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Spatiotemporal optical microscopy of vesicle-mediated hormone secretion in pancreatic cells

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Foreword

This PhD thesis in Nanoscience was conducted at the Scuola Normale Superiore as part of the European Research Council (ERC) program CAPTUR3D (Capturing the physics of life on 3D-trafficking subcellular nanostructures). It presents a biophysical study of pancreatic granules (insulin and glucagon) using advanced optical microscopy.

A foundational component of this work was establishing reliable granule labelling strategies. I benchmarked genetically encoded fusion proteins (C-pep-EGFP for insulin, NPY-EGFP for glucagon) against the chemical probe ZIGIR for zinc-rich granules. This approach enabled robust, high-contrast imaging of both granule types in living cells.

The investigation addressed granule dynamics, providing the first characterization of glucagon granule behaviour compared directly to insulin granules. This analysis was performed under multiple conditions, including cytoskeletal disruption (microtubules and actin) and secretory stimulation.

The focus then shifted to granule luminal pH. By converting EGFP into the ratiometric pH sensor E¹GFP, I enabled calibrated, concentration-independent pH measurements. Using phasor-based FLIM, I measured the luminal pH of insulin granules, revealing a distinct pH difference between membrane-proximal and cytoplasmic granules. With the same approach, I performed the first-ever pH measurements of glucagon granules.

Lastly, I combined single particle tracking with pH sensing to analyse pH fluctuations at high temporal resolution, culminating in a pioneering application of orbital tracking.

This research provides fundamental insights into insulin and glucagon granule biology, laying the groundwork for future basic research and medical applications. I am grateful to the Scuola Normale Superiore and my colleagues for their support and for enriching my knowledge in Nanoscience.



Chapter contents

Chapter 1 introduces the pancreatic hormones investigated in this work (insulin and glucagon) and explores their relationship with the cellular environment, highlighting the disparity in understanding between the extensively studied insulin and the comparatively overlooked glucagon.

Chapter 2 details the labelling strategies and employs iMSD (for dynamics) together with STORM (for size) to validate and benchmark the different labelling methods.

Chapter 3 delves into the dynamical aspect of these hormone granules, starting with basal conditions. This chapter provides the first classification of glucagon granules trajectories and analyses the effects of cytoskeletal alteration. To this end, microtubules and actin filaments are disrupted to elucidate the relationship between granules and these cytoskeletal structures. Furthermore, the impact of secretory stimuli on granule dynamics is investigated.

Chapter 4 shifts the focus to the luminal composition of these granules by changing the labelling probe. Using the ratiometric sensor (E^1GFP) in combination with fluorescence lifetime imaging microscopy (FLIM), a calibrated measurement of insulin granules pH is achieved. This reveals a significant pH gradient between the granule lumen and the cytosol, which persists even after secretory stimuli. This chapter also reports the first pH measurements of glucagon granules.

Chapter 5 presents a combined experimental approach to study the granule acidification process and pH fluctuations. Using confocal two-photon microscopy and orbital tracking, pH variability is monitored, revealing fluctuations that could be overlooked by averaging or slower imaging techniques.

Chapter 6 provides details on Material and Methods.

Chapter 7 includes the list of references.

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Publications included in this Thesis

- Spatiotemporal analysis of glucagon secretory granule dynamics. **Samuele Ghignoli**, Valentina De Lorenzi, Gianmarco Ferri, Licia Anna Pugliese, Marta Tesi, Piero Marchetti, Stefano Luin, Francesco Cardarelli. 24 September 2025 on Traffic journal. DOI: <https://doi.org/10.1111/tra.70019> (Chapters 2-3)
- Measuring pH in insulin secretory granules by phasor-based fluorescence lifetime imaging of a genetically encoded sensor. Valentina De Lorenzi, **Samuele Ghignoli**, Mario Bernardi, Giulia Matteoli, Gianmarco Ferri, Barbara Storti, Ranieri Bizzarri, and Francesco Cardarelli 25 February 2025 on Communications Biology. DOI: <https://doi.org/10.1038/s42003-025-07758-w> (Chapter 4)
- Feedback-based orbital tracking of single insulin granules to monitor pH fluctuations at millisecond temporal resolution. **Samuele Ghignoli**, Valentina De Lorenzi, Gianmarco Ferri, Ranieri Bizzarri, Melissa Santi, Francesco Cardarelli. *Manuscript in preparation*. (Chapter 5)

Publications not included in this Thesis

- Unveiling nanoscale optical signatures of cytokine-induced β -cell dysfunction. Licia Anna Pugliese, Valentina De Lorenzi, Mario Bernardi, **Samuele Ghignoli**, Marta Tesi, Piero Marchetti, Luca Pesce & Francesco Cardarelli. 16 August 2023 DOI: <https://doi.org/10.1038/s41598-023-40272-9> Scientific Reports
- Structuring lipid nanoparticles, DNA, and protein corona into stealth bionanoarchitectures for in vivo gene delivery. Serena Renzi, Luca Digiacomo, Daniela Pozzi, Erica Quagliarini, Elisabetta Vulpis, Maria Valeria Giuli, Angelica Mancusi, Bianca Natiello, Maria Gemma Pignataro, Gianluca Canettieri, Laura DiMagno, Luca Pesce, Valentina De Lorenzi, **Samuele Ghignoli**, Luisa Loconte, Carmela Maria Montone, Anna Laura Capriotti, Aldo Laganà, Carmine Nicoletti, Heinz Amenitsch, Marco Rossi, Francesco Mura, Giacomo Parisi, Francesco Cardarelli, Alessandra Zingoni, Saula Checquolo & Giulio Caracciolo 23 October 2024 DOI: <https://doi.org/10.1038/s41467-024-53569-8> Nature Communications
- Pre-condensed plasmid DNA Enhances CAR-T Cell Generation via Lipid Nanoparticles. Andrea Pirrottina, Serena Renzi, Luca Digiacomo, Francesca Giulimondi, Valentina De Lorenzi, **Samuele Ghignoli**, Luca Pesce, Francesco Cardarelli, Francesco Mura, Giacomo Parisi, Luca Buccini, Chiara Cassone, Alessandra Zingoni, Daniela Pozzi, Giulio Caracciolo 4 July 2025 DOI: <https://pubs.acs.org/doi/10.1021/acsomega.5c04308> ACS Omega

Conferences & Workshops

- INTERNATIONAL SCHOOL OF BIOPHYSICS «ANTONIO BORSELLINO» 48th Course: Memos for biophysics into the future: Lightness, quickness, exactitude, visibility, multiplicity, and consistency. Erice (Sicily) 16-22 October 2023.

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With poster contribution: “A biophysical Study of the Dynamic Properties of Glucagon Granules in α Cells by Imaging-Derived Mean Square Displacement and Single Particle Tracking Approaches”.

- MOLECULE IN(ter)ACTION XXVIII school of Pure and Applied Biophysics. Venice 5-9 February 2024.

With oral contribution: “A biophysical study of the dynamic properties of glucagon granules in α cells by iMSD and SPT approaches”.

- XXVII Congresso Nazionale (National Congress) SIBPA 2024. Genova 16-20 June 2024.

With oral contribution: “A biophysical study of the dynamic properties of glucagon granules in α cells by iMSD and SPT approaches”; which became the article “Spatiotemporal analysis of glucagon secretory granule dynamics”.

- XV EBSA 2025. Rome 30 June-04 July 2025.

With poster contribution: “Capturing vesicle pH fluctuations with high temporal resolution using orbital tracking” which was partially already published in the article “Measuring pH in insulin secretory granules by phasor-based fluorescence lifetime imaging of a genetically encoded sensor”.

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0.1 Acronyms

ATP: Adenosine TriPhosphate	RRP: Readily Releasable Pool
cAMP: cyclic Adenosine MonoPhosphate	SNARE: Soluble N-ethylmaleimide-sensitive
EM: Electron Microscopy	fusion protein Attachment protein REceptors
ER: Endoplasmic Reticulum	SPT: Single Particle Tracking
ESPT: Excited State Proton Transfer	STICS: Spatio-Temporal Image Correlation
FCS: Fluorescence Correlation Spectroscopy	Spectroscopy
FLIM: Fluorescence Lifetime Imaging	STORM: Stochastic Optical Reconstruction
Microscopy	Microscopy
GLP: Glucagon-Like Peptide	TGN: Trans Golgi Network
GLUT: Glucose Transporter	TIRF: Total Internal Reflection Fluorescence
GRPP: Glicentin-Related Pancreatic	
Polypeptide	
GSG: Glucagon secretory granule	
iMSD: image-derived Mean Square	
Displacement	
IP: Intervening Peptide	
ISG: Insulin Secretory Granule	
LAP: Linear Assignment Problem	
Lat-B: Latrunculin-B	
LoG: Laplacian of Gaussian	
MPGF: Major Proglucagon Fragment	
MSD: Mean Square Displacement	
MSS-TAD: Moment Scaling Spectrum -	
Transient Arrest of Diffusion	
MT: Microtubule	
NCZ: Nocodazole	
OT: Orbital Tracking	
PC: Prohormone Convertase	
PSF: Point Spread Function	
ROI: Region Of Interest	

1. Introduction

Diabetes is a worldwide spread pathology which affects nearly 8.5% of the adult population (data from the World Health Organization). Rather than a single disease, diabetes comprises a heterogeneous group of metabolic disorders with different etiologies, pathogenetic mechanisms, and clinical manifestations but with hyperglycaemia as common feature^{1,2}. The main forms include Type 1 Diabetes, resulting from autoimmune destruction of pancreatic β cells; Type 2 Diabetes, which is the most prevalent form, and it is characterized by insulin resistance and progressive β cell dysfunction; Gestational Diabetes, occurring during pregnancy; as well as monogenic, secondary, and several hybrid or atypical Diabetes forms¹⁻⁷. Despite this heterogeneity, all types of diabetes share the hallmark feature of impaired glucose regulation, leading to persistent hyperglycemia and its associated acute and long-term complications^{1,2,6,7}.

The maintenance of glucose homeostasis relies on a finely tuned hormonal interplay involving multiple regulators. Among them, insulin and glucagon are the two key hormones controlling blood glucose concentration. They are secreted by the pancreatic islets of Langerhans which are little organs composed of distinct endocrine cell populations, with β cells responsible for insulin secretion and α cells for glucagon secretion. In addition, the islets contain δ cells, which release somatostatin, ϵ cells producing ghrelin, and pancreatic polypeptide (PP) cells, thereby establishing a complex paracrine network essential for coordinated islet function and systemic glucose regulation⁸⁻¹⁰. Islet architecture indeed is designed to promote paracrine interaction: insulin inhibits glucagon release, somatostatin broadly suppresses both insulin and glucagon, and α cells, by producing glucagon, are not passive components; rather, they actively contribute to intra-islet regulation through secretion of molecules that mediate paracrine crosstalk¹¹. Proper islet function relies on extensive intercellular communication, where numerous paracrine signals, combined with extrinsic neural inputs, coordinate and tune cellular responses to glucose and other metabolites⁹⁻¹². This intra-islet crosstalk is essential for maintaining glucose homeostasis and becomes dysregulated in diabetes. The tissue organization of the islets of Langerhans vary from species to species. In rodents, the inner core is formed by β cells (80%) with α cells and δ cells distributed surrounding the core of the islet (Fig. 1.1A, C); instead, in humans about 60-70% are β cells mixed with a smaller, but conspicuous, portion of α cells (20-30%) and the other remaining cells^{13,14} (Fig. 1.1B, C). Beyond their structural organization, the function of pancreatic islets is largely determined by the counterbalancing actions of insulin and glucagon¹⁵. Insulin, a well-characterized anabolic hormone, lowers blood glucose levels after meals by promoting

glucose uptake and storage. In contrast, glucagon serves as a catabolic hormone, released during hypoglycaemia to mobilize glucose through hepatic glycogenolysis and gluconeogenesis^{16–18}. Thus, post-prandial increase in glucose triggers β cells to secrete insulin and restore normoglycemia, whereas declining glucose levels stimulate α cells to release glucagon, ensuring a continuous supply of glucose between meals¹⁹. The synergic action of both hormones is necessary to properly maintain the right glucose concentration in the bloodstream.

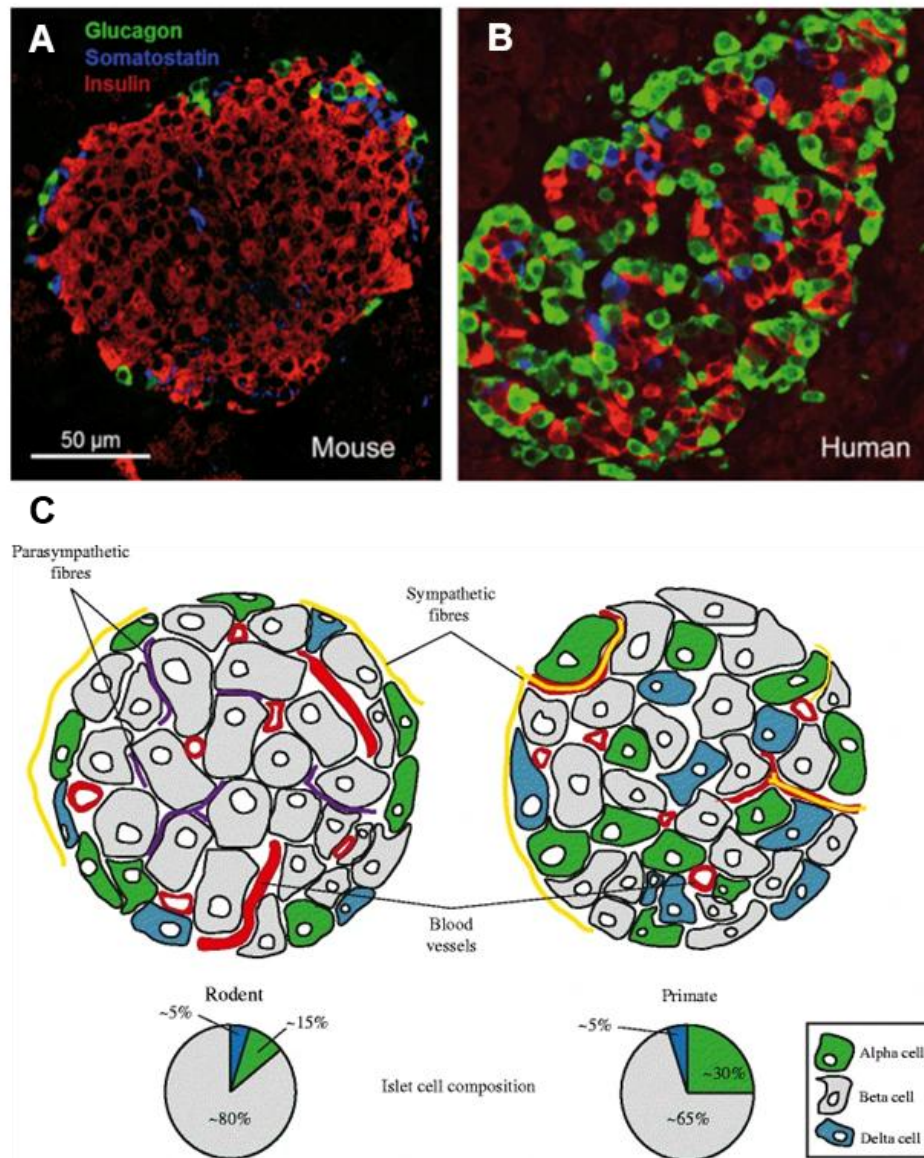


Figure 1.1: Differences in pancreatic islet architecture between rodents and primates

(A) Distribution of endocrine cells in mouse pancreatic islets, showing a core rich in β cells (insulin, red) surrounded by α cells (glucagon, green) and δ cells (somatostatin, blue) mainly located at the periphery. (B) In human islet, endocrine cells are more heterogeneously distributed, with α , β and δ cells intermingled throughout the islet. (C) Schematic representation and relative proportions of the different endocrine cell types in rodent and primate islets. Adapted from:^{13,14}

1.1 Hormones synthesis and maturation

1.1.1 Insulin

Insulin is a 51-amino acid peptide hormone produced and secreted by β cells. Its synthesis begins in the endoplasmic reticulum (ER) as the precursor molecule pre-proinsulin^{20,21}. Pre-proinsulin is converted into proinsulin, through removal of the signal peptide. Inside the ER lumen, proinsulin attains its proper conformation by folding and forming disulfide bonds that connect the A and B chains. Subsequently it is transported to the trans-Golgi network (TGN) where it is packaged into immature secretory granules^{22,23}. Within the secretory granules, specific prohormone convertase (PC) enzymes cleave the C-peptide from proinsulin, generating insulin monomers (Fig. 1.2A). These monomers are further assembled into hexamers coordinated by zinc ions, whose concentration progressively rises during the maturation process through the action of specialized zinc transporters generating the mature form of insulin inside the insulin secretory granules (ISGs)^{22,24,25}. Beyond their structural role, zinc ions also act as extracellular signalling molecules with autocrine and paracrine effects on neighbouring cells once secreted. Proinsulin is sorted into immature secretory granules after it exits the TGN. Although entry-based sorting into ISGs is the primary mechanism directing cargo from the TGN into immature granules, it is not completely specific (Fig. 1.2B). To ensure fidelity, additional quality-control steps take place during maturation. These involve sorting receptors and ion transporters, which help retain the appropriate cargo within the granules while allowing misrouted proteins to be removed through exit pathways²². During maturation, other crucial ions are accumulated, most notably calcium [Ca^{2+}], leading to high luminal calcium concentrations. Regarding this, it is believed that Ca^{2+} ATPases and related ion pumps actively maintain these gradients counteracting passive leak and diffusion^{22,26}. In parallel, proinsulin packaged in budding granules aggregate in a process facilitated by soluble and membrane-associated proteins as well as by lipids such as cholesterol²¹. Acidification is another key event: lowering the luminal granules pH is essential both for proper sorting and for the activation of PC enzymes^{21,22,27}. This acidification process is achieved by the vesicular proton pump V-ATPase, which drive H^+ uptake thus promoting the cleavage of the C-peptide from proinsulin to generate mature insulin (Fig. 1.2B)^{22,27}. Once the proinsulin is processed, the C-peptide fragment remain inside the secretory granules and it is released with the rest of the ISG content, acting as bioactive peptide^{20,22,23}.

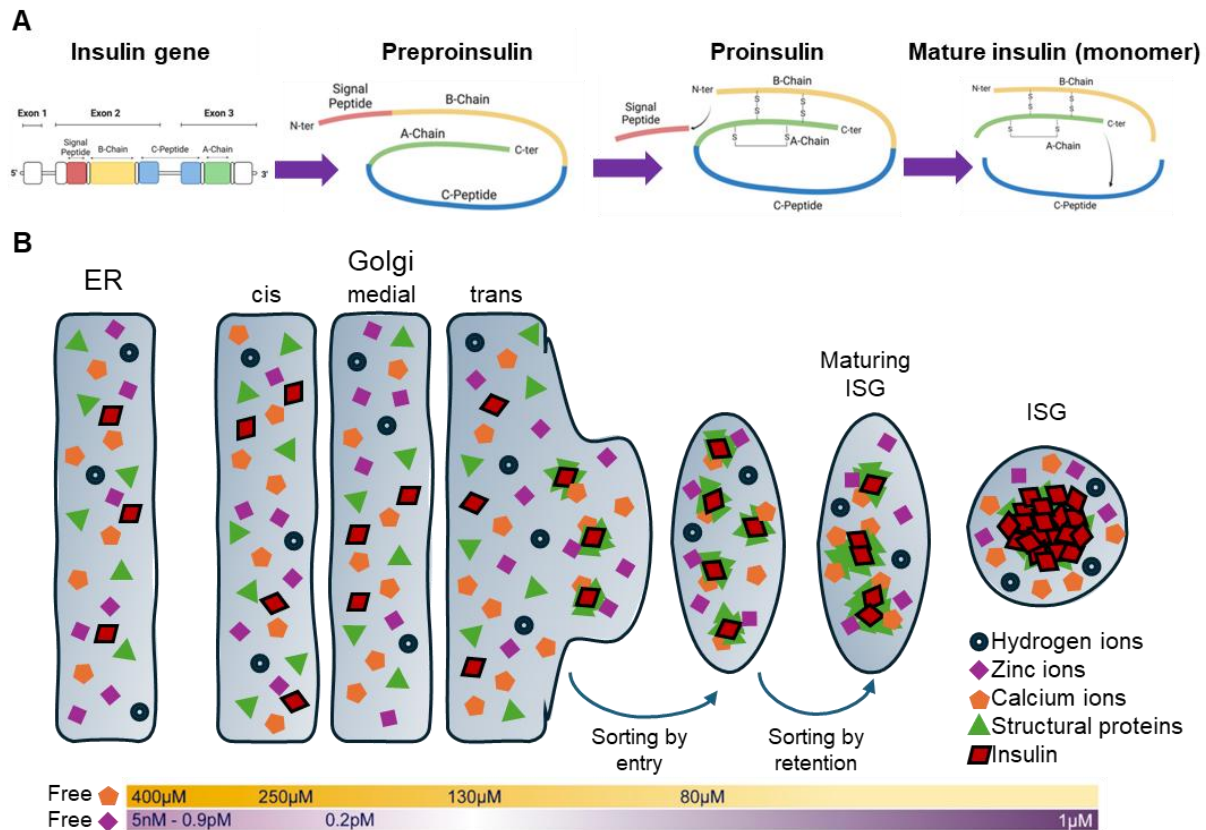


Figure 1.2: Overview of insulin biosynthesis, processing, and granule maturation

(A) Schematic representation of insulin biosynthesis. The insulin gene is transcribed and translated into pre-proinsulin, which enters the rough endoplasmic reticulum (ER), where the signal peptide is cleaved to produce proinsulin. Within the ER lumen, proinsulin folds and forms disulphide bonds linking the A- and B-chains. Subsequent cleavage of the C-peptide in secretory granules yields mature insulin monomers. **(B)** Insulin trafficking and granule maturation. Proinsulin synthesized in the ER is transported through the Golgi apparatus to the TGN, where vesicles destined for secretion bud off as immature secretory granules. Sorting occurs through Ca^{2+} - and H^{+} -dependent aggregation of granule proteins. Non-granular proteins may transiently enter ISGs but are later removed via receptor-mediated “sorting by exit.” Concurrently, uptake of Zn^{2+} , Ca^{2+} , and H^{+} drives protein condensation and retention within granules. During maturation, free Ca^{2+} levels decrease, while granules accumulate 50-100 mM bound Ca^{2+} and 20-30 mM Zn^{2+} bound to luminal proteins. Adapted from:^{22,159}.

1.1.2 Glucagon

As mentioned above, glucagon is a 29-amino acid peptide hormone secreted by α cells and stored within glucagon secretory granules (GSGs)^{28–30}. The biogenesis of GSGs follows a secretory pathway similar to that of ISGs. Proglucagon is synthesized as a protein precursor in the ER after translation, then transported to the Golgi apparatus reaching the TGN²⁸. The precursor proglucagon, composed by glicentin and major proglucagon fragment (MPGF), is expressed also in intestinal enteroendocrine L cells and in certain neurons, where it undergoes differential processing to generate distinct peptide hormones (Fig. 1.3)³⁰. In pancreatic α cells, proglucagon maturation occurs through the action of specific processing enzymes of the PC family, with cleavage producing glucagon peptide and other peptide products, namely GRPP (glicentin-related pancreatic peptide), intervening peptide-1 (IP-1), and the MPGF (Fig. 1.3)³¹. In detail, to produce mature glucagon, inside α cells, the cleavage operated by prohormone convertase 2 (PC2) of glicentin is essential. As for insulin granules, the sorting of proglucagon into GSGs is crucial for generating functional glucagon. Regarding the maturation and the precise timing of proglucagon cleavage in the pancreatic islets, different models have been proposed^{28,32,33}. The canonical model suggests that proglucagon is sorted into the granules and subsequently processed by both PC2 and PC1/3 (prohormone convertase 1/3) to glucagon and GLP-1 (glucagon-like peptide 1), respectively. This model is based on the evidence that significant amounts of GLP-1 are produced in healthy human α cells³⁴. An alternative model proposes that proglucagon is first cleaved into glicentin and major proglucagon fragment before sorting, with glicentin then encapsulated inside GSGs, and further processed to glucagon, while the remaining fragments are not packaged. Importantly, proglucagon processing profile changes in diabetes with a significant increase in GLP-1 production observed both in human and rodent islets²⁸. Once generated, glucagon undergoes structural stabilization inside the GSGs, where it is stored at high concentration in an acidic environment, a process supported by granule acidification mechanisms that ensure proper peptide condensation and stability³⁵.

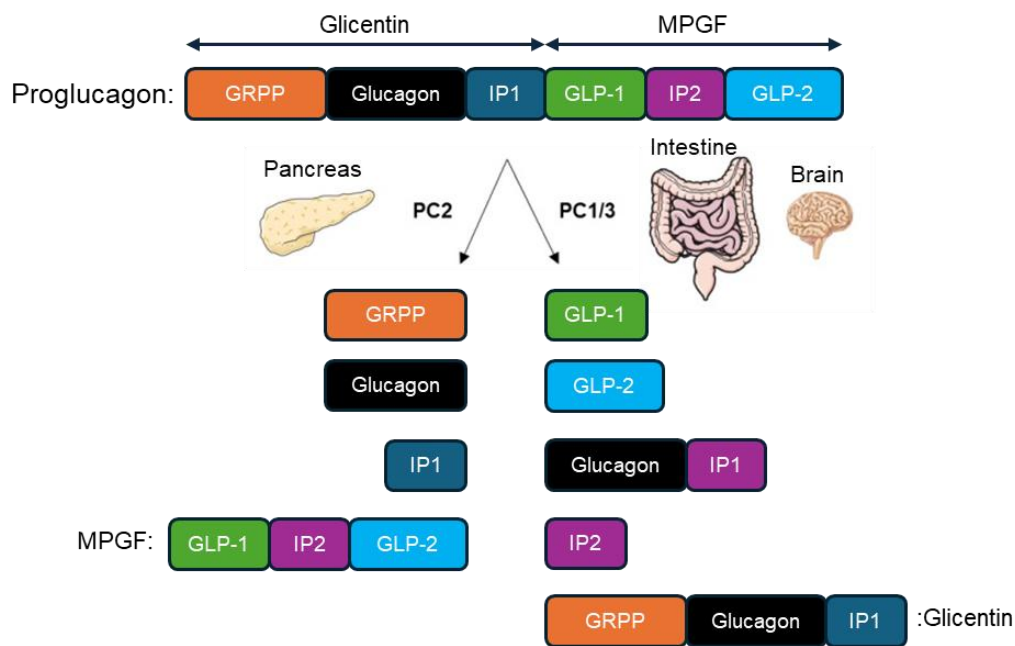


Figure 1.3: Tissue-specific processing of proglucagon into glucagon and related peptides

Schematic representation of proglucagon processing in different tissues. In pancreatic α cells, proglucagon is cleaved by prohormone convertase 2 (PC2) to generate glucagon, glicentin-related pancreatic polypeptide (GRPP), intervening peptide 1 (IP1), and the major proglucagon fragment (MPGF). In contrast, in intestinal L cells and in the brain, prohormone convertase 1/3 (PC1/3) mediates cleavage to produce glucagon-like peptide 1 (GLP-1), glucagon-like peptide 2 (GLP-2), oxyntomodulin, intervening peptide 2 (IP2), and glicentin. Adapted from:^{30,160}.

1.2 Pancreatic hormone secretion from β and α cells

1.2.1 Insulin secretion

During post-prandial state, the increasing glucose in bloodstream triggers insulin secretion in the form of insulin granules release³⁶. Insulin secretion in response to a step increase in glucose concentration occurs in a biphasic manner composed of a transient first phase, lasting only a few minutes, followed by a prolonged, sustained second phase related to the inner organization of ISGs and sustained by the newly synthesized granules (Fig. 1.4A). Inside the β cells, ISGs are arranged into two distinguishable population: a reserve pool, which comprises most of the granules and whose intracellular transport is largely mediated by microtubule-associated motors (i.e. stop-and-go motion or bidirectional) and a readily releasable pool (RRP) which represents a subset of secretory vesicles that are already docked at the plasma membrane and primed with a fully assembled exocytotic machinery. Their availability is regulated by cytoskeletal components, including the cortical actin network, which influences docking and access to release sites^{21,37}. Each pool has specific release kinetics, defining the biphasic insulin secretion. Once glucose is sensed by the β cells, a rapid release of insulin granules takes place, leading to the depletion of the RRP^{38–40}. Subsequently, a slower and sustained phase of secretion arises from the mobilization of distal reserve granules along microtubules from inner cytoplasmic regions to the cell membrane (Fig. 1.4B)^{37,41,42}. The secretion of the two distinct pools of granules is regulated by two pathways: the triggering pathway, which is Ca^{2+} -dependent and initiates the first phase, and the amplifying pathway, which is driven by metabolic and incretin signals sustaining the second phase. These mechanisms are not completely independent but rather interdependent, as both require Ca^{2+} . The amplifying pathway enhances the efficacy of the exocytotic machinery without further raising intracellular Ca^{2+} . At the molecular level, the process begins with glucose uptake into β cells through facilitated diffusion mediated by glucose transporters (GLUTs) localized on the cell membrane which are able to sense and internalize glucose from the bloodstream⁴³. Regarding GLUTs, a clear species-specific difference exists between rodents and humans. In rodent β cells, glucose uptake is primarily mediated by GLUT2. In contrast, the situation in human β cells is less straightforward: GLUT1 appears to be the predominant glucose transporter, GLUT3 is also appreciably expressed, while GLUT2 is present only at very low levels^{44,45}. Once inside the cells, glucose is converted into pyruvate through glycolysis and then further metabolized to generate ATP (adenosine triphosphate) via the Krebs cycle (also known as tricarboxylic acid cycle, TCA cycle). The raise in ATP/ADP ratio closes ATP-sensitive potassium channels (K_{ATP} -channels) triggering membrane depolarization and the opening of voltage-dependent Ca^{2+} channels^{21,37}. The resulting Ca^{2+} influx initiates the rapid release of RRP granules in the so-called “triggering phase”, which covers

approximately the first 10 minutes circa of the secretion. Moreover, SNARE proteins (e.g. syntaxins, VAMP2, SNAP-25) are key to the final fusion step. The assembly/disassembly dynamics of SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) complexes is regulated by cytoskeletal rearrangements and GTPase signalling⁴⁰. The majority of ISGs fusion events proceed via complete vesicle fusion at the cell membrane to ensure the release of insulin⁴⁶. To sustain the subsequent phase of insulin release, additional stimuli are involved such as GLP-1, glucagon, GIP (gastric inhibitory polypeptide). Moreover, during the second phase, granules from the reserve pool fuse directly without passing through the RRP, following an exocytotic route distinct from that of the first phase⁴⁷. This “amplifying pathway” is mediated by incretin receptors and cAMP (cyclic adenosine monophosphate), which enhance the effectiveness of Ca^{2+} on the exocytosis machinery, thereby supporting secretion over prolonged periods³⁶. Rho-family GTPases (especially Cdc42 and Rac1) are implicated in triggering actin remodelling in response to glucose stimulation. These rearrangements are necessary for enabling second-phase granule recruitment⁴⁰.

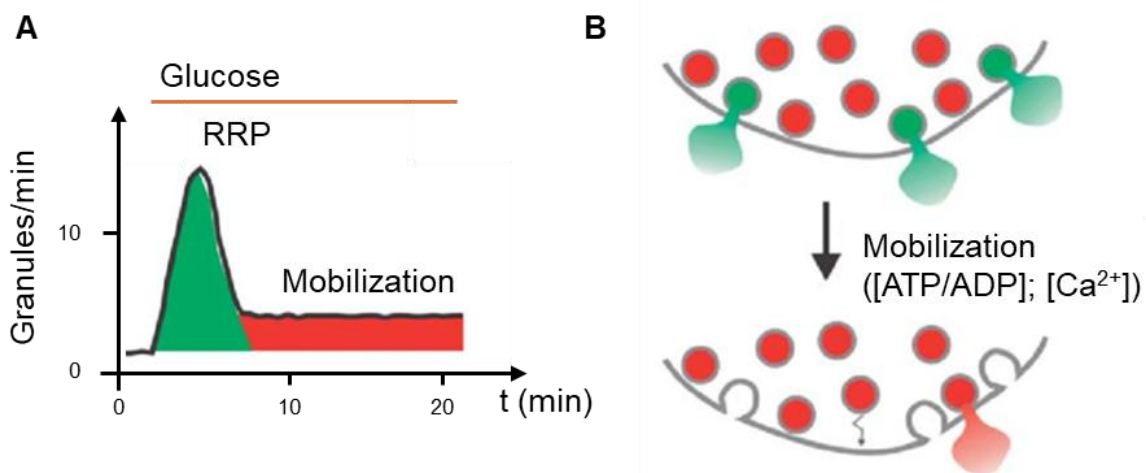


Figure 1.4: Secretory granule pools underlying the biphasic pattern of insulin release

(A) Schematic representation of the biphasic insulin secretion induced by glucose stimulation. The initial rapid spike corresponds to the RRP discharge, followed by a sustained second phase supported by granule mobilization from the reserve pool. **(B)** β cells contain two main pools of secretory granules: a readily releasable pool (RRP, <5%), responsible for the first phase of insulin secretion, and a much larger reserve pool (>95%) that must be mobilized to sustain release. Adapted from:³⁷.

1.2.2 Glucagon secretion

In contrast to β cells, which are activated by rising glucose levels, α cells are intrinsically active at low glucose concentrations and secrete glucagon under these conditions thus maintaining glucose homeostasis by stimulating hepatic glycogenolysis and gluconeogenesis. When glucose levels rise, their activity is suppressed, providing a counter-regulatory balance to insulin secretion. In addition to glucose levels, glucagon secretion from α cells is strongly stimulated by elevated amino acids and free circulating fatty acids and is further modulated by paracrine signals from neighbouring β and δ cells, as well as by autonomic inputs^{10–12,30}. At the cellular level, α cells sense glucose primarily via the transporter GLUT1 and associated metabolic pathways that modulate ATP-sensitive K^+ channels, thereby shaping electrical activity, leading to Ca^{2+} influx through voltage-gated channels and subsequent exocytosis of glucagon granules. Although this pathway resembles that of β cells, the outcome is inverted, with high glucose leading to suppression rather than stimulation of secretion^{35,48}. In details, at high glucose concentrations, K_{ATP} channel activity leads to membrane depolarization beyond the threshold for Na^+ and Ca^{2+} channel activation. This inactivation of voltage-gated channels reduces Ca^{2+} and Na^+ influx, thus suppressing glucagon exocytosis and limiting its release into the circulation^{30,35}. In α cells, the K_{ATP} current is already small at low glucose, keeping the membrane slightly depolarized and allowing low-threshold Na^+ and Ca^{2+} channels to drive action potential firing and glucagon secretion. In contrast, when glucose levels rise, further closure of K_{ATP} channels causes excessive depolarization, inactivating these low-threshold channels. This prevents action potential generation, reduces Ca^{2+} entry, lowers cytosolic Ca^{2+} concentration, and ultimately suppresses glucagon release^{49,50}. Importantly, glucagon release is tightly regulated by inhibitory factors such as insulin, somatostatin, and incretin hormones, which suppress secretion under hyperglycaemic conditions. This finely tuned balance ensures rapid adaptation of glucagon output to metabolic demand. Glucagon secretion into the systemic circulation guarantees the right blood glucose level during hypoglycaemia, by stimulating gluconeogenesis, and glycogenolysis while inhibiting glycolysis and glycogenesis⁵¹. The action of glucagon seems to be evanescent on hepatic glucose production, with a rapid release peak followed by a plateau^{52,53}. The necessary balancing between insulin and glucagon has been known since the last century (1921)⁵⁴ but during the years most of the effort was done to study insulin because of its ability to mitigate the symptoms in diabetic patients⁵⁵. Despite the crucial importance of insulin, a growing interest is rising to understand the role of glucagon in both physiological and pathological contexts. Understanding the intracellular dynamics of these granules is essential for deciphering the regulatory mechanisms that maintain glucose homeostasis and their dysfunction in metabolic diseases such as diabetes^{37,56–58}.

1.3 Secretory granules dynamics of principal pancreatic islet cells in relationship with cytoskeletal components

Granule trafficking and overall motility are orchestrated by cytoskeletal elements, with microtubules (MTs) and actin filaments (F-actin) providing the principal tracks for motor-driven transport. Rather than serving merely as passive pathways, both cytoskeletal systems actively contribute to the regulation of both insulin and glucagon granule dynamics. MTs provide tracks for long range motion and support granules delivery^{59–62}. In contrast, F-actin is thought to be predominantly involved in short range movements beneath the membrane, facilitating granule docking and promoting fusion through the SNARE complex^{21,40}. More in detail, the transport of secretory granules to the plasma membrane depends on long-range movement driven by kinesin-1 molecular motors along MTs, carrying the granules from their site of synthesis in the TGN to the cell periphery^{61,63}. Once at the periphery, granules are transferred to myosin Va motors, which facilitate their positioning and retention within the actin-rich cortical region^{64,65}.

1.3.1 Insulin secretory granules trafficking

In the context of dual secretion pulses of ISGs, F-actin is thought to play a predominant role in the primary phase, regulating secretion from the RRP and mediating short-range movements beneath the plasma membrane, thereby coordinating the docking and release processes (Fig. 1.5A)²¹. By contrast, MTs support the secondary phase of secretion by providing tracks for the long-range transport of both the reserve pool and newly synthesized granules (Fig. 1.5A)^{59–61}. Notably, the granules motility and their relationship with the cytoskeleton is associated to granular age⁶⁶. The shared general idea is that alteration or absence of MTs can lead to decreasing in secretion and inhibiting the transport, probably due to the random meshwork organization^{67,68}. MTs presence guarantees correct localization of granules and their distribution balancing their delivery and removal^{67,68}. When MTs are disrupted, high-glucose stimulation causes granules to build up at the cell periphery, likely because of enhanced glucose-driven priming or docking. In contrast, intact microtubules appear to limit this peripheral crowding, implying that microtubule-mediated transport helps control granule positioning even when the overall movement of granules, is largely random and non-directional⁶⁷. Based on this idea, MTs in β cells, have been hypothesized to play a dual role as negative regulators of insulin secretion following glucose stimulation: first, by actively transporting granules away from the plasma membrane; and second, by exerting forces through MT-associated motors which hinder the docking on the plasma membrane which is the first step of exocytosis^{67–69}. Another perspective on the role of MTs in β cells is not only to drive secretion but rather to restrict the number of insulin granules

positioned for release upon stimulation. The balance of granules at the cell periphery is maintained through their continual removal via non-directional transport along the MT network, a process controlled by glucose-dependent MT destabilization and Golgi-based MT nucleation (Fig. 1.5B)⁶⁸. When this regulatory mechanism is lost, as in nocodazole (NCZ)-treated cells, granules accumulate excessively at the plasma membrane, resulting in a dysregulation in motion and in slowing down the overall motility^{61,68}. NCZ is used to induce tubulin monomers to detach from microtubule structures. Without the pulling force generated by MT-motor activity, granules remain long enough at the cell periphery to become tethered and docked. Microtubules alteration confirmed the kinesin-based ISGs transport⁷⁰. Consequently, partial destabilization of MTs (rather than complete disruption) appears to be a critical regulatory step in glucose-induced insulin secretion, allowing for fine-tuned control of the process. In this framework, granule tethering and docking are viewed as active, glucose-dependent processes that set the pool of RRP of granules, requiring a defined pause during which a granule stays near the docking machinery before fusion can occur⁶⁸. During insulin secretion, glucose-induced destabilization of MTs is counteracted by the generation of new MTs at the Golgi, a mechanism that likely acts to restrain excessive insulin release. The random diffusive motion of ISGs is essential for their directed transport and exocytotic recruitment⁷¹. Regarding ISGs dynamics, a noteworthy study by Tabei and colleagues offers a new interpretation, demonstrating that their motion follows a subordinated random walk rather than simple Brownian diffusion. Their approach was based on stochastic modelling and SPT, in which the authors integrate features of continuous-time random walks and fractional Brownian motion, capturing both transient confinement and correlated motion. This hybrid model convincingly explains the observed anomalous diffusion linking it to the complex cytoskeletal environment⁷². However, the approach remains largely phenomenological, as the molecular origins of “caging” and dwell-time distributions are not fully defined. Furthermore, granule heterogeneity, limited tracking durations, and the use of MIN6 cells may constrain biological interpretation. Despite these limitations, the study offers a powerful conceptual framework that connects the stochastic nature of granule motion to the regulation of insulin secretion and provides a foundation for integrating biochemical parameters, such as granule maturation or acidification, into future dynamic models⁷². Another study was made by Yi et al. which applied total internal reflection fluorescence (TIRF) microscopy to study ISGs dynamics and classified the granules into 5 distinct categories based on their motion: immobile, nearly immobile, run-pause, sub-diffusive, and super-diffusive. Moreover, they sorted ISGs according to their age and interaction with the cytoskeleton. To properly evaluate the relationship with the cytoskeletal components (i.e. microtubules and actin filaments), Yi and colleagues treated β cells (MIN6) with NCZ. Since ISGs transport predominantly depends on MTs through kinesin motors, NCZ treatment suppressed insulin trafficking and led to

granule accumulation in MTs clusters, thereby increasing the immobile population⁷⁰. The authors also employed cytochalasin D (CytoD) to disrupt actin filaments network, resulting in an increasing of super-diffusive granules at the cell periphery, while granule dynamics in the inner cytoplasm remained largely unaffected. Taken together, these observations indicated that the actin network acts as a constrain on granule transport. Upon actin disruption, a clear shift in granule dynamics was observed: the proportions of immobile, nearly immobile, and sub-diffusive subpopulations decreased, while the fraction of super-diffusive granules increased markedly. Overall, Yi et al. concluded that actin bundling, multi-motor dynamics, and its interplay with microtubules collectively enhance active transport behaviour⁷⁰ as previously reported^{73–75}. Under NCZ treatment, ISGs transport increases in actin-rich regions compared to actin-deficient ones, whereas CytoD treatment tended to inhibit transport⁷⁰. Actin disruption experiments, reported in the literature, indicate that the role of the actin filament network remain debated. Actin has been shown to restrain granules under basal conditions by forming a dense meshwork and facilitate secretion by transitioning from F-actin to G-actin during stimulation, thereby loosening this barrier⁶¹. However, what emerged is that actin filaments are not merely passive structural elements, but they also exert active effects on granule transport⁷⁰. In this regard, Polino and colleges in 2023 examined how pro-secretory stimuli such as glucose and/or KCl affect the actin filaments meshwork in β cells. They found that stimulation with glucose and/or KCl caused minimal changes in the cortical actin layer, challenging the idea that a major remodelling is required for insulin secretion⁷⁶. Their observations argue against simple models in which the cortical actin network acts as a passive barrier to granule access. Instead, actin remodelling (or its modulation) may influence insulin secretion via subtler, more localized, or biochemical mechanisms that do not require actin reorganization. More recently, Li and colleagues, reached a different conclusion using a multimodal imaging approach to obtain a structural view of actin filament reorganization during glucose-stimulated insulin secretion. They observed that, upon glucose stimulation, actin filaments anchored at the ventral membrane reorganize from a dense “net-like” pattern into a more open “blooming” architecture, facilitating the transport of ISGs from the cell interior toward the plasma membrane. While the majority of actin filaments remain bundled to maintain cellular stability, a fraction of the network relaxes into a less compact arrangement that confers greater structural flexibility. Within this remodelled architecture, a subset of filaments (about 12%) reorients quasi-orthogonally to the ventral membrane, forming angles of 45-90°, which may provide directional tracks to guide granule movements⁶⁰.

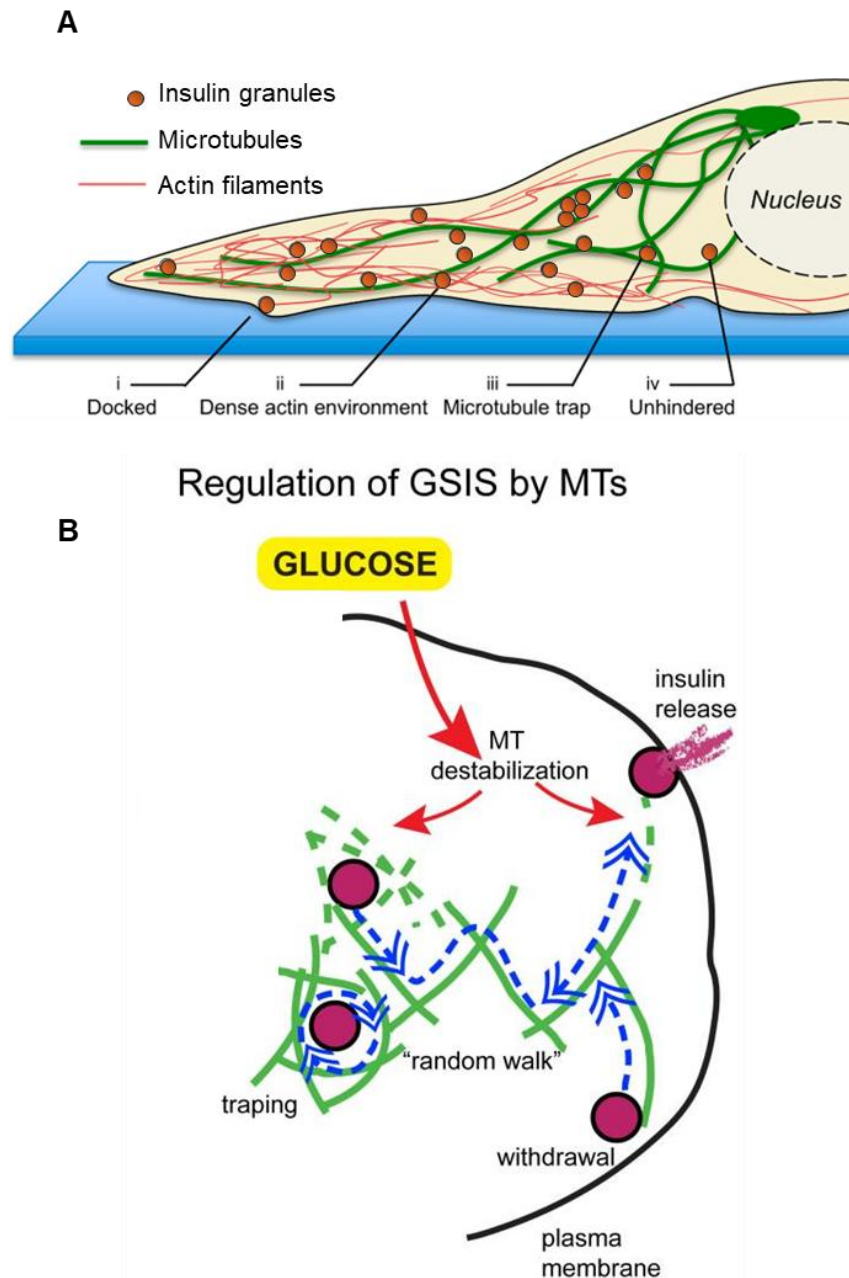


Figure 1.5: Cytoskeletal organization and regulation of insulin granule dynamics in β cells

(A) Schematic vertical cross-section of a β cell, illustrating how cytoskeletal organization shapes insulin granule dynamics. Microtubules (green) extend throughout the cytoplasm, while actin filaments (red) form a dense cortical network near the plasma membrane. This structural heterogeneity generates distinct intracellular environments that influence insulin granule (orange dots) transport, ranging from docking and restricted diffusion to microtubule-dependent transport and unhindered movement. **(B)** Upon glucose stimulation, MTs undergo both nucleation and destabilization. In the cell centre MTs destabilization reduces granule trapping, while peripheral MTs destabilization limits granule withdrawal and enhances free diffusion, promoting docking and exocytosis at the cell membrane. Simultaneously, increased MTs nucleation at the Golgi compensates for peripheral destabilization, achieving a finely tuned “rheostat”-like regulation of granule availability for secretion. Adapted from:^{68,70}.

1.3.2 Glucagon secretory granules trafficking

ISGs structure and trafficking inside β cells have been quite well characterized by high-resolution imaging techniques such as spatiotemporal fluorescence correlation spectroscopy (FCS)⁷⁷, electron microscopy (EM)^{66,78}, single-particle tracking (SPT)^{61,71}, and TIRF microscopy^{46,47,66,68}. In pancreatic α cells, the intracellular trafficking of GSGs is thought to involve both the microtubule and actin cytoskeleton, though relatively few studies have dissected the molecular details as fully as for insulin granules in β cells. MTs provide long-distance transport routes, assisting in the movement of granules from their site of synthesis (e.g. near the Golgi) toward the cell periphery. Regarding the interactions between the cytoskeleton and GSGs, Yokawa and co-workers measured reduced GSGs velocity upon NCZ-induced microtubule depolymerization in α TC6 cells by spinning-disk microscopy⁷⁹. Few data are available on α cells which point to F-actin depolymerization as a pro-secretory stimulus^{80,81}. The limited understanding of GSGs dynamics must be attributed to both the historical focus on β cells (due to their central role in diabetes) as well as to the limited availability of α cell models and suitable live cell labelling techniques for GSGs. Actin has been found to act as a negative regulator of glucagon secretion in α cells, in part by restraining exocytosis: in the study on TMEM55A/PI5P signalling made by Liu and colleagues, F-actin depolymerization was shown to enhance glucagon exocytosis by freeing up GSGs for release⁸¹. Thus, in α cells, microtubules enable long-range or directed transport of glucagon granules, while actin filaments (especially the cortical or sub-membrane actin network) appear to impose constraints under resting conditions, modulating the readiness of granules to dock and undergo exocytosis. Moreover, EphA/ephrin-A signalling acting through RhoA has been shown to increase cortical F-actin density and alter glucagon secretion in α cells, suggesting that remodelling of actin filaments is part of the glucagon release mechanism⁸⁰.

1.4 Motivation of this work

The preceding review of the literature highlights significant and distinct knowledge gaps in our understanding of pancreatic hormones biology, which this thesis aims to address.

As discussed in the introduction, there is a stark disparity in knowledge between insulin and glucagon. As detailed in Section 1.3.2, the intracellular trafficking of GSGs remains comparatively opaque with relatively few studies, standing in sharp contrast to the extensively characterized dynamics of ISGs, even though it is still debated. This gap is largely attributed to a historical focus on β cells and a lack of robust labelling techniques for α cells.

While the literature clearly establishes that granule acidification is an essential step for the maturation of both hormones (as discussed in sections 1.1.1 and 1.1.2), the direct measurement and real-time dynamics of this process remain poorly characterized. The mechanisms governing luminal pH, its stability, and its potential fluctuations at the single-granule level are not fully understood.

This thesis project confronts both gaps through a systematic biophysical approach.

To address the gap of GSG dynamics, this work establishes a comparative framework using α TC1-9 cells as an α cell model and INS-1E cells as a β cell reference, exploiting the ability of the fluorescent Zn^{2+} chelator, ZIGIR, as a novel tool to label both granule types. By employing a suite of spatiotemporal techniques (i.e. iMSD and SPT), I characterized granule mobility under basal conditions. Crucially, I then investigated the response to cytoskeletal perturbations. This is directly motivated by the conflicting reports on the role of actin and microtubules in ISG trafficking (section 1.3.1) and the lack of data for GSGs (section 1.3.2). This comparison aims to define shared versus distinct mechanisms of cytoskeletal regulation.

To address the pH dynamics gap, this thesis moves beyond static assumptions to quantify granular pH in living cells. By combining a ratiometric sensor with advanced microscopy, namely phasor-based Fluorescence Lifetime Imaging Microscopy (FLIM) and Orbital Tracking (OT), I achieved calibrated pH measurements. This approach yielded the first measurements of glucagon granules pH and, critically, enabled the investigation of pH fluctuations down to the millisecond timescale. This provides a new window into the real-time activity of the acidification machinery (like the V-aTPase mentioned in 1.1.1) and lays the groundwork for a detailed analysis of the acidification process itself.

2. Spatiotemporal analysis of granule dynamics

Over the past decades, numerous studies have employed high-resolution and live-cell fluorescence methods such as TIRF, confocal, super-resolution, correlated MSD or spatiotemporal correlation spectroscopy to map the trajectories, fusion modes, mobility states, docking, and the effect of the cytoskeleton on insulin secretory granules in response to stimuli such as glucose or GLP-1^{61,66,70,71,77,82,83}. In contrast, less is known about how glucagon granules behave in α cells, how they diffuse, dock or undergo directed transport. Moreover, the dynamics of glucagon granules have often been inferred from β cell models, assuming comparable compartmentalization and behaviour^{28,84}. This underscores the need for a proper characterization. To fill this gap, I employed a spatiotemporal fluorescence imaging to investigate GSGs dynamics and to compare their mobility patterns with those of ISGs. α TC1-9 cells were employed as α cell model^{85,86} and INS-1E⁸⁷ and MIN6 cells⁸⁸ as β cell references. Throughout the text, α TC1-9 cells will be referred to as α cells and INS-1E cells as β cells; use of MIN6 cells (limited to Section 3.4) is explicitly indicated. α TC1-9 cells are derived from mouse pancreatic adenoma while INS-1E cells are established from a rat pancreatic β cell insulinoma. MIN6 cells, derived from a mouse pancreatic β cell insulinoma, were employed only as a comparative control to exclude possible discrepancies due to species differences. Although both INS-1E and MIN6 are widely used β cell models, INS-1E cells have been reported to reproduce several key features of primary β cells more consistently⁸⁷. By contrast, MIN6 cells may express low levels of non- β islet hormone genes (e.g. glucagon, somatostatin, ghrelin) alongside insulin, which may affect their phenotypic specificity as β cell model⁸⁹. Overall, INS-1E provide a more specific, stable and physiologically relevant *in vitro* model of β cell function, while MIN6 offer a valuable complementary reference for mouse-derived systems. The use of established cell lines offers substantial advantages for dissecting cellular mechanisms: they provide stable and controllable systems, reduce the complexity associated with the morphological and functional heterogeneity of primary islets, and ensure high reproducibility across experiments^{90–92}. These features were essential for a robust characterization of glucagon granule dynamics, ultimately providing a reliable reference framework for comparing granule behaviour under different experimental conditions. Nevertheless, while the α TC1-9 and INS-1E cell lines serve as practical and reproducible models for studying α and β cell biology, they also present limitations compared to primary islet cells, including partial

glucose responsiveness, altered gene expression profiles, and the lack of intercellular paracrine interactions^{93,94}.

Here, I employed a two-step approach to investigate granule dynamics. First, I assessed the average dynamic properties of GSGs using a spatiotemporal fluorescence technique, directly comparing them with insulin granules. Subsequently, I utilized a single granule tracking classification method to examine how individual motility patterns change under different conditions, such as cytoskeletal alteration and secretory stimuli.

2.1 Labelling methods for granules

A crucial step in this study was to reliably label GSGs and ISGs, to track their dynamics with a sufficient signal-to-noise ratio. Traditionally, this task has been addressed using genetically encoded fluorescent reporters, which require the transfection of plasmids encoding granule-associated proteins fused to fluorescent proteins^{77,95,96}. Although well established and widely used, these methods are time-consuming, dependent on variable transfection efficiency, and may introduce unwanted cellular stress⁹⁷. Indeed, cationic lipids in lipofection formulations perturb membrane integrity and trigger the expression of heat shock proteins and stress-related pathways in transfected cells, acting as an insult to cell structure and function^{97,98}. To overcome such limitations, we explored a recently developed chemical probe, ZIGIR, a Zn^{2+} -chelating fluorescent dye that selectively labels zinc-rich secretory granules⁹⁹. GSGs and ISGs are believed to harbour very high Zn^{2+} concentrations relative to the surrounding cytosol, creating an ionic microenvironment distinct from the rest of the cell^{21,28}. ZIGIR is engineered to exploit precisely this differential: upon binding Zn^{2+} , it undergoes a fluorescence enhancement exceeding 100-fold, providing high-contrast visualization. Importantly, ZIGIR affinity to zinc is tuned (with a dissociation constant in the sub-micromolar range) to avoid activation in compartments with lower Zn^{2+} levels, thus favouring specificity for the granules over other cellular organelles or the cytosol. Moreover, ZIGIR is pH-insensitive across physiologically relevant ranges (pH 5-9) and intrinsically membrane-permeable, facilitating rapid labelling of dense granules within living cells⁹⁹. In the following, we compare conventional transfection-based labelling technique with ZIGIR staining, highlighting the advantages in terms of both protocol simplicity and labelling efficiency. As genetically encoded biosensors, we tested NPY-EGFP¹⁰⁰ to label GSGs in α cells and C-pep-EGFP¹⁰¹ to labelled ISGs in β cells. Transfection-based methods depend on plasmid uptake and expression of the fluorescent protein and therefore require at least 24 hours before imaging can be performed (Fig. 2.1A, B). In contrast, ZIGIR staining only involves a simple administration of the dye followed by a short incubation period of approximately 15 minutes, after which granules are immediately visible for live imaging (Fig. 2.1C). Beyond timing-related differences, the overall

labelling efficiency differed substantially between the two methods. With ZIGIR, virtually all cells incorporated the fluorescent probe, resulting in uniform staining across the population. In contrast, transfection produced labelling only in a small subset of cells, expressing the fluorescent fusion proteins. Both approaches resulted in the expected staining pattern, characterized by dot-like signals concentrated predominantly near the plasma membrane. Notably, ZIGIR and NPY-EGFP signals in α cells overlapped in punctate structures throughout the cytoplasm. In addition, sparse NPY-EGFP signal was detected, likely corresponding to fully matured NPY-EGFP proteins within the Golgi network (Fig. 2.2A, B). Since the labelling of ISGs had already been characterized within our group⁷⁷, I focused my attention on the detailed characterization of GSGs. To confirm that the labelled vesicles indeed represent GSGs, we performed anti-glucagon immunofluorescence on NPY-EGFP-transfected α cells (Fig. 2.2C, D and Materials & Methods for further details about immunostaining) and evaluated the co-localization between the NPY-EGFP signal and the glucagon antibody staining. Co-localization was quantified using Pearson's correlation coefficient (0.42 for NPY-EGFP and ZIGIR; 0.53 for NPY-EGFP and immunostaining anti-glucagon) calculated with the BIOP JACoP plugin for ImageJ after applying the Otsu method for thresholding. The measured coefficients confirmed co-localization of the signals, supporting the identification of the labelled vesicles as glucagon-containing granules. It was not possible to perform a similar co-localization analysis between ZIGIR and anti-glucagon antibody staining, since ZIGIR does not retain its fluorescence after fixation.

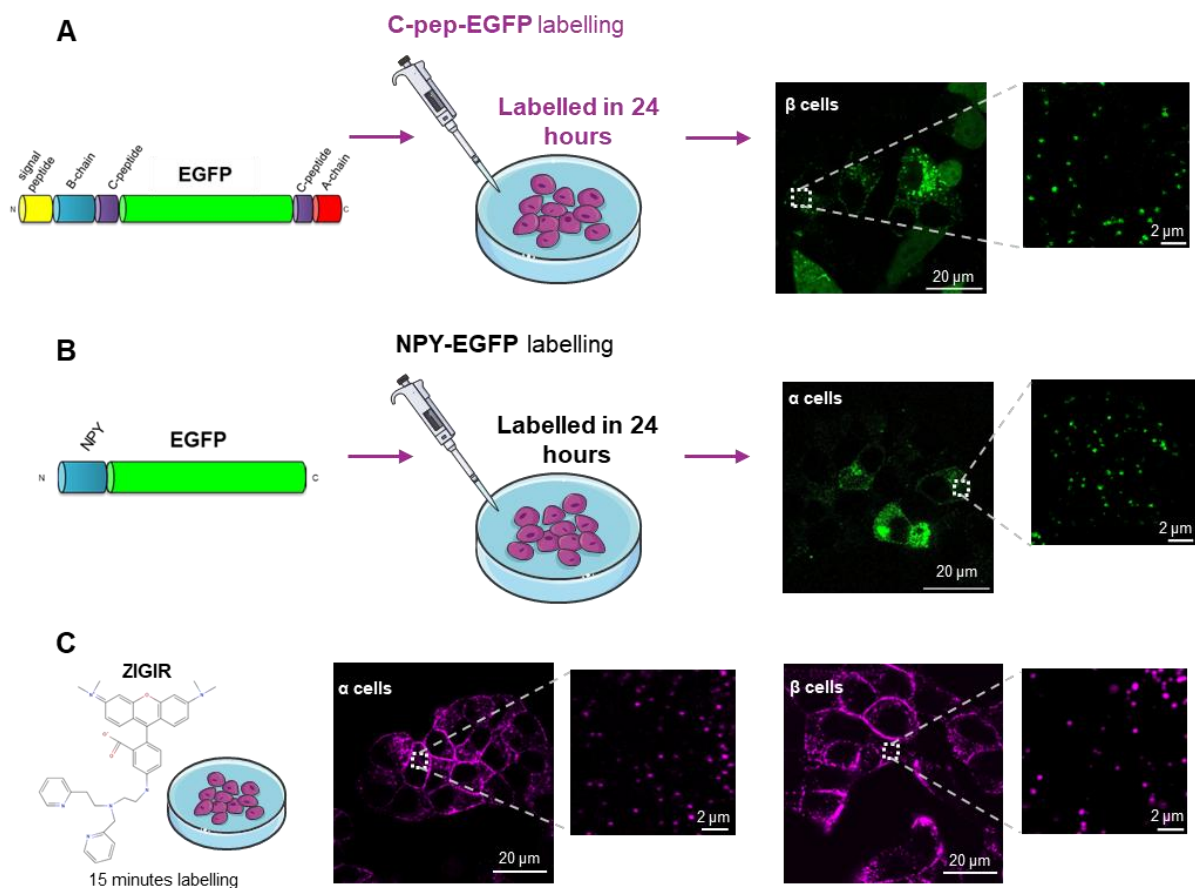


Figure 2.1: Granules labelling strategies employed in this thesis

(A) ISGs in β cells labelled with C-pep-EGFP following conventional transfection (B) GSGs in α cells labelled with NPY-EGFP via transfection (C) ZIGIR labelling performed by single dose administration enabling visualization of both α cells and β cells. Servier Medical Art (<https://smart.servier.com/>) was used to create part of this figure.

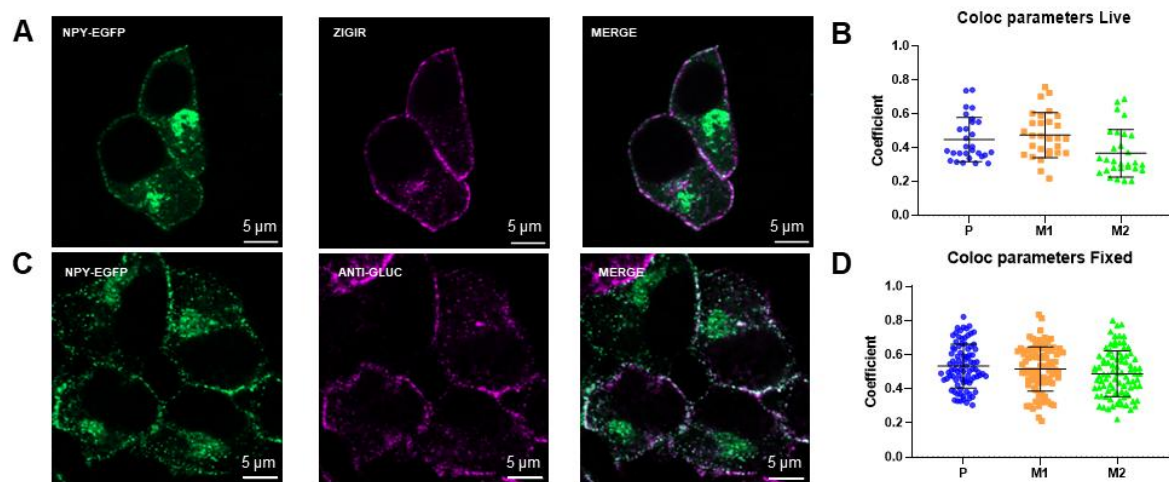


Figure 2.2: Studying glucagon granules colocalization with different labelling methods in a cells

(A) Colocalization between ZIGIR and NPY-EGFP highlights differences in labelling specificity. NPY-EGFP shows partial overlap with ZIGIR but also labels additional structures, likely corresponding to the Golgi apparatus, as visible in the merged image. (B) Quantitative colocalization analysis represented as a scatter plot (mean with 5–95% percentiles) shows an average Pearson’s coefficient (P) of approximately 0.42, calculated using the BIOP JACoP plugin. (C) Colocalization between NPY–EGFP and anti-glucagon immunostaining confirms the specificity of the labelling for glucagon-containing granules. (D) Corresponding colocalization parameters shown as a scatter plot (mean with 5–95% percentiles) indicate an average Pearson’s coefficient (P) of approximately 0.53, computed using the BIOP JACoP plugin.

2.2 Spatiotemporal image correlation spectroscopy to study granules dynamics

Having established reliable labelling strategies for GSGs and ISGs, we next applied quantitative imaging approaches to characterize their spatiotemporal dynamics. We employed the iMSD technique to extract population-averaged parameters of granule mobility. iMSD is a powerful technique based on Spatio-Temporal Image Correlation Spectroscopy (STICS) that transforms time-lapse image stacks into a series of images which represent the spatial and temporal evolution of correlation, thereby extracting dynamic and structural information. The method involves rapid imaging of a region of interest, such as within a cell, over time to track the motion of objects across frames. The technique then correlates successive images, and for each delay interval, the spatial correlation function $G(\xi, \chi, \tau)$ is averaged. As the labelled objects diffuse, the correlation peak broadens between frames¹⁰². A custom MATLAB (MathWorks Inc., Natick, MA) script was adapted to calculate the spatiotemporal correlation function using Fast Fourier methods. The spatiotemporal correlation function is in the form of (Eq. 1):

$$G(\xi, \chi, \tau) = \frac{[I(x, y, t) \cdot I(x + \xi, y + \chi, \tau + t)]}{[I(x, y, t)]^2} - 1 \quad (\text{Eq. 1})$$

Distance between pixels is indicated by ξ, χ in x and y directions, respectively. The time lag is indicated by τ , and the fluorescence intensity is $I(x, y, t)$ defined in a certain spot and time. The numerator of the fraction is the average over spatial and time variables^{77,102}.

$$G(\xi, \chi, \tau) = G_0 + G_1(\tau) e^{\left\{ -\frac{(\xi - v_\xi \tau)^2 + (\chi - v_\chi \tau)^2}{\sigma^2(\tau)} \right\}} \quad (\text{Eq. 2})$$

The function $G(\xi, \chi, \tau)$ (Eq. 2) can be approximated with a Gaussian, where the numerator of the fraction in the exponential quantifies the net movement of particles in a given direction, expressed through their average velocity. The variance ($\sigma^2(\tau)$) (Eq. 3) depicts the mean square displacement of labelled object derived from images^{77,102,103}.

$$\sigma^2(\tau) = \sigma_0^2 + \kappa \tau^\alpha \quad (\text{Eq. 3})$$

where σ_0^2 is an intercept value related to the width of the PSF (point spread function) and the average particle size¹⁰². α in the formula represent the Alpha anomalous coefficient which identify the type of motion. Thus, the analysis of function $G(\xi, \chi, \tau)$ (Eq. 2) performed by iMSD allows us to directly

extract displacement data (intracellular dynamics) without processing individual particle trajectories¹⁰³. The peak of correlation function is centred in the origin where the correlation top is reached if the particle dynamics is governed by diffusion⁷⁷.

The graphical representation of iMSD vs time provides key insight into organelles motion (Alpha anomalous coefficient), diffusion coefficient (D_m), and average organelle apparent size (σ^2 or the y-interceptor of the iMSD plot, offset) (Fig. 2.3). However, it's crucial to note that insufficient temporal resolution can lead to an overestimation of granule size, as granule motion during imaging may not be captured accurately. This effect is detectable by analysing the iMSD offset. The graph resulting from the iMSD script defines the motion law of granules based on three parameters, namely:

- Alpha anomalous coefficient (Alpha anomalous): a diffusion coefficient motion derived from the MSD curve which indicates the type of movement. Values > 1 corresponds to super diffusion, typically associated with active, directed, or guided transport (drifted motion), where the MSD increases faster than linearly with time; values < 1 correspond to sub-diffusion, reflecting hindered or constrained dynamics often caused by molecular crowding or structural barriers, where the MSD grows sub-linearly with time; and for values ≈ 1 the motion follows Brownian diffusion, in which particles undergo random, thermally driven displacements and the MSD increases linearly with time.
- Diffusion coefficient (D_m) represents the effective diffusivity of the tracked granules, quantifying the average rate of their displacement. In the intracellular environment, D_m captures the combined effects of free diffusion, cytoplasmic viscosity, crowding, and transient interactions with cellular structures, thus serving as a robust descriptor of granule mobility across different conditions.
- Offset (σ^2) represents the average apparent size of the organelles under study. While σ^2 does not directly provide the physical granule diameter, it offers a useful comparative metric across experimental conditions, enabling detection of relative changes in apparent granule size. The offset increase with the granular dimension because it corresponds to the squared waist of the spatial correlation function at zero lag time (σ_0^2) and this value contains contributions from both the squared width of the PSF and the squared average particle size^{77,102,103}.

Noteworthy, in the case where Alpha anomalous coefficient is less than 1, a confined motion can be inferred, and the following equation can be used to fit $\sigma^2(\tau)$ (Eq. 4)¹⁰²:

$$\sigma^2(\tau) = 4D_M\tau + \frac{L^2}{3} \left(1 - e^{\left\{-\frac{\tau}{\tau_c}\right\}} \right) + \sigma_0^2 \quad (\text{Eq. 4})$$

Confinement area is defined by L as linear size, and how fast confinement happened is defined by τ_c . In large time scale, D_M (Eq. 5) is the diffusivity and represents $\frac{1}{4}$ of the derivative of σ^2 for $\tau \rightarrow \infty$. D_m represents the diffusivity inside confined areas, and it is calculated by this relationship with τ_c ^{77,103}:

$$D_m = D_M + \frac{L^2}{12\tau_c} \quad (\text{Eq. 5})$$

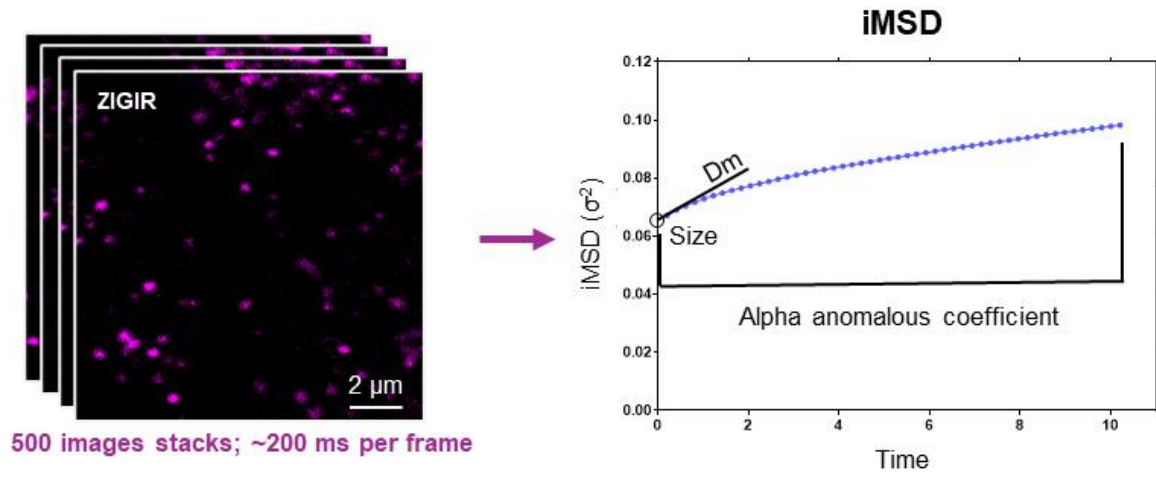


Figure 2.3: iMSD explanatory set up

Confocal image stacks (500 frames, ~200 ms per frame) of labelled secretory granules were analysed using a custom-made iMSD script to extract average dynamic and structural parameters. The resulting iMSD plots report the alpha anomalous coefficient, the effective diffusion coefficient (D_m), and the apparent granule size (offset) obtained from the iMSD fits.

2.2.1 iMSD as a tool to assess the dynamics of insulin and glucagon granules

To perform iMSD analysis, we acquired 500 confocal images of ZIGIR-labelled α and β cells with a temporal resolution of about 200 ms. As in depth described in the previous chapter and Fig. 2.3, iMSD, provides information about granule diffusivity D_m (*D micro*, for confined motion), motion modality (Alpha anomalous coefficient) and average size (via the offset, which is the y axis intercept). Comparing α cells and β cells using iMSD and ZIGIR as granules marker, some remarks can be made:

- ZIGIR proved to be a reliable marker for Zinc-rich organelles, showing consistency in the dynamic properties of control condition with canonical transfection, such as C-pep-EGFP in β cells (Fig. 2.4A)⁷⁷ as well as NPY-EGFP for GSGs in α cells (Fig. 2.4B)¹⁰⁰. In α cells, both labelling methods revealed granules predominantly exhibiting sub-diffusive motion, with anomalous diffusion exponents close to 1, indicative of near-Brownian behaviour. No significant differences were observed between the two conditions, although a few outliers were detected in both datasets (Fig. 2.4A, B). Similarly, in β cells, the comparison between ZIGIR and C-pep-EGFP labelling showed no detectable differences across any of the dynamic parameters evaluated⁷⁷. Consistently, no significant differences emerged in the offset parameter, which reflects apparent granule size, further supporting the comparability of the two labelling approaches for reporting granule dimensions.
- The overall behaviour of these granules is prevalently diffusive in confined areas even though differences in alpha anomalous coefficient might be attributed to a basal inner granules mobilization which seemed to be higher for GSGs compared to ISGs (Fig. 2.5A, B). An alpha anomalous coefficient under 1, generally points to motion that is mostly diffusive but not perfectly Brownian. Such sub-ideal diffusion likely arises from the complex intracellular environment, where barriers like membranes, densely packed organelles, and the cytoskeletal network subtly impede particle movement, resulting in a mildly constrained, sub-diffusive dynamic¹⁰⁴.
- Analysis of offset values revealed a difference between GSGs and ISGs, with the latter exhibiting larger values than glucagon granules (Fig. 2.5C). This indicates that ISGs are larger than GSGs, in agreement with previous reports in the literature^{50,78,105,106}.

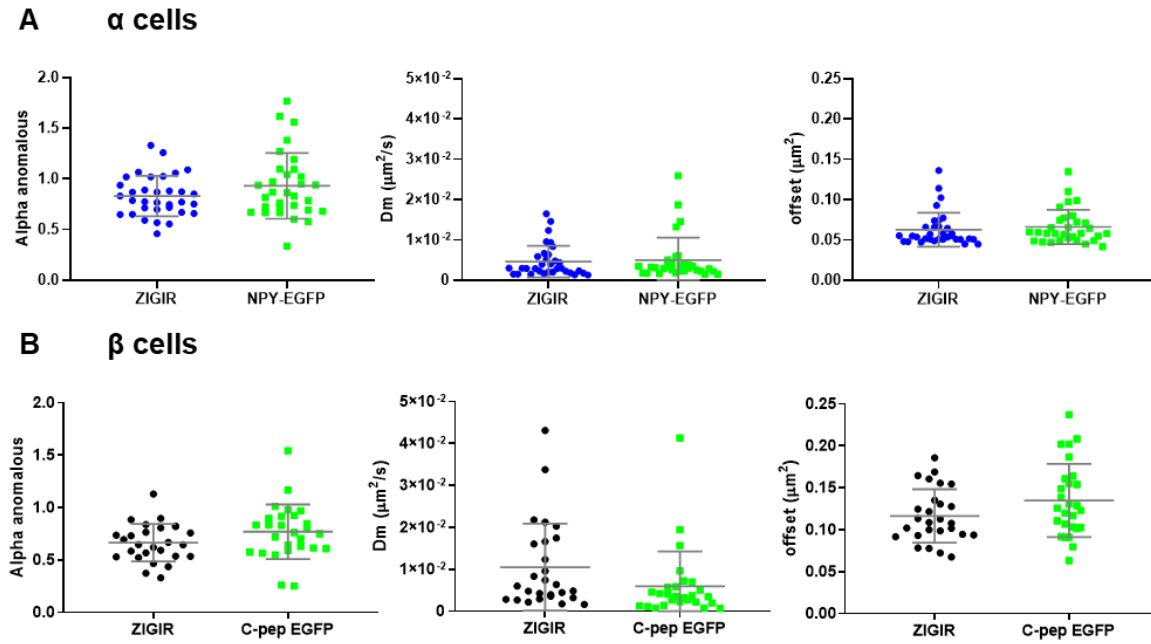


Figure 2.4: iMSD analysis of ISGs and GSGs comparing different labelling approaches (i. e. ZIGIR, NPY-EGFP, and C-pep-EGFP)

(A) Comparison between ZIGIR and NPY-EGFP labelling in α cells. Both labelling strategies revealed predominantly sub-diffusive motion, with alpha anomalous values approaching 1, indicative of near-Brownian behaviour. No significant differences were detected in the diffusion law parameters, although a few outliers were observed in both cases. (B) Comparison between ZIGIR vs. C-pep-EGFP labelling in β cells. All evaluated parameters showed no significant differences between the two labelling approaches. Data normality was assessed using the Shapiro-Wilk test, followed by t-tests for normally distributed data and Kolmogorov-Smirnov tests for non-normally distributed data. The offset parameter, representing the apparent granule size, also showed no significant variation, confirming that both labelling techniques yield comparable granule size estimates.

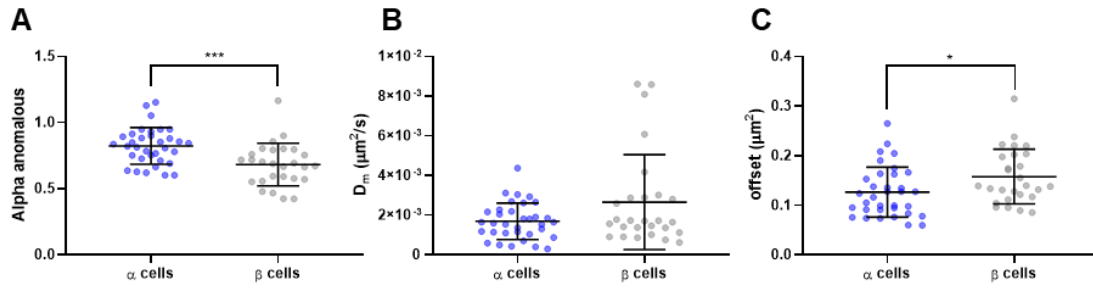


Figure 2.5: iMSD comparison between α cells and β cells under basal conditions

(A-B-C) Comparison of iMSD parameters between α cells ($N = 34$) and β cells ($N = 27$) both labelled with ZIGIR. We checked normality and performed t test for the normally distributed data and Mann-Whitney nonparametric test for the non-normally distributed data. **(A)** Both granule populations exhibited sub-diffusive motion, although GSGs in α cells displayed a higher anomalous coefficient close to 1 (Brownian motion) compared to ISG in β cells. Alpha anomalous coefficient for α cells = 0.82 ± 0.14 and for β cells = 0.64 ± 0.14 (Mean $\pm$ St. Dev.). **(B)** The diffusion coefficients (D_m) showed no significant differences between the two cell types, despite the presence of some outliers particularly among β cells. α cells $D_m = 1.5 \times 10^{-3} \pm 0.9 \times 10^{-3} \mu m^2/s$ and β cells $D_m = 2.3 \times 10^{-3} \pm 1.9 \times 10^{-3} \mu m^2/s$ (Mean $\pm$ St. Dev.). **(C)** A significant difference emerged in the apparent size parameter, indicating that GSGs are, on average, smaller approximately 0.8-fold the size of ISGs.

2.3 STORM to measure granules size

It is noteworthy that, in iMSD analysis, the apparent granule size may be overestimated due to the PSF width and/or suboptimal temporal sampling¹⁰⁷. To further address this point, we measured the size of GSGs and ISGs by Stochastic Optical Reconstruction Microscopy (STORM).

STORM relies on precisely localizing individual fluorophores to determine the centroid of their emission signals. In each acquired frame, only a small subset of fluorophores is activated and localized. By sequentially collecting many such frames and compiling all localization events, a high-resolution reconstruction of the sample is generated, surpassing the diffraction limit¹⁰⁸. To make this process possible, fluorophores must be switched between non-fluorescent and fluorescent states. Generally, two fluorophores conjugated onto an antibody are employed: one serves as activator, and the other as the reporter dye. The final image arises from the precise localization of the reporter dye, which generates the fluorescence signal upon excitation triggered by the activator¹⁰⁹. To allow sequential detection of single molecules, the reporter dye is kept in a non-fluorescent state most of the time by means of a specialized imaging buffer, while stochastically switching to the fluorescent state for imaging. Once the activation laser stimulation randomly activates a group of molecules, the activator dyes induce fluorescence into the neighbouring reporter dyes. This process is repeated until enough localizations are collected to reconstruct the image^{108,110}. The efficiency depends on two factors: the laser intensity and the ratio between reporter and activator dyes. During acquisition, the activation laser controls the number of switched on fluorophores, while the imaging laser both excites fluorescence and drives activated molecules back into their non-active state¹⁰⁸. Commonly STORM applications are based on wide field TIRF systems, in which laser beam is directed onto the sample through a peculiar objective so that the emitted light exits at an oblique angle. When the angle of the laser beam is tuned properly, total internal reflection occurs at the interface between the sample and the coverslip, restricting the imaging region to a very thin zone near the surface of the sample. A common localization method simplify the PSF as a Gaussian in which only the central point is recorded as the centre of the fluorophore. Employing a TIRF-based STORM method maximizes the signal to noise ratio and is convenient to perform, even though TIRF is not a prerequisite for STORM¹⁰⁸. On the negative side, STORM needs quite intense image processing, such as fluorophore localization and identification, which demands sufficiently isolated and non-overlapping signals. Moreover, generating a STORM image requires repeating a single activation frame across tens of thousands of sequential imaging frames making the process time-consuming. Due to this, sample drift or instability in temperature, immersion oil, or other factors may happen. Noteworthy, the spatial resolution is determined by the localization precision, probe size and labelling density^{111,112}.

Heilemann and colleagues in 2008, proposed photo switchable fluorescent dyes able to alternate dark and fluorescent states simply upon irradiation with different wavelengths. This method, called direct STORM (dSTORM), does not require to couple special fluorophores as in conventional STORM^{109,113}. Nowadays, dSTORM is widely applied and it is the method used in this thesis. To collect the STORM images, we employed a Nikon N-STORM TIRF microscope, with a 100× oil immersion objective. A total of 20,000 frames were recorded under TIRF illumination at a digital resolution of 256×256 pixels, corresponding to a pixel size of 158.7 nm. Data acquisition involved 5,000 cycles, each composed of one activation frame using a 405 nm laser, followed by three readout frames excited with a 647 nm laser (for other details see Material & Methods). Our size measurements confirmed the offset difference observed in the iMSD, validating that GSGs are smaller than ISGs. Using dSTORM we obtained a value of 127.6 ± 18.9 nm for GSGs and 163.1 ± 21.4 nm for ISGs (Mean $\pm$ St. Dev.) (Fig. 2.6A-D).

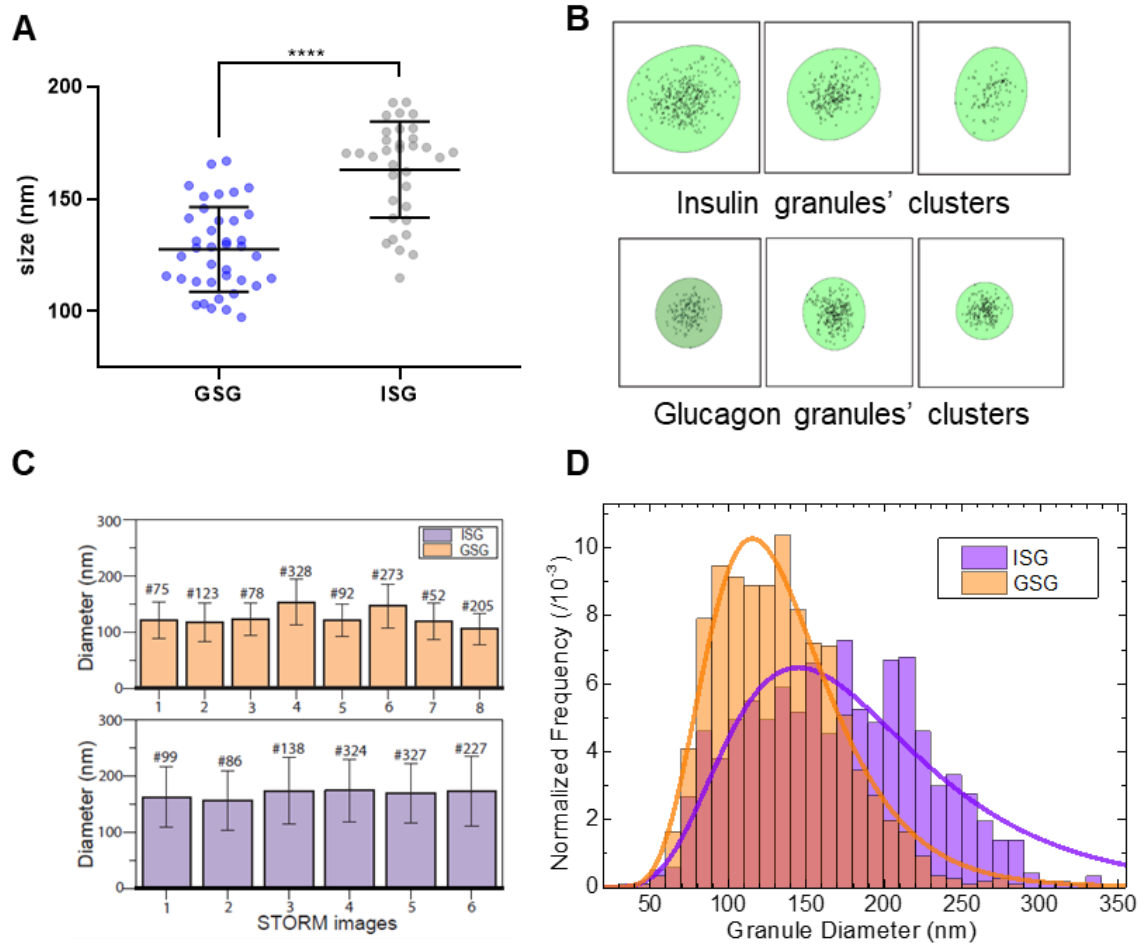


Figure 2.6: STORM imaging and quantitative analysis of ISG and GSG diameter

(A) STORM super-resolution imaging confirmed size difference between ISGs and GSGs, showing average diameters of 127.6 ± 18.9 nm and 163.1 ± 21.4 nm, respectively (Mean $\pm$ St. Dev.). (B) Representative ISGs and GSGs immunostained with an anti-insulin antibody (Alexa Fluor 488 secondary antibody) and a glucagon antibody (Alexa Fluor 647 secondary antibody), respectively. Green shading indicates the granules, which are composed of localized molecules (black dots) identified through DBSCAN clustering. (C) Average diameters of GSGs (orange) and ISGs (violet) are shown as Mean $\pm$ St. Dev., calculated from each STORM acquisition. The numbers above the bars indicate the count of granules identified as clusters of labelled molecules. (D) Histograms showing the distribution of ISG (violet) and GSG (orange) diameters. Overlapping regions are shown with transparency for clarity. All histograms are normalized to an area of 1, and solid lines represent LogNormal fits for each distribution.

2.4 Concluding remarks

In this chapter, we validated robust labeling strategies for studying granule dynamics in living α and β cells. The primary objective was to compare conventional transfection-based methods with the Zn^{2+} -chelating probe ZIGIR⁹⁹, certifying its efficacy as a versatile marker. To achieve this, we implemented a two-tiered validation approach: one functional, one structural. We performed a functional validation using iMSD analysis. This allowed us to extract average dynamic parameters from large granule populations and confirm that the labelling itself did not significantly alter the expected confined and diffusive motion, demonstrating the suitability of these strategies for live-cell studies. This analysis also revealed that the observed variations in the Alpha anomalous coefficient were more pronounced in GSGs, suggesting a higher basal internal activity for this granule type. Then, we conducted a structural validation using super-resolution dSTORM imaging. This technique provided nanometric precision, confirming the morphological features of the labelled granules and, crucially, validating the known size difference between GSGs and ISGs (with the former being smaller on average). Although dSTORM cannot resolve the low-electron-density halo typically observed in EM, the measured dimensions are consistent with pre-existing EM data for the secretory granule dense core^{78,106,114}. This dual strategy not only allowed us to benchmark ZIGIR as a reliable marker for dense-core granules in viable cells but also established a rigorous framework for evaluating new labeling protocols. However, it is important to note that the iMSD algorithm inevitably averages the inherent heterogeneity of granule dynamics. By analyzing all granules collectively, this method masks individual variations between immobile and mobile populations. To overcome this limitation and obtain a more detailed view, the next chapter complements this population-level analysis with Single Particle Tracking (SPT) to extract granule-specific dynamic features.

3. Single Particle Tracking as a powerful tool to investigate and classify granular motion

SPT, first introduced in the mid-1980s for studying biological specimens^{115,116}, has become a widely applied technique to investigate intracellular transport processes. By reconstructing the trajectories of individual fluorescently labelled objects, SPT allows for the classification of motion modes, the identification of transitions between dynamic states, and the distinction of subpopulations with different motility patterns. This approach provides direct access to motility parameters and dynamic heterogeneity with high spatiotemporal resolution, serving as a powerful complement to population-average methods like iMSD. In this thesis, the SPT technique was employed to resolve the motion of individual secretory granules within α and β cells, overcoming the limitations of an averaged analysis to gain a more detailed view of their individual behaviours.

3.1 Technical implementation and data analysis

For the SPT analysis, TrackMate^{117,118}, an open-source ImageJ plugin, was utilized. The analysis was performed on the same sequences of 500 confocal images acquired for iMSD, maintaining a temporal resolution of approximately 200 ms.

The workflow followed two main steps^{117,119}:

1. Granule Identification: Individual fluorescent granules were located using a Laplacian of Gaussian (LoG) detector. Key detector settings included an estimated object diameter of 0.3 μm , a dataset-specific quality threshold, a median filter for preprocessing, and sub-pixel localization.
2. Trajectory Reconstruction: trajectories were generated using an algorithm based on the Linear Assignment Problem (LAP), which was configured to accommodate gap-closing events (temporary signal absences) but not track merging or splitting¹²⁰.

The specific parameters for tracking were set as follows:

- Maximum frame-to-frame linking distance: 0.5 μm .
- Maximum gap-closing distance: 0.5 μm .
- Maximum frame gap: 3.

The raw data exported from TrackMate was subsequently processed using custom MATLAB scripts for trajectory classification (Tab. 1 for parameters). For the analysis, only trajectories spanning more than five frames were considered.

3.2 Trajectories Segmentation and Motion Classification

Once trajectories were reconstructed via the custom-made MATLAB script, their motion were classified into three categories: “blocked”, “diffusive” and “drifted”. All the trajectories were divided into sub-trajectories with a single mode of motion. The MATLAB-based SPT analysis proceeded in two sequential steps:

1. In the first step, a stop-and-go analysis based on a velocity threshold (v_t) was applied^{121–123}. The velocity of each granule was calculated using a sliding 5-frame window across the 500-frame image stack. Whenever necessary, trajectories were divided into sub-trajectories of at least 5 consecutive spots with velocities consistently above or below the threshold. Both complete trajectories and resulting sub-trajectories (collectively referred to as “(sub)trajectories”) were then classified into two groups: those with velocities above the threshold (v_t) were defined as drifted motion (“isdrifted” / go motion)^{103,104,120}, while those with velocities below v_t , often containing segments with different motion types, were considered mixed/stop trajectories. Empirical testing identified an optimal threshold of $v_t = 0.2 \mu\text{m/s}$, which provided the best separation between drifted trajectories and those exhibiting diffusive or confined behaviour, thereby emphasizing the distinction between fast/directed and slow/restricted motion.
2. In the second step, the mixed (sub)trajectories were further analysed using the MSS-TAD (moment scaling spectrum - transient arrest of diffusion) method^{104,119} to discriminate between blocked or slow segments from those that are diffusive or fast. Following previous works^{103,104}, MSD and higher-order displacement moments (1st-6th order) were computed up to a lag time defined as the maximum of either one-quarter of the trajectory length or five frames. If a trajectory exhibited “mixed” features, confinement zones were identified by evaluating the likelihood (L_c) that the local diffusivity was lower than the average. Instead of

applying fixed thresholds for L_c and confinement duration (t_c), a seed-and-threshold segmentation strategy was used¹²⁴ (<https://svi.nl/SeedAndThreshold>):

- Seed zone selection: applying an initial likelihood threshold $L_{c1} = 1.8$ and retaining only zones lasting more than $t_{c1} = 5$ frames (including first and last frames).
- Region growing: extending seed zones using a lower likelihood threshold $L_{c2} = 1.4$ and retaining segments longer than $t_{c2} = 7$ frames.

Drifted sub-trajectories identified in this second step were then combined with the faster drifted trajectories detected in the first step, resulting in a refined classification of blocked, diffusive, and drifted motion modes^{104,119}.

Final (sub)trajectories classification was defined as (Fig. 3.1A):

- Blocked: stationary or nearly immobile trajectories.
- Diffusive: exploratory motion, possibly within confinement. Here, we consider higher limits to discern “immobile” vs. “slow” vs. “fast” (sub)trajectories for diffusivity: 2×10^{-3} and $10^{-2} \mu\text{m}^2/\text{s}$ here (D_{block} and D_{lim} , respectively in Tab. 1).
- Drifted: fast, directional motion associated with active transport, corresponding to drifted trajectories identified in both analysis steps.

Results are expressed as the percentage of total trajectory length corresponding to segments classified as blocked, diffusive, or drifted. In other words, results from SPT classification are expressed in percentage which indicates how much of the total tracked length, granules spend in each type of motion.

SPT PARAMETERS FOR DETECTION AND CLASSIFICATION

TrackMate			
LoG estimated object diameter	0.3 μm	LAP allows gap-closing events	Yes
LAP allows splitting and merging events	No	Maximum frame-to-frame linking distance	0.5 μm
Maximum gap-closing distance	0.5 μm	Maximum frame gap	3 frames
SPT script: options for trajectories and stall parameters			
τ	0.204 or 0.198 s	Moving window	5 frames
v_t	0.2 $\mu\text{m/s}$	Initial likelihood threshold (L_{c1})	1.8
Initial resident time threshold (t_{c1})	5 frames	Secondary likelihood threshold (L_{c2})	1.4
Secondary resident time threshold (t_{c2})	7 frames		
SPT script: limits for TAD analysis			
D_block	$2 \cdot 10^{-3}$	γ_{block}	0.4
v_block	0.6	γ_{drift}	0.7
v_drift	1.5	Lin_min & lin_max	0.95 & 1.05
L_mnw	0.3	L_mxw	1.5
D_lin coefficient	2.58	γ_{lim}	0.5
D_lim	$1 \cdot 10^{-2}$	Lsurmin & Lsurmax	1.25 & 4.75

Table 1: Parameters of TrackMate for granules detection and SPT custom-made MATLAB for trajectories classification

3.3 Classification of α and β cells granules trajectories under basal conditions

The SPT script enabled the classification of both GSGs and ISGs under basal condition, defined as the standard cell culture condition without external alterations. In α cells, the SPT analysis of GSGs showed a predominance of diffusive trajectories ($57.6 \pm 10\%$), above blocked ($35.8 \pm 10.6\%$) and drifted ones ($6.6 \pm 3\%$, Fig. 3.1B, Tab.2). Similarly, ISGs were mainly diffusive ($64.3 \pm 7.7\%$), over blocked ($32.8 \pm 7.2\%$) but with lower propensity for directed motion ($2.9 \pm 1.3\%$) compared to GSGs (Fig. 3.1C, Tab.2). Although the iMSD is less sensitive, a discrepancy in the drifted category was also revealed by this method. Notably, the ISGs results are in line with previous studies which employed similar SPT analyses^{61,66}. In addition to trajectory classification, the SPT script provided us a diffusion-related parameter (γ)¹⁰⁴, formally analogous to the Alpha anomalous coefficient from iMSD, as well as the distribution of short-lag-time diffusion coefficient (D_{12}), which corresponds to D_m for both sub-trajectories and trajectories. As expected, the results reflected the dynamic properties already highlighted by iMSD. In α cells under basal conditions, the γ distribution exhibited a peak just below 0.5, indicating motion that is nearly Brownian but with a slight sub-diffusive tendency (Fig. 3.2A). In contrast, β cells under basal condition showed a peak shifted further below 0.5, indicating a more pronounced sub-diffusive behaviour (Fig. 3.2B). Overall, the γ analysis obtained from SPT is in good agreement with the iMSD-derived alpha anomalous coefficient. Moreover, the comparable distributions and peak positions of D_{12} values in α and β cells corroborate the absence of significant differences in the D_m population.

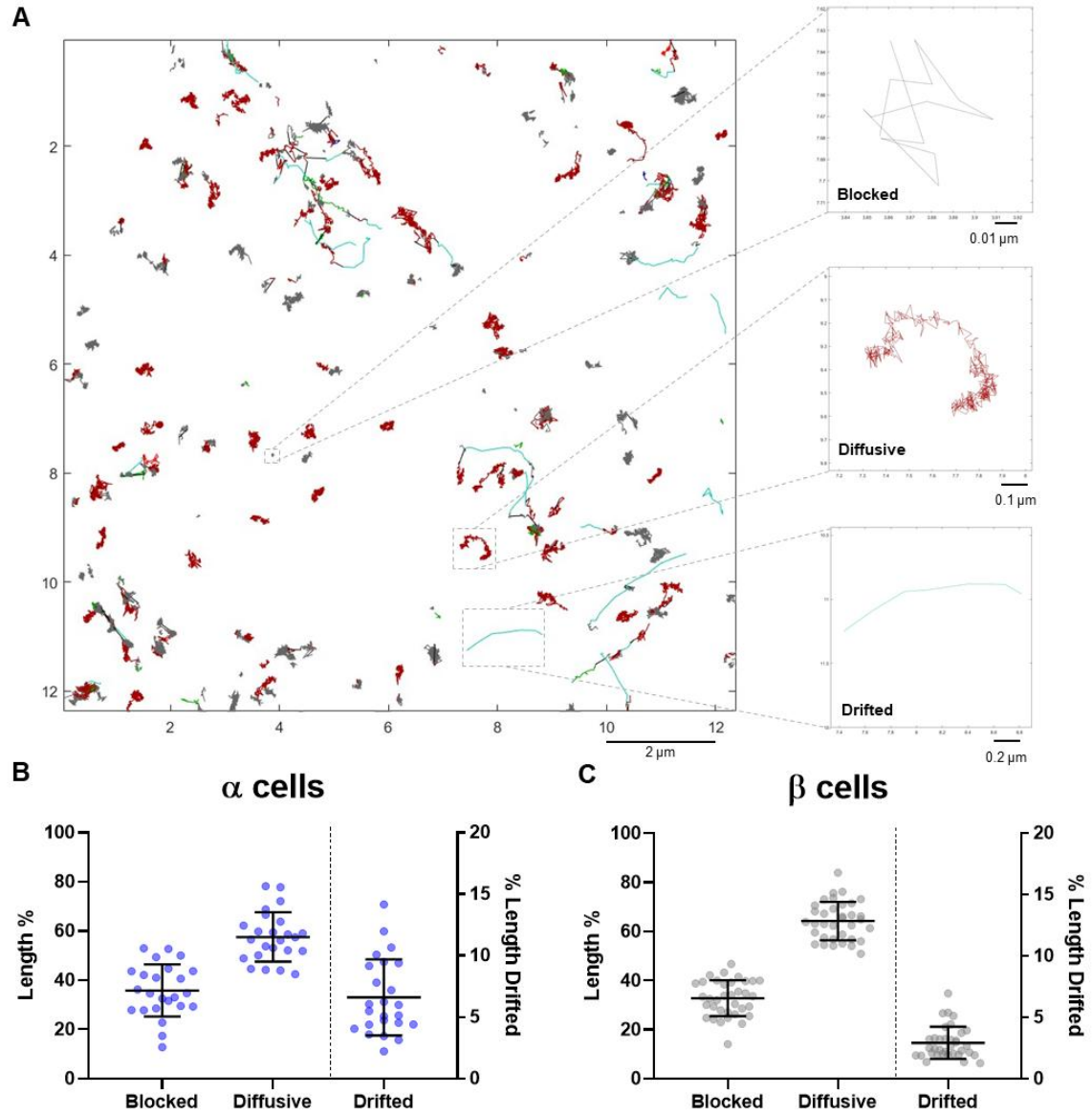


Figure 3.6: SPT analysis and trajectories classification for α cells and β cells under basal condition

(A) Representative output from the custom MATLAB script, showing trajectories classified as blocked, diffusive, or drifted. Example trajectories for each motion type are shown on the right. (B) Scatter plot (Mean $\pm$ St. Dev.; all points shown) summarizing trajectory classifications in α cells. The graph displays the percentage of trajectory length corresponding to each classification, with drifted length indicated on the right y-axis (% Length Drifted). (C) Scatter plot (Mean $\pm$ St. Dev.; all points shown) summarizing trajectory classifications in β cells, displayed as in panel (B).

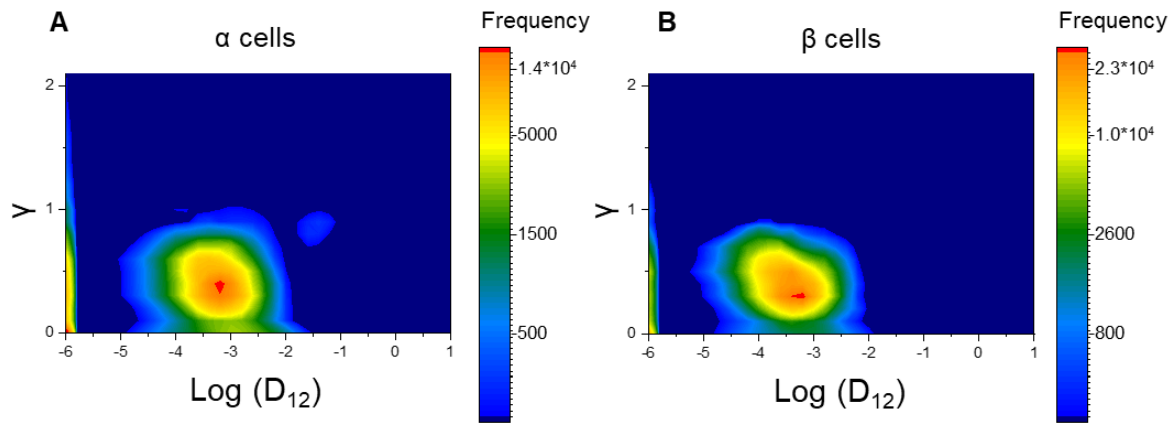


Figure 3.2: Bidimensional distribution of $\log(D_{12})$ on x-axis and γ on y-axis for trajectories obtained by MSS-TAD analysis

(A) α cells under basal conditions: the red peak lies near $\gamma = 0.5$, indicating a mildly sub-diffusive motion very close to the Brownian, reflecting the result obtained in the iMSD analysis. (B) β cells under basal conditions: the red peak falls below $\gamma = 0.5$, indicating a sub-diffusive motion, consistent with iMSD analysis.

CELL MODEL AND CONDITIONS	BLOCKED	DIFFUSIVE	DRIFTED	N
α	35.8 $\pm$ 10.6	57.6 $\pm$ 10	6.6 $\pm$ 3	24
β	32.8 $\pm$ 7.2	64.3 $\pm$ 7.7	2.9 $\pm$ 1.3	35
α NCZ	56.1 $\pm$ 10.7	40.4 $\pm$ 10.2	3.5 $\pm$ 1.7	17
β NCZ	48.2 $\pm$ 9.8	50.1 $\pm$ 9.8	1.7 $\pm$ 0.6	17
α Lat-B	58.3 $\pm$ 7.2	33.6 $\pm$ 8.1	8.1 $\pm$ 4.5	17
β Lat-B	19.2 $\pm$ 6.4	71.0 $\pm$ 6.9	9.8 $\pm$ 3.1	16
α ctrl Hypo (2.5 mM glucose)	28.6 $\pm$ 7.3	65.9 $\pm$ 7.3	5.5 $\pm$ 2	17
β ctrl High Gluc (2.5 mM glucose)	44.8 $\pm$ 10.6	53.1 $\pm$ 10.5	2.1 $\pm$ 0.9	22
α Hypoglycaemia (1 mM glucose)	19.6 $\pm$ 5.2	72.1 $\pm$ 5	8.3 $\pm$ 3.6	19
β High Gluc (16.7 mM glucose)	35.1 $\pm$ 9.2	61.5 $\pm$ 9.3	3.4 $\pm$ 2	25
α ctrl High Gluc (2.5 mM glucose)	28.2 $\pm$ 5.7	67.2 $\pm$ 5.3	4.6 $\pm$ 2.2	23
β ctrl Hypo (2.5 mM glucose)	42.4 $\pm$ 11.1	55.1 $\pm$ 10.5	2.5 $\pm$ 1.8	31
α High Gluc (16.7 mM glucose)	28.4 $\pm$ 6.3	67.1 $\pm$ 6.5	4.5 $\pm$ 1.8	24
β Hypoglycaemia (1 mM glucose)	39.5 $\pm$ 9.8	58.4 $\pm$ 9.4	2.1 $\pm$ 1.7	35
MIN6 ctrl	47.8 $\pm$ 12.2	45.4 $\pm$ 11.1	6.8 $\pm$ 3.4	34
MIN6 NCZ	59.3 $\pm$ 12.7	36.3 $\pm$ 10.9	4.4 $\pm$ 3.3	38
MIN6 Lat-B	27.7 $\pm$ 10.1	51.1 $\pm$ 10.5	21.2 $\pm$ 6.9	36

Table 2: Summary table of motion-classification results across different conditions

α and β cells are alternated with the first on grey rows and the seconds on white rows, MIN6 cells are listed at the bottom. N denotes the numerosity of samples and all the data are expressed as length percentage with Mean $\pm$ St. Dev. Abbreviations: Nocodazole (NCZ), Latrunculin-B (Lat-B), hypoglycaemia condition (Hypo), high glucose stimulus (High Gluc).

3.4 Granules trajectories after cytoskeletal alteration

Cytoskeletal elements, particularly microtubules and actin filaments, are known to regulate intracellular transport and support the movement of organelles in general, and granules in particular, as detailed in paragraph 1.3. In β cells, microtubules support long-range transport sustaining the secondary phase of secretion⁶¹, while cortical F-actin acts as a barrier under basal conditions but remodels upon stimulation to promote exocytosis^{59,60,125}. However, less is known about how these mechanisms operate in α cells. To address this gap, we analysed the effects of cytoskeletal perturbation with nocodazole (NCZ, 10 μ M), a MTs depolymerizing agent (Fig. 3.3A), and Latrunculin-B (Lat-B, 10 μ M), a F-actin depolymerizing agent, on granule dynamics in both α and β cells (Fig. 3.3B). After NCZ treatment, β cells showed an alteration in the trajectory proportions with a significant increase in blocked trajectories compared to control (from $32.8 \pm 7.2\%$ to $48.2 \pm 9.8\%$), accompanied by a consequent decrease in the motile populations. Specifically, the diffusive population decreased from $64.3 \pm 7.7\%$ to $50.1 \pm 9.8\%$ and the drifted population dropped from $2.9 \pm 1.3\%$ to $1.7 \pm 0.6\%$ (all the data in this paragraph are expressed as Mean $\pm$ St. Dev., Fig. 3.3C, Tab. 2). Moreover, β cells reacted as expected to F-actin depolymerization induced by Lat-B, which acts as a prosecretory stimulus. Lat-B treatment led to a marked reduction in blocked trajectories (from $32.8 \pm 7.2\%$ to $19.2 \pm 6.4\%$) with a concomitant increase in motile populations: diffusive trajectories from $64.3 \pm 7.7\%$ to $71.0 \pm 6.9\%$ and drifted ones from $2.9 \pm 1.3\%$ to $9.8 \pm 3.1\%$ (Fig. 3.3D, Tab. 2). Accordingly, a significant increase in D_m was detected in iMSD analysis, from $3.35 \cdot 10^{-3} \mu\text{m}^2/\text{s}$ to $12.8 \cdot 10^{-3} \mu\text{m}^2/\text{s}$ (Fig. 3.4A, B). Following NCZ-induced microtubules depolymerization, α cells exhibited an analogous response to that of β cells, with an increase in blocked trajectories (from $35.8 \pm 10.6\%$ to $56.1 \pm 10.7\%$) and a reduction in the motile populations. Specifically, diffusive trajectories decreased from $57.6 \pm 10\%$ to $40.4 \pm 10.2\%$, while drifted declined from $6.6 \pm 3\%$ to $3.5 \pm 1.7\%$ (Fig. 3.3E, Tab. 2). Notably, α cells responded differently to Lat-B-induced F-actin depolymerization compared to β cells. Indeed, the blocked population of GSGs increased (from $35.8 \pm 10.6\%$ to $58.3 \pm 7.2\%$), with a consequent decrease in diffusive (from $57.6 \pm 10\%$ to $33.6 \pm 8.1\%$) and no significant differences in drifted trajectories (from $6.6 \pm 3\%$ to $8.1 \pm 4.5\%$) (Fig. 3.3F, Tab. 2). Of particular interest is the comparison between granule mobility assessed by iMSD and that obtained from SPT analysis. For ISGs, comparisons between control (β Ctrl, $n = 28$), NCZ-treated cells (β NCZ, $n = 22$), and Lat-B-treated cells (β Lat-B, $n = 15$) showed that all conditions exhibited sub-diffusive motion, as indicated by the Alpha anomalous coefficient. Treatment with Lat-B led to a noticeable increase in the D_m coefficient from $3.35 \cdot 10^{-3} \mu\text{m}^2/\text{s}$ to $1.28 \cdot 10^{-2} \mu\text{m}^2/\text{s}$, whereas NCZ treatment produced no significant change relative to control, demonstrating that even a less sensitive technique such as iMSD can detect the enhanced mobility associated to actin depolymerization (Fig. 3.4A). These observation

are consistent with SPT results, which also revealed enhanced granule diffusivity following actin depolymerization. For GSGs, control (α Ctrl, $n = 34$) and NCZ-treated cells (α NCZ, $n = 25$) both displayed sub-diffusive motion, approaching Brownian behaviour (Alpha anomalous coefficient ≈ 1). In contrast, Lat-B treatment (α Lat-B, $n = 20$) resulted in a significantly lower Alpha anomalous coefficient, reflecting a more constrained dynamics. Indeed, the Alpha anomalous coefficient of α cells decreased from 0.82 to 0.45, pointing out a more sub-diffusive behaviour (Fig. 3.4B). Notably, this reduced sub-diffusive behaviour was not accompanied by a decrease in the D_m coefficient, recapitulating what observed with SPT. Conversely, NCZ exposure led to a reduction in D_m , consistent with impaired long-range granule transport along MTs. As expected, the offset values of the iMSD curves were comparable across all conditions, indicating that granule size remained unaffected by either treatment.

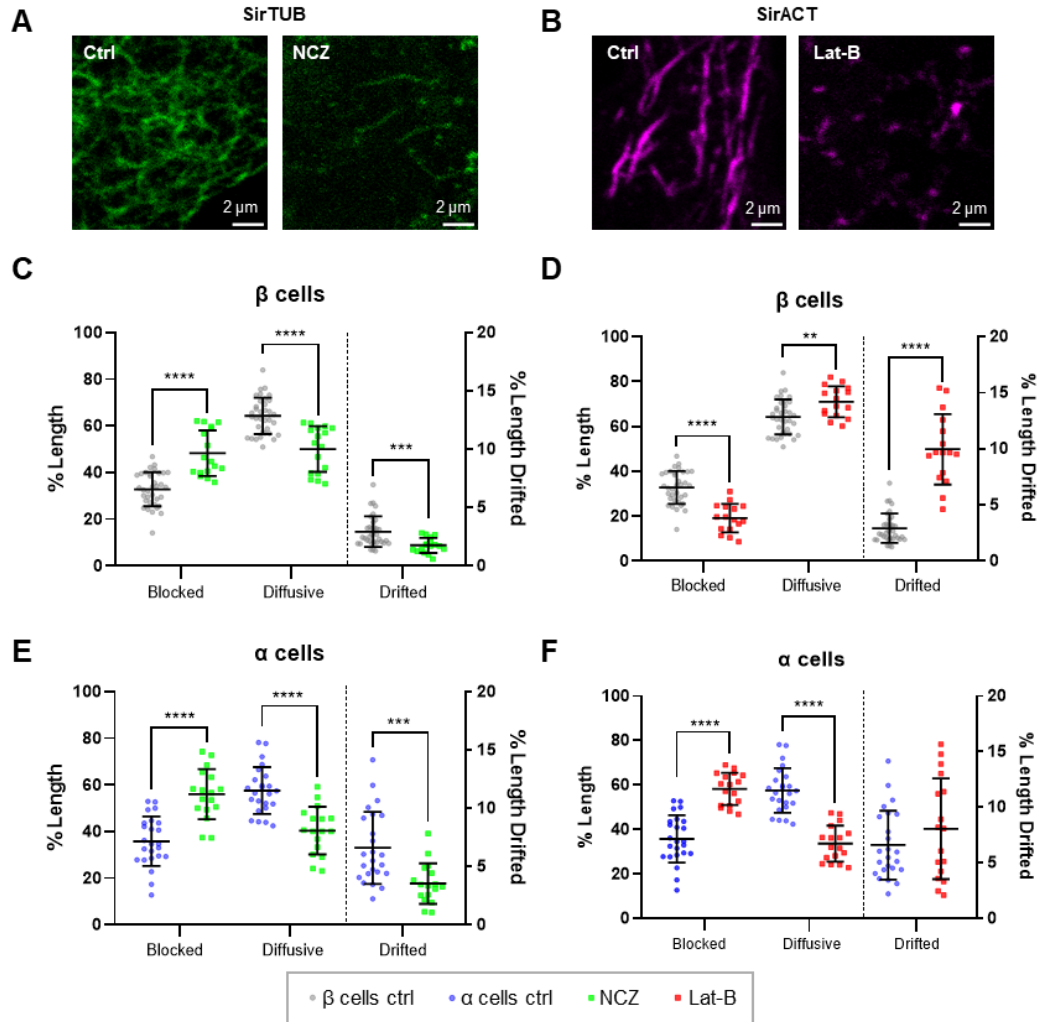


Figure 3.3: SPT analysis of ISG and GSG trajectories after cytoskeleton disruption

(A) Microtubules labelled with SirTubulin (green) in control and NCZ-treated α cells, showing fragmented microtubule structures after treatment. (B) Actin filaments labelled with SirActin (violet) in control and after Lat-B treatment in α cells, showing disrupted actin organization. (C) Scatter plots of ISG trajectory categories comparing Ctrl and NCZ-treated cells. NCZ treatment increased the proportion of blocked trajectories while reducing diffusive and drifted fractions, indicating decreased overall motility. (D) Scatter plots of ISG trajectories comparing Ctrl and Lat-B-treated cells. Lat-B reduced blocked trajectories and increased both diffusive and drifted fractions. (E) Scatter plots of GSG trajectories under Ctrl and NCZ-treated conditions. NCZ increased blocked trajectories and reduced motile fractions. (F) Scatter plots of GSG trajectories comparing Ctrl and Lat-B-treated cells. Lat-B increased blocked trajectories and decreased diffusive trajectories. Drifted length (%) shown on right y-axis.

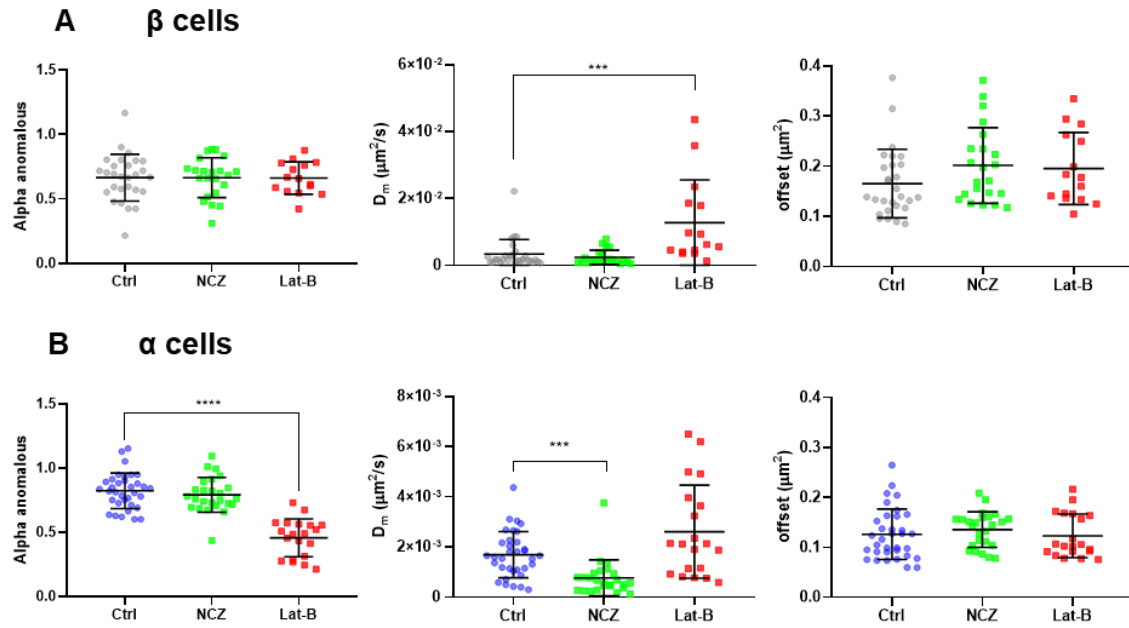


Figure 3.4: iMSD of β and α cells following cytoskeletal perturbation

(A) Scatter plots showing iMSD parameters for ISGs in β cells under Ctrl, NCZ, and Lat-B treatments. All three conditions exhibited sub-diffusive motion as indicated by alpha anomalous parameter below 1. For the diffusion coefficient, we observed an increasing in diffusion for the Lat-B treatment, but no difference were pointed out in the NCZ treatment. As expected, the offset parameter, reflecting apparent granule size, remained unchanged across treatments, consistent with the lack of size-altering effects of these drugs. (B) Scatter plots of iMSD results comparing GSGs control with NCZ and Lat-B. α cells Ctrl condition and NCZ were in a sub-diffusive motion even if were close to 1 (Brownian motion), but the comparison with Lat-B condition highlighted a statistical difference showing a very low sub-diffusive motion. As we can see in the diffusion coefficient, a sub-diffusive motion does not mean a reduction in the diffusion coefficient (Lat-B). Instead, in the case of NCZ the diffusion coefficient D_m is reduced. As expected, the offset parameter remained stable across conditions, confirming that cytoskeletal disruption did not affect granule size. Data normality was assessed using the Shapiro-Wilk test, followed by one-way ANOVA with multiple comparisons.

The β INS-1E and α TC1-9 cells used in this comparison were derived from rat and mouse, respectively. To rule out the possibility that the observed differences were due to species-specific traits, we employed MIN6 mouse cells as a β cell model and subjected them to the same cytoskeletal alterations previously described, allowing a direct comparison with mouse α TC1-9 cells. Upon NCZ treatment, the proportion of blocked granules increased (from $47.8 \pm 12.2\%$ to $59.3 \pm 12.7\%$) accompanied by a decrease in both diffusive (from $45.4 \pm 11.1\%$ to $36.3 \pm 10.9\%$) and drifted trajectories (from $6.8 \pm 3.4\%$ to $4.4 \pm 3.3\%$) (Fig. 3.5A Tab. 2). Following Lat-B treatment, motility changed similarly to what observed for INS-1E β cells, with a decrease in the immobile portion of granules (blocked goes from $47.8 \pm 12.2\%$ to $27.7 \pm 10.1\%$) and an increase in both diffusive (from $45.4 \pm 11.1\%$ to $51.1 \pm 10.5\%$) and drifted (from $6.8 \pm 3.4\%$ to $21.2 \pm 6.9\%$) (Fig. 3.5B, Tab. 2). Notably, the data of MIN6 exhibited greater dispersion compared to INS-1E cells, possibly reflecting the higher degree of variability and heterogeneity previously reported for this cell line⁸⁹. Overall, these results are consistent with those obtained in INS-1E cells and therefore exclude the possibility that the differences observed between α and β cells arise from interspecies variability.

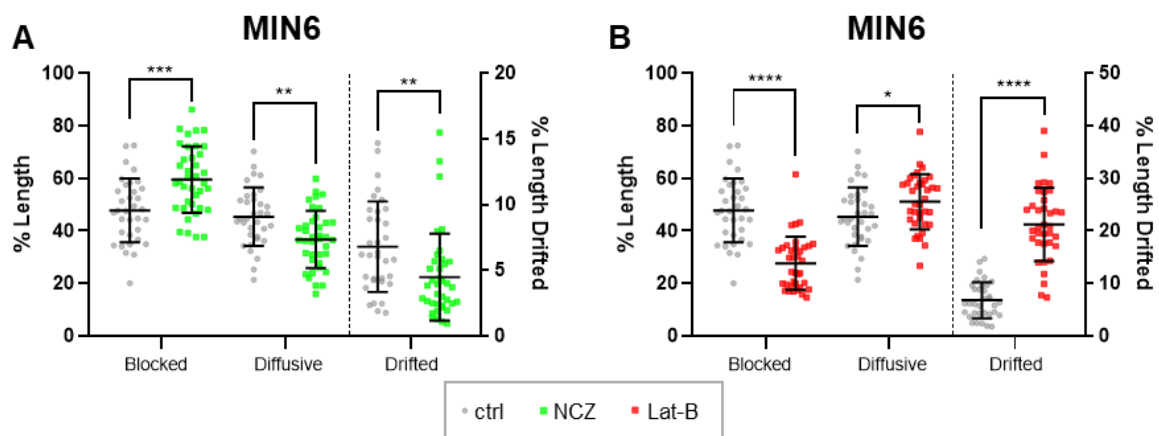


Figure 3.5: SPT classification for ISG after NCZ and Lat-B treatment in MIN6 as β cell model

(A) Scatter plots of ISG trajectory categories comparing Ctrl and NCZ-treated cells. NCZ increased blocked trajectories and reduced diffusive ones, indicating decreased overall motility as shown for drifted trajectories. Drifted length (%) on right y-axis. **(B)** Scatter plots comparing Ctrl and Lat-B-treated cells. Lat-B decreased blocked trajectories while increasing diffusive and drifted fractions. Drifted length (%) on right y-axis. Results in MIN6 cells are consistent with observations in INS-1E β cells, suggesting ISG-specific behaviour rather than cell line-specific effects. Normality was assessed, and multiple t-tests with Holm-Sidak correction ($\alpha = 0.05$) were applied.

3.5 Granules trajectories under secretory stimuli

After identifying differences between GSGs and ISGs in their response to F-actin depolymerization by Lat-B, we next evaluated whether this difference persisted under secretory stimulation. The rationale for this experiment lies in the fact that F-actin depolymerization can be considered as a pro-secretory stimulus for both cell types^{80,81,126,127}. To probe granule behaviour under physiological stimulation, α cells were exposed to low glucose concentrations (hypoglycaemia), while β cells were stimulated with high glucose. The granule population analysed by SPT in this condition, comprises both pre-existing granules and those newly generated in response to stimulation^{66,81,128} and the SPT analysis was performed on this mixed population. For ISGs, increasing the glucose concentration from 2.5 mM to 16.7 mM (in KRBH buffer) led to a decrease in blocked trajectories (from $44.8 \pm 10.6\%$ to $35.1 \pm 9.2\%$), and a concomitant rise in mobile granules, with diffusive trajectories raising from $53.1 \pm 10.5\%$ to $61.5 \pm 9.3\%$, and drifted motion from $2.1 \pm 0.9\%$ to $3.4 \pm 2.0\%$ (Fig. 3.6A, B, Tab. 2). These changes are in line with the effects observed after Lat-B treatment and with previous reports⁶¹. To evaluate GSGs mobility under secretory stimulation, α cells were transferred to KRBH supplemented with 1 mM glucose¹²⁹, and imaging was performed within 30-40 minutes from the beginning of incubation. Under these conditions, SPT analysis showed a reduction in the blocked trajectories (from $28.6 \pm 7.3\%$ to $19.6 \pm 5.2\%$) and consequently a net increase in the diffusive (from $65.9 \pm 7.3\%$ to $72.1 \pm 5\%$) and drifted ($5.5 \pm 2\%$ to $8.3 \pm 3.6\%$) (Fig. 3.6C, D, Tab. 2). As controls, α and β cells were subjected to the opposite glucose condition: α cells to high glucose (16.7 mM) and β cells to hypoglycaemic stimulus (1 mM). Both treatments produces no significant changes in granules dynamics (Tab. 2). The comparative analysis of secretory granule dynamics in α and β cells reveals a surprising dichotomy in the role of the actin cytoskeleton. In response to their respective physiological stimuli, both cell types display a similar enhancement of granule mobility, with an increase in both diffusive and directed motion components (Fig. 3.6A, C). However, an investigation into the underlying mechanisms shows that this common response originates from distinct regulatory pathways. In β cells, the effect of the secretory stimulus on granule dynamics can be mimicked by the pharmacological depolymerization of F-actin. In α cells, however, this equivalence does not hold true. Indeed, while ELISA analyses confirm that Lat-B treatment causes a robust increase in glucagon secretion (Fig. 3.7A, B), this does not translate into a significant change in granule mobility (Tab. 2). These data suggest that while the actin cytoskeleton is implicated in secretion in both cell models, its specific role in modulating granule dynamics is fundamentally different.

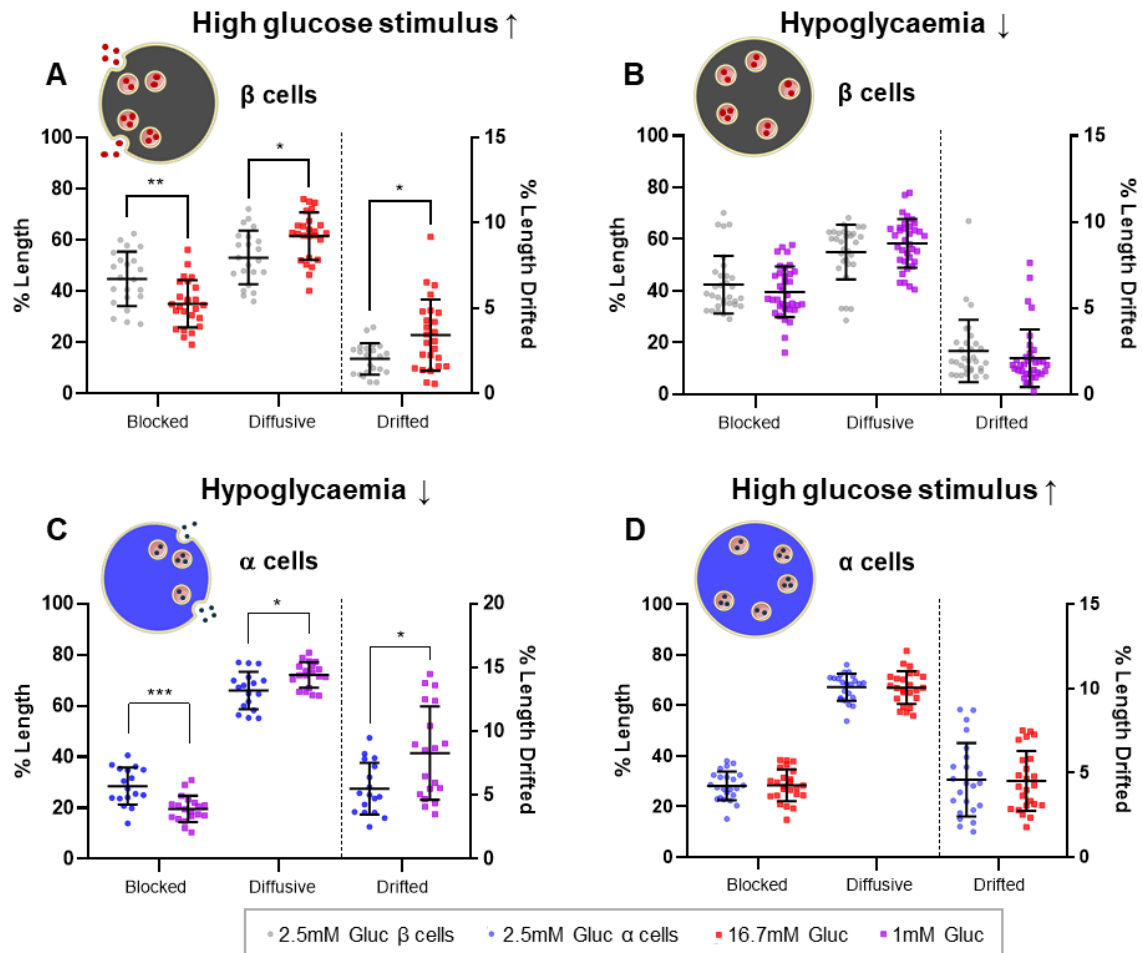


Figure 3.6: Effects of secretory stimuli on granule motility in β and α cells

Scatter plots show ISG and GSG trajectories classified as blocked, diffusive, or drifted, alongside schematics of secretion or no-secretion states. Images were collected within one hour of treatment. Drifted length (%) is indicated on the right y-axis. **(A)** ISGs motility in β cells after high glucose stimulation (16.7 mM) vs. control (2.5 mM), showing increased motility. **(B)** ISGs motility under hypoglycaemia (1 mM) vs. control (2.5 mM), showing no significant changes. **(C)** GSGs motility in α cells under hypoglycaemia (1 mM) vs. control (2.5 mM), showing increased motility with a reduction in blocked trajectories. **(D)** GSGs motility after high glucose (16.7 mM) vs. control (2.5 mM), showing no significant changes. Data normality was assessed, and multiple t-tests with Holm-Sidak correction ($\alpha = 0.05$) were applied. Servier Medical Art (<https://smart.servier.com/>) was used to create part of this figure. Scatter plots with Mean $\pm$ St. Dev. with each dot shown represent the outcome from a single acquisition.

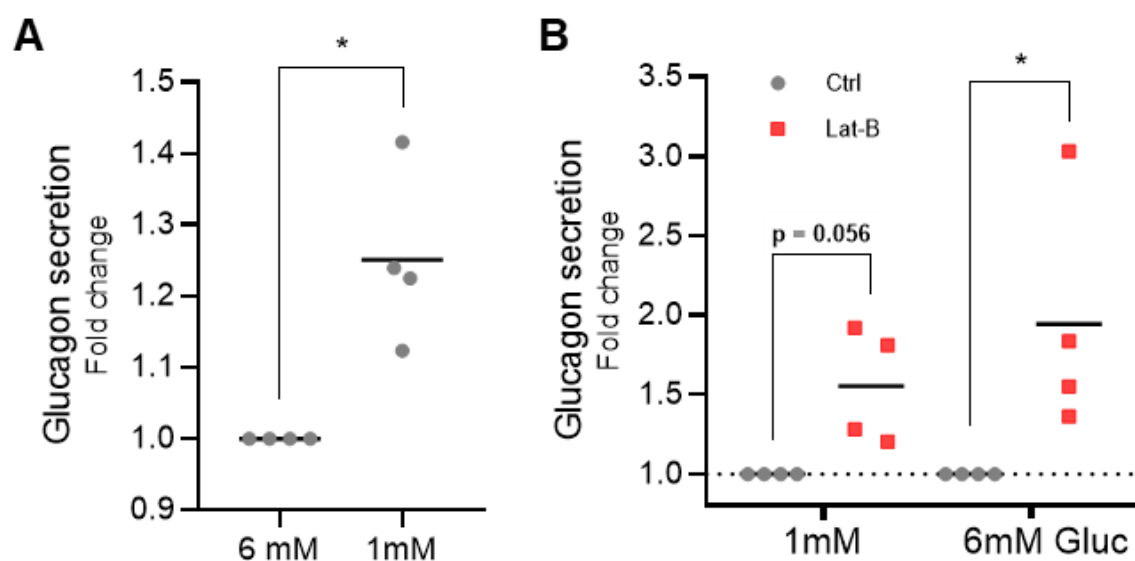


Figure 3.7: Glucagon secretion in α cells, expressed as fold change

(A) Scatter plot of normalized glucagon secretion under control (6 mM glucose) and hypoglycaemic (1 mM glucose) conditions. Secretion significantly increases at 1 mM glucose. **(B)** Scatter plot of normalized glucagon secretion with or without Lat-B at the indicated glucose levels. Lat-B enhances secretion regardless of glucose concentration. Dots represent biological replicates ($n=4$); horizontal bars indicate the mean. The dotted line marks the normalization baseline (fold change = 1).

To gain deeper insight into the temporal evolution of granule behaviour, we re-analysed the single particle tracking data from stimulated cells by segmenting the recordings over time. This approach allowed us to examine how the relative contributions of each motion category changed throughout the secretory response. In β cells, stimulation with high glucose (from 2.5 mM to 16.7 mM) is known to first trigger the release of the readily releasable pool (RRP), followed by the recruitment of additional granules to sustain secretion^{38–40}. In our results, this dynamic was reflected within the first 5 minutes after stimulation (Fig. 3.8A), where granules exhibited a higher proportion of diffusive motion compared to the control, indicating an initial rise in motility. Over time, however, this diffusive fraction progressively decreased, returning to near-basal levels. In parallel, the proportion of blocked granules, initially reduced relative to control, gradually increased until matching control values. Interestingly, the drifted population showed a marked transient increase between 10 and 20 minutes, suggesting a phase of directed transport that likely contributes to sustained secretion once the RRP is depleted. In α cells subjected to hypoglycaemic stimulation, both diffusive and drifted populations increased immediately after treatment, consistent with enhanced granule mobility to support glucagon secretion (Fig. 3.8B). Meanwhile, the blocked fraction remained lower than in control cells. Compared with β cells, α cells displayed overall higher and more persistent motility, with increased granule dynamics lasting longer before gradually approaching control levels. Even after 40 minutes, GSGs remained more active than in unstimulated conditions, displaying a temporal pattern distinct from that of β cells. The drifted population exhibited pronounced variability, rising sharply during the first 25 minutes and subsequently declining toward control levels, reflecting the transient activation of directed transport mechanisms during the secretory response.

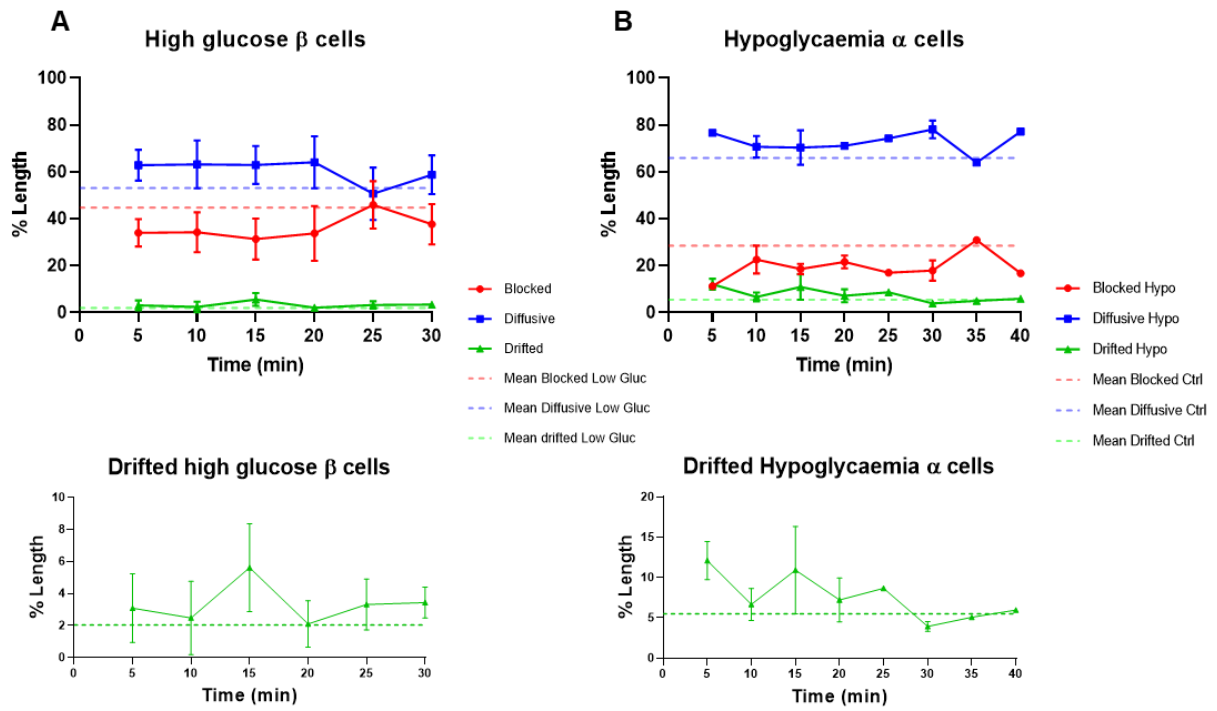


Figure 3.8: Temporal analysis of granule motility in β and α cells under stimulatory conditions

Time points represent 5-min bin; in detail: 5 stands for the first 0-5 minutes, 10 for 6-10 minutes, 15 for 11-15 minutes, 20 for 16-20, and so on. Dashed lines indicates control means. **(A)** In β cells, glucose stimulation transiently increased diffusive and drifted granules, followed by a return to baseline, while the blocked fraction gradually increase. **(B)** In α cells, diffusive and drifted populations increased immediately after stimulation, with higher and more sustained motility than β cells and a transient boost in directed motion within the first 25 min.

3.6 Concluding remarks

Given that intracellular trafficking is a determining factor for effective hormone secretion, this thesis presents the first systematic classification of GSGs dynamics. Using SPT, we developed a robust methodological framework to quantitatively characterize granule mobility patterns in our α and β cells models, classifying them into blocked, diffusive, and directed motion regimes. The analysis revealed both conserved and divergent aspects between the two granule types.

Under basal conditions, both GSGs and ISGs exhibit predominantly diffusive behavior. In response to their respective secretory stimuli (hypoglycemia for α cells and hyperglycemia for β cells), both granule populations increase their mobility, suggesting a shared global response. Furthermore, the depolymerization of MTs confirmed their essential role in directed transport in both cell types, highlighting a conserved transport mechanism.

The most significant divergence emerged in the role of the actin cytoskeleton. Depolymerization of F-actin via Lat-B enhanced granule motility in β cells but had no discernible effect on GSGs dynamics in α cells. This key finding indicates a differential engagement of the actin network, which in β cells acts primarily as a barrier to be removed, while in α cells it appears to have a distinct regulatory role not directly linked to granule mobility. In summary, our findings indicate that while GSGs and ISGs share transport regimes and microtubule dependencies, α and β cells differ in how they leverage the actin network under stimulus conditions. This divergence likely reflects distinct regulatory strategies tailored to the specific secretory demands of each hormone. Further research is needed to clarify whether these mechanistic differences translate into functional disparities in maturation or secretion efficiency. However, this first comparative study lays the essential foundation for future investigations into glucagon granule dynamics, a previously underexplored area.

4. Mapping intragranular pH through phasor-based fluorescence lifetime imaging

Having established reliable methods for granule labelling, tracking, and dynamic characterization, this chapter shifts focus from how granules move to their internal functional state. We aimed to probe their intrinsic physicochemical properties, focusing on luminal pH, which is a key parameter influencing granule maturation, cargo processing, and secretion competence²². To achieve this, we employed phasor-based fluorescence lifetime imaging microscopy (FLIM) combined with an appropriate pH-sensitive probe. This approach provides a direct optical readout of local proton concentration within living cells, adding a crucial physicochemical dimension to the methodological framework previously developed.

4.1 Granule pH measurement: a historical review

High-accuracy measurements of ISGs pH in live cells have long been technically challenging. A pioneering study by Hutton and colleagues published in 1982 highlighted the acidic nature of insulin granules and provided a first evidence on the role of an ATP-dependent mechanism responsible for their acidification¹³⁰. However, these measurements were performed on isolated granules outside the physiological cellular context. The subsequent advent of pH-sensitive fluorescent probes opened the way for direct investigations of intragranular pH in living cells. In 2001, Barg and colleagues employed the fluorescent probe Lysosensor Green DND-189 to monitor granular pH in living cells¹³¹. The fluorescence intensity of the probe is inversely proportional to pH; therefore, by measuring fluorescence intensity, they confirmed that acidification is driven by a V-type H⁺-ATPase located in the granular membrane. Intensity-based sensors are more susceptible to concentration effects, background, dye loading, and environmental artefacts, compared to ratiometric pH sensors. Although they did not obtain quantitative pH values, Barg and colleagues demonstrated that acidification is a crucial step during granules maturation and a prerequisite for exocytosis¹³¹. Few years later, in 2006, Stiernet and colleagues followed a similar approach by employing Lysosensor DND-160 to measure granule pH in Langerhans islets. While this fluorescent probe might be used also as ratiometric

sensor; their results were again expressed as relative pH changes rather than absolute value¹³². Notably, the authors were able to show that an increase in glucose concentration induced a rapid decrease in granular pH in a chloride-dependent manner¹³². By contrast, Eto and colleagues in 2003, reported a mild increase in pH during glucose stimulation, recording an alkalization of 0.016 pH units in ISGs of pancreatic β cells, using Lysosensor Green DND-189¹³³. Taken together, these findings highlight a common limitation: all studies described above relied on non-specific dyes that label various acidic compartments such as lysosomes and endosomes, thereby complicating accurate evaluation of granule-specific pH. To overcome the limitations of non-specific dyes, Tompkins and colleagues devised a more precise genetic strategy: they fused the gene encoding for a pH-sensitive variant of EGFP to the N-terminal leader sequence of human growth hormone (hGH), thereby directing the fluorescent probe specifically into ISGs of the RIN1046-38 insulinoma cell line²⁷. This targeted EGFP sensor allowed them to monitor pH changes within granules *in situ*. Under stimulatory glucose conditions, they observed a decrease in intragranular pH, whereas treatment with inhibitors of the V-type H⁺-ATPase raised the pH and prevented the acidification induced by glucose. The experiment demonstrated not only the feasibility of organelle-specific pH sensing in secretory vesicles but also provided evidence that granule acidification is dynamically regulated and tied to secretory priming mechanisms²⁷. With this approach the authors targeted the insulin secretory pathway but, this sensor was not ratiometric thus allowing only the measurements of relative differences between experimental conditions. Notably, Neukman and coworkers attempted to overcome these limitations by targeting a quantitative pH reporter to secretory granules: they fused eCFP to the ICA512-RESP18 homology domain and expressed it in INS-1 cells, using lifetime changes in eCFP fluorescence to estimate intragranular pH *in situ*. However, this approach remains available as preprint and has not yet undergone peer review¹³⁴. As detailed above, previous studies lack key methodological elements required for accurate and reliable absolute pH measurements within ISGs. Two essential requirements are: a proper calibration of the pH-sensitive probe to measure absolute pH and the specific and validated targeting of the probe to ISGs. Here, to tackle both issues simultaneously, we inserted two-point mutations within the EGFP moiety of the C-pep-EGFP construct to generate the genetically encoded ratiometric pH sensor called C-pep-E¹GFP. Moreover, I extended this approach also on α cells to measure for the first time the pH of GSGs.

4.2 E¹GFP pH sensor

E¹GFP probe employed in this thesis belongs to the Y66-chromophore protein family, whose members contain a tyrosine residue in position 66. The protonated chromophore (A state) has a much higher excitation energy than the deprotonate form (B state); therefore, due to the protonatable phenolic function of the tyrosine lateral chain, the probe is pH sensitive. In proximity to the chromophore, the acid-base equilibrium of E222 is strongly thermodynamically coupled with that of the chromophore, increasing the complexity of the protonation equilibrium. The presence of E222, in E¹GFP, leads to two optically distinguishable states, A and A', which referred to deprotonated and protonated E222, respectively (Fig. 4.1A). E¹GFP exhibits a pK_a close to 6.0. When the pH is higher than pK_a both A and B states become predominant over A' with a fixed mutual stoichiometric ratio. In this first case, when A and B states predominate, the absorption spectrum has two bands, one at 402 nm and the other at 509 nm, respectively. Conversely, at pH values lower than the pK_a, the A' state predominates with an absorption peak at 410 nm¹³⁵. The A' and A states can be excited at nearly the same wavelength, which is 405 nm for single photon excitation. The major photophysical effect on the emission of these two states depends on the different protonation state of E222. Once excited, the protonated form of chromophore undergoes to a proton transfer through an Excited State Proton Transfer (ESPT) to a nearby basic residue in 1-10 ps^{136,137}. The major difference between excited A and excited A' states lies in the proton acceptor: in A state, the proton is discharged to deprotonated E222, whereas in the A' state, it is transferred to adjacent His148^{135,138}. Thanks to this apparent slight difference, both lifetime properties and spectral emission of the two states changes. The emission maximum of the excited state A is 516 nm showing a mono-exponential decay ($\tau = 3.5$ ns) whereas the emission maximum of the excited state A' is 507 nm with a biexponential decay ($\tau = 0.83$ ns) (Fig. 4.1B, C). These differences in lifetime and emission properties enable ratiometric pH sensing using E¹GFP, as previously described¹³⁹. To ensure specific localization to insulin secretory granules, we generated a C-pep-E¹GFP construct by inserting the E¹GFP coding sequence into the proinsulin gene within the C-peptide region. This was accomplished via site-directed mutagenesis of the previously established C-pep-EGFP construct¹⁰⁰.

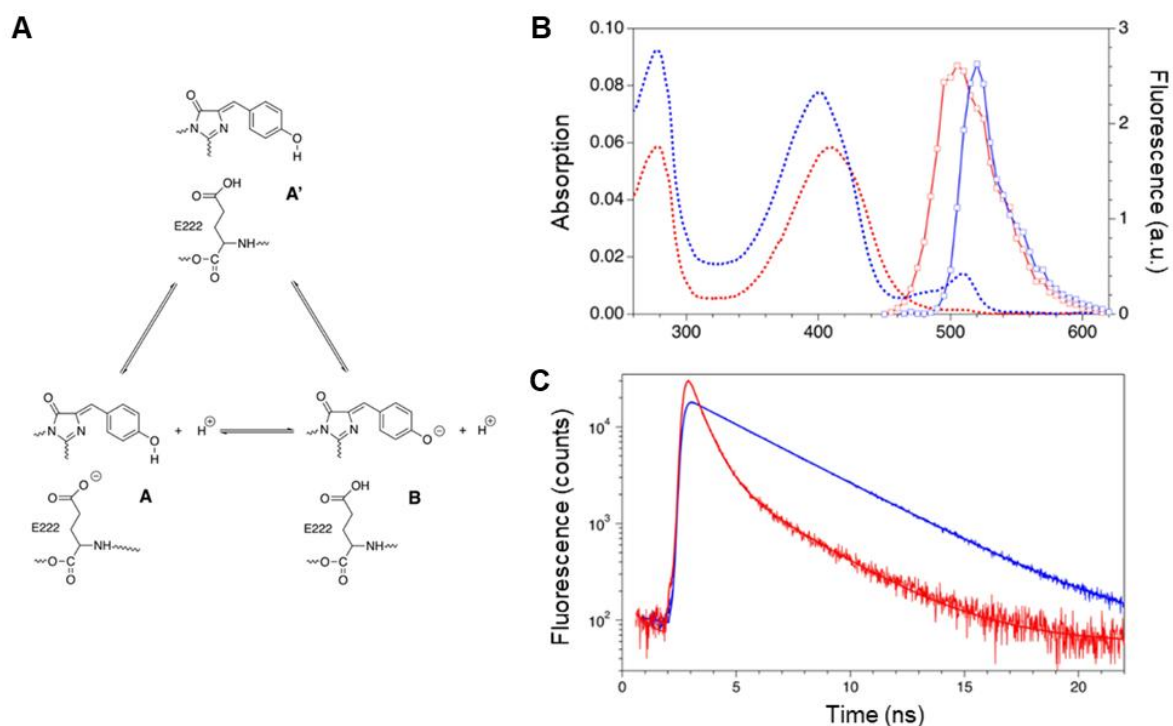


Figure 4.1: Mechanism of chromophore protonation and emission properties of E^1 GFP

(A) Two state (2S)-model of E^1 GFP chromophore protonation. At low pH, only state A' is present, with a protonated chromophore (phenolic tyrosine) and protonated E222. The acid-base equilibrium of E222 is strongly coupled to the chromophore. Increasing the pH shifts populations towards states A and B : in A , the chromophore is protonated and E222 deprotonated; in B , the reverse occurs. A and B exchange protons without net change in free protons, making them insensitive to external pH. A' and A absorb ~ 400 nm; B absorbs at longer wavelengths due to electron delocalization from deprotonation. **(B)** Absorption (dashed lines) and normalized emission ($\lambda_{\text{ex}} = 405$ nm) spectra of E^1 GFP; red and blue traces indicate A' and A , respectively. **(C)** Fluorescence lifetime decays of A' and A upon 405 nm excitation, collected between 480-550 nm.

4.3 Phasor FLIM principles

Fluorescence is a radiative process in which molecules, such as fluorophores, emit light after an excitation phase in which the fluorophore has absorbed photons from an external light source¹⁴⁰. Upon excitation, the fluorophore transitions from the ground state to an excited electronic state at a higher energy level. When returning to the ground state, it releases part of this energy as emitted fluorescence photons. The time spent in the excited state, before photon emission, defines the fluorescence lifetime (τ)¹⁴¹. Emission occurs from the lowest excited level ensuring the independence of the emission spectrum from the excitation wavelength¹⁴⁰. Fluorescence lifetime imaging measures the average time a fluorophore remains in the excited state after excitation, by detecting the emitted photons from the labelled sample¹⁴¹. Measuring the fluorescence lifetime decay of chemical species or cellular structures provides valuable information about their environmental conditions (such as pH, viscosity, ions distribution, and polarity) in a fluorophore concentration-independent manner¹⁴⁰. Several methods are available to determine fluorescence lifetime decay, including frequency-domain techniques, time-sampling approach and time-correlated single photon counting¹⁴². However, challenges may arise when analysing time-domain data using mono- or double-exponential fitting, due to complex decay behaviour of fluorescent proteins and potential correlations between characteristic lifetime and their corresponding amplitude¹⁴². Moreover, such analyses require expertise to correctly interpret molecular contributions. To overcome all these issues, we employed a phasor-based FLIM approach: a robust, fit-free method, to measure luminal pH and to generate a spatial pH map. Starting from the decay function (Fig. 4.2A):

$$I(t) = I_0 e^{-\frac{t}{\tau}} \quad (\text{Eq. 6})$$

Where $I(t)$ is the fluorescence intensity at a certain time t , I_0 is the initial fluorescence intensity at time = 0 and τ is the fluorescence lifetime. In phasor-based FLIM, fluorescence decay data are transformed into a two-dimensional phasor plot which serves as a graphical representation of lifetime distributions within a universal reference system known as the universal semicircle. Each image pixel corresponds directly to a single point in the phasor plot^{140,142}. The two phasor coordinates represent the real and the imaginary components of the fluorescence decay after Fourier transformation (Fig. 4.2A). These coordinates are the cosine (g) (Eq. 7, 9) and sine (s) (Eq. 8, 10) of the angle which identify a position inside the universal semicircle. The following formulas (Eq. 7, 8) calculates the components in the time domain and are the core of the transformation^{140,143}:

$$g = \frac{\int_0^\infty I(t) \cos(\omega t) dt}{\int_0^\infty I(t) dt} \quad (\text{Eq. 7})$$

$$s = \frac{\int_0^\infty I(t) \sin(\omega t) dt}{\int_0^\infty I(t) dt} \quad (\text{Eq. 8})$$

Here, ω corresponds to the modulation angular frequency, indicating how rapidly the fluorescence intensity is modulated during measurement. The value of ω is selected to properly resolve the features of the fluorescence decay, according to the relation $\omega = 2\pi f$, where f is the modulation frequency. The coordinates g and s are expressed as cosine and sine, respectively with these formulas, which relate modulation and phase shift:

$$g = M \cos(\varphi) \quad (\text{Eq. 9})$$

$$s = M \sin(\varphi) \quad (\text{Eq. 10})$$

M (modulation) indicates how strongly the emitted fluorescence varies relative to the excitation signal. The modulation depth depends on the fluorophore's lifetime: longer lifetimes cause weaker modulation. φ (phase) represents the time delay between the modulated excitation and the emitted fluorescence. This delay arises because the emission does not occur instantaneously after excitation. In the phasor space, an infinite lifetime is located at the origin (0,0) and zero lifetime lies on the x axis at (1,0) with the semicircle itself centred at (½,0), and with radius ½ (Fig. 4.2B). Fluorescence lifetime values can thus be inferred from the position of the phasor within the coordinate system. Mono-exponential decays lie directly on the semicircle, whereas multi-exponential decays fall inside it. It is possible to identify molecules and chemical species by their position inside the phasor plot; if two molecules coexist within the same image, their combined contribution lies along the line connecting their individual phasor coordinates (Fig. 4.2B, C)¹⁴². The same is true for three different molecules but in this case a triangle represent the region in which the final distribution lay down depending on the single weight of each component and so on. Since phasor-based FLIM relies on the collection of a sufficient number of photons per pixel to accurately represent the fluorescence decay in the phasor space, signal integration over time is required. As a result, long acquisition times may introduce motion-induced artefacts, such as apparent deformation of dynamic structures like ISGs (Fig. 4.2D). Importantly, combining FLIM with the genetically encoded E¹GFP pH sensor provides a robust alternative to traditional ratiometric methods at the intracellular level. FLIM-based readouts are inherently resistant to artefacts caused by wavelength-dependent scattering and avoid the signal-to-noise loss typically associated with ratio-based calculations.

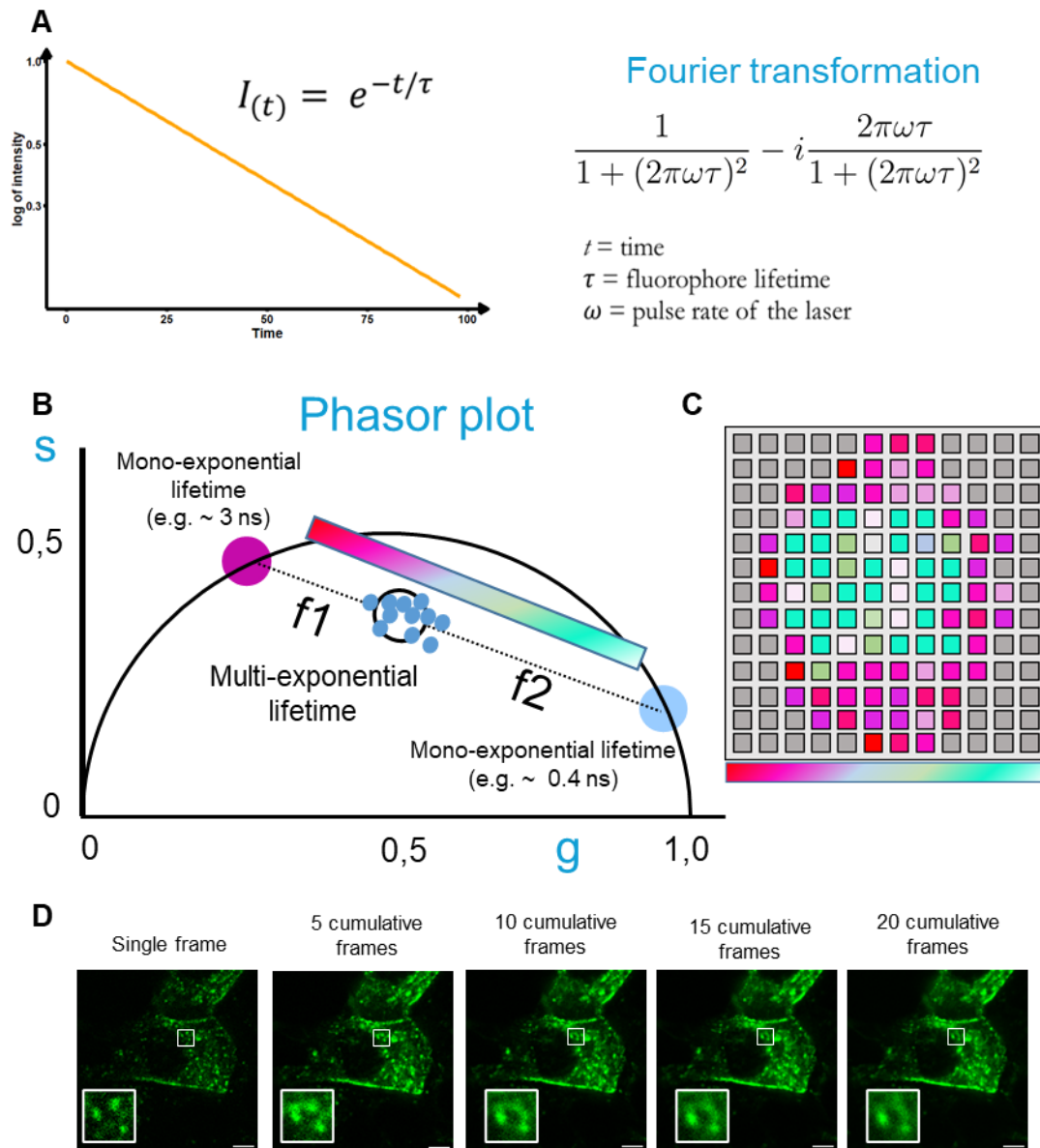


Figure 4.2: FLIM principles

(A) Schematic representation of fluorescence lifetime decay, where fluorescence intensity (log scale, y-axis) is plotted against time (x-axis) (Eq. 6). The fluorescence lifetime (τ) can be measured directly, but a multiexponential fit is typically required to extract detailed information about labelled structures. Alternatively, a fit-free Fourier transform approach, can be used to convert fluorescence decay data into a phasor plot, where each pixel's decay is represented by a point defined by the real and imaginary components of the Fourier transform. (B) Illustration of a phasor plot. An infinite lifetime maps to the origin (0,0), while a zero lifetime lies at (1,0). Mono-exponential decays fall along the universal semicircle (centered at 0.5, radius 0.5), whereas multi-exponential decays appear inside it as linear combinations of mono-exponential components. The phasor position directly reflects the fluorescence lifetime, providing an intuitive visualization of decay behaviour. (C) Each image pixel yields a corresponding point in the phasor plot, allowing direct correlation between spatial and lifetime information. A colour look-up table (LUT) can be applied to map phasor clusters back onto the image, enabling a fully fit-free, pixel-wise visualization of lifetime distributions. (D) FLIM technique is related to the time of acquisition. Prolonged signal integration might lead to apparent changes in granule size or shape. Panels compare single-frame imaging (on the left) with cumulative frame integration over increasing acquisition times, as indicated. Scale bar: 5 μm . Image areas: 42.43 μm^2 . Zoomed region area: 3.07 μm^2 .

4.4 Calibrating the E¹GFP pH sensor

To characterize granular pH inside β cells, we inserted the genetically encoded E¹GFP pH reporter (pK_a close to 6.0) within the C-pep of proinsulin. Methodologically, several aspects deserve attention:

- Employing a pH-sensitive variant of the GFP family offers a versatile and effective strategy, as these genetically encoded sensors (unlike traditional fluorogenic dye) can be targeted to specific subcellular regions through fusion with localization sequences and expressed in living cells, tissues, or entire organisms, enabling the monitoring of pH or other parameters^{136,144} with minimal phototoxicity.
- As abovementioned, FLIM represents a powerful alternative to conventional ratiometric techniques, as it enables accurate measurements that are independent of fluorophore concentration and rely on a single excitation and emission range.
- While FLIM has previously been used to measure intracellular pH with fluorescent proteins^{145,146}, its integration with phasor analysis provides a fast, reliable, and model-free approach for quantifying vesicular pH, while simultaneously offering spatially resolved information.

The goal was to achieve reliable intracellular pH measurements using E¹GFP in combination with FLIM. Before applying the FLIM approach in living cells, we performed an *in vitro* characterization of the E¹GFP probe. This preliminary step was essential, as this study represents the first application of E¹GFP in combination with FLIM, while previous work¹³⁹ relied on ratiometric intensity-based measurements. Although a FLIM analysis has been previously reported for the related E²GFP variant¹⁴⁷, the photophysical properties of E¹GFP had not yet been validated under phasor lifetime-based detection. Therefore, to ensure the reliability of subsequent cellular measurements, we established a complete *in vitro* calibration curve of fluorescence lifetime as a function of pH under controlled conditions, before proceeding with the calibration *in situ* within insulin secretory granules. Phasor-FLIM analysis was performed on purified His-tagged E¹GFP diluted in buffers of known pH (Fig. 4.3A). For this thesis, phasor analysis was conducted using a previously published, cloud-based custom Python 3.6 routine¹⁴⁸. The fluorescence decay, measured pixel-by-pixel, generates a phasor cluster whose position corresponds to the average fluorescence lifetime. This average lifetime was determined by calculating the phasor barycentre. Consistent with a single-site protonation mechanism, the calibration revealed a linear distribution of barycentres across pH values ($R^2 = 0.9873$). At high pH, the barycentres approached the universal semicircle, indicating a nearly mono-exponential decay dominated by the excitation of A state. At low pH, the phasor shifted inside the universal semicircle, reflecting a multi-exponential decay with a calculated lifetime around 1.0 ns, in agreement with A' state emission measured *in vitro* (Fig. 4.3B).

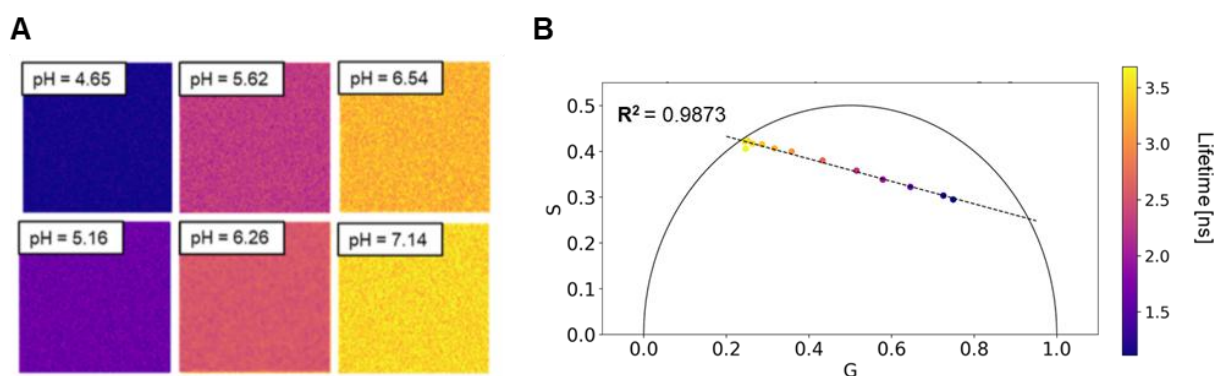


Figure 4.3: In vitro calibration of the C-pep-E¹GFP sensor

(A) Representative images of purified recombinant E¹GFP in buffers with defined pH values. Images represent pseudo-coloured fluorescence images recorded in calibration buffers ranging from pH 4.65 to pH 7.14. (B) Phasor plot representation of the calibration FLIM dataset, where each dot represents the barycentre coordinates of an individual pH condition. A linear regression fit ($R^2 = 0.9873$) illustrates the correlation between fluorescence lifetime and pH confirming the response of the sensor.

4.4.1 Living-cell calibration of E¹GFP within ISGs in INS-1E β cells

Probe calibration in the cellular context was achieved by permeabilizing β cells expressing C-pep-E¹GFP with nigericin, followed by incubation in calibration buffers of known pH and subsequent FLIM analysis (Fig. 4.4A). Under these conditions, phasor plots revealed a linear distribution along a segment within the universal circle ($R^2 = 0.997$), consistent with the behaviour of the purified protein (Fig. 4.4B). The relationship between fluorescence lifetime and pH was accurately modelled with a single-site sigmoidal function ($R^2 = 0.9985$), yielding a pK_a of 5.91. This value closely matches the pK_a previously obtained using fluorescence intensity-based ratiometric imaging (Fig. 4.4C)¹³⁹, indicating that the same A'/A equilibrium observed *in vitro* is preserved when E¹GFP is fused into the C-peptide and expressed in cells. Interestingly, absolute lifetimes measured in living cells were consistently shorter than those recorded in solution, a difference likely arising from the crowded or chemically distinct environment of the ISGs, which enhances non-radiative decay. Despite this reduction, the intracellular titration curve closely overlapped with the *in vitro* calibration, suggesting that these environmental effects have minimal impact on the pH dependence of the probe and, instead, reflect intrinsic differences between the intracellular environment and buffered solutions.

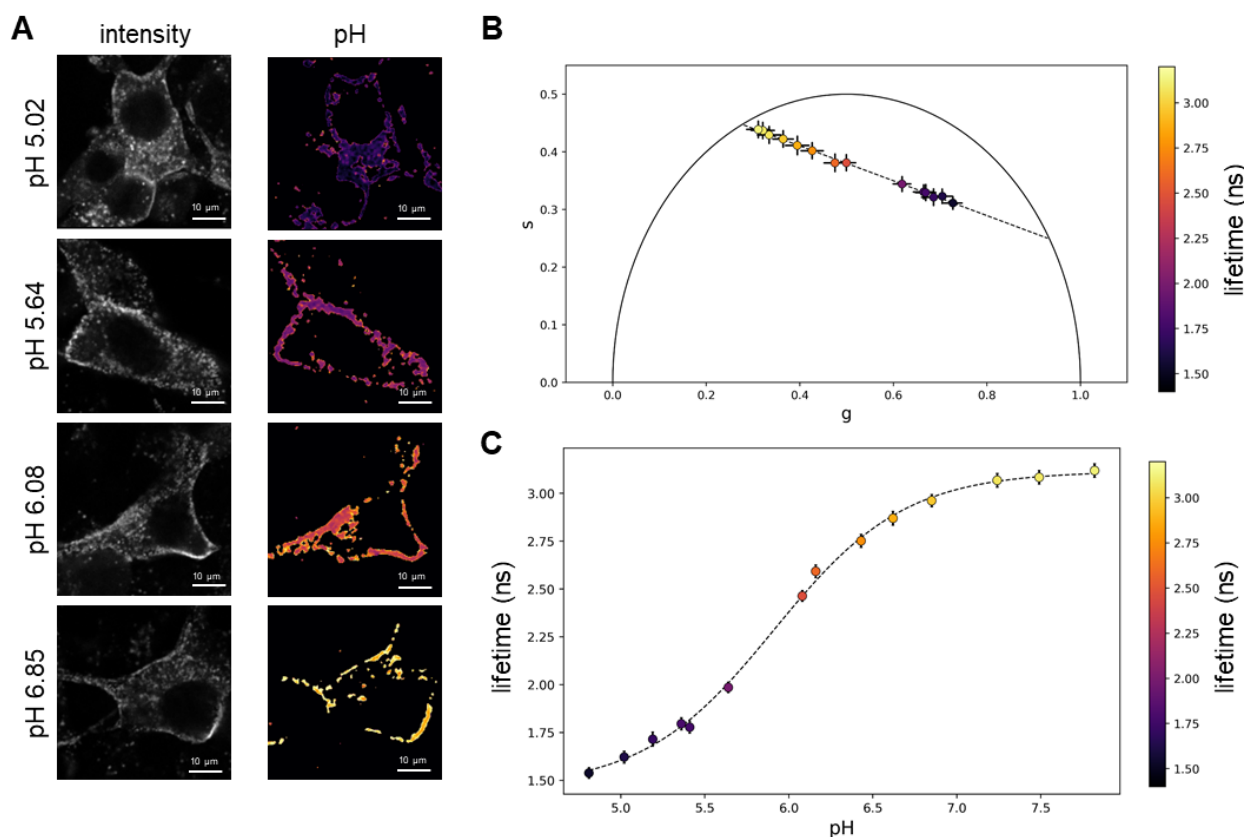


Figure 4.4: Calibration of the C-pep-E¹GFP sensor in β cells

(A) Calibration in living cells in which some representative C-pep-E¹GFP transfected β cells are displayed. Left panels show fluorescence intensity, right panels display pseudo-coloured lifetime maps using the LUT shown in figure. We incubated β cells in buffers of defined pH in presence of 10 μ M nigericin to clamp the pH value. (B) Phasor plot of the calibration data corresponding to (A), with a linear fit within the universal semicircle ($R^2 = 0.997$). (C) Calibration curve showing the relationship between fluorescence lifetime and pH. Data were fitted using a non-linear regression (log[agonist] vs. response - Variable slope (four parameters) equation. This calibration was subsequently used to convert fluorescence ratios acquired in live-cell imaging experiments into absolute pH values for quantitative mapping of granule acidification.

4.5 Evaluation of ISG pH in basal culture conditions

Once calibrated, the C-pep-E¹GFP sensor was expressed in β cells to measure the luminal pH of ISGs under basal conditions. The resulting measurements using the phasor-based FLIM approach, confirmed the acidic nature of ISGs, with an average luminal pH of 5.8 ± 0.06 (Fig. 4.5A, B). Rather than relying solely on the barycentre-derived average pH, the use of a lifetime-dependent LUT allowed the visualization of spatial variations within individual cells. This analysis revealed a gradient in granule acidity, with granules located near the plasma membrane exhibiting slightly lower pH values ($\text{pH} = 5.72 \pm 0.06$) compared to those in the cytoplasm ($\text{pH} = 5.83 \pm 0.09$) (Fig. 4.5B). Notable heterogeneity was observed at the single-cell level: while some cells displayed minimal differences in granule pH, others exhibit variations of up to ~ 0.3 pH units. This variability may reflect true biological differences among individual cells or could partly arise from technical limitations, such as the lower spatial resolution along the z-axis. In such cases, granules located at the plasma membrane might be partially included within cytoplasmic regions of interest (ROIs), potentially leading to an underestimation of the actual pH difference. Nonetheless, the observed trend is consistent with the expected acidification changes that occur during granule maturation and trafficking from the TGN towards the plasma membrane²¹. As discussed in the paragraph 1.1, the acidification process is mediated by vesicular ATP-dependent proton pumps (V-ATPase) which play a key role in activating the prohormone convertase PC1/3 and PC2^{149–151}. Treatment with concanamycin, a V-ATPase proton pump inhibitor shifted the pH towards neutrality ($\text{pH} = 7.07 \pm 0.27$) confirms that proton pump is crucial to properly regulate the acidification of the ISGs. By contrast, inhibition of chloride channels with the channel blocker R(+)-IAA-94 did not significantly alter granular pH ($\text{pH} = 5.82 \pm 0.08$) (Fig. 4.5C), suggesting that other counterions and/or chloride channels insensitive to IAA-94 may contribute to granule pH regulation.

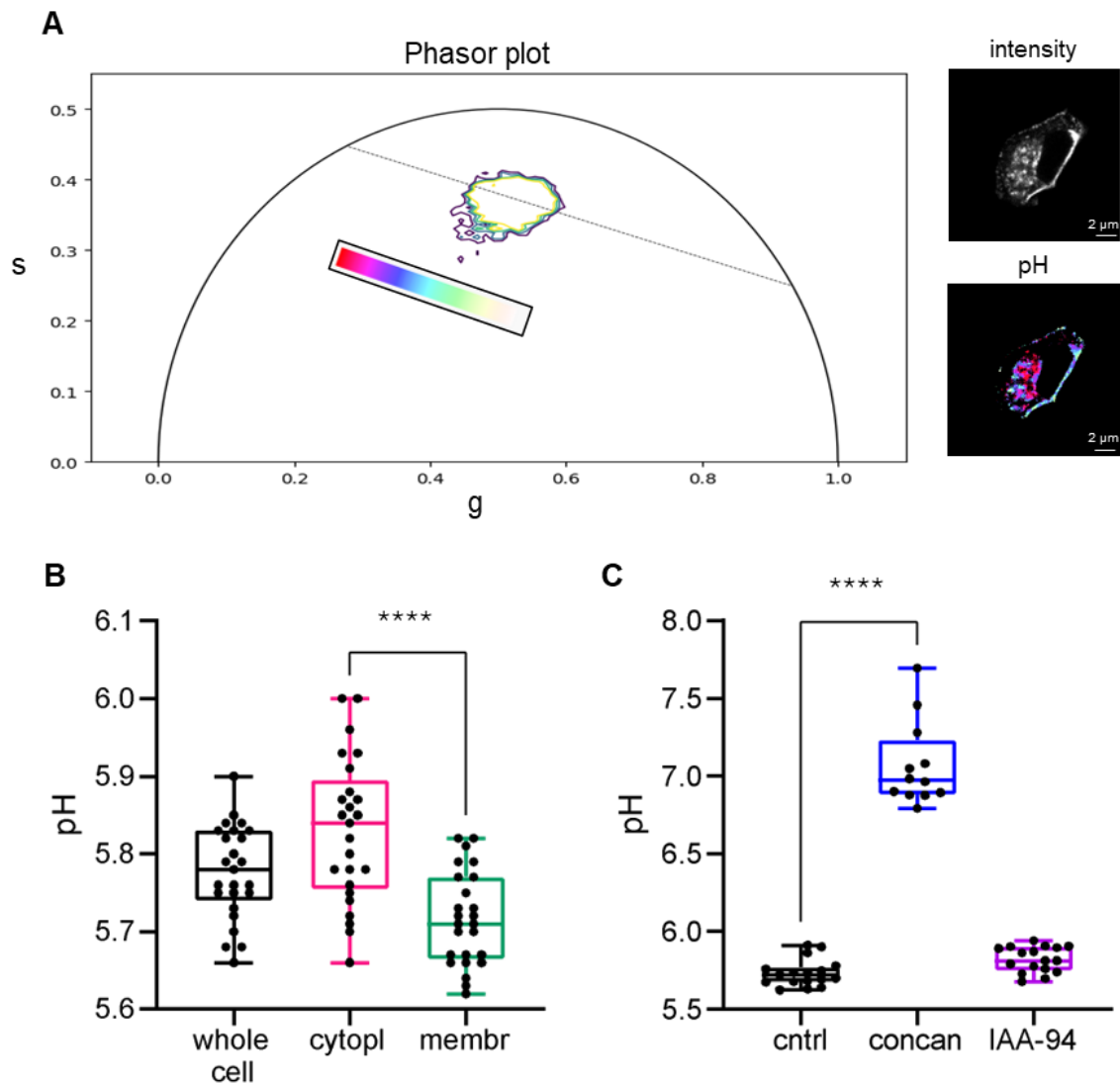


Figure 4.5: Measurement of ISG pH in β cells with controls

(A) Representative β cell expressing C-pep-E¹GFP imaged 48 h post-transfection in standard growth medium. Panels show phasor plot, fluorescence intensity, and corresponding pH map. (B) Box plot (min–max) of average ISGs pH measured in three regions of interest: whole cell, cytoplasm, and plasma membrane ($n = 25$ cells, 4 independent experiments). The analysis on two different zone inside the same cell, highlighted significant differences between the cytoplasmic granules and the ones near the plasma membrane. (C) C-pep-E¹GFP–transfected β cells treated for 1 h at 37 °C with 100 nM concanamycin or 100 μ M R(+)-IAA-94 and imaged by FLIM. Box plots (min–max) show ISGs pH in treated versus control cells ($n = 12$, 17, and 17, respectively; 3 independent experiments). The IAA-94 seemed to have no influence on the calculated pH; instead, concanamycin shifted granule pH towards neutrality.

4.6 ISGs pH after glucose stimulation

Glucose stimulation represents an additional factor reported to influence the luminal pH of insulin secretory granules, though evidence in the literature remains inconsistent. Some studies have proposed that glucose enhances granule acidification^{27,132}, whereas others, notably the work by Eto and co-workers¹³³, observed instead a transient alkalinization of the ISGs lumen following stimulation. To resolve these discrepancies, we assessed the impact of glucose stimulation on ISGs pH in β cells using the phasor-FLIM methodology. β cells were incubated with SAB buffer and first exposed to a low-glucose condition (2.2 mM) for 45 minutes, followed by an acute increase in glucose concentration to a final value of 16.7 mM. To monitor the secretion, FLIM acquisitions were performed on the same cell before glucose addition (0 minutes) and subsequently at 5 and 10 minutes after stimulation (Fig. 4.6A). Upon glucose stimulation, a fraction of the granules are released but, the LUT analysis of the cells highlighted that the remaining granules in proximity to the plasma membrane remains more acidic than the granules in the cytoplasm during the early phase of secretion (Fig. 4.6A). When comparing the granule pH over time, a slight trend toward higher pH was observed, particularly among membrane-proximal granules, but this change did not reach statistical significance. Notably, the pH of membrane-associated ISGs in cells incubated with SAB buffer containing 2.2 mM glucose (5.47 ± 0.17 , in Fig. 4.6B the 0-minute condition) was found to be significantly lower than that measured in cells maintained under standard culture conditions (5.72 ± 0.06 , Fig. 4.6B). To determine whether this acidification resulted primarily from serum starvation or from the reduction in glucose concentration, we conducted a comparative analysis in which cells maintained in complete medium (containing 11 mM glucose) were evaluated alongside cells incubated in SAB buffer supplemented with either 2.2 mM or 11 mM glucose. Interestingly, the pH of membrane-localized ISGs was similarly decreased in cells exposed to SAB buffer even when it was supplemented with 11 mM glucose, indicating that the observed acidification is mainly attributable to the absence of serum components in the buffer rather than to glucose concentration reduction. A possible interpretation of the pH difference observed during the secretory process is that the granules undergo acidification as part of their priming process when translocating to the membrane under glucose stimulation. This observation would be consistent with at least one of the dominant hypotheses already proposed regarding granule maturation and secretion^{131–133}. Our findings demonstrate the reliability and sensitivity of the phasor-FLIM approach combined with E¹GFP in resolving spatially defined pH variations within living β cells, thus providing valuable insight into the acidification dynamics accompanying glucose-induced secretion. However, the limited temporal resolution inherent to FLIM prevents detailed tracking of rapid granule events. This

limitation was addressed in the following chapter by integrating pH sensing with granule tracking to achieve a high-resolution spatiotemporal view of granule dynamics.

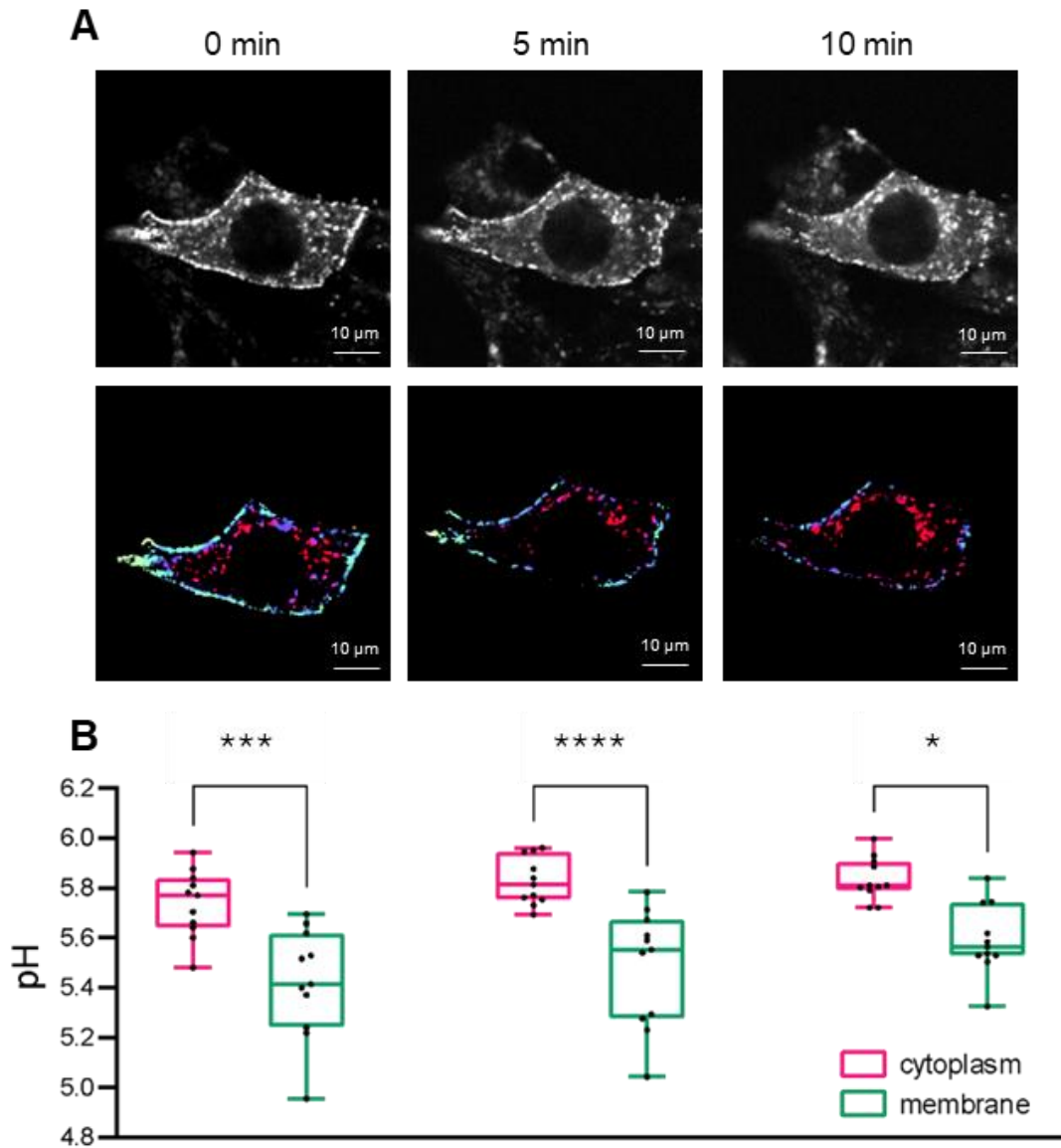


Figure 4.6: ISGs pH measurements during glucose stimulation evaluating the cytoplasm and membrane pH distribution

C-pep-E¹GFP transfected β cells were stimulated with glucose, and the same cell is imaged at 0, 5 and after 10 minutes. **(A)** Intensity images and pH pseudo-coloured images of a representative cell. **(B)** Box plot (min to max) derived from transfected β cells, showing the comparison between granules in cytoplasm (magenta) with granules near the plasma membrane (green) at three different timepoints. N = 11 from 3 independent experiments.

4.7 Evaluation of GSG pH in basal culture conditions

Building upon the experimental framework previously developed to investigate the acidification of ISGs using FLIM, we extended this approach to explore the pH dynamics within GSGs. To our knowledge, this represents the first attempt to directly measure intragranular pH in α cells using a genetically encoded fluorescence lifetime sensor, thereby opening a new avenue for comparative studies of hormone storage and secretion mechanisms. To enable accurate and compartment-specific pH quantification, we engineered a chimeric fluorescent construct by fusing the pH-sensitive E¹GFP probe¹³⁹ to neuropeptide Y¹⁰⁰. The resulting NPY-E¹GFP fusion protein localizes efficiently to GSGs, paralleling the NPY-EGFP construct previously used as a control labelling strategy (see Chapter 2). This design was crucial for ensuring that the fluorescence lifetime signal specifically reflects the luminal pH of glucagon granules rather than cytoplasmic background fluorescence. Experiments were conducted as described for ISGs, using two-photon excitation and phasor-based FLIM analysis. Notably, measuring GSG pH proved technically more challenging than ISG pH. A plausible explanation may be the smaller size of GSGs and their reduced halo⁷⁸, which likely results in lower intragranular sensor accumulation. At this preliminary stage, data were collected from a limited number of α cells ($n = 4$), which precluded statistical determination of population-wide pH averages or spatial comparison comparable to that achieved in β cells. Nevertheless, individual measurements already provided valuable qualitative insights into α cell granule acidification under basal conditions. From the lifetime distributions obtained across all analysed cells, we determined an average fluorescence lifetime of 2.23 ns, corresponding to phasor coordinates $g = 0.562$ and $s = 0.3515$ (Fig. 4.7A). Using the previously established E¹GFP calibration curve (see Fig. 4.4), this lifetime translates into an estimated intragranular average pH of 5.86 for glucagon-containing secretory granules. This value lies within the expected acidic range characteristic of secretory vesicles and is in close agreement with the pH measured in insulin granules under comparable conditions. Interestingly, when examining spatial distributions within individual cells, a slight difference in fluorescence lifetime was detected between cytoplasmic and membrane-proximal granules (Fig. 4.7A, B). Although this variation is not statistically significant at present, due to limited sample size, it may reflect subtle spatial heterogeneities in granule acidification similar to those observed in β cells. Further experiments with larger sample numbers will be required to confirm this trend and to determine whether such differences correlate with distinct stages of glucagon granule maturation or readiness for secretion.

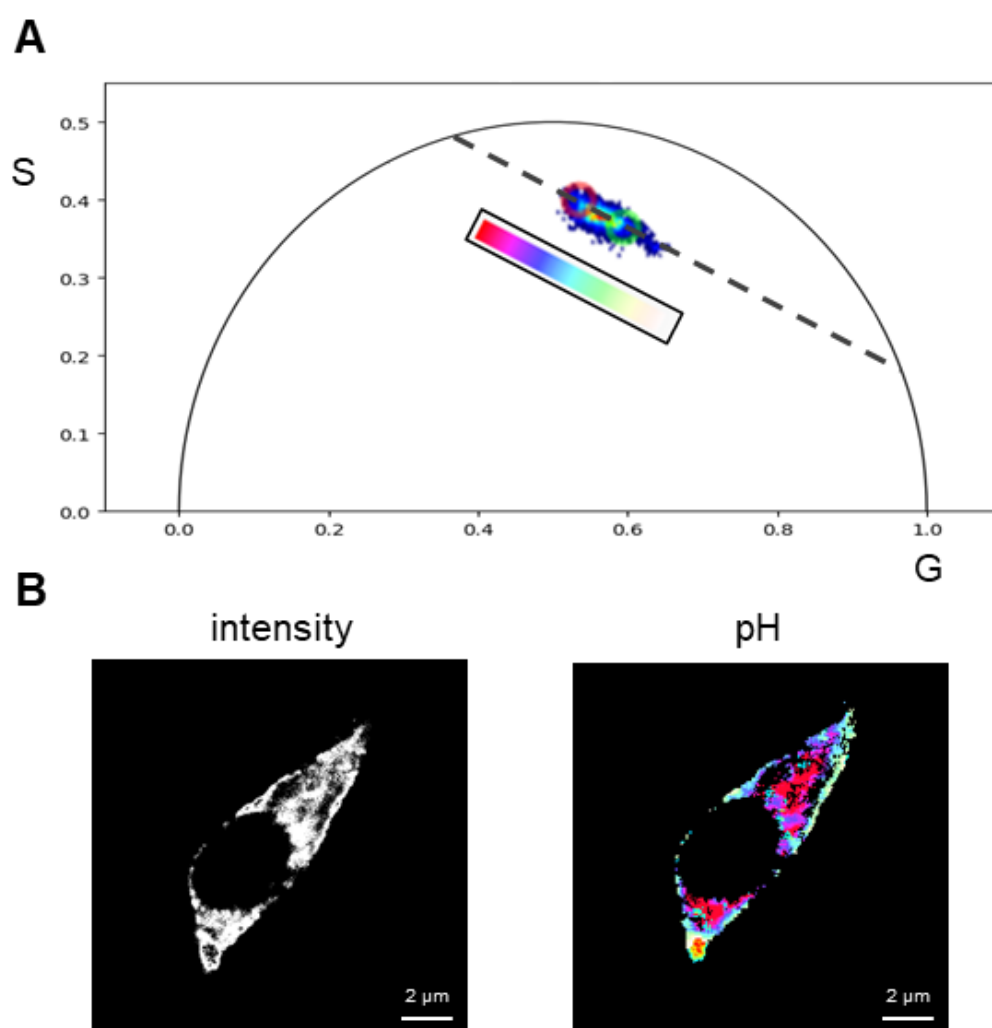


Figure 4.7: FLIM measurements of intragranular pH in NPY-E¹GFP-transfected α cells

(A) Phasor plot of a representative α cell (B). Corresponding fluorescence-intensity image (left) and pseudo-coloured pH map (right).

4.8 Concluding remarks

4.8.1 Calibrated pH measurement of insulin granules

In this study, we established a calibrated and concentration-independent strategy to measure the luminal pH of individual ISGs in living β cells. By combining phasor-FLIM with a genetically encoded, granule-targeted pH sensor (E¹GFP inserted into proinsulin's C-peptide), we obtained robust and quantitative data. Our measurements revealed that insulin granules maintain a distinctly acidic environment, with an average pH of approximately 5.8 under basal conditions. This acidity is actively sustained by the vacuolar H⁺-ATPase, as confirmed by its near-complete neutralization following treatment with the inhibitor concanamycin. Furthermore, we identified a slight but consistent spatial heterogeneity: granules located near the plasma membrane exhibited a more acidic lumen than those deeper in the cytoplasm. This spatial pH gradient suggests that granules may undergo progressive acidification as they traffic toward the membrane, a process likely linked to their maturation and competence for release.

4.8.2 Context and contribution to the field

Our work complements recent findings in the field. Concurrently with our study, a report by Lin and colleagues introduced a novel FRET-based reporter that also highlighted the dynamic and heterogeneous nature of insulin granule pH. Their work effectively pointed out granule subpopulations with distinct pH values. Lin and colleagues developed novel FLIM-FRET sensors (e.g. pHLeon, pHCitron) and targeted them to dense-core vesicles via NPY fusion, enabling subpopulation discrimination and single-granule lifetime readouts. Lin et al. performed in-cell calibration in the cytosol of permeabilized HEK-293T cells which gives valuable sensor characterization but may not fully capture the physicochemical microenvironment of the granule lumen¹⁵². Accordingly, because calibration was performed in the cytosol and targeting relied on an exogenous peptide, direct assignment of absolute luminal pH remains uncertain unless complemented by in-granule calibration and/or quantitative colocalization controls. On the contrary, our approach provide a fully calibrated quantification. The use of a C-peptide-targeted probe ensures high specificity for the insulin granule lumen, addressing potential concerns of mislabeling. Moreover, we performed: quantitative colocalization providing stronger direct evidence of exclusive ISGs luminal localization, negative controls, and both purified-protein and in-cell calibrations.

4.8.3 First pH measurements in glucagon granules

Building on our validated methodology, this study presents the first attempt to measure the luminal pH of GSGs in living α cells. By adapting a similar genetically encoded sensor strategy, we confirmed that GSGs also maintain an acidic luminal environment. The identification of an acidic intragranular pH in glucagon vesicles supports the notion that granule acidification is a conserved feature of hormone maturation and storage across endocrine cell types. The measured pH value (~ 5.9) aligns with the functional requirements of PC and other luminal enzymes that process proglucagon into its active peptide forms^{21,22,27}. Moreover, the observed lifetime heterogeneity suggests potential regulation of luminal pH in response to granule localization or exocytotic competence, a phenomenon documented for β cells but largely unexplored in α cells. While more extensive measurements are needed to fully characterize GSGs acidification, these initial findings open a new avenue for investigating α cell physiology. From a methodological perspective, this proof-of-concept demonstrates that E¹GFP-based FLIM sensing can be successfully adapted to different endocrine cell types, providing a versatile platform for investigating organelle microenvironments *in situ*. Expanding this dataset through quantitative analyses across multiple cells and physiological conditions (e.g., glucose stimulation, paracrine signalling) will allow a deeper understanding of the interplay between granule acidification, trafficking, and hormone release.

4.8.4 Future directions

In summary, our results confirm that the combination of phasor-FLIM and genetically encoded probes is a robust and sensitive method for detecting subtle, spatially resolved pH variations in living endocrine cells. This provides a powerful tool for studying the final stages of secretory granule maturation. While powerful, the intrinsic temporal constraints of FLIM imaging limit its ability to capture rapid pH dynamics. To address this, the next chapter will integrate our pH sensing approach with high-speed granule imaging for a more complete spatiotemporal view. Future developments, such as combining this technique with single-granule tracking, will further enhance our ability to monitor the acidification dynamics throughout the granule lifecycle, from transport to exocytosis.

5. Preliminary observations on granule acidification and future perspectives

A detailed understanding of the acidification pathway is crucial in the context of disease, as defects in granule maturation and acidification are known to impair insulin secretion^{21,27,132}. However, such insights can only be achieved once the underlying mechanisms are thoroughly characterized. The FLIM approach used in the previous chapter is too slow for capturing rapid events, as it requires long integrations that average out fast processes. We therefore adopted a faster confocal strategy to track luminal pH changes in secretory granules at higher temporal resolution. Now, the C-pep-E¹GFP pH reporter was used with a two-channel ratiometric approach, as previously described¹³⁹. The E¹GFP emission was split into two spectral channels, and their intensity ratio was computed pixel-by-pixel to report pH. Specifically, Channel 1 captured 505-570 nm and Channel 2 470-505 nm (implementation details in Material and methods). As in our previous work described in chapter 4, we generated an *in vitro* calibration curve, acquiring E¹GFP in defined pH buffers with this optical configuration, to convert the fluorescence intensity ratios into pH values (Fig. 5.1A, B).

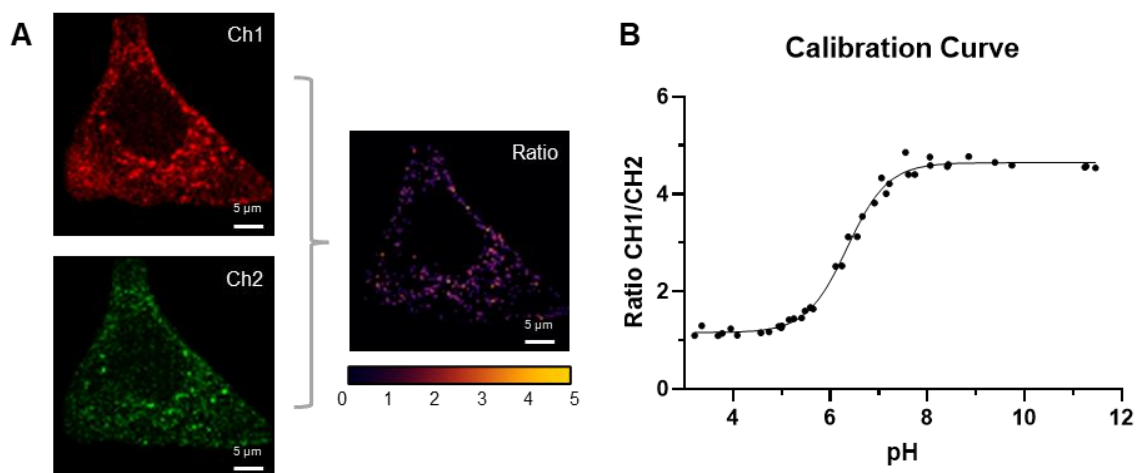


Figure 5.1: Two-channel ratiometric pH imaging with C-pep-E¹GFP in β cells

(A) Explanatory fluorescence images derived from channel splitting represent the emission acquired in Ch1 (red) and Ch2 (green) with the ratio image (Ratio) generated by pixel-wise division of Ch1 and Ch2 signals (using image calculator of ImageJ), producing a spatial pH map of the cell. The ratio was displayed using the Mpl-Inferno LUT in ImageJ. Channel images were processed using the automatic Default threshold to generate binary masks. These masks were then combined with the Image Calculator (using the AND operation) to produce the final mask, which was subsequently applied to the calculated ratio image (Image Calculator with the Divide operation) to obtain the final representation. (B) *in vitro* calibration curve generated by dividing the intensity of two channels measured for recombinant E¹GFP protein in buffer at known pH; Data were fitted using a non-linear regression (log[agonist] vs. response - Variable slope (four parameters) equation).

5.1 Spatial pH heterogeneity in living β cells

Leveraging the ratiometric readout, we next explored spatial patterns of estimated pH in live β cells. Using INS-1E cells expressing C-pep-E¹GFP, we acquired short stacks of images (higher frame rate than FLIM) and generated spatial pH maps. For a coarse spatial stratification, cells were segmented into perinuclear, cytoplasmic, and membrane-proximal regions (Fig. 5.2A-C). The average pH of perinuclear (PN) and cytoplasmic (C) regions were similar with no significant differences (PN pH = 6.02 ± 0.57 n = 160, and C pH = 5.96 ± 0.58 n = 138), while membrane-proximal (M) granules were significantly more acidic (M pH = 5.74 ± 0.6 n = 142) (Fig. 5.2D). The reported numerosity corresponds to individual granules, with approximately 3 to 5 granules detected per region in each analysed image. This analysis revealed a progressive decrease in average pH from the cell interior toward the plasma membrane, consistent with the maturation-dependent acidification trend previously observed by FLIM. In addition, these results highlighted pronounced heterogeneity among granules, each exhibiting distinct pH values (PN Δ pH range = 3.95, C Δ pH range = 3.85, M Δ pH range = 3.37). Examination of individual images further confirmed spatial heterogeneity in pH distribution across granules (Fig. 5.3A, B). Acquisition of a 10-frame time series enabled temporal mapping of granule dynamics. Although membrane-proximal granules generally displayed lower pH values, significant variability persisted throughout the acquisition period (Fig. 5.3C). To capture these rapid and localized pH fluctuations with higher temporal precision, we employed the spectroscopic technique orbital tracking (OT) to monitor individual granules at millisecond resolution, albeit with a reduced cellular field of view.

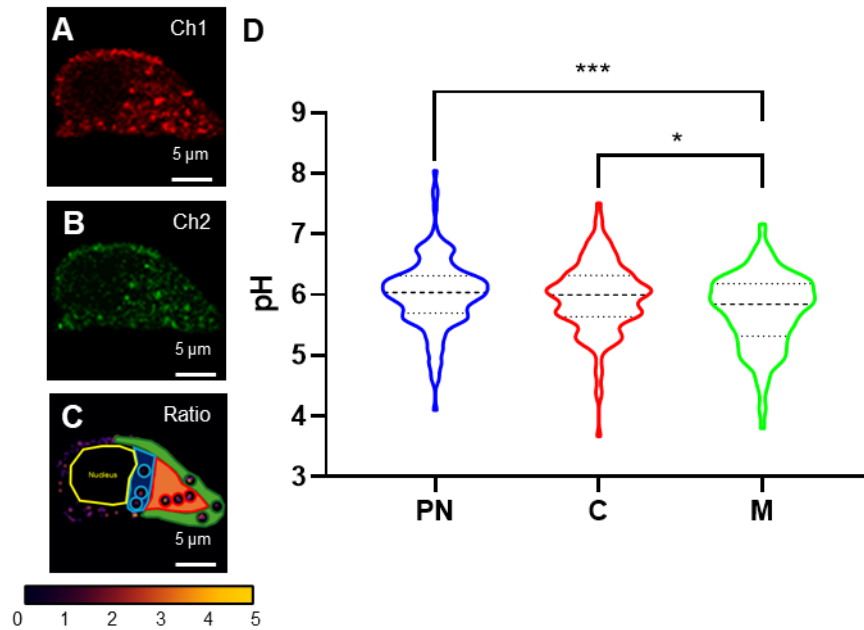


Figure 5.2: Spatial pH heterogeneity resolved by ratiometric confocal imaging

(A-B) Representative β cell imaged in Ch1 (red) and Ch2 (green). (C) The ratio was displayed using the Mpl-Inferno LUT in ImageJ with the three analysis regions delineated: perinuclear (blue), cytoplasmic (red), and membrane-proximal (green). Dots mark example granules. Channel images were processed using the automatic Default threshold to generate binary masks. These masks were then combined with the Image Calculator (using the AND operation) to produce the final mask, which was subsequently applied to the calculated ratio image (Image Calculator with the Divide operation) to obtain the final representation. (D) Violin plots from 50 single-cell frames. Results are perinuclear pH = 6.02 ± 0.57 (n = 160), cytoplasmic pH = 5.96 ± 0.58 (n = 138), and membrane pH = 5.74 ± 0.6 (n = 142) (Mean $\pm$ St. Dev.). This spatial segmentation highlights a decrease in pH towards the membrane; tracking of individual granules over time further reveals fast fluctuations superimposed on this trend.

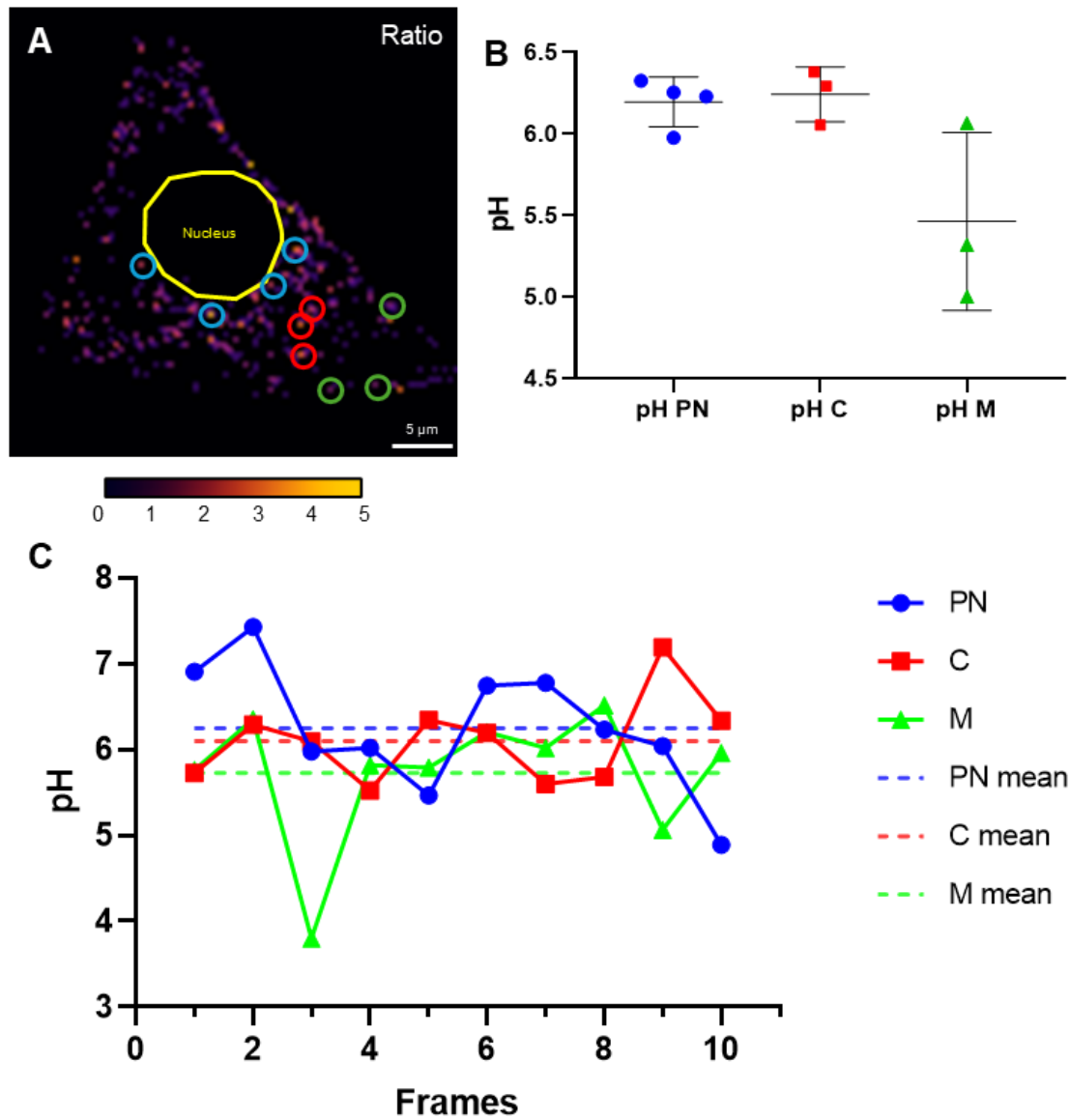


Figure 5.3: Single cell analysis contest and pH change in time

(A) The ratio was displayed using the Mpl-Inferno LUT in ImageJ with representative granules marked in the three different regions. Blue, perinuclear; red, cytoplasm; green, membrane proximity. Channel images were processed using the automatic Default threshold to generate binary masks. These masks were then combined with the Image Calculator (using the AND operation) to produce the final mask, which was subsequently applied to the calculated ratio image (Image Calculator with the Divide operation) to obtain the final representation. (B) Corresponding pH values of single measurement for the highlighted granules, illustrating marked heterogeneity within the same cell. (C) Time series from one β cell showing pH fluctuations for three exemplary granules (one per region). Dashed lines indicate the regional mean values (scan time per frame ~ 0.43 seconds).

5.2 Towards millisecond sampling with feedback-based single granule orbital tracking

To capture rapid dynamics at single-granule level, we combined the ratiometric readout with OT. OT microscopy enables the monitoring of fluorescently labelled particles by steering a laser beam along a circular orbit around a labelled target object and analysing the emitted fluorescence. This approach makes it possible to reconstruct the particle's trajectory, offering valuable information on its motion and interactions within the system. OT functioning is based on a circular motion of the microscope's laser beam around the fluorescent particle (Fig. 5.4A), instead of a raster scan as traditionally happens for standard confocal imaging^{153,154}. To achieve this, a continuous feedback system adjusts in real-time the laser position over the fast-moving labelled object¹⁵⁴. As the particle shifts position inside the orbit, the fluorescence signal measured along the scanning path varies accordingly. In this manner, the feedback-based modulation related the centre of the orbit with the relative position of the particle (Fig. 5.4A)¹⁵⁴. OT can be implemented in both two and three dimensions; in the latter case, the orbit is switched between different axial (z) positions allowing the 3D reconstruction of particle trajectory¹⁵³. This technique enables particle localization with nanometre-scale accuracy while capturing dynamics at millisecond temporal resolution. Moreover, it is quite versatile for different microscopes (i.e. confocal, two-photons) and for a broad range of biological applications such as: intracellular transport, mitochondrial dynamics, inner granule composition and other motility-driven processes. In OT experiments, the fluorescence signal collected along each orbit generates a circular line-scan that serves as a space-time map of the local environment; by applying correlation analyses to these scans, it becomes possible to resolve temporal dynamics and probe interactions with nearby structures¹⁵³. Here, the OT technique is pushed further by combining the tracking ability with the pH ratiometric sensor C-pep-E¹GFP to track granule acidification with a focus on fast pH fluctuations. In this mode, we do not acquire conventional images but “carpets”: the circular orbit is sampled as a line and repeated over time to form a space-time map (see details in Material and Methods). The detection pathway is identical to that used for spatial pH mapping; signals from the two channels are combined (Ch1/Ch2) to compute a ratio-intensity carpet (Fig. 5.4B). As before, an *in vitro* calibration converts carpet values into estimated pH (Fig. 5.4C). OT allowed us to perform high temporal resolution measurement of pH at the single-granule level, providing one pH value per millisecond (Fig. 5.5A). Initial recordings over about 10 seconds revealed a scattered yet consistent dynamic profile, suggesting that the ratiometric signal effectively reflects the rapid changes occurring within individual granules. These traces, converted via the *in vitro* calibration, produced broad pH distributions centred within the expected acidic range with an average pH of 5.5 with a broad

dispersion (pH range width = 6.38, n granules = 36) (Fig. 5.5B). As an internal control, we compared the extent of fluorescence ratio fluctuations observed in live-cell experiments with those measured for the purified E¹GFP protein in calibration buffer. This qualitative comparison revealed markedly larger fluctuations in the intracellular sensor than in the buffer, supporting the idea that the observed variability may arise from biological processes, such as local environmental changes (Fig. 5.5C). Given current numerosity and *in vitro* calibration constraints, these distributions are presented as proof-of-principle rather than definitive measurements. Of note, in small organelles, both reporter protonation kinetics and shot noise can impact apparent pH. Consequently, absolute values and fluctuation amplitudes must be interpreted with caution pending the control suite described below.

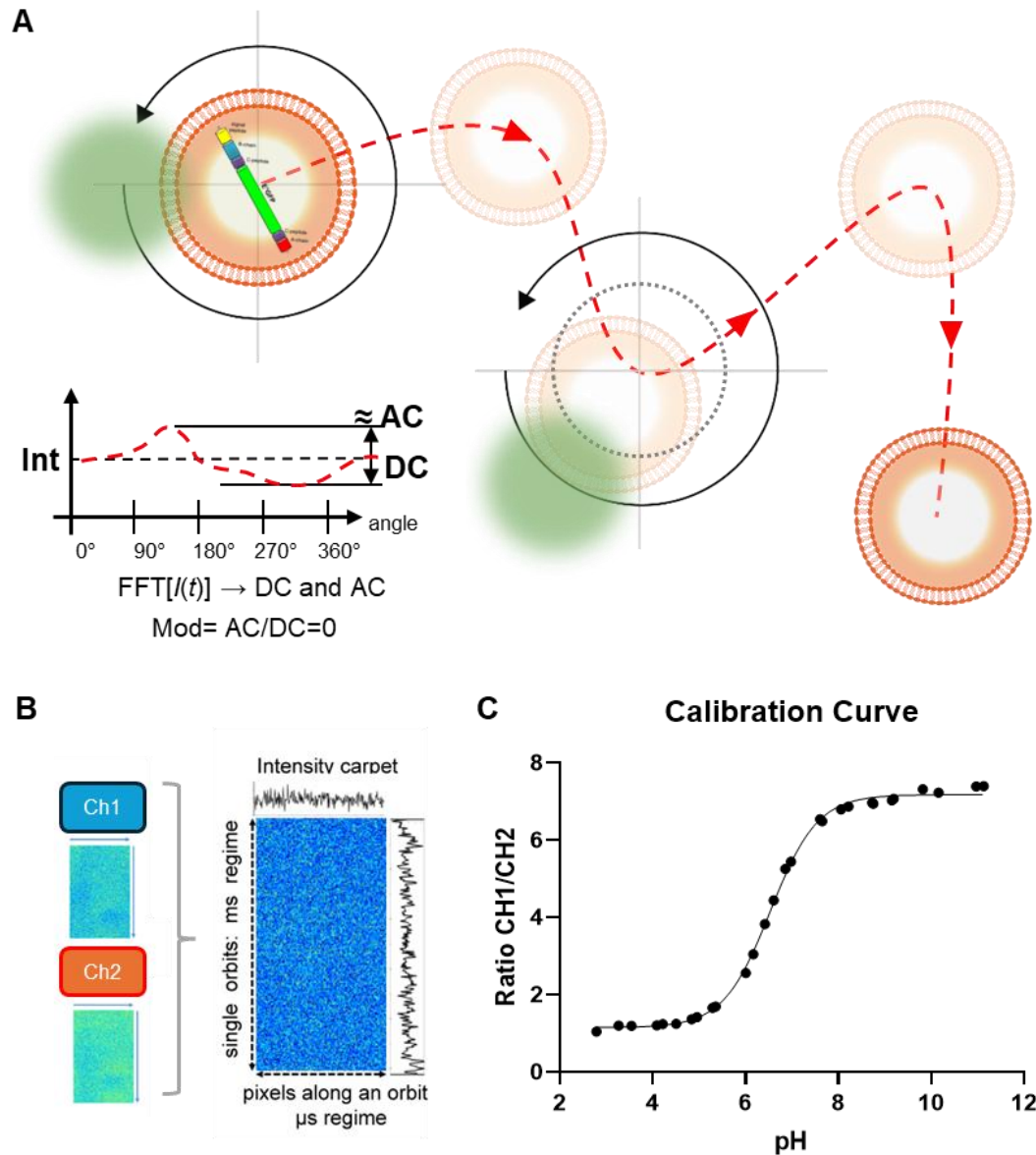


Figure 5.4: Schematic representation of the workflow and *in vitro* E¹GFP calibration curve

(A) Schematic representation of the OT approach used here to follow E¹GFP-labelled ISGs in space and time by feedback-based imaging. The red dotted line indicates the granule trajectory, and the green circle represents the laser beam around the granule. When the granule move away from the central position (grey dotted circle), the feedback restores the centrality of the laser beam orbit. (B) The ratio of the two channels lead to an intensity carpet which decodify the information about the ratio (Ch1/Ch2) in time. (C) *in vitro* calibration curve made with E¹GFP protein in buffer at defined pH. Data were fitted using a non-linear regression (log[agonist] vs. response - Variable slope (four parameters) equation.

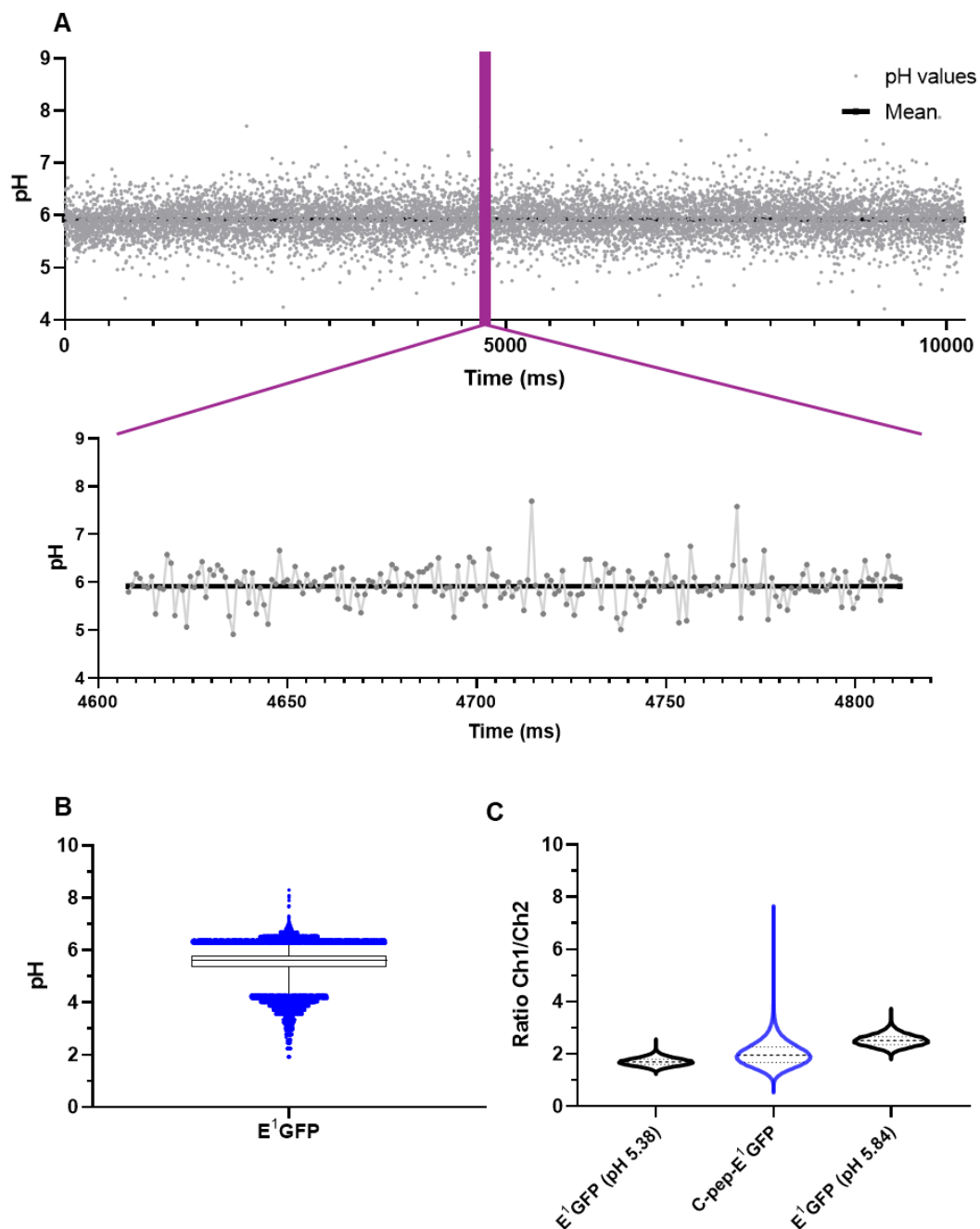


Figure 5.5: Orbital tracking preliminary results

(A) Example ratio (Ch1/Ch2) time series from a single granule acquired with orbital tracking with corresponding mean value (mean pH = 5.9); zoomed segment illustrate millisecond-scale fluctuations. (B) Ratio traces converted post hoc to pH values using the *in vitro* calibration yield distributions centred in the acidic range (i.e., ~5.5) with a broad dispersion (pH range width = 6.38, n granules = 36 imaged for 10 seconds). (C) Comparison of ratio fluctuations between live granules (C-pep-E¹GFP) and the recombinant protein in buffer references at nearby pH values shows larger variance in cells than in calibration points, indicating contributions beyond calibration/geometry (likely biological dynamics).

5.3 Future controls and validation plan

To reinforce the quantitative reliability and reproducibility of our findings, we will perform the following prospective controls.

5.3.1 Acquisition and analysis robustness

We will maintain fixed acquisition parameters (laser power and dwell time) and periodically validate the ratio-to-pH conversion using calibration buffers. A full calibration will be repeated at regular intervals or if instrument drift is detected, ensuring all datasets remain comparable on a consistent pH scale. Furthermore, an *in vivo* calibration using buffers and nigericin will be performed to corroborate the established conversion curve.

To minimize sampling bias, we will pre-define the number of granules to be analysed per region (membrane-proximal vs. interior) and select fields using random or grid-based criteria.

To differentiate biological variability from measurement noise, we will quantify the variance expected from shot noise and apply corresponding confidence bands to each trace. Fluctuations confined within these bands will be attributed to noise; significant excursions beyond them will be interpreted as biological events.

Finally, τ (the E¹GFP protonation/deprotonation time constant) will be estimated *in silico* and, where feasible, cross-checked *in vitro* using independent measurements. This will allow us to define the fastest resolvable changes. Fluctuations faster than $\sim\tau$ cannot be faithfully tracked by the sensor and will be binned or down weighted in the analysis.

5.3.2 pH-insensitive control (C-pep-E⁰GFP), V-ATPase inhibition and liposome-encapsulated E¹GFP

To isolate biological variability from instrumental or analysis artifacts, we will express the pH-insensitive variant E⁰GFP (inserted within the C-peptide). We will then acquire ratiometric images and OT carpets using the identical setting employed for E¹GFP. If the apparent fluctuations collapse to a flat, calibration-consistent baseline, this will confirm that the variability observed with C-pep-E¹GFP reflects genuine biology, not optical or processing artifacts.

To determine if proton-pump activity contributes to the pH dynamics, we will acutely inhibit the V-ATPase (using concanamycin A or bafilomycin A1) under matched imaging conditions. We will record baseline ratio carpets and then monitor the response post-inhibition. We expect a progressive luminal alkalinization and, critically, a sharp reduction in the amplitude of fast, ratio-derived pH

fluctuations. Quantitatively, we will compare variance before and after treatment. A reproducible decrease in amplitude will demonstrate that V-ATPase activity is a primary driver of short timescale pH variability.

In the end, we will employ an *in vitro* control: purified E¹GFP encapsulated at a defined pH in liposomes (size-matched to granules). We will then record ratio carpets under stationary conditions. Lacking pumps and channels, these liposomes provide an ideal control system where any fluctuation arises solely from measurement noise. The minimal fluctuations in this system will define the instrumental baseline, allowing any excess variance in live cells to be confidently attributed to genuine granule pH dynamics.

5.4 Concluding remarks

In this final chapter of the thesis, we assembled a workflow to probe granule acidification, by combining spatial ratiometric imaging and high temporal resolution OT. By exploiting the C-pep-E¹GFP sensor, we generated spatial pH maps in living β cells. This allowed the observation of dynamic processes occurring at the subcellular level and revealed a gradual acidification gradient from the perinuclear region toward the plasma membrane, consistent with previous evidence of progressive granule maturation.

At the same time, we consistently observed substantial granule-to-granule variability within the same cell and along the explored time window, suggesting that individual granules may experience distinct local microenvironments. This heterogeneity suggested that insulin granule may experience larger-than-expected pH fluctuations, overlaid on the gradual acidification process involved in granule maturation; however, the magnitude of these fluctuations in such confined organelles remains unclear.

A theoretical analysis on small unilamellar vesicles (SUVs) suggests that pH fluctuations increase as vesicle size decreases and are influenced by buffering capacity and surface potential¹⁵⁵. This would tentatively point to non-negligible pH fluctuations in insulin granules, which are small and contain a dense core that limits accessible lumen. Conversely, a recent work on stochastic simulations of vesicle acidification¹⁵⁶ reports modest dispersion (typically ~ 0.1 - 0.3 pH units), arguing that much of the apparent variability in optical readouts may arise from dye protonation kinetics and photon shot noise rather than true proton-number changes.

Our OT traces, which reveal broad distributions but also depend on sensor photophysics and counting statistics, likely sit between these two viewpoints. Given these constraints, we interpret our current data as proof of principle: OT coupled to ratiometric sensing can follow fast pH dynamics at single-

granule level, but absolute amplitudes must be treated with caution. The planned controls discussed above (pH-insensitive C-pep-E⁰GFP, V-ATPase inhibition with concanamycin and/or bafilomycin, and liposomes with purified E¹GFP) along with noise propagation and sensor-kinetics estimation, will be essential to disentangle biological variability from measurement limit. These measurements represent, to our knowledge, the first experimental evidence of fast pH fluctuations in individual confined organelles, a behaviour not captured by conventional imaging, with magnitude bounds to be finalized upon completion of planned controls.

To conclude, the dual approach (spatial maps and OT traces) provides a practical framework to study granule acidification across space and time. With the forthcoming validation steps and increased replication, this platform will enable quantitative, confidence-bounded estimates of pH dynamics and, ultimately, this work should clarify how local luminal conditions relate to granule maturation and β cell function, with potential extension to other organelles and cell types.

6. Materials and Methods

6.1 Cell culture

Alpha TC1 clone 9 (α TC1-9) cells (ATCC, CRL-2350)^{85,86} were cultured at 37°C in a humidified atmosphere with 10% CO₂. They were maintained in DMEM low-glucose medium (Gibco, cat# 11880036) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 15 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin-streptomycin, 0.1 mM non-essential amino acids (NEAA), and 0.02% bovine serum albumin (BSA). For β -cell-like cultures, we used INS-1E cells⁸⁷, kindly provided by Prof. C. Wollheim (University of Geneva, Medical Center). INS-1E β cells were grown in RPMI 1640 medium (Gibco, cat# 11835105) containing 11.1 mM D-glucose, supplemented with 10 mM HEPES, 100 U/mL penicillin-streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% heat-inactivated FBS, and 50 μ M tissue culture-grade β -mercaptoethanol. They were maintained at 37°C with 5% CO₂ in a humidified atmosphere. MIN6 cells⁸⁸ were grown in DMEM high-glucose medium (4.5 g/L = 25 mM) (Gibco, cat# 31053) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin. The day before the experiment MIN6 cells were switched to DMEM supplemented as above but containing 11 mM glucose.

6.1.1 Transfection protocol and treatments

For transfection, α TC1-9 cells were transfected with NPY-EGFP (EGFP-tagged neuropeptide Y plasmid, a gift from Justin Taraska; Addgene plasmid #74629 [<http://n2t.net/addgene:74629>; RRID: Addgene_74629])¹⁰⁰. INS-1E β cells were transfected with C-pep-EGFP¹⁰¹. The transfections were performed by plating α TC1-9 cells on 22-mm glass-bottom dishes (WillCo Wells, cat# HBST-3522) and INS-1E β cells on ibiTreat μ -Dish 35 mm plates (Ibidi, cat# 81156) and 1.5 μ g of plasmid DNA per well was used for both the transfections. The following day, transfection was carried out using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Cells were imaged 24-48 h post-transfection. Drugs were supplemented to the standard culture medium as follows: Concanamycin (MedChemExpress cat #HY-N1724) was used at 100 nM for 1 h at 37 °C and R(+)-IAA-94 (MedChemExpress cat #HY-12693) at 100 μ M for 1 h at 37 °C.

6.1.2 Plasmids construction

E¹GFP protein harbours two mutations (T65S and T203Y) compared to EGFP¹³⁹. Therefore, to generate the C-pep-E¹GFP and the NPY- E¹GFP constructs, C-pep-EGFP plasmid¹⁰¹ and NPY-EGFP

plasmid were subjected to two rounds of site-directed mutagenesis using QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies). The forward sequence for the T65S mutation was 5'-CCCACCCTCGTGACCACCCTGAGCTACGGCGTGCAGTGCTTC-3', for the T203Y 5'-CAACCACTACCTGAGCTACCAGTCCGCCCTGAG-3'. For the bacterial expression of recombinant His-tagged E¹GFP, the E¹GFP sequence was amplified from C-pep-E¹GFP construct by PCR and cloned NdeI/BamHI in pET28c+ to generate pET-His-E¹GFP plasmid.

6.1.3 Expression and purification of recombinant E¹GFP

pET-His-E¹GFP construct was transformed in BL21 (DE3) competent cells (Invitrogen). Cells were grown at 37 °C till OD600 of about 0.6, and protein expression was induced by the addition of 250 µM IPTG for 24 h at 28°C. Cells were harvested by centrifugation and frozen at -20°C. Cell pellet was resuspended in ice-cold lysis buffer (40 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM MgCl₂ supplemented with EDTA-free protease inhibitor cocktail (Roche) and DNaseI) and lysed by sonication on ice followed by 1 h treatment with 1% Triton-X100 at 4°C. The bacterial lysate was cleared by centrifugation and filtered through a 0.2 µm filter before loading onto a Bio-Scale Mini Profinity IMAC Cartridge (Bio-rad cat# 7324614) in a fast protein liquid chromatography system (AKTApur, GE Healthcare). The His-tagged protein was eluted using a 0-500 mM imidazole gradient. Imidazole was then removed by buffer exchange in 20 mM diethanolamine pH = 8.5. Protein purity was evaluated by SDS-PAGE, and the concentration was determined by UV absorption measurements using the extinction coefficient calculated in¹³⁵. The absorption spectrum was recorded at RT on a Jasco V550 spectrophotometer (JASCO, Easton, MD, USA) with the following collection parameters: bandwidth 2 nm, scanning speed 1000 nm/min, and data resolution 1 nm.

6.2 Live-cell imaging

For iMSD and SPT experiments, glucagon and insulin granules were labelled with ZIGIR, a fluorescent probe that targets zinc-rich granules⁹⁹. αTC1-9 and INS-1E β cells were plated on 22-mm glass-bottom dishes and ibiTreat µ-Dish 35 mm plates, respectively, and allowed to adhere overnight before labelling and imaging. For labelling, cells were incubated with 1 µM ZIGIR in maintaining medium and after 15 minutes of incubation, unless otherwise specified, imaging was performed. For cytoskeletal labelling, cells were treated with 10 µM SiR-Tubulin (Spirochrome, cat# SC002¹⁵⁷) for 30 minutes, followed by 10 µM Nocodazole (Sigma-Aldrich, cat# 487928) and 1 µM ZIGIR in complete growth medium for an additional 30 minutes before imaging. Similarly, actin filaments were labelled with 10 µM SiR-Actin (Spirochrome, cat# SC001¹⁵⁷) for 30 minutes, followed by treatment with 10 µM Latrunculin-B (Sigma-Aldrich, cat# 428020) and 1 µM ZIGIR in complete

growth medium for another 30 minutes before imaging. In both cases, image acquisition was completed within 1 hour and 30 minutes. For iMSD labelling comparison, image stacks were acquired using a Leica SP5 inverted confocal microscope, exciting ZIGIR at 514 nm and EGFP at 488 nm. A 100× (N.A. 1.3) oil-immersion objective was used, with the pinhole aperture set to 1 Airy. Each stack consisted of 500 frames, acquired with a 204 ms acquisition time per frame and a 1400 Hz line frequency. iMSD and SPT experiments were conducted using two confocal microscopes with equivalent tracking capabilities. Imaging was performed on an inverted Zeiss LSM 800 confocal microscope (Jena, Germany), exciting ZIGIR at 561 nm and collecting fluorescence between 590-700 nm. The pinhole aperture was set to 1 Airy (53 μ m), using a 63× (N.A. 1.4) oil objective. Additionally, a Leica Stellaris 8 confocal microscope was used, exciting ZIGIR at 545 nm and collecting fluorescence between 565-732 nm. Each stack consisted of 500 frames at 256×256 pixels resolution, with an acquisition time of approximately 200 ms per frame. Live-cell imaging was performed at 37°C under controlled CO₂ conditions.

6.3 Immunostaining and colocalization analysis

α TC1-9 cells were plated on glass coverslip and transfected with NPY-EGFP. After 24 hours, the cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature (RT), followed by permeabilization with PBS containing 0.1% Triton X-100 (PBST) for 10 minutes at RT. To block non-specific binding, cells were incubated with 2% bovine serum albumin (BSA) for 30 minutes at RT. For immunostaining, cells were incubated with a mouse anti-glucagon primary antibody (Boster, cat# MA1047) diluted 1:200 for 2 hours at 37 °C, followed by incubation with an anti-mouse Alexa Fluor 647 secondary antibody (ThermoFisher, cat# A31571) diluted 1:500 for 1 hour at RT in the dark. For STORM imaging, we applied the same fixation protocol described above to both α TC1-9 cells and INS-1E cells. For STORM immunostaining, α TC1-9 cells were incubated with anti-glucagon primary antibody as previously described, while INS-1E cells were incubated with an anti-insulin primary antibody (Immunological Sciences, cat# AB-84377) diluted 1:200 for 2 hours at 37°C. Both primary antibody incubations were followed by incubation with an anti-mouse Alexa Fluor 647 secondary antibody (ThermoFisher, cat# A31571), diluted 1:500, for 1 hour at RT in the dark. To validate the specificity in labelling glucagon granules, colocalization analysis was performed. Colocalization comparison was performed into two steps because ZIGIR cannot be used on fixed samples⁹⁹. First it was performed between NPY-EGFP and ZIGIR in living α TC1-9 cells, followed by a comparison between anti-glucagon and NPY-EGFP in fixed α cells. Manders and Pearson coefficients were calculated using the BIOP JACoP plugin for ImageJ, applying the Otsu method.

6.4 Insulin and glucagon secretory granules dSTORM imaging

A Nikon N-STORM TIRF microscope (Nikon Instruments), equipped with a 100× oil immersion objective (CFI Apo TIRF 100×, NA 1.49, oil; Nikon), was used to acquire 20,000 frames in TIRF illumination mode. Images were captured at a digital resolution of 256×256 pixels, with a pixel size of 158.7 nm. Acquisitions consisted of 5,000 cycles, each including one activation frame with a 405 nm excitation laser, followed by three readout frames with a 647 nm excitation laser. Excitation intensities, measured at object to ~ 0.5 – 1 KW/cm² for the 647 nm readout laser (MPB Communications) and ~ 35 W/cm² for the 405 nm activation laser (Oxiux LBX-405). Images were detected using an EMCCD camera (Andor iXon DU-897; Andor Technologies) with EM gain set to 300 and a temporal resolution of 30 ms. The STORM imaging buffer was prepared by filtering and storing at 4°C a solution of 690 μ L of 50 mM Tris buffer (pH 8.0), containing 10 mM NaCl and 10% w/v glucose. Immediately before use, 3.5 μ L of GLOX solution and 3.5 μ L of 2-mercaptoethanol were added. The final solution was then applied to the petri dishes, which was sealed with aluminium tape. The GLOX solution was prepared by dissolving 14 mg of glucose oxidase and 50 μ L of catalase (17 mg/mL) in 200 μ L of STORM imaging buffer. It was stored at 4°C for a maximum of 14 days. Acquired dSTORM stacks were processed using ThunderStorm, a Fiji (ImageJ) plugin for PALM and STORM data analysis. First, acquisition properties were configured in the “Camera setup” menu: pixel size = 158.7 nm, photoelectrons per A/D count = 2.5, base level = 100 counts, and EM gain = 300. Localization was performed using the default algorithm, and results were refined through post-filtering in ThunderStorm, including a) Removal of the first 500 frames; b) drift correction by correlation; c) merging of reactivated molecules (max distance: 20 nm, max off frames: 1, limited frames per molecule) d) Removal of localizations with sigma > 130 nm OR uncertainty > 40 nm. Filtered molecule localizations were first analysed using a ClusDoC algorithm for clustering¹⁵⁸ installed in MATLAB, based on DBSCAN algorithm for clusterization. The parameters for clusterization were set as: epsilon = 50 nm and minPts = 8, respectively representing the maximum distance between two points for them to be considered neighbours and the minimum number of neighbouring points required to form a cluster. Each identified cluster was considered a secretory granule. The cluster contour is determined by creating a 2D histogram of localization points within the cluster as described in ClusDoc reference paper. The boundary of this region is used as the cluster contour for measuring single granule diameter, approximating it as a circle through a custom-made MATLAB script.

6.5 Secretion assays

6.5.1 INS-1E cell insulin secretion assay

INS-1E cells were seeded onto Ibidi plates and cultured until they reached 75-80% confluence on the day of the experiment. Cells were washed twice with KRBH supplemented with 2.5 mM glucose and pre-incubated in this low-glucose medium with 1 μ M ZIGIR for granule labelling for 30 minutes at 37°C and 5% CO₂. KRBH working buffer was based on the Krebs-Ringer physiological buffer, composed of 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 10 mM HEPES, 5 mM NaHCO₃, 1.5 mM CaCl₂, 0.1% BSA, at pH = 7.4. Following pre-incubation, confocal imaging was performed under low-glucose conditions for up to 30 minutes. To induce secretion, glucose was added to the KRBH medium to reach 16.7 mM (high-glucose condition), and imaging started 3 minutes post-stimulation for an additional 30 minutes. For a negative control, the same protocol was applied to α TC1-9 cells, which were plated on WillCo plates instead of Ibidi plates. Moreover, for FLIM experiments, C-pep-E¹GFP transfected INS-1E cells were incubated for 45 min in SAB buffer (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 1.16 mM MgSO₄, and 20 mM HEPES (pH 7.4)) containing 2.2 mM glucose prior to microscope acquisitions, then glucose was added to reach a final concentration of 16.7 mM for glucose stimulation.

6.5.2 α TC1-9 cell glucagon secretion assay

For glucagon secretion experiments, α TC1-9 cells were seeded on WillCo plates two days before the experiment (Day 0) to reach 75-80% confluence by the day of the experiment.

- Day 1 (Starvation): Growth medium (DMEM, see Cell Culture section) was replaced with the same formulation of DMEM used for cell growth, except for a reduced glucose concentration (2.5 mM).
- Day 2 (Experiment): Cells were washed twice with KRBH + 2.5 mM glucose and pre-incubated in this medium at 37°C and 5% CO₂ for 45-60 minutes. Image stacks were acquired during this period for the control condition, with 1 μ M ZIGIR used for granule labelling.

For the treatment condition, after pre-incubation, cells were washed with KRBH (0 mM glucose), then incubated in KRBH supplemented with 1 mM glucose to induce glucagon secretion, along with 1 μ M ZIGIR for granule labelling. Image acquisition was completed within 30-40 min to pair the stimulation of insulin granule release. For the negative control, the same protocol was applied to INS-1E cells. To evaluate glucagon secretion, α TC1-9 cells were seeded in 24-well tissue culture plates

and subjected to the above-described stimulation protocol. A 1 mM glucose concentration was used to mimic hypoglycaemic condition, while 6 mM glucose served as control. After 45 minutes of stimulation, cell cultures supernatants were collected, and glucagon levels were quantified using a Glucagon ELISA kit (Mercodia, cat #10-1281-01). To account for differences in cell number and protein content, glucagon secretion values were normalized to total protein content measured by Pierce BCA protein assay kit (ThermoFisher, cat #23225).

6.6 pH sensor calibration *in vitro* and in living cells

Titration of E¹GFP lifetime versus pH was performed by diluting recombinant E¹GFP to a final concentration of 2.1 μ M in a citrate (2 mM)/phosphate (10 mM) buffer adjusted to the desired pH by addition of 1 M NaOH. WillCo-dish[®] Glass Bottom Dishes (WillCo Wells, cat# HBST-3522) were incubated with 2% BSA for about 40 min at 37°C to avoid protein sticking to the dish surface. For sensor calibration in living cells, C-pep-E¹GFP transfected INS-1E cells were incubated for 10 min with an enriched K⁺ buffer containing 120 mM potassium gluconate, 40 mM sodium gluconate, 20 mM HEPES, 0.5 mM CaCl₂, 0.5 mM MgSO₄ adjusted to the desired pH with NaOH and supplemented with 10 μ M nigericin. The final pH value used for the calibration curve was measured right after the FLIM acquisition.

6.7 Spectroscopic and lifetime measurements of pure low-pH and high-pH states *in vitro*

Absorption spectroscopy of purified recombinant E¹GFP was carried out according to the procedure reported in ref¹³⁵. Almost pure A' and A forms (see text for details) were addressed by setting the pH to 4.85 or 8, respectively by a citrate (2 mM)/phosphate (10 mM) buffer adjusted to the desired pH by addition of 1M NaOH. Emission spectra were obtained by exciting the protein at 405 nm and collecting emission in the 450-650 nm range with a 5 nm step. Lifetime decays of the protein were obtained by exciting at 405 nm and collecting emission in the 480-550 nm range. In both cases, measurements were carried out in a Leica TCS SP5 SMD inverted confocal microscope (Leica Microsystems AG, Buffalo Grove, IL) equipped with an external pulsed diode laser for excitation at 405 and 470 nm and a time correlated single photon-counting acquisition card (PicoHarp 300; PicoQuant, Berlin, Germany) connected to internal spectral detectors. The laser repetition rate was set to 40 Hz. Acquisitions lasted until 100-200 photons per pixel were collected, at a photon-counting rate of 100-500 kHz.

6.8 Two-photon microscopy, phasor-FLIM measurements, and data analysis

Phasor-FLIM measurements were carried out on an Olympus FVMPE-RS microscope coupled with a two-photon Ti:sapphire laser with an 80-MHz repetition rate (MaiTai HP, SpectraPhysics) and FLIMbox system for lifetime acquisition (ISS, Urbana Champaign). Measurements were performed at 37 °C in a humidified 5% CO₂ atmosphere. E¹GFP was excited at 800 nm, and the emission was collected by using a 30× planApo silicon immersion objective (NA = 1.0) in the 500-540 nm range. Calibration of the ISS FLIMbox system was performed by measuring the known mono-exponential lifetime decay of Fluorescein at pH = 11.0 (i.e. 4.0 ns upon excitation at 800 nm, collection range: 500-540 nm). To prepare the calibration sample, 100 mM Fluorescein solution in EtOH was diluted 1:5000 in 0.1 M NaOH. For each measurement, a 512 × 512 pixels image of FLIM data was collected until about 100k counts in the brightest pixel were achieved. The phasor analysis of experimental lifetime acquisitions was performed by using custom dedicated routines implemented in Python 3.6 as described in ref¹⁴⁸.

6.8.1 Two-photon microscopy for spatial pH and orbital tracking

To perform spatial pH analysis and Orbital tracking (OT) the same Olympus FVMPE-RS microscope [coupled with a two-photon Ti:sapphire laser with an 80-MHz repetition rate (MaiTai HP, SpectraPhysics)] in combination with FLIMbox system (ISS, Urbana Champaign) employed for FLIM, was used. To acquire OT carpets, we employed track particle module of SimFCS in combination with Olympus two-photon microscope. Spatial pH images were acquired with a dwell time of 40 μs/pixel and a pixel size of 400 nm. Each image was cropped to variable frame sizes (ranging from 70 × 70 to 90 × 90 pixels) to include a single cell, resulting in a variable acquisition time per frame between 0.277 s and 0.428 s. For each cell, 10 sequential images were recorded. In OT experiments, each orbit was sampled over a 1024 μs period with 128 measurements, using on the Olympus set up a dwell time of 8 μs/pixel to match the mandatory parameter of SimFCS. A radius of 4 and a search radius of 28 were selected in track particle module of SimFCS based on preliminary trials. For both experimental conditions, the optical pathway was configured to split the fluorescence signal into two channels, exploiting the ratiometric properties of the C-pep-E¹GFP sensor. A beam splitter first removed emitted light above 570 nm, and the remaining signal was then divided at 505 nm by a second beam splitter. Wavelengths between 505 nm and 570 nm were directed to Channel 1, while shorter wavelengths were further filtered with a 510 ± 40 nm bandpass filter, isolating the 470-505 nm range for Channel 2.

6.8.2 Orbital tracking carpet analysis

To properly analyse the carpets resulting from orbital tracking acquisitions, a custom-made MATLAB script was developed by our collaborator and divided in two main steps:

1. **Main_batch**: this initial step enables the selection of the “.b64” files exported from the FLIMbox system and processes them in pairs, each representing the two detection channels.
2. **Carpet_operation**: in this step, the total number of photons along each orbit is collapsed to obtain a single value per each orbital scan (i.e. per carpet row), providing a temporal resolution of approximately one millisecond, while the intensity ratio between channel 1 and channel 2 is calculated.

Finally, **Main_batch** merges the results from all processed datasets into an Excel file summarizing mean, standard deviation, variance, and median ratio values for each recording.

6.9 Statistical analysis

To evaluate data distribution, Shapiro-Wilk and Mann-Whitney tests were applied. Normally distributed data were analysed using an unpaired t-test, whereas Mann-Whitney or Kolmogorov-Smirnov nonparametric tests were used for non-normal datasets. For multiple group comparisons of trajectory categories (Chapter 3), multiple t-tests with Holm–Sidak correction ($\alpha = 0.05$) were performed, analysing each data row independently without assuming equal standard deviations. In multiple comparisons for FLIM analyses (Chapter 4) and pH spatial analysis (Chapter 5), one-way ANOVA was conducted after verifying normality. All analyses were performed using GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). A p-value < 0.05 was considered statistically significant. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; non-significant results are not shown. All experiments were repeated at least three times (except for the preliminary study on glucagon granule pH) and reproducibility was confirmed.

7. References

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