VISUALISATION OF EXOCYTOSIS OF INSULIN-CONTAINING SECRETORY VESICLES IN PANCREATIC ß-CELLS USING TIRFM

One of life science's primary challenges is to understand and define the role of a number of proteins in cellular processes associated with health and disease of living organisms. Understanding this vast array of diverse biochemical processes of the living cell is paramount to not only gaining an insight into the living organism as a whole, but also discovering when and why these cellular processes break down, thus causing disease. Whilst innovative and specialised techniques have been instrumental for many advances in molecular biology, many questions concerning the structure and function of cells can only be answered by observing living cells. Live-cell imaging offers the power of capturing the dynamics of biological action in live cells and in real-time, something not previously available with biochemical approaches.

The development of a wide range of specific fluorescent probes that can be used to non-invasively interrogate living cells, such as dyes and fluorescent proteins, has facilitated the study of complex cellular processes. Furthermore, the development of new microscope technologies and powerful computer software and hardware for digital image processing and analysis has played a key role in the emergence of live-cell imaging as a viable laboratory tool, as well as a routine methodology that is practised in a wide range of biomedical research disciplines.

As a result there have been an increasing number of studies employing live-cell microscopy and imaging techniques at cellular and sub-cellular levels. Microscopy has progressed from the purely structural characterisation of fixed cells towards the investigation of processes in living cells. Static morphological observation can now be complemented by the characterisation of the three-dimensional (3-D) architecture of cellular structures. In turn, these innovations provide the ability to monitor dynamic cellular activities in living tissues with sub-micrometer resolution in real-time, and offer critical insights into the fundamental nature of cellular and tissue function, which are not possible using fixed cell techniques.

A REVOLUTION IN RESOLUTION

Cellular processes are dynamic, and investigating how cells function allows for a better understanding of physiological and pathophysiological interactions. Such experiments have to be performed on living cells under relevant physiological conditions and have been significantly enhanced through recent developments in fluorescent microscope technologies over the last 20 years. Since the invention of confocal microscopy and more recent advances such as confocal laser scanning spinning disk microscopy, and Total Internal Reflection Fluorescence Microscopy (TIRFM), today's live-cell imaging systems have dramatically improved the ability to detect biological specimens and offer the high spatial resolution required to image living cells without destroying them.

SHEDDING NEW LIGHT

Among the many optical tools that enable the capture of these cellular and molecular events, TIRFM illumination imaging offers important opportunities. TIRFM enables total internal reflection of the laser illumination within the glass of the slide or vessel below the sample. This creates an evanescent wave, which penetrates a thin surface layer of the sample, enabling very high signal-to-noise (S/N) ratio imaging of fluorescently labelled molecules in this layer. TIRFM is essentially contact microscopy, and by using this unique property, scientists can clearly visualise the specific regions where cells adhere to their neighbours and substrates.

TIRFM can shed new light on distinct cell structures and processes, whilst making use of already established cell preparations and conventional methods of processing. Furthermore, TIRFM is easy to apply, very flexible and the results generated can be interpreted quickly. TIRFM is compatible with the widely established epi-fluorescence microscopy, enhancing the applications of non-laser microscopy. To facilitate interpretation of the very characteristic, and at first unfamiliar, image of the cell in TIRFM, it is easy to directly compare conventional and TIRFM images of the same sample. Thus, TIRFM positions itself as a powerful method in the fields of modern cell biology, as well as tissue engineering and pharmaceutical screening. Furthermore, the TIRFM method is particularly useful for investigation of a wide-spectrum of cell surface events, such as endo/exocytosis, cell adhesion and the formation of junctions between cells.

EXPLORING THE POTENTIAL

This article describes how we have used a combination of electrophysiology and TIRFM to explore the potential of TIRFM illumination image analysis for live-cell microscopy. Together, these techniques may provide a further insight into the visualisation of sub-cellular organisation and the role of micro-domains in the mechanisms behind the secretion of insulin from pancreatic β-cells, and the role of vesicles in the fusion events of insulin exocytosis.

INSULIN RELEASE BY EXOCYTOSIS

Insulin regulates the balance of energy for the whole human body. Its secretion from the endocrine pancreatic islet β-cells is stimulated by increases in blood glucose and modulated by neuronal and hormonal input. Insulin acts primarily on the liver, muscle and fat to promote energy storage. The major effects of insulin include the promotion of glucose uptake and storage, as well as the inhibition of glycogen breakdown. Along with insulin, β-cells secrete a host of peptides and small molecules, such as transmitter molecules. Among these, ATP and γ-aminobutyric acid (GABA) are thought to mediate important auto- and paracrine communication within the islets of Langerhans. This secretion occurs via the regulated fusion of vesicles with the plasma membrane, a process known as exocytosis [1].

Exocytosis is a regulated, Ca^{2+} -dependent, multi-stage process involving the transport of vesicles to the plasma membrane, as well as the docking of the vesicles, with fusion then causing the release of its contents. Insulin is transported in pancreatic β-cells in large dense core vesicles (DCV). In order for the DCVs to undergo exocytosis, they must be in close proximity to the plasma membrane and in close association with the voltage-dependent Ca^{2+} channels that mediate calcium entry [1].

Exposure of the islet β-cells to high blood glucose levels activates a number ofsignalling pathways, stimulating the insulin-containing DCVs to fuse with the plasma membrane. This leads to a small proportion of total insulin being released into circulation. Conversely, to maintain membrane homeostasis, the added vesicular membrane must be recaptured. Therefore, it can be said that the stimulated insulin secretion is coupled to the re-uptake of vesicles.

Glucose increases the intracellular free Ca^{2+} concentrations and is the key trigger for insulin release from the β-cells. These Ca^{2+} changes are

Microscopy Focus

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tightly regulated and it is thought that locally high [Ca^{2+}] microdomains may control exocytosis via interactions with key proteins present at the site of exocytosis.

TYPE 2 DIABETES

A healthy β-cell maintains in excess of 10,000 vesicles, although not all of these are functional. However, type 2 diabetes can develop as a result of impaired β-cell function and is closely associated with increased plasma free fatty acid (FFA) concentrations. Whereas FFAs enhance the β-cell response on acute application, long-term exposure exerts lipotoxic effects and leads to a decrease in glucose-stimulated insulin secretion, as shown in *Figure 1*, as well as some decreased cell viability. Despite this knowledge, it has been hard to pinpoint the exact nature of this dysfunction at the cellular level, as organelle and ion channel function appears normal (or even improved).

ELECTROPHYSIOLOGICAL MEASUREMENTS OF ß-CELLS

Pancreatic β-cells are electrically excitable, and changes in membrane potential link variations of blood glucose concentrations to increases or decreases in insulin secretion. The mechanism by which insulin secretion is regulated by glucose suggests that ATP-sensitive potassium ion (K^+) channel activity maintains a negative β-cell membrane potential at sub-stimulatory glucose concentrations, and therefore prevents electrical activity and insulin secretion. Elevation of glucose stimulates β-cell glucose uptake and metabolism, which in turn increases intracellular [ATP] and closes the KATP channel. This closure leads to membrane depolarisation, opening of voltage-gated Ca^{2+} channels, elevation of intracellular free [Ca^{2+}] and ultimately exocytosis of insulin-containing secretory vesicles.

The plasma membrane of the cell separates the cytosol from the exterior bathing solution and acts as an electrical insulator due to its lipid composition. It is therefore, appropriately modelled as a parallel plate capacitor. With all other properties of the cell membrane, such as thickness, remaining unchanged, changes in the capacitance of the membrane is directly proportional to membrane area. Thus, membrane capacitance (C_m) measurements allow the direct detection of changes in membrane area that occur during the processes of exocytosis (increases in C_m) and endocytosis (decreases in C_m) [2, 3]. Surprisingly, measurements of exocytosis as measured by capacitance were higher in single cells chronically exposed to FFAs. This is, of course, in direct contrast to our measurements of hormone secretion, as shown in *Figure 1*.

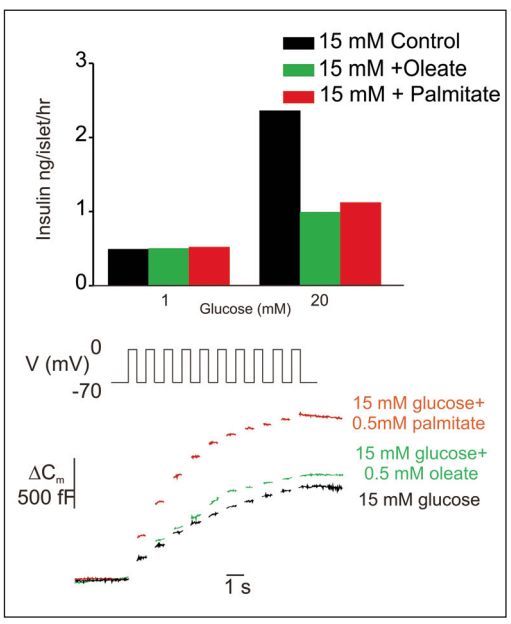
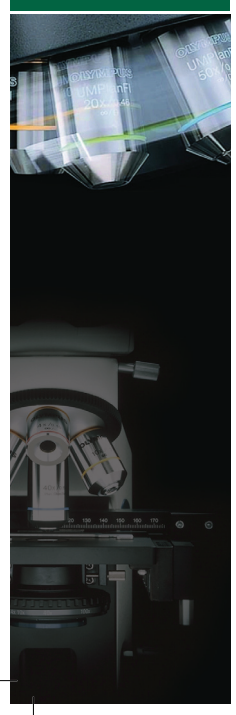


Figure 1: Measurements of cellular function of β-cells after long-term (72h) culture in high glucose and lipids. Effects of long-term culture in high glucose and lipids on insulin secretion (above). D: Depolarisation-elicited capacitance increases (ΔC_m) evoked by a train of depolarisation (V; indicated schematically above capacitance trace) in β-cells (below) [4].



"KISS-AND-RUN" EXOCYTOSIS

The classic mechanism of exocytotic release of insulin is generally believed to involve the complete merger of the secretory vesicle with the plasma membrane, and is also known as "classical" exocytosis. However, another mechanism of exocytosis has been recently proposed, known as "kiss-and-run" exocytosis [5].

The "kiss-and-run" events suggest that vesicles release only part of their contents through a transiently opened fusion pore. During this type of event the vesicle fuses with the plasma membrane only transiently and is re-internalised intact. Our measurements of the fusion pore have found that it is too narrow to allow the exit of insulin hormone [6]. Therefore, a shift towards cells using "kiss-and-run" fusion would prevent hormone secretion while maintaining exocytosis rates in the β-cell.

BENEFITS OF USING TIRFM

As previously described, membrane capacitance can be used for the estimation of passive cell parameters. However, this method is restricted in its application because it does not allow for the identification of the type of fusion occurring in the cell. These results create quite a discrepancy, but this could possibly be explained with a shift in exocytotic mechanisms, such as "kiss-and-run". However, as this cannot be explored using electrophysiology alone, we have explored this concept further by using the advanced Olympus TIRFM imaging system, to enable visualisation, as well as to obtain direct parameter measurements.

Advances in fluorescent imaging approaches have made it possible to track a single DCV within the β-cell before, during and in some cases after exocytosis as in "kiss-and-run", shown in *Figure 2*.

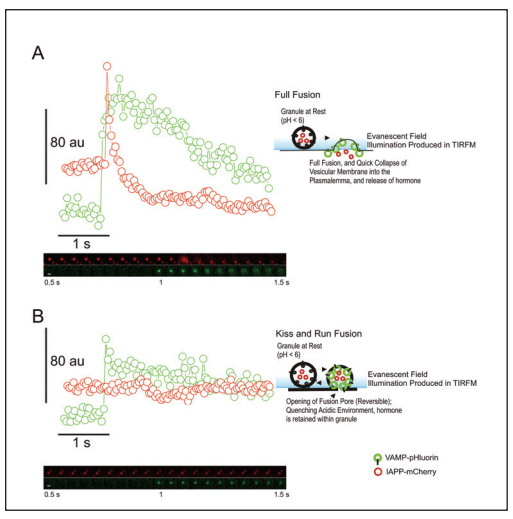


Figure 2: Measurements of single exocytotic events from a mouse β-cell, expressing both VAMP-pHluorin and IAPP-mCherry. (A) Relationship of fluorescence for VAMP-pHluorin (green) and IAPP-mCherry (red) plotted against time for a full fusion event. Graphic representation is shown to the right, and a montage of consecutive images (0.5-1.5 s) of the event is shown below. (B) Similar to above, except displaying a kiss-and-run fusion event from the same cell. Scale bar = 500 µm. Exocytosis was elicited using a 500 ms depolarisation from a voltage-clamped cell.

By employing a highly advanced fluorescence imaging system and exploiting specific fluorescent probes, we used TIRFM illumination imaging to facilitate the observation of cellular structures down to single molecule fluorescence at surfaces and interfaces. This was enabled by imaging individual insulin vesicles at the base of the cell, where the plasma membrane approached the coverslip, as shown in *Figure 3*.

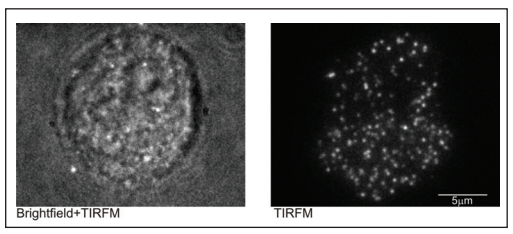


Figure 3: TIRF illumination of mouse β-cells expressing IAPP-mCherry to visualise vesicles in close proximity to the plasma membrane. Brightfield and TIRF images are shown for comparison.

This may provide new insights into the potential role of not only vesicles and their cargo, but also protein and ions, such as Ca^{2+} microdomains, in the local environment of the cellular membrane in β-cells, especially when combined with electrophysiology, as shown in *Figure 4*.

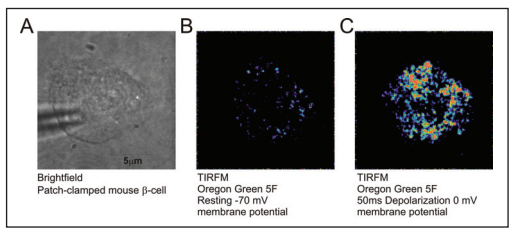


Figure 4: Measurements of localized Ca^{2+} influx from a patch-clamped mouse β-cell infused with a fluorescent indicator. The cell is shown in (A) brightfield, (B) TIRF held at a resting membrane potential of -70 mV, and (C) TIRF at 0 mV, to measure localized influx for voltage sensitive L-type Ca^{2+} channels.

DISCUSSION

Employing the Olympus TIRFM system has enabled us to explore the potential of laser-based fluorescence imaging technology for live-cell microscopy by monitoring the exocytosis of insulin-containing secretory vesicles in pancreatic β-cells, which is crucial for the maintenance of plasma glucose levels.
They fuse with the plasma membrane in a regulated manner to release their contents and are subsequently recaptured. Thus, the aim of our ongoing studies is to make simultaneous measurements of multiple-targeted fluorescent reporter constructs with the direct visualisation of the dynamics of insulin exocytosis in pancreatic β-cells. We hope to show in future studies that the insulin secretion defect that develops following long-term exposure of β-cells to high glucose, results from insufficient expansion of the fusion pore rather than a reduction of exocytosis. Furthermore, our ultimate aim is to study how this defect may contribute to the pathophysiology of Type 2 diabetes in humans.

MATERIALS & METHODS

Experiments performed:

Preparation of ß-Cells~

Pancreatic islets were isolated from cd-1 mice.
The islets were then dissociated into single cells and plated onto conventional glass coverslips for imaging. Expression of fluorescent protein chimaeras was achieved by adenoviral infection.

Calcium measurements~

Single cells were infused with calcium dye (Invitrogen, USA) to visualise microdomains of calcium infux in the evanescent field.

Equipment used:

These experiments were conducted using the Olympus laser-based TIRFM illumination module integrated with an Olympus Cell^R imaging system and an Olympus IX-81 inverted microscope (Olympus, UK) customised for evanescent field microscopy (see *Figure 5*), using a high numerical aperture objective (1.49 N.A., Apo x 60 Oil, Olympus, UK) or (1.45 N.A., Apo × 150 Oil, Olympus UK). All excitation was achieved using a 488 nm line laser (100mW argon ion laser, Melles Griot, Bensheim, Germany) for GFPs and calcium dyes or a solid state 561 nm line laser for RFPs (Olympus, UK).

Experimental execution and data evaluation was facilitated by the advanced Olympus cell^R software (Olympus, UK) combined with analysis using Metamorph Software (Pennsyvania, USA).
Images were acquired at 20Hz with 8ms of exposure time, and captured using a Cascade II 512b CCD camera (bin 1x1).

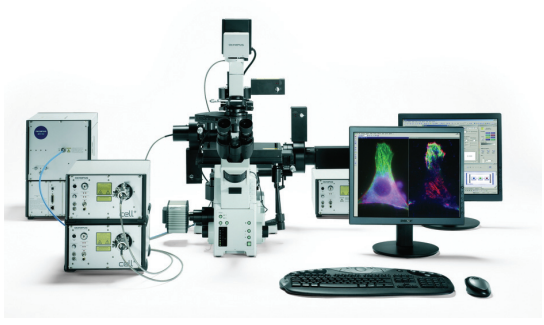


Figure 5: Olympus laser-based TIRFM illumination module integrated with an Olympus Cell^R imaging system and an Olympus IX-81 inverted microscope

REFERENCES

[1] MacDonald PE and Rorsman P. 2007. The ins and outs of secretion from pancreatic β-cells: Control of single-vesicle exo- and endocytosis. Physiology. 22: 113-121.

[2] Neher E and Marty A. 1982. Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. Proc. Natl. Acad. Sci. U.S.A. 79:6712-6716.

[3] Lindau M. 1991. Time-resolved capacitance measurements monitoring exocytosis in single cells. Quart. Rev. Biophys. 24:75-101.

[4] Olofsson C.S., S. Collins, M. Bengtsson, L. Eliasson, A. Salehi, K. Shimomura, A. Tarasov, C. Holm, F. Ashcroft, and P. Rorsman. 2007. Long-term exposure to glucose and lipids inhibits glucose-induced insulin secretion downstream of granule fusion with plasma membrane. Diabetes. 56:1888-97.

[5] Rizzoli SO and Jahn R. 2007. Kiss-and-run, collapse and "readily retrievable" vesicles. Traffic. 8 (9): 1137-1144.

[6] MacDonald PE, Braun M, Galvanovskis J and Rorsman P. 2006. Release of small transmitters through kiss-and-run fusion pores in rat pancreatic beta cells. Cell Metabolism. 4 (4): 283-290.