Super-resolution single molecule localization microscopy of the exocytotic machinery underlying insulin secretion.

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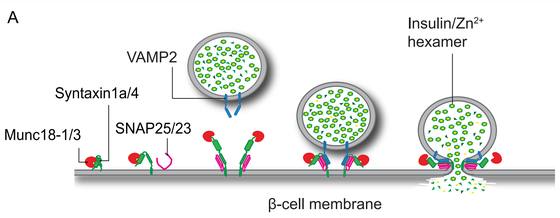
ABSTRACT

Insulin secretion requires the fusion of insulin-containing vesicles with the plasma membrane through the process of exocytosis. Membrane fusion is driven by the action of SNARE proteins. In β-cells, various SNARE isoforms exist; these include: syntaxin1a, 3 and 4 and SNAP-23 and SNAP-25 (t-SNAREs) at the plasma membrane, together with VAMP2 on the vesicular membrane. Type 2 diabetes (T2DM) occurs when β-cells can no longer compensate for the prolonged high elevations of glucose, leading to insufficient insulin secretion.

Single molecule localization microscopy (SMLM) utilizes photoswitchable fluorescent probes to control the density of fluorescent emitters. By imaging photoswitching events repeatedly over thousands of frames we can build up a precise map of fluorophore positions. Combining SMLM with diffraction-limited microscopy we have examined the co-location of t-SNARE proteins and insulin vesicles in HIT-T15 cells. Using GFP tagged insulin, or immunostaining for insulin, we were able to observe the relative spatial distribution of vesicles to individual t-SNARE molecules.

Applying a Bayesian approach of quantitative cluster analysis, we systematically compared t-SNARE membrane clustering following normal and elevated glucose exposure. We observed that t-SNARE proteins form clusters of low and high molecular density with a non-random spatial distribution on the plasma membrane. Furthermore, we do not observe t-SNAREs clustered directly underneath insulin vesicles, noting only partial overlap between the two. Overall, these results provide new insight into the molecular organization of t-SNARE proteins at the plasma membrane of β-cells.

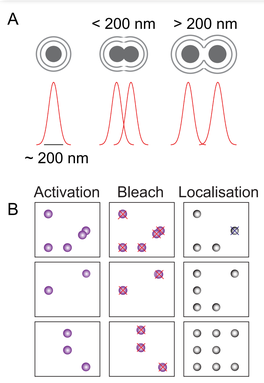
Introduction



A.Insulin secretion requires the action of the highly conserved SNARE protein family, that catalyse the final steps of membrane fusion. Syntaxin-1a/4, SNAP-23/25 and VAMP-2 mediate insulin secretion in β-cells.

B.The SNARE proteins share a common motif of 60 amino acids, known as the SNARE motif. The proteins form a highly stable core complex composed in a 1:1:1 ratio.

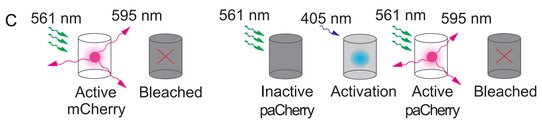
Method



A.The limit of resolution in light microscopy governs that a single object has a PSF (red) and is visualized by an Airy pattern due to light diffraction and chromatic aberration. Objects <200 nm apart are visualized as one object (centre) and >200 nm are resolved as two (right).

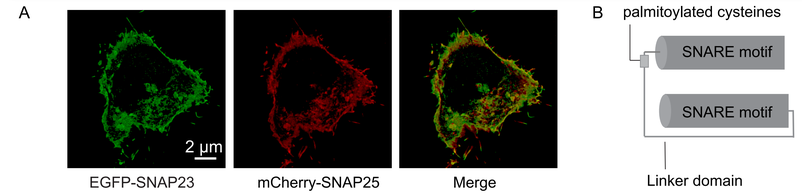
B.SMLM overcomes the diffraction limit by sequentially imaging sparse sets of fluorophores over thousands of frames. The location of molecules > 200 nm apart is recorded and used to recreate a precise map of fluorophore positions.

C.Photoactivable Localisation Microscopy (PALM). mCherry is fluorescent under 561 nm light, emitting light at 595 nm until it bleaches. PA-mCherry is not fluorescent under 561 nm light until it is activated by UV light. Once activated pa-mCherry is fluorescent until it is bleached.



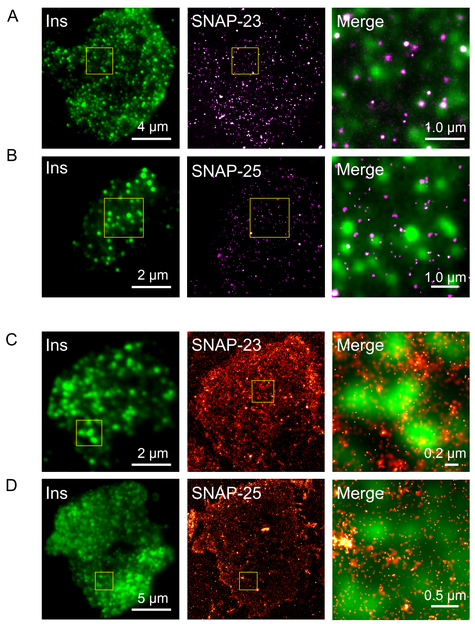
Results

1.SNAP-25 and SNAP-23 expression in pancreatic beta cells



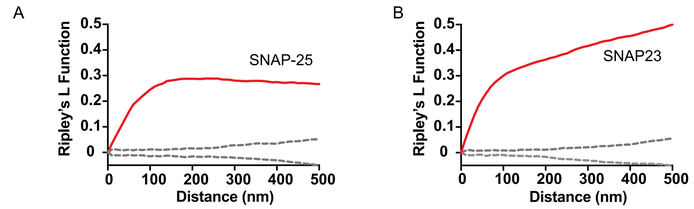
A. Confocal slice of a β-cell expressing EGFP-SNAP23 (left) and mCherry-SNAP25 (centre). These are the main functioning SNAP isoforms which play a direct role in insulin secretion. SNAP-25 functions exclusively in regulated secretion. SNAP-23 functions in both regulated and constitutive secretion. B. Structurally each protein contains two SNARE motifs and a linker domain consisting of a membrane anchor domain comprising palmitoylated cysteine residues. The sequences are 59% identical and 72% similar at the amino acid level.

2.Plasma membrane organisation of SNAP-25 and SNAP-23



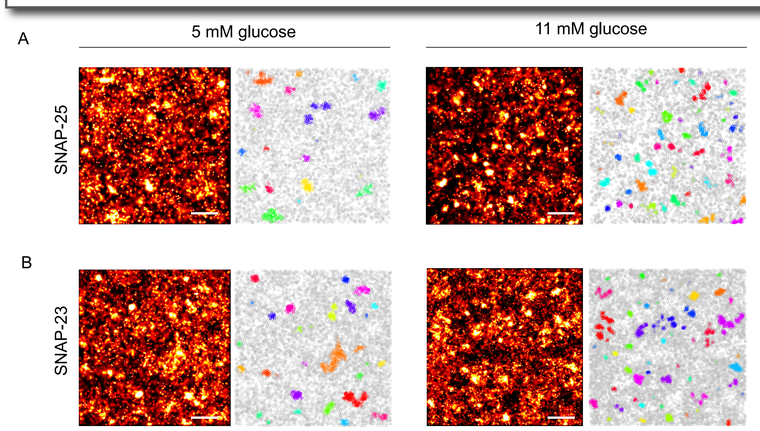
Organization of SNAP-23 and SNAP-25 on the β-cell plasma membrane. **A.** Stochastic Optical Reconstruction Microscopy (STORM) image of endogenous SNAP-23 in HIT T15 cell. Representative cell in TIRF showing immunostained insulin vesicles (left green panel) with rendered image of single SNAP-23 molecules (centre magenta panel). ROI highlighted in yellow is a channel merge of SNAP23 molecules and insulin vesicles (right). **B.** STORM imaging of SNAP-25 with layout as in A. **C.** PALM data of paCherry fused SNAP-23 in a HIT T15 cell. EGFP tagged insulin vesicles (left green panel), rendered image of single SNAP-23 molecules (red centre panel). ROI highlighted in yellow is an overlap of insulin vesicles (green) with PALM data (red). **D.** PALM imaging of SNAP-25 with layout as in C.

3.Non-random spatial distribution of SNAP-25 and SNAP-23



Ripley's analysis of SNAP-23 and SNAP-25 confirms they have a non-random clustered distribution at the plasma membrane. Using the coordinate information obtained from PALM, Ripley's analyses compares the observed molecular spatial distribution to randomised simulations. **A.** Ripley's plot of SNAP-25 molecules within a region of interest shows a clustered organization with L function values (red line) outside minimum and maximum randomized simulation envelopes (grey dashed lines). **B.** SNAP23 molecules also exhibit a clustered organization at the plasma membrane.

4.Clustering behaviour of SNAP-25 and SNAP-23



Comparison of the clustering behaviour of SNAP-25 and SNAP-23 in HIT T15 cells at low (5 mM) or elevated (11 mM) glucose levels. Cells were exposed to low or high glucose for 72 hours prior to fixation in 4% PFA. Using a Bayesian method of cluster identification, we found an altered behaviour in SNAP-25 and SNAP-23 clusters at elevated glucose levels. **A.** Rendered PALM image of ROI from a HIT T15 cell expressing SNAP-25 molecules with corresponding cluster plot for those molecules at low glucose (left) and at elevated glucose (right) levels. **B.** Same layout as shown in A, but for SNAP-23 molecules. Scale bar 500 nm.

References

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