DYNAMIC IMAGING OF INTRACELLULAR Ca^{2+} CONCENTRATION IN INSULIN SECRETING MIN6 B-CELLS USING WIDE FIELD AND TIRF MICROSCOPY

Shiv Kumar Mishra*, Priyanka Sharma, Vinita Pawar, Archana Tiwari
*School of Biotechnology, Rajiv Gandhi proudyogiki vishwavidyalaya (RGPV) University of Technology of Madhya Pradesh, Airport Bypass Road, Gandhi Nagar, Bhopal-462033, India
E-mail of Corresponding Author: skmibt@gmail.com

Abstract
Since 1920's major medical advance based on animal research i.e. Insulin for diabetes. Diabetes is world’s most common metabolic disorder characterised by pancreatic β-cells dysfunction. Normally these cells respond to high glucose concentrations following exocytosis of stored insulin trigger by the combined action of store-operated calcium entry (SOCE) and calcium-release activated current (CRAC) channel, with an elusive mechanism. To illustrate it, dynamic imaging may used as power full tool as, it offers power of capturing the dynamics of biological action in live cells. In this review we focuses on dynamic imaging of pancreatic MIN6 cells, an appropriate model used as an alternative to animal experiments in diabetes research to understand what is occurring, in and among pancreatic β-cells and influence their behaviour in desired ways, using advanced wide field and total internal reflection fluorescence microscopy (TIRFM). This may empower the principles of optimization of diabetes research.

Keywords: β-cells; Dynamics; Glucose; Imaging; Insulin and Insulinoma

1. Introduction
Dynamic Imaging is a powerful approach to look inside the cell in order to retrieve the proper functioning which facilitate and characterise the regulatory dynamics of the cell and their various organelles and capable to place principles of alternatives to animal experiments in diabetes research by facilitating the use of cells and tissues. Diabetes is a group of metabolic diseases (Also known as metabolic syndrome and a slow poison) in which a person has high blood sugar, either because the body does not produce enough insulin due to loss of β-cells, or because cells do not respond to the insulin that is produced i.e. due to development of insulin resistance (Figure 1). Type-1 insulin dependent diabetes mellitus (IDDM) is characterized by loss of the insulin-producing β-cells of the islets of Langerhans in the pancreas leading to insulin deficiency. Type-2 non-insulin dependent diabetes mellitus (NIDDM) is result of the malfunctioning of pancreatic β-cells due to insulin resistance and some genetic factors. It is evident in previous research works, that Ca^{2+} is a key regulator of many normal cellular functions of cells and also involves in the release of insulin from pancreatic β-cells. Although glucose stimulated insulin secretion largely depends on the closure of ATP-sensitive K^+ channels, followed by voltage-activated Ca^{2+} influx, the uptake and mobilization of ion from intracellular stores is also important for normal β-cell to respond elevated glucose. Few researchers hypothesized role of store-operated calcium entry (SOCE) pathway and calcium-release activated current (CRAC) channel in the regulation of the dynamics of insulin exocytosis. From many years the molecular basis of store-operated entry was unknown. There was a suspicion that Ca^{2+} channels were involved, but what these were and how they were gated was unclear. These all cellular events are associated with type-2 diabetes mellitus in both the humans and rats. The dynamic imaging method comprises illuminating at least a portion of cell and multivariate analysis of spectral features of the images thus obtained can yield the location and chemical identity of components in the field of view. This information can be combined or overlaid with other spectral data (e.g. a visible microscopic image) Obtained from the field of view.

Fig. 1- Pancreatic β-cells produce insulin in response to elevated glucose level and lose of these cells results in development of metabolic disorder Diabetes.
In this review we deal with insulinoma MIN6 cells, derived from the transgenic mice (C57BL/KSJ mice) have a morphological and functional similarity to pancreatic β-cells, widely used in the diabetes research experiments to study the regulation dynamics of insulin release and the various components involved in the stimulation of insulin release capable of replacing use of experimental animal mice to carry out this study. In another words MIN6 cells are an appropriate model or best alternatives to replace animals in diabetes research experiments which would leads to reduction in implement of various social and ethical issues and greatly facilitates refinement of new insights in diabetes research. This can be achieved by dynamic imaging of live cell enable us to find out the possible insight related to the mechanism underlying the movement of Ca²⁺ inside the pancreatic β-cells, facilitating or triggering insulin release in order to compensate the higher blood glucose level and helpful to treat diabetic condition. This will greatly help us to make an attempt in order to optimize diabetes mellitus research and made it free from use of experimental animals as well.

2. MIN6 Cell

Human pancreas composed of five type of subcellular composition namely α-cells, β-cells, δ-cells, γ-cells and ε-cells all are engaged in secreting- glucagon, insulin, pancreatic polypeptide and ghrelin respectively collectively known as “Pancreatic Islet”. Importantly, they engaged in maintaining glucose-insulin homeostasis. Defective glucose stimulated insulin secretion (GSIS) from pancreatic β-cells is main cause of type 2 diabetes mellitus (non insulin dependent) many investigations are carried out to reveal the mechanism insulin secretion in response to higher extracellular glucose level. However, the precise mechanism is yet elusive. The main factors responsible for this are cellular heterogeneity and restricted availability of pancreatic islet. Recent progression in the field of gene manipulation technology and use of alternative to animal experiments, made possible to reveal cellular mechanism more precisely. The development of clonal cell lines are also proved as a milestone to support and made advancement in gene manipulation technology. Various researchers utilized X-rays or viruses to provoke insulinomas, in vitro transformation, derivation of cells from transgenic mice or even use non islet cells to produce immortalised beta cell lines to fulfill the narrow availability of primary beta cells to make an advancement in diabetes research. Relating to this context, by using primary cultured β-cells in biochemical and molecular research numerous insulin producing cell lines have been established as an alternative to animal experiment, among them recently developed MIN6 cell line derived from transgenic mouse (C57BL/KSJ mice), secretes 6-7 fold higher insulin at 25 mmol/l glucose than that obtained at 5 mmol/l glucose. Showing that secretion of insulin in MIN6 cells is depends on the concentration of glucose as in parent islet cells. In contrast, some other insulin producing cell lines are also cloned i.e. HIT, β-TC (Poitout et al., 1995), RIN, INS-1 and IgSV195 that do not respond and respond at very lower glucose concentration(1-5 mmol/l). In case of INS-1 cell line there is 2.2-fold increase in insulin secretion is reported at elevated glucose concentration under physiological conditions. In HIT cells secretion and degradation of glucagon takes place which is essential for positive differentiation of insulin secreting mouse pancreas. NIT-1 is insulin secreting pancreatic β-cell line established from a transgenic NOD/Lt mouse. Therefore, among all insulinoma cell lines, only MIN6 cell line popularly used as β-cells specific cell line greatly facilitate refinement in diabetes research. MIN6 cell lines have an islet like structure and secrete insulin as parent islet cells, hence they are also known as pancreatic MIN6 β-cells possessing ability of highly active self-renewal. Transplantation study are carried out using MIN6 cells as an alternative to animal experiment in order to rectify the diabetic state in mouse, and information obtained from this study we can try to apply it on humans in order to short out the problems like allograft rejection and fulfils the shortage of human pancreata sufficient to reverse the diabetic state. In this study, transplanted cells form tumour so further study should be done to reveal this problem. Another study result shows that the first Phase of Glucose-Stimulated Insulin Secretion from MIN6 Cells does not always require extracellular calcium influx as the study diazoxide and verapamil used to completely inhibit tolbutamide- or glibenclamide-induced insulin secretion and Only partial suppression of the peak of the first phase of GSIS, significant amount of insulin secretion remained even in the combined presence of verapamil and dantrolene. The voltage gated Na⁺ channel blocker tetrodotoxin nearly completely inhibited the first phase release. These results suggest that the first phase of GSIS from MIN6 cells depends on both Ca²⁺ dependent and - independent mechanisms. In another study a
new imidazoline compound, S-21663 reported to stimulate insulin release from the MIN6 cell line \(^{29}\). As we know that diabetes type 2 is associated with decrease in \(\beta\)-cells mass which is characterised by increased apoptosis and/or decreased proliferation. Hence, MIN6 cells are used as a model to reveal the mechanism of cellular events like apoptosis and proliferation which may gives the major therapeutic implications to type 2 diabetes mellitus by identifying the novel molecular targets in order to increase the \(\beta\)-cells proliferation and/or avoid \(\beta\)-cells from apoptosis.

3. Dynamic Imaging

Dynamic imaging or live cell imaging offers the power of capturing the dynamics of biological actions, mechanism of actions, metabolism, storage/release, components and factors involved in pathway regulation and physiological and pathophysiological action of cell. In other words it is the technique enables us to look inside the cell to monitor or compare different cellular events at different physiological conditions. The dynamic imaging method comprises illuminating at least a portion of cell with substantially monochromatic light and the light scattered from the illuminated portion at a plurality of discrete times. Multivariate analysis of spectral features of the images thus obtained can yield the location and chemical identity of components in the field of view. This information can be combined or overlaid with other spectral data (e.g. a visible microscopic image) Obtained from the field of view. With the progress of microscopy, static morphological observation can now be complemented by the ability to monitor dynamic cellular activities in living tissues with sub-micrometer resolution in real time\(^ {30,31}\). This offers valuable insight into the nature of cellular and tissue function, not previously possible using fixed cell techniques. Researchers investigating calcium signalling have particularly benefitted from live-cell imaging, and new discoveries in the field continue with new developments in imaging technology \(^ {32,33}\). The advent of advance microscopy allows us to visualize, deep within intact organs and tissues while minimizing photodamage in real-time. At least nine types of cellular close encounters have now been visualized by live-cell immunoimaging in the lymph node, providing fresh insight on modes of cell communication during basal migration, antigen capture, antigen recognition, cell activation, differentiation, and death\(^ {34,35}\). The application of live-cell in vivo imaging to the immune system has just begun, and we can anticipate that technological advances in microscopy and design of improved morphological and functional probes will provide new opportunities. Dynamic cellular imaging of cellular activity can be accomplished with the use of various fluorescent probes. For example, Ca\(^{2+}\) indicator dyes can provide detailed spatial and temporal information about Ca\(^{2+}\) entry through voltage-gated channels in excitable cells or patterns of Ca\(^{2+}\) release from intracellular stores\(^ {36}\). So dynamic imaging is now become a very essential technique in order to make an advancement in the field of cellular as well as molecular biology. Which may further leads the progression of the biomedical or therapeutic outcomes to improve the efficacy accuracy of the treatment of the various cellular consequences (like diabetes) at both the cellular and molecular level and able to facilitate the use of Alternatives to animal experiments. This can be achieved by use of highly advanced imaging system described below.

4. Wide Field and TIRF Microscopy

Wide field and Total internal reflection fluorescence Microscopy (TIRFM) utilize the only one of its kind properties of an induced field in a limited specimen region immediately adjacent to the interface between two media having different refractive indices facilitate fast screening of single-molecule orientations without the necessity for scanning or of excitation modulation\(^ {24,37}\). A surface electromagnetic field, known as 'evanescent wave', can selectively excite fluorescent molecules in the liquid near the interface so it is also known as an evanescent wave microscopy\(^ {38}\). In TIRFM contact area utilized between a specimen and a glass coverslip or tissue culture container interface which reduces background fluorescence from fluorophores either in the bulk solution or inside the cells (i.e. autofluorescence and debris). Moreover, TIRF facilitate the much enhanced healthy survival of the culture during imaging procedures because of its minimum cell interior exposure to light, which is not in standard epi- (or trans-) illumination. Generally, TIRFM has potential advantage in any application requiring imaging of minute structures or single molecules in specimens having large numbers of fluorophores located outside of the optical plane of interest, such as molecules in solution in Brownian motion, vesicles undergoing endocytosis or exocytosis, or single protein trafficking in cells\(^ {24,37}\). A live cell imaging set-up
that enables one to switch rapidly between TIRFM and widefield epi-fluorescence microscopy, the latter for deeper penetration of the excitation into the bulk of the sample, signifies a flexible and powerful approach for studying intracellular transport mechanisms. Wide-field epifluorescence imaging of live cells can give an excellent overview of protein dynamics within a cell, and facilitates rapid acquisition of images\(^{39}\). Laser scanning and spinning disc confocal microscopy widely used techniques with various disadvantages like accept emission from a specific and precisely controlled plane within the sample; the acquisition of z-stacks can generate impressive 3-dimensional images entire field of view is exposed to excitation laser light during acquisition, overexposure can result in phototoxicity of live samples and photobleaching effects on fluorophores. The problem remains, however, that dynamic proteins studied in live cell imaging can move in and out of the focal plane. Furthermore, when studying protein movements that exist within a 5-nm plasma membrane, to avoid all above, we recommend the use of TIRFM with a sensitive charge-coupled device (CCD) camera in studying the biology of ion channels at the plasma membrane. TIRF microscopy utilise specially angled light and exploits the difference in refractive indexes of aqueous medium and a glass coverslip to obtain extremely high vertical spatial resolution. In brief, an excitation laser beam is directed at the specimen at an angle of incidence greater than or equal to the critical angle of refraction, whereby the excitation laser light is reflected. Confocal imaging will capture mainly cytoplasmic signals\(^{40,41}\). An exponentially decaying evanescent wave is generated at the point of reflection and penetrates the specimen. Because of exponential decay, only those fluorophores within 10 nm to 100 nm of the glass/medium interface are excited, provide high-resolution images of the nearest plasma membrane and closer subcortical regions (Figure 2)\(^{24,41}\). Given the limited depth of fluorophore excitation, signal-to-noise ratios can be high enough to image either single ion channels or their clusters more efficiently. TIRFM have only a limitation that is the interface of the cell and coverslip may be imaged, the focal plane cannot be moved upward as in confocal microscopy. Low level of energy from the evanescent wave causes markedly less phototoxity and photobleaching compared with the toxic effects of confocal and epifluorescence imaging\(^{38,40}\). Recent improvements in TIRF technology now allow for fully automated multiwavelength systems, greatly improve consistency and facilitate the use of techniques such as Fluorescence recovery after photobleaching (FRAP) And fluorescence resonance energy transfer (FRET) in combination with TIRF microscopy\(^{24,39}\).

**Conclusion**

Highly advanced imaging system is a powerful tool for the basic cell biologist and may lay down important insight to decode the mysteries behind the precise working of store-operated calcium entry (SOCE), calcium-release activated current (CRAC) channel across the plasma membrane. So, as we know well that Ca\(^{2+}\) is a key regulator of the variety of cellular events including “EXOCYTOSIS OF INSULIN” following membrane depolarisation due to increased extracellular concentration of Ca\(^{2+}\), may give valuable information about mechanism of glucose stimulated insulin secretion(GSIS). If it is so, we are on the way to find out permanent cure from chronic metabolic disease diabetes mellitus-type...
2 (non insulin dependent). In order to achieve this goal an insulinoma MIN6 cells a best alternatives to experimental animals (or an appropriate model) may play great help, as it have all characteristics similar to normal β-cells, used to perform the various experimental study. Use of advanced imaging system facilitates understanding of the intracellular calcium dynamics across the plasma membrane as well as intracellular store. Also characterize role and effects of the interaction of various cellular components and consequences associated with them helps in revealing mechanism behind SOCE/CRAC channel. It is expected that live cell imaging may lay down a breakthrough of actual mechanism involved in dynamic regulation of Ca\(^{2+}\) across intracellular Ca\(^{2+}\) stores. This breakthrough made advancement in further research and development at both cellular and molecular level to find out permanent cure from diabetes mellitus and lay down MIN6 cells as a best alternative to experimental animal in diabetes research.

Acknowledgments
I would like to express my special gratitude towards Dr. Mahavir Yadav, Mr. Susmit kosta and Mr. Deepesh gupta for your kind support and guidance.

References
1. Walker, S., Cunniffe, N., Bootman, M., Llewelyn Roderick, H. Dynamic Imaging of Calcium and STIM1 in the Same Cell Using Wide-field and TIRF Microscopy. BioTechniques 2008; 45:347-348.
2. Stutzmann, G.E., Parker, I. Dynamic Multiphoton Imaging: A Live View from Cells to Systems. Int.Union.Physiol. Sci. /Am. Physiol. Soc 2005; 20:15-21.
3. Parekh, AB. On the activation mechanism of store-operated calcium channels. Pflugers Arch - Eur J Physiol 2006; 453:303-311.
4. Carafoli, E., Santella, L., Branca, D., & Brini, M. Generation, control, and processing of calcium signal. Crit. Rev. Biochem Mol 2001; 36:107-260.
5. Chang, W.C. Store-operated calcium channels and pro-inflammatory signals. Acta Pharmacologica Sinica 2006; 27:813-820.
6. Parekh, AB. Store-operated channels. Mechanisms and function J Physiol 2008; 13:3033
7. Axelrod, D. Total Internal Reflection Fluorescence Microscopy in Cell Biology. Munksgaard International Publishers 2001; 2:764-774.
8. Varadi, A. and Rutter, G.A. Dynamic Imaging of Endoplasmic Reticulum Ca\(^{2+}\) Concentration in Insulin-Secreting MIN6 Cells Using Recombinant Targeted Cameleons. Diabetes 2002; 151:S190-S20.
9. Skelìn M., Rupnik M., Cencic A. Pancreatic Beta Cell Lines and their Applications in Diabetes Mellitus Research. Altex 2007; 27:105-113
10. Kinosita, K. “Real time imaging of rotating molecular machines” FASEB J 1999, 13; S201-S208.
11. Curry, DL. Bennett, LL. and Grodsky, GM. Dynamics of insulin secretion by the perfused rat pancreas. Endocrinology 1968; 83:572-584.
12. Sunderland, HB. Sinauer, MA. Ionic Channels of Excitable Membranes. Nature 2002; 417:515-522
13. Nakashima, K., Kanda, Y., Hirokawa, Y., Kawasaki, F., Matsuiki, M., & Kohei Kaku. MIN6 is not a pure beta cell line but a mixed cell line with other pancreatic endocrine hormones. Endocrine Journal 2009; 54:45-53.
14. Butler, AE, Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, RA., & Butler, PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 2003; 52:102-110.
15. Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inui, K., Anai, M. et al. Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. Diabetologia 1993; 36:1139-1145.
16. Miyazaki, J-I., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y.et al. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology 1990; 127:126-132.
17. Poitout, V., Stout, LE. Armstrong, MB. Walseth, TF, Sorenson, RL., & Robertson, RP. Morphological and functional characterization of βTC-6 cells-insulin secreting cell line derived from transgenic mice. Diabetes 1995; 44:306-313.
18. Gilligan, A., Jewett, L., Simon, D., Damjanov, I., Matschinsky, FM., Weik, H., C Pinkert, C., & Knowles, B. Functional pancreatic β-cell line from SV40 T-antigen transgenic mouse. Diabetes 1989; 38:1056-1062.
19. Eliasson, L., Abdulkader, F., Braun, M., Galvanovskis, J., Hoppa, M.B., & Rorsman, P. Novel aspects of the molecular mechanisms controlling insulin secretion. J Physiol 2008; 14:3313-3324.
20. Rorsman, P. and Renstrom, E. Insulin granule dynamics in pancreatic beta cells. *Diabetologia* 2003; 46:1029-1045.

21. Diem, P., Walseth, TF., Zhang, H-J., & Robertson, RP. Secretion and degradation of glucagon by HIT cells. *Endocrinology* 1990; 127:1609-1612.

22. Prasadan, K., Daume, E., Manna, P., & Gittes, GK. Glucagon is required for early insulin-positive differentiation in the developing mouse pancreas. *Diabetes* 2002; 51:3229-3236.

23. Hamaguchi, K., Gaskins, HR. and leiter, EH. NIT-1 a pancreatic beta cell line established from a transgenic NOD/Lt mouse. *Diabetes* 1991; 40:842-849.

24. Smyth, J.W. and Shaw, R.M. Visualizing ion channel dynamics at the plasma membrane. *Heart Rhythm Society* 2008; 5:S7-S11.

25. Hughes, C.B., Gaskins, HR. and leiter, EH. Solid organ pancreas transplantation: a review of the current status and report of one institution’s experience. *Am. Surg* 1994; 60:669-673.

26. Rorsman, P., Eliasson, L., Renstrom, E., Gromada, J., Barg, S., & Gopel, S. The Cell Physiology of Biphasic Insulin Secretion. *News Physiol. Sci* 2000; 15:72-77.

27. Ohara-Imaizumi, M., Aoyagi, K., Nakamichi, Y., Nishiwaki, C., Sakurai, T., Nagamatsu, S. Pattern of rise in subplasma membrane Ca2+ concentration determines type of fusing insulin granules in pancreatic beta cells. *Biochem Biophys Res Commun* 2009; 385:291-295.

28. Shigeto, M., Katsura, M., Matsuda, M., Ohkuma, S., & Kaku, K. First Phase of Glucose-Stimulated Insulin Secretion from MIN 6 Cells Does Not Always Require Extracellular Calcium Influx. *J Pharmacol Sci* 2006; 101:293-302.

29. Brigand, L.L., Virsolvy, A., Peyrollier, K., Manechez, D., Godfroid, J-J., Guardiola-Lemaître, B, et al. Stimulation of insulin release from the MIN6 cell line by a new imidazoline compound, S-21663: evidence for the existence of a novel imidazoline site in β cells. *Br. J. Pharmacol* 1997; 122:786-791.

30. Roos, J., DiGregorio, P.J., Yeromin, A.V., Ohlsen, K., Lioudyno, M., Zhang, S. et al. STIM1, an essential and conserved component of store-operated Ca2+ channel function. *J. Cell Biol* 2005; 169:435-445.

31. Meisslitzer-Ruppitsch C., Rohrl, C., Neumuller, J., & Ellinger, PM. A Photooxidation technology for correlated light and electron microscopy. *J Microsc* 2009; 235:322-335.

32. Miller, M.J., Wei, S.H., Parker, I., & Cahalan, M.D. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 2002; 296; 1869-1873.

33. Spiegelhalter, C., Tosch, V.R., Hentsch, D., Koch, M., Kessler, P., Schwab. et al. From Dynamic Live Cell Imaging to 3D Ultrastructure: Novel Integrated Methods for High Pressure Freezing and Correlative Light-Electron Microscopy, *PLoS ONE* 2010; 5:e9014.

34. Van Rijnsoever, C., Oorschot, V. and Klumperman, J. Correlative light-electron microscopy (CLEM) combining live-cell imaging and immunolabeling of ultrathin cryosections. *Nat Methods* 2008; 5:973-980.

35. Partiseti M., Ledeist, F., Hivroz, C., Fischer, A., Korn, H., & Choquet, D. The calcium current activated by T cell receptor and store depletion in human lymphocytes is absent in a primary immunodeficiency. *J Biol Chem* 1994; 269: 32327-32335.

36. Cahalan, M. D. and Parker, I. Choreography of Cell Motility and Interaction Dynamics Imaged by Two-Photon Microscopy in Lymphoid Organs. *Annu Rev Immunol* 2008; 26:585-626.

37. Bohmer, M. and Enderlein, J. Orienstation imaging of single molecules by wide-field epifluorescence microscopy. *J Opt. Soc. Am* 2003; 20:554-559.

38. Toomre, D., Manstein, DJ. Lighting up the cell surface with evanescent wave microscopy. *Trends Cell Biol* 2001; 11:298-303.

39. Axelrod, D., Burghardt, TP. and Thompson, NL. Total internal reflection fluorescence. *Methods Enzymol* 1999; 307: 3-20.

40. Webb R. Theoretical basis of confocal microscopy. *Methods Enzymol* 1999; 307: 3-20.

41. Darcy, K.J., Staras, K., Collinson, L.M., & Goda Y. An ultrastructural readout of fluorescence recovery after photobleaching using correlative light and electron microscopy. *Nat Protoc* 2006; 1:988-994.

42. Takemura, H., Hughes, AR., Thastrup, O., & Putney, JWJ. Activation of calcium entry by the tumour promoter thapsigargin in parotid acinar cells. Evidence that an intracellular calcium pool and not an inositol phosphate regulate calcium fluxes at the plasma membrane. *J Biol Chem* 1989; 264:12266-12271.