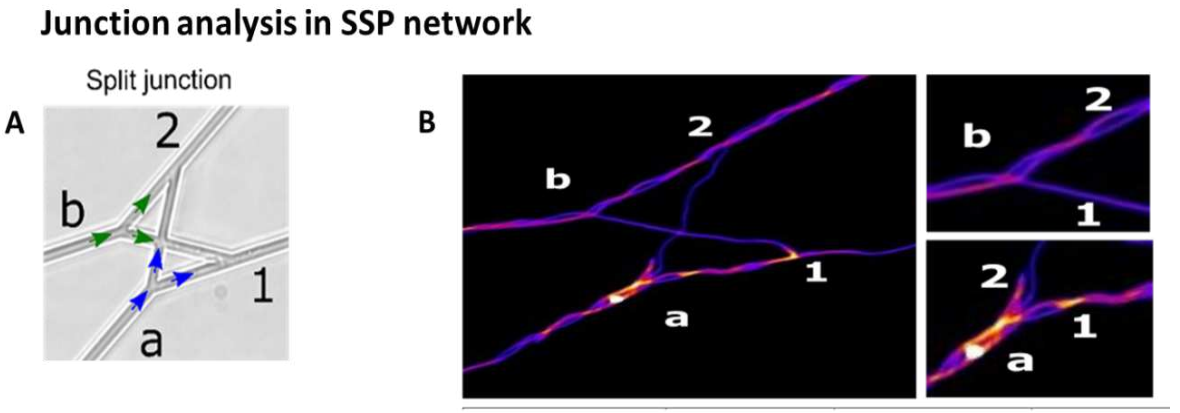
Figure 17: Schematic showing an instance of a computation network. The agents (euglenids) enter from the top-left corner. Schematic of A. split and B. pass and C. join junction in the network.

The motile biological agents are allowed to autonomously explore the microfluidic network encoded with the combinatorial problem; agents pass through a sequence of junctions in order to report for solutions at the exit. As the agents enter the network from the designated entrance point, they are guided unidirectionally downward along the defined channel. The network features two types of junctions, split junction and pass junction. Split junction is designed to distribute agents ideally, evenly along the network and the agents are allowed to choose randomly between the two paths. The pass junction is where the agents are guided to continue along the initial direction and do not change their swimming path. In the network encoding the subset sum problem, the directed movements of agents are akin to the basic addition operation. The vertical spacing between rows of split junction corresponds to integers from the given set S. As the agents move straight down from the split junction, it excludes the corresponding integer from the summation, while diagonal movement includes it. The channel-guided, "ideally" unidirectional motility of the biological agents and any returning on switching of swimming direction in the opposite that is towards the entry is deemed as an error and defeats the purpose. Similarly, change in swimming path at the pass junction is also an error. For a successful run and reliable readout of computation, there must be sufficient agents facilitating exploration of all paths, all the agents and their respective trajectories are tracked at high precision in space and time from the point of entry to exit. Additionally, the operational function for a computation strategy using autonomous

biological agents should meet specific criteria: Agents are abundantly available at a minimal cost, (ii) independent operation that is agents are autonomous and self-propelled, (iii) agents move rapidly for efficient computation time, (iv) move predominantly in forward direction to minimize errors in the network, (v) replicate for parallel computation with exponentially increasing agents.

6.3.1 Junction analysis

Junction analysis and error rate in SSP network – the computing network that encodes combinatorial problems comprises of entry, exits, nodes, junctions, and channels. The preliminary analysis started by optimizing the experimental setup and junction analysis A low-error network is a critical factor for biological computation, particularly due to the stochastic movement of biological agents in the network.



C **Error in the junctions**

Split junction	a1+b1	a2+b2	Wrong turn
Total	1748	1500	56
% Traffic	53	45	1.7

Figure 18. Optical image of split junction with green and blue arrows indicating the correct direction. B. Density maps of the distribution of euglenids in the junction. C. Quantitative data of directional preference at each junction and the wrong turn in the junctions and channel.

The purpose of the split junction is to equally distribute the agents visiting the junction for optimal traffic throughout the network. Figure 18 A shows the correct path in the split, and 18B shows the qualitative data of the first model organism employed in this study *Euglena gracilis*. Quantified data, (Figure 18 C) of the spilt junction shows a near equal distribution, i.e., 53% and 45% in *E. gracilis*. Although this is not perfectly 50-50 distribution of the agents the distribution was still at a tolerable margin. The error rate where the number of cells that took a wrong turn, that is cells that were swimming back towards the entry was 1.7% which is still very high. An alternative design to improve the error rate would be to add a bridge at the junction to minimize the error.

Efficiency of the network-based biological computation with euglena.

Considering clock frequency of a "good" modern computer to be 2.5 GHz.

Clock Frequency of a Single Euglena

The "clock frequency" of a single Euglena depends on its velocity and cell length. It is calculated using the formula: fEuglena=length (microns)/velocity (microns/sec)

Example Calculation:

- **Velocity**: Euglena swims at an average speed of ~83 microns/second.
- **Length**: Euglena is ~47 microns long (mean ± standard deviation of 4 microns).

Length, width, velocity based on calculations from our experiments

Using the formula, the clock frequency is approximately **0.57 Hz**.

Assuming, each Euglena cell performs at **0.57 Hz**. To match the **2.5 billion FLOPs of a computer**, we need approximately **1.41 billion Euglena cells**

Exponential growth formula,

$$N(t)=N_0 \times 2^{t/T}$$

Where: N(t) is the number of cells at time t, N0 is the initial number of cells (usually starting with T is the doubling time (4,500 seconds or 75 minutes).

Number of Euglena Cells Over Time

Euglena divides exponentially with a doubling time of approximately 75 minutes (4,500 seconds) under optimal conditions. To achieve cells (matching 2.5 GHz FLOPs): It takes approximately **37.2 hours** for the Euglena population to match 1.41 billion euglena cells.

Total FLOPs of the Euglena Biocomputer

At cells, each operating at Hz: 0.57 Hz

Volume of the Euglena Biocomputer

Total Volume of Euglena Cells:

- **Width of a single Euglena cell**: ~8 m
- **Concentration**: 10%.
- **Volume required** – 0.2 mL

Summary of Calculations

1. **Clock Frequency per Euglena**: ~ 0.57 Hz.
2. **Time to Reach 1.4 Billion Cells**: ~37.2 hours.
3. **FLOPs at Peak**: ~2.5 GHz FLOPs.
4. **Volume Required (10% concentration)**: ~0.2 mL.

These calculations provide a preliminary estimate of the biocomputation capacity of Euglena. It takes approximately 37 hours for euglena to reach the required quantity of cells, however, the biological computation at this point operates with 2.5 GHz while the computer works with 1. This approach also offers benefits such as replying on natural energy sources such as solar and water which makes it an avenue to explore for sustainable computing.

6.3.2 Replication

When the cells were exploring the network, we noticed replication of a single euglenid into two cells in the network (Figure 19). We calculated the replication time of the 32 cells in the network, and the replication time was between 75 ± 15 minutes per replication.

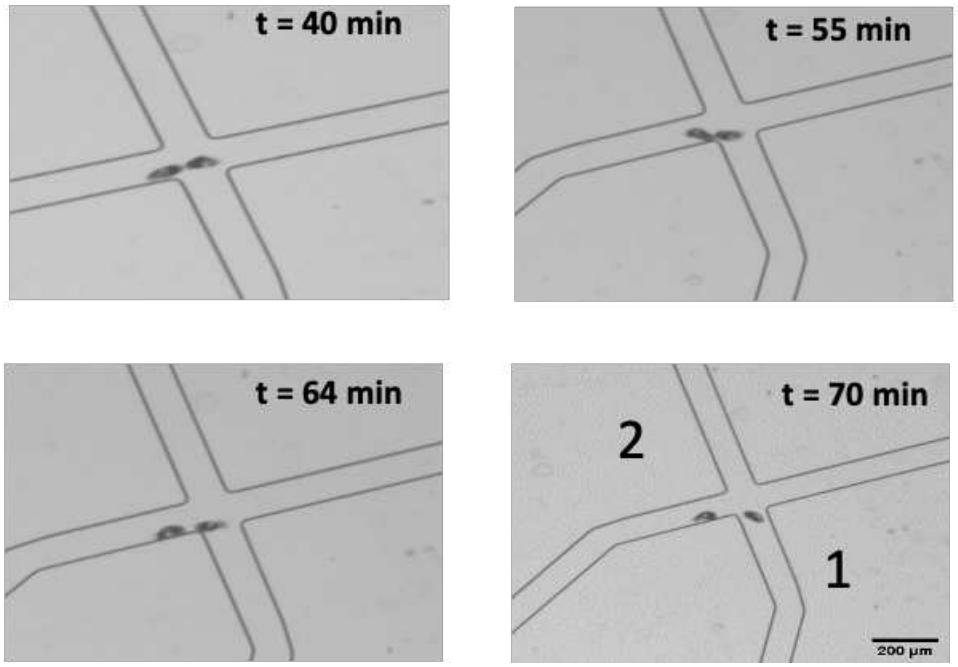


Figure 19: Optical images showing agents successfully replicating at the junction. Euglena divides into two cells while exploring the network, here at a pass junction and continues into two separate route, demonstrating parallel computation.

The proposed method aims to eliminate exponentially increasing time needed by electronic computers with exponentially growing independent agents. Figure 19 shows successful replication of Euglena gracilis in the network. We demonstrate the effectiveness of this approach by showing replication of agents while exploring the network showing a proof-of concept of biological computation with increasing number of agents in the network. Additionally, the device was running for more than 12 hours with only water when device was sealed to a coverslip. When the device was sealed to a glass petri-dish with and immersed about 40% of its total height in water the device ran successfully for 12 days. On day 0 – 1 µl culture (1-10 cells) was allowed to enter the network, the experimental observation was at 1hr, 2hr, 3hr, 4hr, 8hr, 24hr, 48hr, day 3, 4, 5, 6, 7, 9, 11 and 13. After which the population of cells were high and several cells were getting overfilled in the exit channel and returning to the device through the exit channel. Based on the preliminary results we modified the design and structure (not given here) of the device where we designed a connecter channel from the exit to the entry so cells that exit are recycled back to the channel.

6.4 Conclusion

This work attempts to demonstrate a proof-of-concept photosynthesis driven bio computation with euglenids as biological agents. We successfully observed the operation of a computation device driven by photosynthetic euglenids. Each euglenid as an autonomous, independent computing agent explored the microfluidic network encoding an instance of mathematical problem. The cell multiplication translates to growing resources establishing parallel computation. The proof of concept of a photosynthesis-driven biological computation holds the potential although modifications required, to develop a computing device that functions independently with longer run times and successfully scale up.

Chapter V

Patterns of bacterial motility

The preliminary study of my Ph.D. took off with analyzing the motility patterns of five bacterial species namely, *Escherichia coli, Vibrio natriegens*, *Pseudomonas putida*, *Vibrio fischeri*, and *Magnetococcus marinus* in microfluidic-based geometrical confinements. Specifically, experimental data analysis by tracking individual bacterial movement in three-dimensional (3D) space, that is, along the x, y, and z plane of the microfluidic channel using Fiji ImageJ software. The analysis included tracking trajectories and plotting of their x, y, and z position. The trajectories of a single bacterium were tracked with point-and-click tracking using Manual tracking and the x-y-z-time coordinates from the trajectories were used for extracting their spatial position. In the presence of a wall or a physical boundary, the motility behaviour of microswimmers swimming in low Reynold's number is highly influenced by several factors including hydrodynamics, steric effects, mechanical forces, and biological responses. We report that bacterial motility is the result of "tug-of-war" between hydrodynamics and local nano mechanics. In less confining spaces, bacterial motility is governed by hydrodynamics and in tightly confining environments, movement is mainly controlled by the steric interactions between flagella and the surrounding wall. This work was published in the Proceedings of the National Academy of Sciences (PNAS) journal, the DOI for the paper is, https://doi.org/10.1073/pnas.2013925118 and is also attached as a supplemental file additional to the thesis.

This chapter encompasses a collaborative manuscript to which I have contributed as a co-author during my Ph.D. journey. However, this chapter specifically highlights the section of the paper that I have contributed towards.

7. Patterns of bacterial motility in microfluidics-confining environments

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Abstract

Understanding the motility behaviour of bacteria in confining microenvironments, in which they search for available physical space and move in response to stimuli, is important for environmental, food industry, and biomedical applications. We studied the motility of five bacterial species with various sizes and flagellar architectures (*Vibrio natriegens*, *Magnetococcus marinus*, *Pseudomonas putida*, *Vibrio fischeri*, and *Escherichia coli*) in microfluidic environments presenting various levels of confinement and geometrical complexity, in the absence of external flow and concentration gradients. When the confinement is moderate, such as in quasi-open spaces with only one limiting wall, and in wide channels, the motility behaviour of bacteria with complex flagellar architectures approximately follows the hydrodynamics-based predictions developed for simple monotrichous bacteria. Specifically, *V. natriegens* and *V. fischeri* moved parallel to the wall and *P. putida* and *E. coli* presented a stable movement parallel to the wall but with incidental wall escape events, while *M. marinus* exhibited frequent flipping between wall accumulator and wall escaper regimes. Conversely, in tighter confining environments, the motility is governed by the steric interactions between bacteria and the surrounding walls. In mesoscale regions, where the impacts of hydrodynamics and steric interactions overlap, these mechanisms can either push bacteria in the same directions in linear channels, leading to smooth bacterial movement, or they could be oppositional (e.g., in mesoscale-sized meandered channels), leading to chaotic movement and subsequent bacterial trapping. The study provides a methodological template for the design of microfluidic devices for single-cell genomic screening, bacterial entrapment for diagnostics, or biocomputation.

Significance

Understanding bacterial movement is crucial for health, agriculture, environment, and industry. Studying the motility of five bacterial species in microfluidic environments showed that bacterial motility behaviour is the result of a “tug-of-war” between hydrodynamics and local nanomechanics. In less confining spaces, bacterial motility is governed by hydrodynamics and can be approximately predicted by modeling developed for the simplest species. Conversely, in tightly confining environments, movement is mainly controlled by the steric interactions between flagella and the surrounding walls. Intriguingly, in mesoscale-sized geometries, hydrodynamics and bacterium– wall interactions overlap, either “constructively,” leading to smooth movement in straight channels, or “destructively,” leading to trapping. Our study provides a methodological template for the development of devices for single-cell genomics, diagnostics, or biocomputation.

7.1 Introduction

Many motile bacteria live in confining microenvironment (e.g., animal or plant tissue, soil, waste, granulated, and porous materials) and consequently are important to many applications like health (infectious diseases [202, 203] , pharmaceuticals [204], and nutrition[205]), agriculture (veterinary [206]and crops[207]), environmental science (photosynthesis [208], biodegradation[209] , and bioremediation [210]), and industrial activities (mining [211]and biofouling [212]). Bacterial motility is essential in the search for available physical space as well as for enabling bacterial taxis in response to external stimuli, such as temperature [213], chemical gradients [214], [215], mechanical cues [216], or magnetic fields [217]. To thrive in environments with diverse geometrical and physical characteristics, from open spaces to constraining environments, motile bacteria have evolved a multitude of propelling mechanisms [218], with flagellum-driven being the most common [219, 220]. Flagellum-based machinery features various numbers of flagella [221] and designs: monotrichous, lophotrichous, amphitrichous, or peritrichous. The mechanics of this machinery, coupled with cell morphology [222] (e.g., coccus, rod-like, or curved) translates into several motility modes (e.g., turn angle, run-and-tumble, or run-and-flick [223] and various motility behaviours (e.g., swimming, tumbling, and swarming) [218, 224]

Environmental factors [17] (e.g., chemical composition, viscosity, temperature, pH, and the chemistry and the roughness of adjacent surfaces) also influence bacterial motility. “Pure” bacterial motility, unbiased by chemotaxis or fluid flow, was reported near simple flat surfaces [197] [225]and in channels [226-228]. Simulations of model bacteria in analogous conditions were also undertaken [201, 229-234] but owing to the complexity of bacterial mechanics [235], modeling from first principles did not provide sufficient understanding to accurately predict movement patterns of different species in complex, confined environments. Consequently, studies of the effects of bacterial geometry in confined geometries were limited to models of simple, monotrichous bacteria with an assumed rigid flagellum [230, 236]. Microfluidic devices [237, 238] are commonly used for the manipulation of individual or small populations of cells in micrometre-sized channels for medical diagnostics [239], drug screening [240], cell separation [241, 242], detection and sorting[243], and single-cell genomics [244]. While microfluidic structures are used for the study of the motility of mammalian cells [245, 246], and microorganisms (e.g., fungi [247, 248], algae [249], or bacteria [227], [146, 250-252]), these studies typically focus on a single species.To make progress toward a more general understanding of the motility of individual bacterial cells in confining microenvironments, as well as to assess the extent to which the behaviour of bacteria with complex architectures can be assimilated with that of the more predictable monotrichous bacteria, the present work investigated the movement of five species (i.e., *Vibrio natriegens, Magnetococcus marinus, Pseudomonas putida, Vibrio fischeri, and Escherichia coli*) in microfluidic geometries with various levels of confinement and geometrical complexity.

7.2 Results and Discussion

The modulation of motility behaviour by confinement was assessed by observing, by three-dimensional (3D) imaging, the movement of individual bacteria, presenting various characteristics (Figure 20. A and SI Appendix, Figure S1) in microfluidic structures with high (6 μm) or low (4 μm) ceilings (Figure 20. B) and with various geometries (Figure 20. C and SI Appendix, Figure S2) as follows: 1) large chambers with quasiopen spaces (“plazas”), 2) linear channels with various widths, 3) channels presenting lateral exits at various angles, and 4) meandered channels with various widths. In the absence of pressure and concentration

gradients, this approach allowed the study of the interaction between hydrodynamics and the steric interactions of bacteria with the walls, unobscured by other external factors (e.g., rheo- and chemotaxis). Experimental, image analysis, and simulation protocols are fully described in SI Appendix.

7.2.1 Motility in Large Chambers

Impact of the distance between horizontal planes. To minimize the possible coupling of the impact of horizontal planes, the designs of microfluidic chambers, made of polydimethylsiloxane (PDMS), had to find a compromise between their height and fabrication and operation issues. From the design perspective, it was found that a height of 6 μm (Figure 20. A and SI Appendix, Table S1) allows, conservatively, the unencumbered bacterial motility. Furthermore, preliminary experiments comparing motility in both types of microfluidic structures presented evidence (Movie S1) of the coupling of the impact on both horizontal planes on bacterial motility for those with 4 μm heights. Consequently, 6 μm–tall microfluidic structures were used for all further experiments. A detailed discussion is presented in SI Appendix.

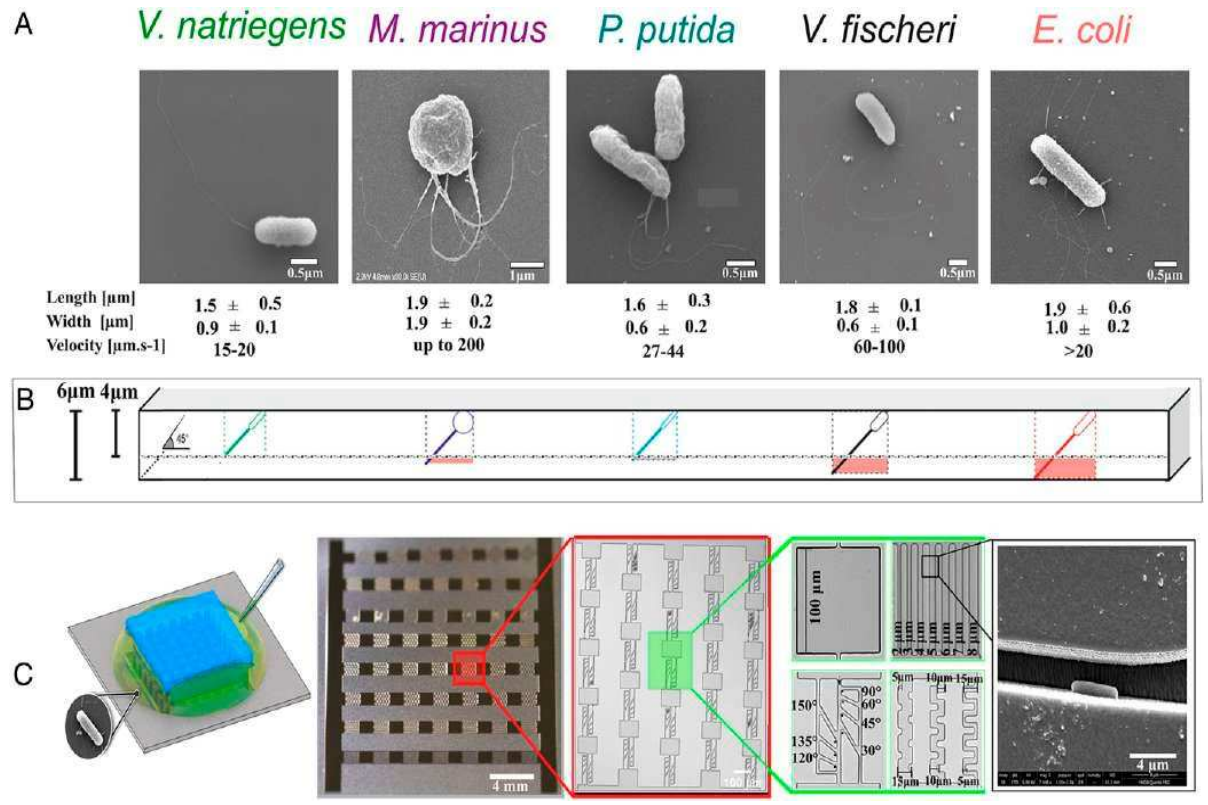

Figure 20: Microfluidics chip for testing microbial motility. (A) Scanning electron micrographs (SEM) images of bacteria studied with various architectures and dimensions (full details in SI Appendix, Table S1). (B) Graphical projection of the fit of the total bacterial length (body plus flagella) positioned at 45° versus the height of the microfluidic structures for 6 µm and 4 µm heights. (C) Sequential, from left to right, zoom-in images of the experimental device: 1) the bacterial suspension is introduced from the side of the chip attached to the cover slide; 2) the overall architecture of the chip; 3) zoom-in of one lane of experimental structures (sequence of angled channels separated by plazas); 4) detailed image of the experimental structures used in this study (i.e., plazas) and linear channels (top row), angled, and meandered channels (bottom row); and 5) SEM image of a bacterium (here, *E. coli*) in a channel.

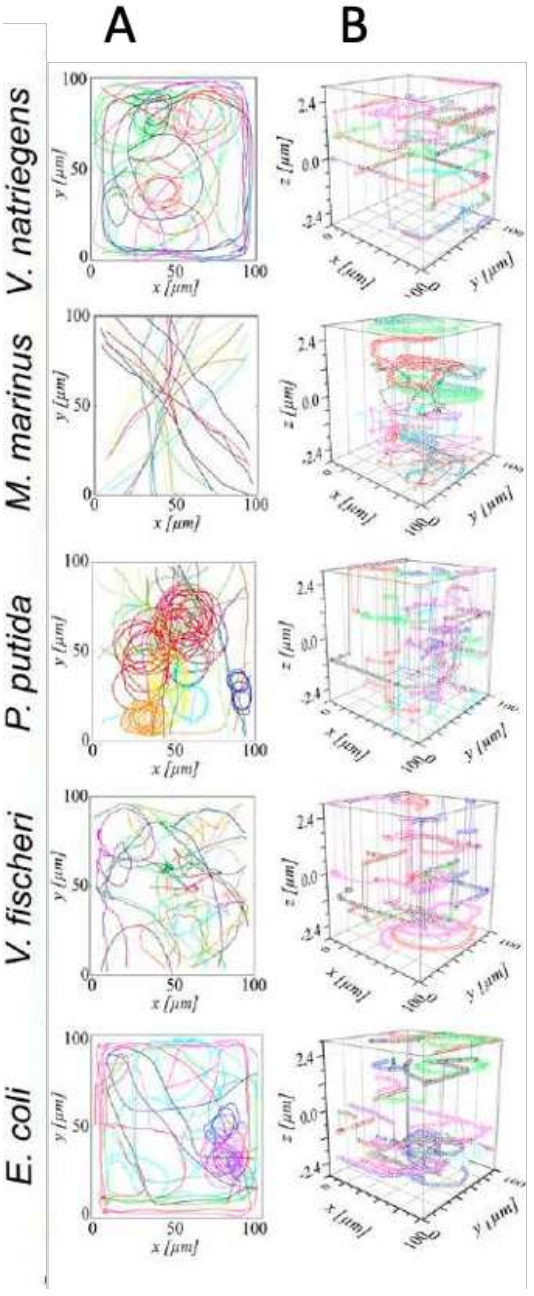

Figure 21: Motility in plazas with 6 μm high ceilings. (A) Characteristic long2D projections of bacterial trajectories. (B) 3D bacterial trajectories. By rows, from top to bottom, are the following: *V. natriegens* (average count of bacterial positions in each frame, n = 14/frame); *M. marinus* (n = 12/frame); *P. putida* (n = 15/frame); V. fischeri (n = 15/frame); and *E. coli*

(n = 13/frame).Movie S1 presents the bacterial movement in plazas, with representative trajectories (similar to C).

The 3D imaging and z-stack sectioning of bacterial trajectories in 6 μm–tall plazas (Figure 21. D and SI Appendix, Figures S6–S8) revealed a similar behaviour in the central area close to the horizonal walls (i.e., free of the possible edge effects from the vertical walls). *V. natriegens*, *V. fischeri*, and *E. coli* presented trajectories in proximity to—and parallel with—the horizontal walls. This was not the case for *P. putida* and *M. marinus*, which frequently fluctuated between z-planes (SI Appendix, Figures S7 and S8). Statistical analysis of the bacterial positions (SI Appendix, Figure S9) showed that *V. natriegens*, *V. fischeri*, and *E. coli* moved preferentially in a parallel plane to the horizontal walls and that *P. putida* and *M. marinus* presented a rather uniform distribution of positions on the vertical axis. Theoretical classification of bacterial motility behaviour. For bacteria that are propelled by a flagellum or flagellar bundle behind the cell, the fluid flow generated by swimming has a dipolar structure: the fluid is pushed backward by the flagellum and pulled forward by the cell body. This flow has been shown to attract swimmers to solid walls, causing them to remain close to the wall for long time periods despite rotational Brownian motion [253]. A separate effect of swimming near surfaces is that hydrodynamic interactions between the wall and rotating flagellum and between the wall and counter-rotating cell body, respectively, lead to bacteria swimming in circular orbits when they are close to a wall [195].

Detailed hydrodynamic modelling of monotrichous bacteria showed that the geometrical parameters of the cell (length and width) and of the helical flagellum (length, helical amplitude, and wavelength) determine the motility behaviour near a single flat surface [230]. Based on this modeling framework, correlated with the experimental observations from the present study, three classes of behaviour were observed, depending on the geometry of the bacterium. “Wall accumulators” descend to the walls and exhibit a strong propensity for swimming in the closest vicinity to the wall (with a separation of tens of nanometers between the bacterium and the surface), where steric interactions are likely, thus making difficult the precise prediction of motility behaviour even for the simplest monotrichous bacteria. When bacteria swim at distances further than this from the wall but at a nearly constant separation, exhibiting the characteristic circular orbits predicted by simpler

analysis, they are classified as “stable swimmers parallel to the wall.” It was observed [197] that dynamical interactions are negligible before collisions with the walls, but once bacteria swim on parallel planes a few micrometers away from surfaces, hydrodynamic forces maintain long residence times in this region. Finally, when hydrodynamic interactions result in bacterial movement away from surfaces, they are classified as “wall escapers.” The demarcation between these classes is approximate, due to the inherent stochasticity of bacterial motility.

Two key geometrical parameters determining whether a particular bacterium is an accumulator, escaper, or moving parallel to the wall are 1) the cell body aspect ratio and 2) the length of the flagellum. Higher aspect ratios (more rod-like) and shorter flagella encourage escape from walls. For geometries at the boundary between parallel motion and escapers, it is possible for a bacterium to exhibit either stable motion close to the wall or escape depending on the angle of approach to the wall. It is useful to first determine the behaviour of bacteria near a single wall because this is indicative of motility in more complex environments. For example, simulations showed that parallel–stable swimmers and escapers had different characteristics when placed between parallel walls [232] and in corners of rectangular channels [236] . However, the variability of characteristic bacterial dimensions adds to the inherent stochasticity of movement. This in turn makes the demarcation between motility classes approximate. Details of the modeling used in Figure 22 are given in SI Appendix, and the characteristic dimensions of bacteria are presented in SI Appendix, Table S2.

While these theoretical studies were based on a model with a single, polar flagellum, it was demonstrated that such models accurately reproduce the experimentally observed radius of curvature of near-wall tracks for *E. coli*, which swim with several flagella [229]. Therefore, it is expected that this classification serves as a useful conceptual background for the characterization of motility behaviour in relation to a solid surface, even though most of the species in the current study are architecturally more complex than the monotrichous model (here, *V. natriegens*). Indeed, the propensity to move near surfaces was observed experimentally for several nonmonotrichous bacterial species, for instance (extensively) for *E. coli* [197, 225, 228, 229, 253], but also for Serratia marcescens and Pseudomonas aeruginosa [254]. Comparison of experimental and theoretically predicted behaviour. By

comparison with monotrichous model bacteria of equivalent dimensions, *M. marinus* is predicted to be a wall accumulator, but (B) Example of a “wall escaper” bacterium: *M. marinus*.it is actually near the boundary between accumulators and escapers (Figure22)

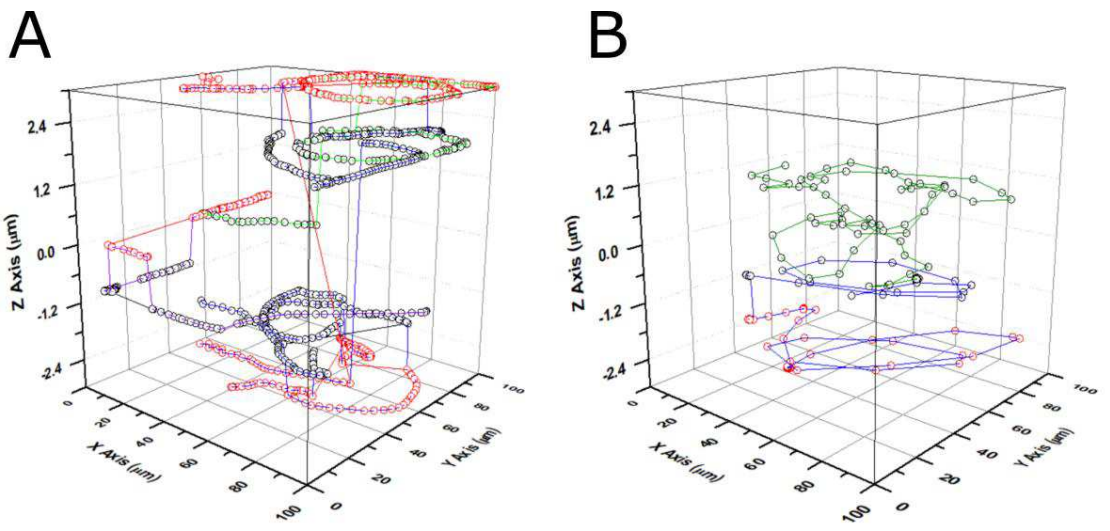

Figure 22: Comparison of two bacterial species with opposite motility behaviour. **A**. *E. coli* moves in planes parallel to the walls, at different distances. **B**. *M. marinus* does not move in a parallel plane, thus flipping from a wall accumulator to a wall escaper behaviour.

All other species are expected to maintain stable motion parallel to and near the walls, although variability within populations is sufficient for some individuals to be classified as escapers. There are elements that correlate well with the predicted motility behaviour of simple bacteria with that of the more complex geometries studied as well as explanations for the deviations from this general "motility landscape" (Figure 22):

1) Our experiments showed that *M. marinus* did not exhibit stable motion parallel to the wall but rather a "ping-pong"–like movement, with abrupt approaches to the walls alternating with equally abrupt breakouts. Recently, a model of the movement of a polar biflagellate bacterium [255], based on *M. marinus*, showed that such wall escaping (scattering) behaviour could occur for certain arrangements of the two flagella. Additionally, it was recently reported that *M. marinus* swims with one flagellar bundle in front of the cell body and one behind [256], a mode of motility that is fundamentally different from the monotrichous model.

2) The long trajectories, represented as two-dimensional (2D) projections and in 3D (Figure 21. A, B, respectively) for *P. putida* and *E. coli*, showed characteristics of both

escapers, more apparent for *P. putida*, and movement parallel to the wall, more apparent for *E. coli*. The persistent circular orbits indicate motion close to the horizontal walls, and for *E. coli*, the long trajectories along the vertical walls also highlight boundary accumulation. In contrast, the long, relatively straight trajectories through the middle of the chamber and frequent transitions between z-planes represent wall escaping behaviours. These seemingly contradictory observations are, in fact, consistent with the variability found in the measured cell shapes and flagella lengths. While the average values for both *P. putida* and *E. coli* lie within the movement parallel to the wall regime (Figure 22), the spread of parameters extends considerably into the wall escaper region.

3) The 2D projections and 3D bacterial trajectories (Figure 21. A, B, respectively), are consistent with the placement of *V. natriegens* and *V. fischeri* deep in the movement parallel to the walls, according to the theoretical predictions in Figure 22. Both species showed circular trajectories (more prominent in *V. natriegens*) and high densities around the perimeter of the chamber. Interestingly, *V. natriegens* was often observed swimming parallel to the vertical walls but at distances of around 3.5 μm from the wall rather than keeping almost in contact with the wall. This type of parallel motion was found in simulations of boundary accumulators in corners of channels [236].

7.2.3 Motility patterns

The longest trajectories of bacterial motility in plazas had characteristics that were the most species specific (Figure 21. A and Movie S1, top row). *V. natriegens*, *E. coli*, and *V. fischeri* presented, to various degrees, two classes of trajectories: 1) movement along the vertical and horizontal walls and, when detached, 2) circular motions, until again attaching to the walls. *M. marinus* exhibited a "ping-pong"–like motility pattern, generally following relatively straight paths until it approached and scattered off a vertical wall, resulting in a statistically higher density localized near the walls (due to frequent collisions). There was little discernible movement along the vertical or horizontal walls of the plaza, and no complete circular orbits were observed. Two classes of behaviour were present in the longest trajectories of *P. putida*. Some were relatively straight, spanning from one side of the chamber to the other, whereas other trajectories were circular and persisted for many overlapping cycles. Long trajectories around the perimeter of the chamber, as observed for *V. natriegens*, *E. coli*, and even *V. fischeri*, were uncommon for *P. putida*.

7.2.3.1 Circular motion

The circular motion of bacteria near surfaces was previously reported for *E. coli* both at air–liquid [225] and solid–liquid interfaces [26], [257] and for *P. putida* at solid–liquid interfaces [231],[258]. Counterintuitively, despite their very different flagellar arrangements (Figure 20. A and SI Appendix, Figure S1 and Table S1), circular patterns were also observed here for *P. putida*, to a lesser extent for *E. coli*, and for *V. natriegens* (Figure 21. A and B). Theoretically, the hydrodynamic interactions between a flat surface and a bacterium swimming on a parallel plane to it are indeed able to explain this curved pattern of trajectories [195], [254]. In summary, in quasi-open spaces, such as plazas, when the movement is limited only by parallel vertical or horizontal walls placed at distances considerably larger than the size of bacteria, their motility can be approximately characterized as stable movement parallel to the wall, wall escapers, or—rarely—as wall accumulators, as derived from bacterial geometric parameters and hydrodynamics-based modeling of the movement near surfaces of monotrichous bacteria.

7.2.3.2 Motility in channels

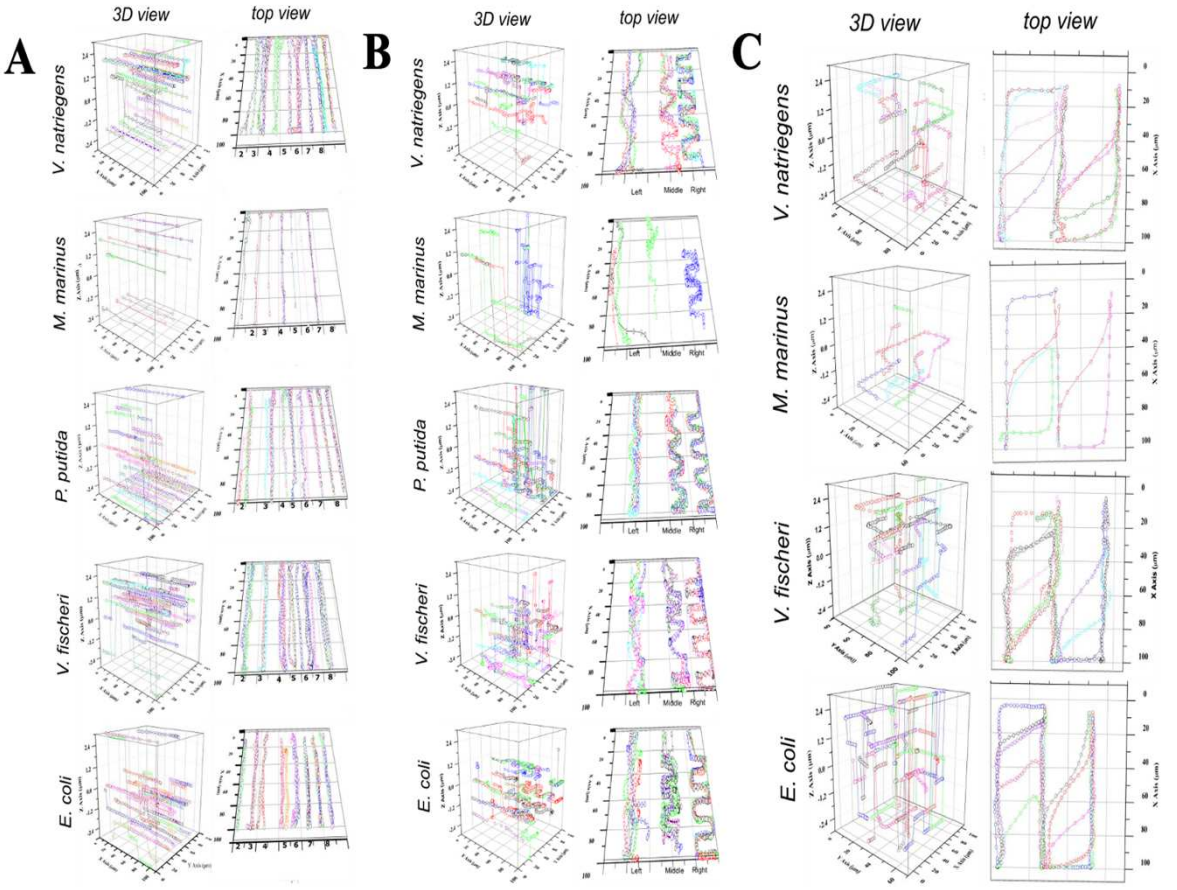

Figure 23: Cumulative representation of trajectories of five different bacterial species projected in 3D space using z-stack imaging in A. Linear channels, B. Meandered channels, and C. Angled channels.

Linear channels.

Figure 23. A, in wider channels (6–8 µm), *V. natriegens* and *E. coli* exhibited a strong preference for moving along the walls, consistent with their motility patterns observed in plazas and their alignment parallel to vertical and horizontal surfaces. *P. putida* displayed

sinusoidal movement, particularly in larger channels, while *E. coli* also demonstrated sinusoidal characteristics, with wavelengths increasing proportionally to channel width. This relationship resembles the larger radii of circular movements seen in plazas with higher ceilings compared to those with lower ceilings. *M. marinus* also exhibited sinusoidal-like behaviour in wider channels.

In narrower channels (3–6 μm), tighter confinement predominantly forced bacteria (except *M. marinus*) to move along the channel axis, diverging from their motility in open spaces. In these confined environments, bacterial movement appeared to benefit from both hydrodynamic effects and steric interactions with the walls, which synergistically directed their motion along the straight channels.

Meandered channels.

The meandered system consisted of three channels with varying gap widths between the "teeth" edges: 5 μm, 10 μm, and 15 μm (Figure 23. B). The tightly confined 5 μm channels induced complex motility patterns across all species, with M. marinus experiencing frequent trapping and lower success rates due to elastic-like collisions. Notably, the 90°-angled corners acted as traps, particularly for *E. coli* and *V. natriegens*. Larger gaps allowed for hydrodynamic-driven movement, while tight confinement channeled bacteria via steric interactions. The mesoscale 10 μm gaps lacked synergistic interaction mechanisms, increasing trapping probabilities, especially for compact species like *E. coli* and *V. natriegens*. Conversely, *V. fischeri* and *P. putida* demonstrated higher success rates due to their morphological attributes, suggesting bacterial shape, not just size, modulates steric interaction-driven motility. To conclude, in complex geometries, such as meandered channels, hydrodynamics-driven motility is prevalent in wider channels, and the local steric interactions–based mechanism governs bacterial motility in narrow channels. However, in the mesoscale region, these two mechanisms do not act in synergy, resulting in trapping bacteria, with high efficiency for species swimming parallel to the walls, finely modulated by their characteristic shape ratios.

Angled channels.

In the structures featuring angled exits (Figure 23 C), all bacterial species exhibited a strong preference for moving in straight trajectories along the central axis of the channel, as demonstrated by representative 3D bacterial trajectories. Even at the smallest exit angle of 30°, the likelihood of bacteria maintaining a straight trajectory rather than exiting laterally (quantified as the ratio of bacteria continuing straight to the total reaching the intersection) ranged from 72% for *P. putida* to 58% for *M. marinus*.

Overall, the probability of lateral exit decreased as the exit angle increased, but species-specific variations were observed. For *V. natriegens*, *E. coli*, and *P. putida*, there was a pronounced decline in exiting probability with increasing angle. In contrast, this trend was less evident for *V. fischeri*, and *M. marinus* showed an almost indifferent response to exit angle, apart from an abrupt decrease at angles exceeding 30°. Notably, all species except *M. marinus* displayed a relatively higher probability of exiting at 90° angles.

7.3 Conclusion

We here provided a comprehensive account of the motility of individual bacterial cells, belonging to five species with considerably varied dimensions and morphologies, in microfluidic networks and with various levels of confinement and complexity. For lesser confining geometries, such as facing one limiting wall, the motility behaviour of the five species studied can be assimilated, with qualifications, to that of monotrichous bacteria with similar dimensions. However, when increasing confinement complexity, as for instance in straight channels with various widths, in networks with exits at various angles, and meandered channels, the classification as swimming parallel to the walls for *V. natriegens*, *E. coli*, *V. fischeri*, and *P. putida* and as escapers, partially, for *E. coli*, *P. putida*, and *M. marinus* is increasingly inaccurate, as a result of the increase of the impact of local steric interaction of species-specific morphology with the tightly confining geometry. The study can be also used as a methodological template for the optimization of the design of microfluidic devices with specific functions (e.g., motility-based cell selection for single-cell genomic screening, detection of rare cells, bacterial entrapment devices for diagnostics, or biocomputation).

7.4 Materials and Methods

All experimental, modeling, and simulation data analysis protocols are presented in SI Appendix.

Data Availability. All study data are included in the article and/or supporting information.

ACKNOWLEDGMENTS. This work was financially supported by the Defense Advanced Research Projects Agency under grant agreements HR0011-16-20028 and N66001-03-1-8913, by the Natural Sciences and Engineering Research Council of Canada under grant agreements RGPIN-2016-05019 and RGPIN-2018-04418, by the New Frontiers Research Fund of Canada under grant agreement NFRFE-2019-00129, by the Czech Science Foundation under grant agreement GACR, project 17-11851Y, and by Australian Research Council Future Fellowship FT180100698. We thank the researchers from the Facility for Electron Microscopy Research at McGill University, from the Faculty of Dental Medicine at

the University of Montreal, and from the Department of Geology of the University of Quebec at Montreal, for help in electron microscopy characterization of bacteria.

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Chapter VI

8. Discussion

8.1 Summary

This thesis explores the motility of biological microswimmers, specifically euglenids, with a focus on their responses to external stimuli, propulsion mechanisms, and behavioural patterns under varying conditions. Key findings include the effects of confinement on motility patterns and swimming efficiency (Chapter 2) and the relationship between flagellar modulations and behavioural responses (Chapter 3). These findings were supported by innovative experimental approaches, computational modeling, and microfluidic applications. The research concludes with a comprehensive discussion of its broader implications, limitations, and prospects for the field. Additional contributions, including advancements in experimental techniques, are discussed in chapter 4.

This thesis discusses two research works focused on understanding motility pattern and behavioural responses of euglenids in microfluidic-based structures with varying confinements. The first work investigates the motility patterns of two euglena species. When subjected to confinements the cells change their motility patterns, swimming trajectories, swimming efficiency, and exhibit various behavioural responses. The velocity and residence times of the cells changed with the type of confinement. The cells switched from helical swimming to various behaviours like metaboly and backward swimming showcasing the ability of cells to self-adapt by mechanical self-regulation of their body. The second work shows the interaction between the flagellum and its surroundings that reflects on the motility and behavioural responses of the cell to escape confinements, in other words, we study how the modulations of flagellar beat patterns were integrated into the overall behaviour of the cell. The flagellum undergoes a series of shape excursions, allowing the cell to regulate the conventional flagellar beating and consequently select the most efficient responses reflecting on changes in behaviour to free itself from tight spaces. In less confined spaces the cell chose alternative swimming modes such as exploiting vertical inclination spinning, and gliding.

The observed patterns reflect the complex interplay between the cell's physiology, external cues, and the need for efficient motility or escape. Our findings also suggest that metaboly, is highly related to the compromised flagellum or the inability of flagellum to conventionally, which brings us closer to a better understanding of metaboly adaptation. In conclusion, the research here provides critical insights into underlying characteristics and strategies of flagellar motility and a comprehensive understanding of the plasticity of cells to escape external stressors on a need-basis highlighting an embodied intelligence in euglenids. The findings can aid in designing a methodological template for developing biocomputational devices and other domains such as bio-inspired robotics and biomedical applications, leading to the development of more efficient and effective solutions for various applications.

The motility patterns of euglenids were extensively studied in the microfluidic channels with varying geometrical confinements namely, linear channel, quasi-open space (plaza), wavy, and zig-zag channel systems (W1-W6) with three complexity dimensions, 1. turn angle complexity, 2. number of turn angles, and 3. channel member variety. Euglenids are typically ellipsoid (cigar-shaped) bodies that swim rotating counterclockwise along their long axis, the flagellar swimming in euglenids is propelled by the lateral beating of a single emergent flagellum located at the anterior end of its body. Swimming under these “ideal” (flagellum-driven, ellipsoid shape) conditions, the motility pattern is a characteristic sinusoidal path or a periodic swinging motion [31]. The swimming propulsion mechanism can be summarized as a complex sequence of non-planar flagellum beats with a characteristic movement mostly dubbed to figure “8” [31]. In the presence of a wall or a physical boundary, the motility behaviour of microswimmers swimming in low Reynold’s number is highly influenced by several factors including hydrodynamics, steric effects, mechanical forces, and biological responses. Based on these factors a microswimmer (depending on the swimming type) either swims in closer vicinity to the wall (wall accumulator) or away from the wall (wall escaper) [20]. To illustrate further, when thrust is generated behind the cell body otherwise classified as a pusher, the cell tends to swim closer to the wall showing wall accumulating behaviour, and when the thrust is generated in front of the cell body (puller), the cell swims away from the wall showing wall escaping behaviour [24]. Our previous work explicated such behaviours in five bacterial species [20]. Alternatively, several studies reported this type of swimming behaviour in sperm cells [15, 21-23], bacteria [20, 24-26], and ciliates [27, 28].

In quasi-open structures (O1-O3), the simplest geometric constraint bound by vertical and horizontal walls, *E. gracilis* presented an uneven or broken near-wall density and exhibited abrupt wall-bouncing reflections like ping-pong ball, however, cells swam in closer vicinity to the wall for short distances (wall accumulator) before abruptly scattered by the wall (wall escaper). *E. gracilis* oscillates between a wall accumulator and a wall escaper, a report on the flow field analysis of swimming behaviour reported *E. gracilis* exhibit mixed puller-pusher behaviour during a stroke [160]. The scattering dynamics of *E. gracilis*, initiated by short flagellar interactions (steric effects) with the wall, when cells collided head-on and the initial response was a shock mechanical response, the swimming behaviour was a result of short steric interactions with the wall until hydrodynamic forces dominate. The impact with the wall instigated mechanical responses where cells immediately scatter, yet remain trapped in close proximity to the wall showing a brief hydrodynamic escape, this type of phenomenon of hydrodynamic escape was also observed in ciliates [28]. The initial mechanical responses when scattered add a critical factor in memory of the incidence angle and symmetry of the exit angle, when the turn/scatter was sharp after collision it resulted in an asymmetrical exit angle, when the scattering was smooth the exit angle was symmetrical, and swimming was dominated by the steric effects until cells escape the wall. In *E. acus*, cells scattered off quickly with a sharp turn upon collision without being trapped near the wall, the scattering was predominantly by mechanical shock responses from the collision. *E. acus* is predominantly a wall escaper, the cells scattering was primarily dominated by mechanical forces upon collision, resulting in sharp turns when scattered by the wall. However, in all cases the cell course corrects its trajectory parallel to the wall maintaining at least 200 µm distance from the wall and continues to swim after course correction. The scattering angle decreased with flagellum length, showed the scattering angle predominantly depends on the flagellum length. Additionally, when cells collide head-on with the wall the scattering is initially dominated by the mechano-elastic response of the cell and later the steric or hydrodynamic forces take over to influence the motility. In open channels, as the wall corner is bound by two walls, cells appeared to be trapped in the corners. Upon entrapment, cells began to gyrate in the channel corner for several seconds and this continued until the cells can negotiate a way around the obstruction. The duration of entrapments varied depending on the species, *E. gracilis* exhibited a longer entrapment time, which can be attributed to its

longer flagellum length. Conversely, *E. acus* displayed a shorter entrapment time, almost half the duration, as the shorter flagellum of the cells facilitated a fast escape strategy.

In *E. acus*, when cells exit relatively complex and confining W1, W2 structures they opted for long, circular trajectories with longer residence time in the open plaza, when they exit the least complex and confining W3 structures their trajectory patterns were predominantly linear in the corresponding open space, with a shorter residence time and quick transition to the next structure. It appeared that the structures they were previously subjected to have a temporary impact on their motility pattern in the corresponding open space. Euglenids contain epiplasmic pellicle strips that serve as an elastic motor system to adapt highly coordinated bodily deformations or metaboly. Some studies showed an adaptable mode of locomotion in Euglena by mechanical self-regulation of the pellicle strips under confinement [14]. The channel design in the structures W1 and W2, imposed agitation, severely restricted forward motion that prompted cells to adapt various biological responses mainly metaboly in *E. acus*. The mechanical self-regulation and adaptability of the active pellicle (mechanical machinery) to the dynamics of the constraining microenvironment could presumably contribute to this distinctive behaviour. Although, this behaviour was not distinctively observed in *E. gracilis*, the percentage of cells that opted for straight trajectories was slightly higher when they exited W3 compared to the other four structures. Some cells that exit the relatively complex channels showed erratic behaviour immediately after exiting the complex channels. Euglenids are shown to modulated their flagellum beat patterns to change distinctive trajectory patterns in response to light stimuli [10]. The mechanical stress induced on the flagellum and pellicle envelope could be the contributing factors for the difference in this behaviour between the two species. Overall, these observations highlight the adaptation of each species in response to their environment.

8.2 Flagellar shape excursion to achieve helical swimming

To investigate the intricate interplay between swimming motion and flagellar beat patterns, we conducted high-resolution imaging of cells at a higher magnification and frame rate. This study highlights the pivotal role played by the mechanical interaction between the flagellum and the surrounding environment in governing the dynamics of flagellum beating and the development of escape strategies. With the flagellum present at the anterior end steering the

body, with each sharp bend, the flagellum as well as the body is obligated to collide with the wall. In most cases, this collision results in hindered free-swimming resulting in behavioural changes. Cells swimming in the channels adapted various flagellar beat pattern modulations as a result of mechanical shock from the collision with the wall followed by malfunction of flagellar beating. We observed that the flagellum undergoes a series of shape changes to regulate the conventional beating. In free-swimming cells, the conventional flagellum beating can be summarized as nonplanar, asymmetrical waveforms mimicking the number 8 or a swinging lasso [169]. When the cell's free swimming was hindered due to confinement, we observed that controlled regressive behavioural responses were triggered. Molecular motors play a crucial role in driving the movement of eukaryotic flagella, these motors aid in driving flagellum movement by facilitating bending, [177] while the flagellar elasticity counters the bending. Other than the bending and opposing forces, the fluid forces of the surrounding liquid act on the flagellum, the coordinated action and balance between the three forces governs the flagellar motility and swimming of the cell. In addition to the above, the flagellum-body mechanical sensing or interaction with the surroundings plays a critical role in the movement of the flagellum.

8.3 Sensory cues in eukaryotic flagellates

Eukaryotic ciliates and flagellates use sensory cues to navigate in their habitat, when cells need to change the swimming direction, it becomes challenging as unsynchronized movements are required to produce the appropriate torque for the changes in swimming direction. Reports on sensory reception in eukaryotic organisms date back 100 years, this sensory reception includes mechanosensation, phototactic, and chemotactic sensation etc., Microswimmers are known to respond to physical stimuli using mechanosensitive channels, these channels function as molecular detectors and are found in most living organisms ranging from microbes to humans, they play a critical role in multiple functions including photo, sound, gravity perception, taste, touch and pain sensation, heat sensation, fluid flow, etc., *Paramecium* showed to reverse ciliary beating as an avoidance response upon collision with the wall. [178]. Recent studies on mammalian cells have identified mechanosensitive channels in endothelial and vascular muscle cells [179], as well as immotile cilia or crown cells in the nodal cavity that respond to mechanical stimuli and sense the magnitude and

direction of flow [180]. These channels play an important role in protecting bacteria against osmotic shock and varying membrane tension [181]. Mechanosensitive channels in *Euglena gracilis* aid in changes in the movement and course correction of the flagellum during gravitaxis, and it is assumed that these channels are located at the front end of the cell underneath the flagellum as the modulated signals occur when the flagellum points downwards [182]. The avoiding reaction was first reported in *Paramecium* [19, 183], when the cell collides with a solid object, it briefly reverses swimming direction and orientation, then resumes forward motion, this continues until the organism successfully avoids the obstacle. It was later discovered that the underlying mechanism for this avoiding reaction was the initial contact with the obstacle triggered a Ca^{2+}-based depolarizing mechanoreceptor potential a temporary rise in intraciliary Ca^{2+} concentration [184-187]. This change in the Ca^{2+} action potential initiates a temporary reversal of axonemal beating, resulting reversal in the swimming direction. A similar mechanoreceptor-mediated Ca^{2+} influx was also observed in *Chlamydomonas* [188, 189], furthermore, it has also been reported that *Chlamydomonas* is equipped with other flagellum-mediated sensory roles [190-192]. The sensory function of the motile flagellum in protozoan *Heteromastix* showed the flagellum movements constantly feeling for obstructions, and upon encounter seizes the hold of it [193]. It's important to note that the specific flagellar-based functions can vary among different eukaryotic cells and organisms. The observed complex dynamics of flagellar beat pattern modulations in our study suggest that the flagellum's intricate movements are not random but follow a purposeful and regulated process aimed at optimizing the cell's locomotion. With the flagellum present at the anterior end steering the body, with each sharp bend, the flagellum as well as the body is obligated to collide with the wall. In most cases, this collision results in hindered free-swimming resulting in behavioural changes. We observed that the modulations exhibit several repeated patterns, despite their seemingly haphazard appearance. Remarkably, the flagellum is capable of entirely unwinding itself in order to restore optimal beating and facilitate efficient locomotion to escape confinement. These repeated patterns, particularly, the attempt to resume the conventional figure-eight pattern characterized by double loops on either side of the cell, indicate a deliberate effort to reconfigure the flagellum's shape during the beating. Furthermore, the observed capability of the flagellum to entirely unwind itself, extend, and sense the confined space around suggests an adaptive

mechanism employed by the cell to overcome confinement and facilitate escape. Different shapes may provide varying propulsive forces and directional control, allowing the cell to navigate and understand the space effectively. As iterated earlier, perhaps the changes in the flagellum shape could be a result of such mechanoreceptors present at the anterior end of the cell. Perhaps, the functional significance of the flagellum extends beyond mere propulsion, encompassing multifaceted roles within the organism's physiological framework.

8.4 Intriguing behavioural responses

Metaboly, also known as euglenoid movement refers to highly coordinated bodily deformations exhibited by euglenids. Despite extensive research, the precise function of this interesting phenomenon remains elusive. However, our experimental observations have led to an intriguing theory that sheds light on its potential initiation and purpose. The most recent studies on metaboly reported that metaboly was prompted by culture crowding or confinement [98], however, we report that metaboly appears to be predominantly related to the agitation at the anterior end and the inability to beat the flagellum conventionally for free swimming. Confinement-induced hindrances to the flagellar motion result in changes in the cell orientation and position in 3D space, ultimately when the flagellum fails to resume conventional beating, metaboly takes over as an alternative swimming strategy. The complex feedback between the flagellum beat patterns and the accompanying space sensing (stimulated by flagellum unwinding and modulating) enables the selection of an optimal response and facilitates strategic decision-making for effective navigation through complex confinements/environments. The most fascinating observation here was although metaboly is a quick and faster escape strategy this does not seem to be an immediate response to confinement. Rather, the cells first attempt to unwind the flagellum and modulate its beating, and perform various trials to regulate the beating, in this process, sensing the availability of space or level of confinement to resume free helical swimming, when unsuccessful ultimately adapt metaboly. In other words, metaboly is not a first response to confinement, rather a series of alternative methods are adopted by the cell which when failed leads to metaboly. Fewer cells were observed to spontaneously adapt a reverse or backward swimming as an avoidance response to the confined channel. The cells swimming through the confined channel first adapted modulations in flagellar beating and very quickly

transitioned to a brief backward or reverse swimming. The principal observation here was that the cell unwinds the flagellum, however, the cell itself held its original shape (ellipsoid) while swimming in the backward direction, the flagellum beat patterns continued to vary as the cells descended the channel. It can be hypothesized that there was an altered wave propagation in cells swimming backward. The propulsion in euglenids can be summarized as non-planar waveforms resulting in helical trajectories coupled to body rotations [169]. These waves are generated from the root and extend to the tip of the flagellum with each beating stroke thus pushing the swimming cell forward. It is noteworthy that backward swimming is not a predominant trait in euglenids, however, some studies report a reversed flagellar motion with a shift in curvature and wave propagation in euglena as a photoinduced response [194]. Microswimmers show interesting responses to confined spaces, such as, bacteria showing circular swimming near boundaries due to hydrodynamics [195], accumulation near solid surfaces [196], reverse the swimming direction by flagellar reorientation at an obstacle [16], cell-surface scattering and long residence time on surfaces [197]. Eukaryotes like sperm cells also exhibit such circular swimming due to hydrodynamics [198], accumulation near flat surfaces [15], and preferential swimming such as boundary following navigation [17]. *Paramecium* also exhibits avoidance [19], meandering and bent or circulating navigation in confined spaces [18]. Our study did not involve an external stimulation such as phototaxis, however, it is evident that confined spaces act as a physical cue that triggers very intriguing self-organized responses in euglenids which can be exploited for the design and control of biomimetic soft robots. The free-swimming cells transition from helical swimming to spinning to avoid an obstacle. The reference to spinning here is adapted from [90], during spinning-type motility, the flagellum is twisted into a single loop in comparison to two loops while helical swimming. Spinning as a behavioural change was observed in *Euglena gracilis* as a phototactic response to changes in light intensity [90]. In our study, this response was a result of behavioural changes or mechanical responses to obstacles, particularly in relatively unconstrained spaces. Decisively, the flexibility of the flagellum coupled with prompt stimulus to physical cues trigger an active change in switching of swimming behaviour resulting in avoidance reaction without exertion. In addition to spinning, we also observed cells exploiting their innate gravitactic ability [199] to escape or switch swimming direction. The cell swiftly vertically

tilts the anterior end at 180° in the Z-direction changing the swimming direction to swim away and back in the horizontal plane. In this context, the term 'gravitaxis' is used solely for descriptive purposes and not suggest the existence of a taxis behaviour resulting from gravitational force. Euglenids perform both negative and positive gravitaxis [199] to optimally position themselves for efficient photosynthesis and cellular multiplication [259]. What is intriguing is that most of these biological behaviours, although instigated by external stimuli such as light or gravity, imprint a lasting influence on these organisms. Gliding, cells when trapped in the substrate interface exhibited gliding type motility, substrate-dependant, whole-cell motility with or without instigation of bodily deformations. Gliding or bio adhesion, predominately substrate-associated motility, is ubiquitous among biological microswimmers. A variety of microorganisms including bacteria, and eukaryotic microalgae, have developed adhesion strategies to form dense populations or biofilms for survival [260-263]. Chlamydomonas when triggered by light, switches from free swimming to surface-bound states through light-induced flagella adhesion [264]. Under favourable light conditions conducive to photosynthesis, the cells transition into gliding motility forming interconnected microbial communities for effective light harvesting [265]. Gliding motility is not largely reported in euglenids capable of free helical swimming, however, this type of motility was reported in *Euglena mutabilis*, this species lacks the emergent flagella [200]. As the flagellum does not play a role in motility the cell resorts to an alternative mode of movement which is, gliding. This type of motility is facilitated by the secretion of localized mucus while the cell attaches itself to the substrate. In context to our study, although the cell has an emergent flagellum, being trapped at the substrate interface and inability to beat the flagellum or propel forward, the cell switches to an alternative mode to facilitate movement. These observations underscore the remarkable plasticity and versatility of euglenids in adopting alternative modes of movement as a survival strategy. It reflects the cell's capacity to adapt and respond to external challenges on an as-needed basis, thus enabling it to thrive in diverse and fluctuating conditions.

8.5 Prospective work and applications

Considering the immediate yet controlled avoidance reaction after the collision, and instead of a full body deformation the cell attempts to create additional space from the wall to resume

conventional beating, it is safe to hypothesize that this feedback response could perhaps be due to the mechanosensitive channels present in the anterior end of the cell. As observed in this study, the controlled regressive behaviour to retract the anterior end just to make more space to beat the flagellum, or the continued effort to entirely unwind and rewind the flagellum to regulate the beating while continuously sensing the space indicates a complex layer of characteristics to the flagellum. This implies that the flagellar beat pattern modulations are not arbitrary but rather purposeful adjustments aimed at ensuring the cell's effective movement in response to surrounding challenges. However, a pivotal question that warrants consideration is whether the eukaryotic flagellum is an organelle exclusively used for motility/locomotion or does it possess many additional functions such as serving as an active sensory organelle. Further research and experimentation would be necessary to provide more detailed insights into the underlying mechanisms and functional significance of flagellar shape modulations in specific euglenids. To understand the mechano-receptors particularly responsible for these behavioural responses.

Micro and nanorobots have potential and employed in biomedical applications such as targeted drug delivery, and microsurgery due to their controllable motion, size, and biocompatibility [266-268] [269, 270] although these robots hold potential, it is still challenging to maneuver them into narrow spaces for complicated biomedical tasks. Soft robots pose new possibilities for task execution in narrow and complicated spaces where most rigid microrobots fail due to a lack of deformability and adaptability. Organisms found in nature have evolved over millions of years to exhibit high adaptability and various motility patterns to various environmental changes by altering their shapes and direction of motion for survival. Inspiration from these organisms has led to the design and development of soft robots using various actuation mechanisms [271-275]. Leveraging insights from motility studies to design and develop bioinspired soft robotic systems capable of autonomous navigation and sensing in complex environment, also applicable to understanding dynamic motility regime, targeted drug delivery, biofilm prevention. The efficient designs of soft robots depend on successful embodiment involving clever morphology, selection and utilization of material properties. The set of principles that can be explicitly utilized from our work includes, the cell’s behaviour is influenced by its internal control and external niche, physical constraints shape the dynamics of interaction between the cell and its surroundings,

the flagellum and body embodiment are linked to information procession with sensory cue activities. Considering a cell as an embodied agent, the complex dynamics of the system allows application of concept like self-organization, adaptability, flexible interactions offering opportunities for stability and maneuverability instead of top-down control of bio-inspired robots.

8.6 Biological computation

Parallel computing in biology is not a new phenomenon, most living beings process signals presented to them in analog forms that help them make decisions to either react or flee, comparable to zero's and one's used in the binary form of electronic computing. Such parallel computing has been demonstrated in an *in vitro* set-up to approximate solutions for computation using several agents such as *Physarum* [44], nucleic acid-based DNA [45-47] computing and recently by our team using molecular motor proteins [48], and bacteria (currently explored by our team). The earliest form of biological computing using DNA demonstrated to solve an instance of Hamilton path problem [157], although an excellent and convincing parallel computing approach, it has hardly scratched the surface of the complexity of these NP-complete problems due to their key scalability issues meaning demanding incredibly large resources for solving larger size problem. For example – DNA computing suffers from a fundamental drawback in raw materials, i.e., the requirement of a large number of DNA fragments. It has been shown that even the recent high-end, state-of-the-art quantum computing (though not biological computing) is limited in scale to solve such intractable, exponentially growing NP-complete problems [158]. To address this problem Prof. Nicolau and his team proposed a new computational paradigm, in solving the combinatorial problem by allowing a very large number of motile biological agents, here molecular motors to explore the microfluidics network [48]. These self-propelled agents explore the network independently and parallelly through channels by brute force to solve the graphically represented mathematical problem. This approach was successful, and the experiment-based computer aided simulations revealed the proof-of-concept that parallel computation using the biological agents has the capability to efficiently solve the NP-complete problem of medium to higher sample sizes. However, the approach employing

molecular motor proteins faced challenges like detachment of molecular motor proteins from one another, handling of molecular motor proteins, and scalability associated with readout for computing. Using photosynthesis microbes (euglenids) as biological agents offer several advantages such as self-propelled, easy to handle, low-cost maintenance, replication, and the most appealing factor about euglenids are that they are photosynthetic and require minimal nutrients to survive. Microorganisms, though memory-less, exhibit dynamic responses to their environment and appear to have recognition and decision-making abilities in receiving and processing signals like chemical, light, etc., as either an independent or collective entity resulting in efficient space-searching, adaptation, and colonization. Euglenids are photosynthetic, unicellular flagellated protists abundantly found in aquatic ecosystems. They are unique in that they are both heterotrophic (derive nutrients and energy by consuming other organisms) and autotrophic (can produce their own food via photosynthesis - the most appealing factor), making them easily culturable microorganisms that live stably without stringent culture conditions.

The operation of our work is that the motile biological agents are allowed to autonomously explore the microfluidic network encoded with the combinatorial problem, agents pass through sequence of junctions in order to report for solutions at exit. In this process, the agents are flooded at the entry port of the device and the movement of the agents is optically recorded along the network at high precision. The key to addressing the exponential increasing solutions with problem size is employing growing resources – establishing massively parallel computation. Based on motility patterns and performance parameters, we design and develop graph-theory based microfluidic network encoding of mathematical problems for biocomputation. This physical network encoding the mathematical problem serves as a rigid master for fabricating and replicating microfluidic devices, particularly by the soft lithography process. The microfluidic structure comprising entry, exits, nodes, junctions, channels are the computing network that encodes combinatorial problems of interest. To make this device setup photosynthesis based, the network is submerged in sterile water to wet the device and allowed to explore under uniform light. The preliminary results showed decent distribution of cells with tolerable but not zero error at the spilt junction, we showed replication in the network, studied the duration of replication and investigated the span of photosynthesis-driven biocomputation set up. We showed euglenids explores the

microfluidic network encoding instances of a benchmark combinatorial problem, multiply in the network translating to growing resources for an exponentially growing problem size – establishing a potential for parallel computation. The proof-of-concept of a photosynthesis-driven biological computation holds the potential to develop a computing device that is independent with longer run times and hold the potential to successfully scale up however with more design corrections.

9. Conclusion

The work presented in this thesis has provided insights into to motility patterns and behavioural responses of euglenids, highlighting their remarkable adaptability and resilience in response to varying physical constraints.

The first manuscript underscores the impact of different confinements on the motility patterns and swimming efficiency of euglenids within microfluidic-based structures. We elucidated how different types of confinements induced distinct changes in motility, with cells exhibiting remarkable adaptability by transitioning from helical swimming to alternative behavioural states like backward motility and metaboly. The study highlights how the structure that the cells were previously subjected to has a temporary impact on the swimming patterns or trajectories of the cell. We explored the scattering dynamics of cells near boundaries and reported an interesting correlation between flagellar length and biological behaviours including scattering dynamics and show scattering was initially dominated by the mechano-elastic response of the cell and later by the steric or hydrodynamic forces. Next, we studied the correlation between flagellar modulations and behavioural responses of the cell, more precisely observation of flagellar beating and how the changes in flagellar beats were integrated into the overall behaviour of the cell. This work unveiled the influence of flagellar shape excursions on the motility dynamics of the cell and the pivotal role played by mechanical interactions between the flagellum and its surroundings in orchestrating the escape of the cell from confinement. This study highlights the multifaceted functionality of the flagellum beyond propulsion, suggesting its sensory perception and active response mechanisms. Our findings indicate that metaboly or euglenoid movement represents a complex escape response associated with the compromised flagellum, this constitutes a substantial stride towards unravelling the intricacies of metaboly adaptation marking a noteworthy advancement in understanding this elegant phenomenon Although other findings reported behavioural responses under phototactic or gravitactic conditions, our study reports the responses were triggered by physical confinements and mechanical responses from the collision with a wall or physical boundary. We observed how

elegantly these cells adapted these responses on a need basis highlighting an embodied intelligence in euglenids. These findings collectively underscore the complexity and diversity of behavioural responses in euglenids and their extraordinary physical plasticity. Our findings lay the groundwork for future research in various interdisciplinary domains including bio-inspired robots, develop structures for biocomputation employing euglenids, and biomedical applications.

We also performed initial experiments to dictate a proof-of-concept biocomputation using euglenids as biological agents. The preliminary experiments started with investigating if the euglenids follow the rules in the network, their replication in the network, error rate, and testing out the span of photosynthesis-driven biocomputation. The preliminary work of a photosynthesis-driven biological computation holds the potential to develop a computing device with longer run times and holds the potential to successfully scale up.

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