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PROBES FOR MONITORING REGULATED EXOCYTOSIS

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Abstract

Regulated secretion is a fundamental cellular process that serves diverse functions in neurobiology, endocrinology, immunology, and numerous other aspects of animal physiology. In response to environmental or biological cues, cells release contents of secretory granules into extracellular medium to communicate with or impact neighboring or distant cells through paracrine or endocrine signaling. To investigate mechanisms governing stimulus-secretion coupling, to better understand how cells maintain or regulate their secretory activity, and to characterize secretion defects in human diseases, probes for tracking various exocytotic events at the cellular or sub-cellular level have been developed over the years. This review summarizes different strategies and recent progress in developing optical probes for monitoring regulated secretion in mammalian cells.

Graphical abstract

Introduction

Secretion is a fundamental cellular process that are essential for diverse biological processes and functions. Cell secretion can be broadly classified into two major types. The constitutive secretory pathway is used by all eukaryotic cells and involves transporting secretory vesicles to the cell surface and releasing them independently of a biochemical stimulus [1]. The regulated secretory pathway, on the other hand, becomes activated only in response to environmental or biological cues. It mainly operates in specialized secretory cells such as neurons, endocrine cells, hematopoietic cells and exocrine cells [2–4]. The size of secretory granules of the first three secretory cells is normally in the range of 50 nm to 300 nm. In the

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exocrine cells, secretory vesicles are much bigger and many of them have diameters larger than 1 μm .

Regulated secretion is an essential component of the intercellular communication network in a multicellular organism. Secretory cells release the content of their secretory granules into the extracellular medium or the blood circulation upon biochemical or electrical stimulation. The released biomolecules, among others, may include neurotransmitters, peptide or protein hormones, growth factors, cytokines, and digestive enzymes. These molecules modulate a wide variety of signaling events either locally or systemically through paracrine or endocrine mechanisms. To study mechanisms regulating stimulus-secretion coupling and to perform functional analysis of exocytosis at cellular and sub-cellular level, there have been great interests in developing methods and probes for monitoring specific exocytotic events in the secretory cells of interest [5]. Traditional electrical methods including patch-clamp capacitance measurement or electrochemical methods such as carbon fiber amperometry are capable of monitoring regulated secretion at the cellular level with high temporal resolution [6, 7], yet they are limited in their spatial resolution and can only be applied to a single cell at a time. Fluorescence microscopy, on the other hand, provides a number of benefits including high sensitivity of detection, high spatial and temporal resolution, and the ability of monitoring multiple cells simultaneously in cell populations including tissues or organs. Moreover, laser scanning microscopy by either one photon or two photon excitation makes it possible to visualize cells in three dimensions (3D) both at the surface and in the interior of tissues. The combination of these advantages has made optical methods a versatile and popular approach for assaying cell secretion in different biological preparations.

This review summarizes optical tracers and related imaging methods for monitoring exocytosis, focusing on recent developments from exploiting two conceptually different strategies, namely labeling secretory vesicles with fluorescent probes and tracking the release of specific secretory products using customized fluorescent sensors.

Tagging secretory vesicles with fluorescent probes for monitoring exocytosis

There are two general approaches of tagging secretory granules for monitoring regulated secretion. Both approaches exploit the fact that, when the secretory granule fuses with the plasma membrane, there is rapid exchange of small molecules through the fusion pore between the granule lumen and the extracellular medium. In the first approach, secretory granules undergoing fusion are revealed by small fluorescent dyes that can rapidly diffuse into or out of granules. In the second approach, fluorescent pH sensors are targeted to the secretory vesicle to report the equilibrium of luminal and extracellular pH at the moment of granule fusion.

Small fluorescent tracers diffusible through the fusion pore

FM1-43 is an amphiphilic, membrane impermeable styryl pyridinium dye that was developed for tracking activity-dependent synaptic vesicle cycling (Figure 1) [8]. FM1-43 can be introduced into the secretory vesicles through compensatory endocytosis following

cell stimulation and exocytosis. Once incorporated into the inner leaflet of the vesicle membrane, the dye exhibits much higher fluorescence intensity than in aqueous solutions. This enables imaging the dynamics of single labeled vesicles. Subsequent triggering of exocytosis promotes the fusion of the labeled vesicular membrane with the plasma membrane, during which FM1–43 dissipates into the extracellular solution and loses fluorescence over a period of a few seconds [8]. In addition to synaptic vesicles, FM1–43 has been applied to label different types of secretory granules in non-neuronal cells, though it has been noted that certain secretory granules do not take up FM1–43 reliably [9]. Besides fluorescence imaging, electron microscopy (EM) can also be applied to map the location of FM dyes at nanoscale dimension after photo-conversion in the presence of diaminobenzidine [10, 11]. When combined with serial-section EM, this offers the possibility of characterizing the ultrastructure of secretory vesicles and their 3D organization and function relationships.

In addition to the granular membrane, the lumen of secretory granules can also be marked with fluorescent probes. In this case, small fluid phase fluorescent tracers that can rapidly diffuse through the fusion pore are taken up by the granule undergoing exocytosis. Sulforhodamine B (SRB, Figure 1), for example, is such an extracellular polar-tracer that has been applied to label synaptic vesicles through activity dependent uptake [12]. Other fluid phase tracers including dextran-dye conjugates have also been used to image exocytosis [9, 13]. Since fluid phase tracers are present in the bulk solution during cell stimulation, it would be necessary to reject signals out of the focal plane in order to monitor the dynamics of secretion and dye filling. Two photon fluorescence microscopy offers high spatial resolution by restricting the excitation at the focal point, hence effectively eliminating signals from dyes away from the focal plane. Two photon extracellular polar tracer imaging was hence developed to track exocytic events in the plane of focus within the intact tissues [14]. When a secretory granule fuses with the plasma membrane, it forms an Ω -shaped profile that could be detected by two photon laser scanning microscopy as a small fluorescent dot along the cell surface. The capture of these transient events at the cell-cell interface were interpreted as individual exocytotic events. A recent application of this technique in isolated pancreatic islets suggested a polarized distribution of insulin release sites towards the β -cell membrane facing the vasculature [15].

Fluorescent false neurotransmitters (FFNs) represent a class of cell specific imaging labels for visualizing monoamine neurotransmitter release [16, 17]. FFN200 (Figure 1), for instance, enters cells and gets enriched in the secretory granule of dopaminergic neurons by the vesicular monoamine transporter 2. Subsequent cell activation released FFN200 from the labeled secretory granule, and the loss of FFN200 fluorescence signal from the granule was used as a surrogate marker of dopamine release [17]. The study revealed the prevalence of presynaptically silent synapses that showed little dopamine release despite a reliable Ca^{2+} influx upon electrical stimulation, suggesting functional heterogeneity of dopamine release sites in the central nervous systems.

In addition to small fluorescent dyes, fluorescent or bioluminescent proteins have also been applied to mark secretory cargos for monitoring hormone secretion. Superfolder green fluorescent protein (SfGFP), for example, was fused with human proinsulin C-peptide [18]. The resulting fusion protein CpepSfGFP was expressed in mouse insulin granules at a low

level without exerting an observable metabolic impact. In transgenic mice expressing CpepSfGFP, intravital imaging revealed that only a subset of islets lost their stored CpepSfGFP (insulin) content upon glucose challenge, whereas most islets showed little loss of CpepSfGFP. The observation suggested inter-islet heterogeneity and the presence of “first responder” islets post blood glucose elevation [18]. Besides fluorescence, bioluminescent probes provides another modality for assaying hormone secretion. To develop a high throughput screening of insulin release, *Gaussia* luciferase was inserted into the C-peptide portion of proinsulin [19]. When expressed in beta cells, the engineered fusion protein was processed in the insulin granule to produce luciferase, which was co-released with insulin when cells were stimulated.

In addition to imaging the spatiotemporal dynamics of regulated secretion, small fluorescent tracers that can diffuse into the fusing granules may also be used for tagging and separating cells with distinct secretory activities. To study the functional heterogeneity of islet b-cells, we recently developed a zwitterionic dendrimer conjugate, G2-Cy3 (Figure 1) [20]. G2-Cy3 is derived from a generation 2 PAMAM dendrimer (G2 dendrimer) whose surface amino groups are partially neutralized with carboxylates. G2-Cy3 contains a Cy3 fluorophore and is brightly fluorescent. The molecule has a shape of a small spheroid with a diameter less than 4 nm. Its small size is expected to facilitate diffusion through the fusion pore into the lumen of the secretory granules that undergo partial fusion events such as kiss-and-run or cavicapture [21]. In the event of full fusion where secretory vesicles completely collapse into the plasma membrane, G2-Cy3 can be taken up by cells when they subsequently retrieve the exocytosed granular membranes to keep the cell volume constant. This exoendocytotic coupling has long been observed in a variety of secretory cells during stimulated secretion [22–25]. We exploit these processes to develop a strategy termed ACEE-Tagging (activation coupled exocytosis/endocytosis tagging) to selectively mark islet b-cells with robust insulin release activity by exposing cells to G2-Cy3 transiently during stimulation.

Compared to other commonly used fluid phase tracers including SRB or dextran-dye conjugates, G2-Cy3 displays a negligible level of non-specific cellular staining [20]. This unique property is highly desirable for applying fluorescence activated cell sorting (FACS) to separate cells based on their level of dye uptake through compensatory endocytosis following stimulated secretion. Since endocytic vesicles of most secretory cells are small (<50 nm), cells containing labeled endocytic granules (hence higher secretory activity) can be selected by FACS most reliably when dyes like G2-Cy3 produces little non-specific cell staining other than marking endocytic granules through ACEE. Further, G2-Cy3 can be used in live animals to mark islet b-cells during glucose stimulation. In vivo application of ACEE-Tagging in mice identified sub-populations of islet b-cells with distinct insulin secretin activities [20].

Since ACEE-Tagging can be applied to cell populations, it also provides an efficient and convenient assay to screen for individual cell clones manifesting robust secretory response, which should be of interest to stem cell researchers who are engineering insulin-releasing beta cells from human stem cells for the cell replacement therapy of diabetes.

pH sensors for imaging exocytosis

The lumen of secretory granules in mammalian cells has acidic pH down to ~5.5. Since extracellular fluids are neutral, secretory granules experience alkalization during exocytosis. This phenomenon forms the basis for an imaging assay of exocytosis through engineering pH-sensitive green fluorescent proteins (GFP) termed pHluorins [26, 27]. When fused with a vesicle membrane protein such as synaptobrevin (synapto-pHluorin), pHluorins were sorted to synaptic vesicles where they resided in the vesicle lumen and remained weakly fluorescent at acidic pH. Fusion of the vesicle with the plasma membrane led to a sudden rise of vesicular pH and a concomitant increase in pHluorins' fluorescence intensity, providing an instant readout of membrane fusion. pHluorins have since been applied to tag a number of vesicle proteins to monitor exocytic events in different cells. Among others, these include synaptagmin [28], syncollin-pHluorin for pancreatic acinar cells [29], phogrin or neuropeptide Y for insulin release islet beta cells [30].

pH sensitive fluorescent proteins emitting at longer wavelengths would be advantageous for tissue imaging as light scattering and autofluorescence are sizably attenuated at red or infra-red wavelengths. A number of red-shifted fluorescent protein have since been reported, including pHTomato [31], td-mOrange2 [32], and pHuji [33]. Similar to pHluorins, these pH sensors have pK_a above 7.1, so they are weakly or nonfluorescent when targeted to the secretory granules, yet restore strong fluorescence signal upon granule fusion with the plasma membrane. Another advantage of red-emitting pH sensors is their compatibility with optogenetic actuators that can be efficiently switched on at blue or green excitation wavelengths [34] to enable an all-optical approach for simultaneous controlling and monitoring of cell excitability and secretion.

While pH sensitive fluorescent proteins remain to be a versatile and generally applicable tool for imaging exocytosis, it should be cautioned that expressing fluorescent proteins in granules nevertheless can potentially perturb the biogenesis, trafficking, and/or localization of native secretory granules in certain cells [35]. These observations highlight the needs of performing appropriate controls and combining different approaches and probes to monitor dynamics of secretion when investigating the regulation or function of stimulated exocytosis.

Monitoring exocytosis using fluorescent sensors of the released cargos

The second strategy for imaging exocytosis employs fluorescent indicators that are responsive to the secreted products. Compared to the first strategy discussed above, this approach tracks the release of the granular content to the extracellular medium, hence providing a direct measure of the secreted molecule that may actually play a role in paracrine or endocrine signaling. The success of this approach relies on engineering fluorescent indicators that are specific for the analytes of interest. To achieve higher sensitivity of detection, these sensors are expected to respond to the analytes robustly with large dynamic range. In general, these indicators are targeted to the outer leaflet of the plasma membrane where the local concentration of the released products are highest. Efforts in probe engineering thus far have been most successful for crafting fluorescent sensors of small molecules including Zn^{2+} and certain neurotransmitters.

Zn²⁺

A number of mammalian cells, including pancreatic islet beta cells, prostate epithelial cells, certain glutamatergic neurons, Paneth cells, pituitary cells, and mast cells, contain a high level of Zn²⁺ in their secretory granules [36]. Impairments in Zn²⁺ secretion from these cells are associated with disorders such as diabetes, infertility, and cancer [37]. Zn²⁺ plays important roles in cell signaling and there have been strong interests in understanding how Zn²⁺ functions both intracellularly and extracellularly [38, 39]. Once secreted, Zn²⁺ can act locally as an autocrine and/or a paracrine signal [40], and it can affect distal tissues or organs through an endocrine mechanism. For instance, Zn²⁺ released from islet beta cells circulates to liver through the portal vein to suppress hepatic insulin clearance by inhibiting clathrin-dependent insulin endocytosis [41]. Moreover, since Zn²⁺ is frequently co-released with other cargos residing in the same secretory granule, it can also serve as a surrogate marker for monitoring secretion of other signaling molecules or growth hormones such as insulin. Hence, developing fluorescent Zn²⁺ probes and suitable cellular delivery and targeting strategies will provide powerful sensing technologies for investigating the regulation and function of Zn²⁺ release [42].

To maximize the sensitivity of detecting Zn²⁺ secretion, we have explored three complementary approaches to target fluorescent Zn²⁺ probes to the cell surface (Figure 2). ZIMIR is an amphipathic fluorescent zinc indicator for monitoring induced exocytotic Zn²⁺ release [43]. It contains a pair of dodecyl alkyl chains for membrane tethering, yet it is also highly charged near neutral pH so it cannot diffuse across cell membrane by itself. When ZIMIR is added to cells at low micromolar concentration, the dodecyl chains quickly anchored the molecule to the outer leaflet of the plasma membrane through hydrophobic interactions (Figure 2A). The labeling is non-invasive and does not appear to have any observable side effects on cell growth or function. ZIMIR binds Zn²⁺ with a K_d of 0.45 μM and displayed a 70-fold enhancement in fluorescence intensity upon Zn²⁺ binding. In addition to cultured cells, ZIMIR also labeled cells in tissues such as pancreatic islets and prostate glands [43–45]., Confocal ZIMIR imaging in isolated islets revealed that beta cells were fairly heterogenous in their secretory responses to glucose stimulation, with small clusters of islet beta cells exhibiting potent insulin/Zn²⁺ secretion activity surrounded by non-secretory cells [43].

While anchoring fluorescent probes to cell surface with lipophilic tails is convenient and is of general utility [46–48], the strategy nevertheless lacks cell specificity. Targeting cell surface receptors offers an alternative strategy for delivering small fluorescent sensors selectively to the cells of interest. Glucagon like peptide 1 receptor (GLP-1R), for examples, is a G-protein coupled receptor (GPCR) that is selectively and abundantly expressed in the b-cells of pancreatic islets. To target fluorescent Zn²⁺ sensors to the b-cell surface through GLP-1R, ZIMIR-Ex4(9–39) was developed (Figure 2B) [49]. Exendin4(9–39) (Ex4(9–39)) is a high affinity peptide antagonist of GLP-1R. ZIMIR-Ex4(9–39) conjugate binds to GLP-1R with nanomolar affinity without activating the receptor, so it remains on the cell surface for a prolonged period of time. When added to beta cells at submicromolar concentration, ZIMIR-Ex4(9–39) rapidly binds to GLP-1R and labels cell membrane in minutes to report the dynamics of insulin/Zn²⁺ release with high sensitivity and

spatiotemporal resolution [49]. Since numerous GPCRs and other types of cell surface receptors are expressed in a tissue- or cell-specific manner, and because high affinity ligands for these receptors already exist, conjugating fluorescent indicators with these ligands ought to represent a generalizable approach for the targeted delivery of imaging probes to cells of interest for monitoring regulated secretion and other cell functions.

Integrating chemistry with genetics offers yet another approach to achieve cell-specific delivery of synthetic imaging probes. Since genetically encoded protein labels can be expressed in cells of choice with exquisite specificity by using tissue-specific promoters, it enables targeted delivery of small imaging probes in vivo in transgenic animals expressing genetically encoded protein labels. ZIMIR-HaloTag is a ZIMIR homologue containing a hexyl chloride moiety that acts as the substrate of HaloTag enzyme (Figure 2C). HaloTag is a genetically encoded protein tag based on a modified haloalkane dehalogenase from bacterium *Rhodococcus*. [50, 51] At low micromolar concentration, ZIMIR-HaloTag reacted rapidly with HaloTag expressed on the cell surface to form a covalent bond and became permanently attached to the protein. Once immobilized to the outer cell membrane, ZIMIR-HaloTag displayed an impressive 15-fold fluorescence enhancement after it complexed with Zn^{2+} [52]. Because of its excellent dynamic range, ZIMIR-HaloTag was able to detect small secretion events at subcellular resolution. Combining channelrhodopsin (ChR2) with ZIMIR-HaloTag and a red-emitting Ca^{2+} sensor R-GECO1.2 [53] allowed concurrent manipulation and imaging of cell excitability and cell secretion (Figure 3) [52]. Photo-activation of ChR2 depolarized cell membrane and raised cellular Ca^{2+} in MIN6 beta cells, and ZIMIR-HaloTag revealed subcellular sites displaying high activity of insulin/ Zn^{2+} release. Interestingly, quantification of cellular Ca^{2+} activity (R-GECO1.2) revealed rather uniform elevation of subplasmalemmal Ca^{2+} activity during photostimulation (Figure 3B), demonstrating a disconnection between cellular Ca^{2+} rise and exocytosis, and suggesting other biochemical events such as the localized assembly of membrane fusion proteins and/or targeted trafficking of insulin granules to selected membrane patches might underlie the observed heterogeneity in exocytotic activity along the cell membrane. Moreover, at each release sites, it often took several photoactivations and cellular Ca^{2+} rises before the next exocytic events took place (Figure 3B). This probably reflected the limited number of readily releasable insulin granules near the cell membrane, such that after each release, it would take some time for the cell to replenish the pool before it was capable of launching the next round of granule fusion and exocytosis at the same release zone.

Besides membrane tethered Zn^{2+} sensors, fluid phase Zn^{2+} sensors have also been applied to image Zn^{2+} secretion. LZ9 consists of a green emitting Zn^{2+} sensor covalently linked to sulforhodamine B. Since SRB is insensitive to Zn^{2+} fluctuations, it serves as an internal reference to calibrate local dye concentration and excitation intensity so a ratiometric readout can be generated. [54] LZ9 imaging in the dorsal cochlear nucleus demonstrated that synaptic zinc is phasically released from synaptic zinc-rich parallel fibers during action potentials.

Neurotransmitters

The mammalian nervous systems contain numerous neurotransmitters and messengers. Development of fluorescent sensors of neurotransmitters has largely been focused on glutamate, an excitatory amino acid involved in diverse neuronal signaling events and the formation of synaptic plasticity. To engineer fluorescent glutamate indicators, a number of naturally occurring glutamate-binding proteins have been chosen as the templates to generate the sensing module of glutamate. Besides genetically encoded glutamate sensors based on fluorescent proteins, semisynthetic glutamate indicators comprising of glutamate binding proteins and small organic dyes have also been developed. Progress in these areas has been reviewed recently [5, 55, 56].

Release of neurotransmitters can also be tracked with cell based sensors - so called sniffer cells or biosensors [5]. These cell based biosensors exploit the exquisite sensitivity and selectivity of a native receptor to its cognate ligand. The assay involves expressing a neurotransmitter receptor in a cell line and placing the cell ("sniffer cell" or "biosensor") in juxtaposition to the biological preparation containing the secretory cells. For instance, to monitor acetyl choline release from human pancreatic islets, CHO cells expressing the muscarinic acetylcholine receptor M3 were loaded with the Ca^{2+} indicator Fura-2 and pressed against the isolated pancreatic islets [57]. Secretion of acetyl choline activated PLC- IP_3 - Ca^{2+} signaling pathway that could be revealed by Fura-2 Ca^{2+} imaging. The spatial resolution of the biosensor approach is typically low, since in principle any cells surrounding the biosensor could release the neurotransmitter to activate Ca^{2+} signal. In addition, this approach requires a series of control experiments to ensure that the event detected by the biosensor is specifically caused by the released analyte of interest instead of from other secretory products.

Summary

A variety of strategies have been developed over the years for imaging regulated secretion, and there has been recent progress in engineering fluorescent indicators of several secreted molecules including Zn^{2+} and glutamate. Given the large variety of biomolecules released from different secretory cells, there are still enormous needs for crafting fluorescent indicators capable of sensing specific secreted products including hormones, cytokines, and neurotransmitters. Desired properties of these imaging probes include high specificity, suitable binding affinity and kinetics, and high responsivity or large dynamic range of signal change. To apply these sensors to monitor regulated secretion in physiological preparations including primary tissues, 3D organotypic cultures, or live animals, appropriate targeting and delivery strategies also need to be developed. Genetically encoded fluorescent sensors hold great potential to meet the demands, yet they do not necessarily replace small indicators prepared from synthetic chemistry. These small chemical sensors can be applied to label cells in situ without viral infection or genetic expression so they can be particularly useful when studying freshly isolated tissues from higher mammals including primates.

Besides cellular imaging applications, new strategies and probes for assaying regulated secretion at the level of cell population would be valuable for developing high throughput screens of genes or large compound libraries. Conventional radioimmunoassay or enzyme-

linked immunosorbent assay (ELISA) of hormone release is time consuming, labor intensive and costly. Homogenous cell population based assays capable of rapid detection and measurement of hormone secretion would greatly facilitate high throughput studies. Several homogenous assays were developed recently to meet the demand, including bioluminescence measurement of luciferase targeted to the secretory granules [19], AlphaScreen (Amplified Luminescent Proximity Homogeneous Assay Screen) [58], and homogenous time-resolved fluorescence (HTRF) assay that combines fluorescence energy transfer and time resolved luminescence [59].

Future development and application of new probes and strategies for monitoring regulated secretion will continue to enhance our ability to investigate how cells control the timing, pattern and sites of granule release in different tissues or organs. To study how secretory cells integrate inputs from other cells or systems including the nervous systems to achieve precise and timely control of secretion to meet the physiological demand, it would require monitoring regulated cell secretion in vivo. Since light penetration or scattering may become a limiting factor for optical imaging in live animals, novel concepts and strategies would be required. Some emerging approaches have yielded promising results for in vivo studies, including intravital microscopy or microendoscopy [18, 60, 61], in vivo ACEE-Tagging followed by postmortal analysis [20], and magnetic resonance imaging [45, 62]. Future exploration of these and other imaging technologies will offer exciting opportunities for investigating physiological regulation of stimulus-secretion coupling in vivo.

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HIGHLIGHTS

- Probes that are capable of monitoring regulated secretion with high sensitivity and spatiotemporal resolution are invaluable tools for studying the regulation and mechanism of stimulus-secretion coupling.
- There are two conceptually different strategies for crafting probes for monitoring regulated secretion.
- One strategy involves labeling secretory vesicles with fluorescent probes.
- The other approach is to engineer customized fluorescent indicators that can report the release of specific secretory products.

Figure 1.
Structures of representative fluorescent tracers for marking secretory granules and for reporting exocytosis. The size of G2 dendrimer was not drawn to scale.

Figure 2.

Structures of membrane tethered fluorescent Zn^{2+} sensors and their modes of action. **(A)** ZIMIR adhere to the plasma membrane through hydrophobic interaction with membrane lipids. **(B)** ZIMIR-Ex4(9–39) binds to the cell surface GLP-1 receptor with high affinity without activating the receptor. **(C)** ZIMIR HaloTag reacts with HaloTag protein at the cell surface to become permanently attached to the protein. All three ZIMIRs sense local Zn^{2+} fluctuations immediately adjacent to the plasma membrane when cells release Zn^{2+} granules. GLP-1R and HaloTag protein are not drawn to scale.

Figure 3.

Activation of channelrhodopsin (ChR2) led to Zn^{2+} /insulin secretion events in discrete subcellular sites in beta cells. **(A)** A MIN6 beta cell expressing ChR2 and a red Ca^{2+} sensor R-GECO1.2 was photo-activated. Zn^{2+} /insulin release was detected in a microdomain at the plasma membrane (arrow head). Scale bar = 5 μm . **(B)** Time courses of R-GECO1.2 and ZIMIR-HaloTag in three example regions of interest (ROIs 1, 2, and 3 as outlined in A) during photostimulation of ChR2 (1 $\times$ 9 repetitions, represented by blue bars at the top of the plot. Adapted from Ref [52].