Imaging dynamic insulin release using a fluorescent zinc indicator for monitoring induced exocytotic release (ZIMIR)

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Current methods of monitoring insulin secretion lack the required spatial and temporal resolution to adequately map the dynamics of exocytosis of native insulin granules in intact cell populations in three dimensions. Exploiting the fact that insulin granules contain a high level of Zn2+, and that Zn2+ is coreleased with insulin during secretion, we have developed a fluorescent, cell surface-targeted zinc indicator for monitoring induced exocytotic release (ZIMIR). ZIMIR displayed a robust fluorescence enhancement on Zn2+ chelation and bound Zn2+ with high selectivity against Ca2+ and Mg2+. When added to cultured β cells or intact pancreatic islets at low micromolar concentrations, ZIMIR labeled cells rapidly, noninvasively, and stably, and it reliably reported changes in Zn2+ concentration near the sites of granule fusion with high sensitivity that correlated well with membrane capacitance measurement. Fluorescence imaging of ZIMIR-labeled β cells followed the dynamics of exocytotic activity at subcellular resolution, even when using simple epifluorescence microscopy, and located the chief sites of insulin release to intercellular junctions. Moreover, ZIMIR imaging of intact rat islets revealed that Zn2+/insulin release occurred largely in small groups of adjacent β cells, with each forming a "secretory unit." Concurrent imaging of ZIMIR and Fura-2 showed that the amplitude of cytosolic Ca2+ elevation did not necessarily correlate with insulin secretion activity, suggesting that events downstream of Ca2+ signaling underlie the cell-cell heterogeneity in insulin release. In addition to studying stimulation-secretion coupling in cells with Zn2+-containing granules, ZIMIR may find applications in β-cell engineering and screening for molecules regulating insulin secretion on high-throughput platforms.

Design, Syntheses, and in Vitro Characterization of Zinc Indicator for Monitoring Induced Exocytotic Release. To develop a robust imaging assay for monitoring insulin secretion and to boost the sensitivity of Zn2+ detection near the plasma membrane, we designed a membrane-anchored fluorescent Zn2+ indicator, zinc indicator for monitoring induced exocytotic release (ZIMIR; Fig. 1). ZIMIR consists of three moieties: a fluorophore based on fluorescein, a Zn2+ binding motif derived from dipicolylamine (17–19), and a pair of dodecyl alkyl chains for membrane tethering. In the absence of Zn2+, the fluorescence of ZIMIR is quenched by the photo-induced electron transfer from the amino group to fluorescein. When ZIMIR binds Zn2+, the lone pair electrons of the nitrogen atom of 6-aminofluorescein coordinate around Zn2+, resulting in the dequenching of ZIMIR fluorescence. At physiological pH, ZIMIR is an amphiphilic molecule containing four negative charges, preventing its diffusion across hydrophobic cell membranes by itself. This restricts the probe to the outer leaflet of the lipid bilayer after the insertion of its two alkyl chains into the plasma membrane. During granule exocytosis and insulin secretion, the elevation of local Zn2+ concent-

The proper regulation of insulin secretion is essential for maintaining the normal homeostasis of blood glucose (1). To understand better how insulin secretion becomes impaired in pancreatic islet β cells in diabetes, there is increasing interest in studying how the mechanisms that govern insulin release are regulated (2). Techniques that can track the dynamics of regulated secretion with high sensitivity and high spatial and temporal resolution would be invaluable for such studies (3, 4).

Monitoring insulin release at the cellular level was made possible with the development of electrophysiological approaches, including amperometry (5) and measurement of membrane capacitance (6). Although these techniques provide remarkable temporal resolution, they are very limited in spatial aspects of insulin release, are applicable only to a single cell at a time, and can disrupt plasma membranes. To track the location, amplitude, duration, and frequency of insulin secretion at cellular and subcellular levels, fluorescence imaging offers numerous advantages, including high spatial and temporal resolution, superb sensitivity, and noninvasiveness, provided that fluorescent probes for monitoring the secretory activity of pancreatic β cells can be developed (3, 4). Ideally, such probes should be applicable to cultured cells, dissected tissues (islets), or even intact pancreas to track the dynamics of insulin release in three dimensions (3D) over time. In addition, it would be desirable to follow stimulus-secretion coupling by imaging the release of native granules or cargos without the requirement of expressing artificial reporters, because such expressions could potentially perturb the biogenesis, trafficking, and/or localization of native secretory granules (7, 8).

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groups (ZIMIR-C2, Fig. S1). The absorption maximum of which two dodecyl alkyl chains were replaced by a pair of ethyl chains to facilitate characterizing the fluorescent properties in aqueous solutions. The two lipophilic alkyl chains (wavy lines) anchor ZIMIR to the outer leaflet of the membrane lipid bilayer.

The fluorescence readout of ZIMIR should be highly sensitive to β cells’ secretory activity. We synthesized ZIMIR in a total of eight steps (Fig. S1). To facilitate characterizing the fluorescent properties, we also prepared a highly soluble ZIMIR homolog in which two dodecyl alkyl chains were replaced by a pair of ethyl groups (ZIMIR-C2, Fig. S1). The absorption maximum of ZIMIR-C2 centered around 493 nm with an extinction coefficient of 73,000 M⁻¹cm⁻¹. At low nanomolar [Zn²⁺], ZIMIR-C2 was nearly nonfluorescent [fluorescence quantum yield without Zn²⁺, or Q₀ (0 Zn²⁺) = 0.0032] (Fig. 2A). Its fluorescence intensity increased with [Zn²⁺] and reached a plateau at micromolar [Zn²⁺], with an overall fluorescence enhancement of 78-fold on Zn²⁺-bound ZIMIR-C2, providing a Zn²⁺ binding dissociation constant of 0.45 μM (Fig. 2B). To confirm that ZIMIR-C2 binds Zn²⁺ selectively against interfering divalent cations present in physiological salines, we measured its fluorescence in the presence of Ca²⁺ and Mg²⁺. At millimolar concentrations, neither Ca²⁺ nor Mg²⁺ affected ZIMIR-C2 fluorescence, nor did they affect [Zn²⁺]-dependent fluorescence enhancement displayed by ZIMIR-C2 (Fig. 2C).

**ZIMIR Uptake and Zn²⁺ Response in Live Cells.** To examine the cellular uptake of ZIMIR, we used a mouse insulinoma cell line, MIN6 (20). When ZIMIR was incubated with cells at a concentration of 1 μM, it rapidly adhered to the cell surface of intact living cells (Movie S1). To test the specificity and stability of membrane labeling, we used CLSM to follow the cellular uptake and distribution of ZIMIR in MIN6 cells (Fig. S2). Within 5 min after probe addition, there was already a clear accumulation of ZIMIR along the plasma membrane (Fig. S2 A and B). By 20 min, the cellular uptake of ZIMIR appeared to reach completion (Fig. S2 C and D). Subsequent repetitive washings did not change membrane fluorescence intensity, suggesting strong association between ZIMIR and membrane lipids once ZIMIR was anchored to the plasma membrane (Fig. S2E). It is worth noting that another ZIMIR homolog containing a pair of nonyl alkyl chains, ZIMIR-C9, also showed membrane enrichment during loading but failed to adhere to cell membranes on washing, suggesting that a minimum of two decyl chains is required for ZIMIR to remain stably anchored in the plasma membrane. Once taken up by cells, there was a gradual internalization of ZIMIR into the intracellular compartments, yet a sizable portion of ZIMIR still remained on the cell membrane (Fig. S2F). The intracellular distribution of ZIMIR appeared to overlap extensively with that of rhodamine-transferrin, a marker of endocytic vesicles (21), suggesting that ZIMIR internalization may be at least partially mediated through endocytosis (Fig. S3). We routinely loaded cells with ZIMIR (0.5–1 μM) for ~20 min before washing and imaging. Further testing of ZIMIR in other β cells, including the rat insulinoma cell line INS-1 and primary β cells isolated from mouse, rat, or human islets, as well as in other types of cell lines, such as HeLa, HEK-293, and COS cells, confirmed the same cellular uptake and membrane labeling properties, suggesting its high efficiency of membrane labeling to be general among cultured mammalian cells. Moreover, β cells labeled with ZIMIR showed the same growth rate (Fig. S4A), comparable apoptosis (Fig. S4B), and identical insulin secretion in response to different secretagogues compared with unlabeled cells (Fig. S4 C and D), suggesting that ZIMIR labeling caused very little cytotoxicity or perturbation toward cell functions.

Membrane-anchored ZIMIR reliably reports fluctuations of [Zn²⁺] in the extracellular medium. After labeling cells with ZIMIR, we varied extracellular [Zn²⁺] ([Zn²⁺]e) from nanomolar to micromolar levels. Intensity of ZIMIR fluorescence along the plasma membrane declined as expected from the reversibility of Zn²⁺ binding. Membrane-anchored ZIMIR reliably reports fluctuations of [Zn²⁺] in the extracellular medium. After labeling cells with ZIMIR, we varied extracellular [Zn²⁺] ([Zn²⁺]e) from nanomolar to micromolar levels. Intensity of ZIMIR fluorescence along the plasma membrane declined as expected from the reversibility of Zn²⁺ binding.

ZIMIR Imaging of Insulin/Zn²⁺ Release and Correlation with Membrane Capacitance. To image insulin/Zn²⁺ secretion, we used wide-field epifluorescence microscopy and stimulated MIN6 cells with a high KCl concentration (40 mM) to depolarize the cell membrane and to activate the voltage-operated Ca²⁺ channels. Subsequent Ca²⁺ influxes triggered insulin release and, as expected, caused a robust enhancement in ZIMIR fluorescence (Fig. 3 A–E and Movie S2). During the experiment, a small amount of EDTA (10 μM) was included in the solution to chelate the residual Zn²⁺ present in the physiological saline and to reduce the baseline signal. In β cells isolated from WT animals (C57BL/6 mouse), successive stimulation with high glucose (17 mM), KCl (30 mM), and the ATP-sensitive K⁺ channel blocker tolbutamide (0.2 mM) caused repetitive ZIMIR fluorescence increases (Fig. 3F and Movie S3). In the case of stimulation with the physiological secretagogue glucose, the fluctuations were frequently highly localized at specific regions of the plasma membrane and are likely to reflect individual (or a small number of) exocytotic events, given the relative infrequency of these events under these conditions (22). In contrast, β cells extracted from mice lacking the granular zinc transporter ZnT8 (coded by the Slc30a8 gene) (23) failed to display similar enhancements in ZIMIR fluorescence signal following the same set of stimulations (Fig. 3 G and H and Movie S4). ZnT8 is highly expressed on the granular membrane of islet β cells (24), and ZnT8 KO mice showed defects in cellular Zn²⁺ transport, insulin crystallization, and the formation of dense core granules, suggesting that ZnT8 represents a key Zn²⁺ transporter responsible for accumulating the ion in insulin granules (25). Consistent with these studies, the drastically reduced ZIMIR fluorescence in response to stimuli observed in the ZnT8 KO β cells likely reflected a much decreased level of granular Zn²⁺ content in these cells.

**Epifluorescence ZIMIR Imaging of Insulin/Zn²⁺ Release and Correlation with Membrane Capacitance.** To image insulin/Zn²⁺ secretion, we used wide-field epifluorescence microscopy and stimulated MIN6 cells with a high KCl concentration (40 mM) to depolarize the cell membrane and to activate the voltage-operated Ca²⁺ channels. Subsequent Ca²⁺ influxes triggered insulin release and, as expected, caused a robust enhancement in ZIMIR fluorescence (Fig. 3 A–E and Movie S2). During the experiment, a small amount of EDTA (10 μM) was included in the solution to chelate the residual Zn²⁺ present in the physiological saline and to reduce the baseline signal. In β cells isolated from WT animals (C57BL/6 mouse), successive stimulation with high glucose (17 mM), KCl (30 mM), and the ATP-sensitive K⁺ channel blocker tolbutamide (0.2 mM) caused repetitive ZIMIR fluorescence increases (Fig. 3F and Movie S3). In the case of stimulation with the physiological secretagogue glucose, the fluctuations were frequently highly localized at specific regions of the plasma membrane and are likely to reflect individual (or a small number of) exocytotic events, given the relative infrequency of these events under these conditions (22). In contrast, β cells extracted from mice lacking the granular zinc transporter ZnT8 (coded by the Slc30a8 gene) (23) failed to display similar enhancements in ZIMIR fluorescence signal following the same set of stimulations (Fig. 3 G and H and Movie S4). ZnT8 is highly expressed on the granular membrane of islet β cells (24), and ZnT8 KO mice showed defects in cellular Zn²⁺ transport, insulin crystallization, and the formation of dense core granules, suggesting that ZnT8 represents a key Zn²⁺ transporter responsible for accumulating the ion in insulin granules (25). Consistent with these studies, the drastically reduced ZIMIR fluorescence in response to stimuli observed in the ZnT8 KO β cells likely reflected a much decreased level of granular Zn²⁺ content in these cells.

**Fig. 1.** Design of ZIMIR. (A) Chemical structure of ZIMIR in the Zn²⁺-free (nonfluorescent) and Zn²⁺-bound (strongly fluorescent) states. (B) Mode of action of ZIMIR for reporting local Zn²⁺ elevation at the membrane surface during exocytotic insulin granule fusion. The two lipophilic alkyl chains (wavy lines) anchor ZIMIR to the outer leaflet of the membrane lipid bilayer.
We next examined how the increase in ZIMIR fluorescence and [Zn\(^{2+}\)] on the cell membrane is related to the granule exocytosis induced by direct membrane depolarization. To this end, we used standard whole-cell patch-clamp configuration and monitored changes in membrane capacitance simultaneously with respective changes in ZIMIR fluorescence. To ensure that “full-fusion” events dominated over partial events (“cavity recapture” or “kiss-and-run”) (26, 27), we imposed 10 pulses of 2.5-s depolarizations, which resulted in a dramatic increase in capacitance [Fig. S5; corresponding to the release of 109 ± 37 granules per 10-pulse train, assuming the single-granule capacitance of 3.5 fF (28)]. This was followed almost immediately by an increase in ZIMIR fluorescence. Judging from the proportional elevation in capacitance and membrane ZIMIR signal, we concluded that ZIMIR imaging provided sensitive and faithful readout of exocytotic activity of insulin granules in β cells over a wide dynamic range.

On membrane depolarization, the ZIMIR signal increased immediately and reached a plateau before it gradually declined (Fig. 3E and F). Unexpectedly, however, the ZIMIR signal remained elevated even after we lowered KCl concentration to the resting level (5 mM) (Fig. S6). This prolonged elevation of fluorescence suggested that Zn\(^{2+}\), once released from the insulin granules and complexed by ZIMIR, only gradually dissociated from the membrane-anchored probe before it escaped into the bulk solution. Because such a slow membrane dissipation of Zn\(^{2+}\) could artificially lengthen the duration of the observed insulin/Zn\(^{2+}\)
membranes in both cultured MIN6 cells (Fig. 4B). DPAS is not expected to diffuse across cell membranes by itself. Like other dipicolylamine-based chelators, DPAS binds Zn\(^{2+}\) with good selectivity against Ca\(^{2+}\) and Mg\(^{2+}\), and it chelates Zn\(^{2+}\) with much faster kinetics than EDTA in physiological solutions (Fig. S7). In INS-1 β cells labeled with ZIMIR, after recording ZIMIR fluorescence in solutions containing ∼1 μM free Zn\(^{2+}\), we perfused cells with a nominally Zn\(^{2+}\)-free solution containing only EDTA (10 μM) or with both EDTA (10 μM) and different concentrations of DPAS. In the absence of DPAS, membrane ZIMIR signal declined slowly on Zn\(^{2+}\) washout (decaying half-lifetime = 138.7 ± 13.8 s, Fig. 4A), likely because the EDTA–Ca\(^{2+}/Mg\(^{2+}\) complex had to dissociate first (a slow process with an off rate ≤ 2.8 s\(^{-1}\)) (29) before EDTA could complex with Zn\(^{2+}\). In contrast, DPAS, even at low micromolar concentrations, increased the rate of Zn\(^{2+}\) dissipation from the plasma membrane by about one order of magnitude (Fig. 4A).

Consistent with the role of DPAS in accelerating Zn\(^{2+}\) dissipation into the bulk solution, ZIMIR imaging in the presence of DPAS displayed a more dynamic picture of insulin release activity, revealing repetitive fluorescence spikes along the plasma membranes in both cultured MIN6 cells (Fig. 4B and Movie S5) and primary human β cells (Movie S6). Interestingly, there appeared to be preferred sites of insulin release along the cell-cell contacts, where pulses of insulin release were observed repeatedly. Whether those sites correspond to subcellular domains that favor the formation of readily releasable pools of insulin granules remains an interesting question for future investigation.

Confocal imaging of ZIMIR uptake and insulin/Zn\(^{2+}\) release in pancreatic islets. In addition to cultured cells, ZIMIR rapidly and noninvasively labels cells in such preparations as dissected pancreatic islets. After loading islets with ZIMIR for 25 min, we used CLSM to track the distribution of ZIMIR in 3D. We observed that ZIMIR was taken up by cells throughout the islets, from the mantle to the core (Fig. S4 [mouse islets] and Fig. S8 [human islets]). In contrast, loading cells with cytosolic dyes, such as calcein acetoxyethyl ester (calcein/AM), only labeled cells of the superficial layers (Fig. 5B). The result confirmed that ZIMIR readily diffused through the interstitial space to reach interior cells, whereas calcein/AM was trapped by cells in the outer layer and unable to penetrate any deeper. After labeling, we depolarized cells with high concentration of KCl. A robust and synchronized enhancement of ZIMIR fluorescence was detected in many cells within the islet (Fig. S9 and Movie S7), demonstrating ZIMIR’s ability to capture the dynamics of insulin granule release in physiological preparations or tissues.

To examine the pattern and the timing of glucose-stimulated insulin secretion (GSIS) at cellular and subcellular resolution in intact islets, we monitored ZIMIR response by CLSM at multiple confocal sections. Following glucose (20 mM) stimulation, ZIMIR fluorescence displayed a robust enhancement in small clusters of β cells (Fig. 6, Movie S8 showing one confocal section and the corresponding time courses of four regions of interest, and Movie S9 showing ZIMIR response of the same islet in 3 confocal sections 10 μm apart). These clusters of glucose-responsive β cells were scattered throughout islets and were surrounded by β cells that showed little insulin secretory activity (Movies S8 and S9). The onset of insulin/Zn\(^{2+}\) release from these clusters was synchronized; however, the onsets of GSIS of separate responsive cell clusters of the same islet were frequently out of sync (e.g., Fig. 6 and Movies S8 and S9). Of the 14 rat islets examined, 10 of them displayed a response similar to the one shown in Fig. 6 and the remaining 4 islets displayed a more synchronized ZIMIR response among separate cell clusters (Fig. S10 and Movie S10).

In rodent islets, most β cells cluster in the islet core, which is shelled by a mantle of other types of endocrine cells, including α, δ, and PP cells (30). Our ZIMIR imaging thus far in isolated islets has revealed that insulin secretion could occur both near the mantle and in the interior of islets (Movies S7–S10). It is not clear, however, whether β cells release insulin exclusively at the homologous cell-cell contact (β–β) or if they can also secrete hormone at the heterologous cell-cell contact (e.g., β–α). Answers to this question may become important in investigating the cellular organization of secretory machinery in islets and in understanding paracrine and/or autocrine signaling among islet cells (31). As the initial step to address this question, we performed immunohistochemistry after ZIMIR imaging to identify α and β cells. Correlation of glucose-stimulated ZIMIR responses with immunohistochemical images confirmed that GSIS occurred at both homologous and heterologous cell-cell contacts in islets (Fig. 7).

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**Fig. 4.** DPAS accelerates Zn\(^{2+}\) dissipation from membranes and facilitates revealing oscillatory activity of insulin/Zn\(^{2+}\) release. (A) Time courses of ZIMIR fluorescence intensity along the plasma membrane \(I_{ZIMIR(PM)}\) decay after washing INS-1 cells (initially bathed in HBS containing 1 μM Zn\(^{2+}\)) with HBS containing EDTA (10 μM) and DPAS (0–8 μM). (B) ZIMIR imaging of insulin/Zn\(^{2+}\) release of MIN6 cells bathed in SAB containing EDTA (10 μM) and DPAS (4 μM). Time courses of \(I_{ZIMIR(PM)}\) fluctuation of four separate regions of interest (ROIs) and example ZIMIR images at different time points are shown (a–e; arrowheads highlight local ZIMIR increases in separate ROIs at different times). F, fluorescence. Scale bar, 10 μm.

**Fig. 5.** ZIMIR labels cells throughout islets. Confocal images of mouse islets labeled with ZIMIR (A) or calcein/AM (B). Scale bar, 20 μm.
mast cells, and certain excitatory neurons, contain a high level of including submandibular salivary gland, prostate epithelial cells, freshly isolated primary

developing and using ZIMIR derivatives with appropriate Zn2+

spatiotemporal resolution. Importantly, the probe can be imaged

of granular Zn2+ release in intact cell populations with high

Fig. S4), it is ideally suited for studying insulin secretion in

importance of the organization and timing of GSIS at

Fig. 6. ZIMIR imaging of GSIS in intact islets. (A) Confocal ZIMIR images of an islet before (Left) and at different time points (β, a–d) after (Right) stimulation with 20 mM glucose. (Right) Images are enlarged views of four subareas containing four example regions of interest (ROIs) along cell membranes that showed strong ZIMIR response. (B) Time courses of ZIMIR fluorescence of ROI-1 through ROI-4.

Discussion

We describe here a unique fluorescent Zn2+ sensor, ZIMIR, that possesses a number of salient features for imaging the dynamics of granular Zn2+ release in intact cell populations with high spatiotemporal resolution. Importantly, the probe can be imaged on a simple epifluorescence microscope without the requirement for sophisticated optical devices (e.g., TIRF microscope, multi-photon microscope). Further, because the use of ZIMIR does not rely on cell transfection to express a fusion protein and does not affect cell viability or the release of the endogenous hormone (Fig. S4), it is ideally suited for studying insulin secretion in freshly isolated primary β cells, intact islets, and possibly even in the intact pancreas.

In addition to pancreatic β cells, a variety of mammalian cells, including submandibular salivary gland, prostate epithelial cells, mast cells, and certain excitatory neurons, contain a high level of Zn2+ in their secretory granules. On stimulation, these cells likewise corelease Zn2+ together with other secretory granules' contents, into the extracellular medium (32). These different biological systems may also represent fertile areas to apply ZIMIR or its homologs to study stimulus-secretion coupling and Zn2+ homeostasis (33). Depending on the cell type and/or the biological preparation, local [Zn2+] fluctuations near the cell surface likely vary over a wide range during secretion. Developing and using ZIMIR derivatives with appropriate Zn2+ affinities will certainly improve the sensitivity and the resolution for imaging Zn2+ release in different biological systems.

We demonstrate here that ZIMIR can be used to image the exocytotic activity of a variety of insulin-secreting cell preparations, ranging from dispersed cells to the intact islet, reporting changes across the entire cell surface in each case (in contrast to TIRF microscopy) (10, 16). Each approach revealed interesting features of the organization and timing of GSIS at both the cellular and subcellular levels. First, we show that within intact rat islets, β cells are heterogenous in their exocytotic activity following glucose stimulation, such that only a subpopulation of β cells displays robust secretion at any one time. The above findings may reflect the metabolic heterogeneity of individual β cells (34), possibly attributable to differences in the expression of “glucose-sensing” enzymes, notably glucokinase (35), and the heterogeneity in glucose-induced cytosolic Ca2+ ([Ca2+]i) increases previously observed in 11% of WT mouse islets (36). Interestingly, our results showed that rat β cells manifesting robust GSIS tended to aggregate in small clusters and that cells within individual clusters appeared to act together as a “secretory unit” to release insulin in synchrony. These secretory units are scattered throughout islets among other β cells that show much weaker secretory activity; following glucose challenge, the onset of exocytosis between individual secretory units is frequently desynchronized. This “short-range synchronization” in exocytotic activity contrasts with the “long-range synchronization” in [Ca2+]i, that has been reported to occur among β cells throughout the islet in mice and humans (37, 38). The difference suggests a disconnection between [Ca2+]i elevation and insulin release in at least some islet β cells and further highlights the complexity of mechanisms underlying the heterogeneity of cellular exocytotic activity.

Second, we demonstrate that the sites of insulin/Zn2+ release at the subcellular level include both homologous cell-cell contacts (β-β) and heterologous (β-α) cell-cell contacts. By contrast, Zn2+/insulin release was rarely observed (in cell clusters) at other sites. In this context, our earlier use of a highly pH-sensitive granule-targeted probe (NPY-Venus) (39), capable of reporting “all” exocytotic event types [including rapid kiss-and-run events in which only small molecules, such as ATP, γ-aminobutyric acid, or H+, are released (40)], did not provide any evidence for the localization of such transient events at cell-cell contact points in MIN6 cell clusters. These findings lead us to speculate that more “complete” fusion events, leading to insulin dissolution and Zn2+ release (27), occur at different sites on the plasma membrane from those leading to kiss-and-run (41).

To uncover further mechanisms and factors governing regulated granule release, it would be desirable to establish a moment-to-moment correlation between cellular signaling and exocytotic activity. Combining ZIMIR with other biochemical fluorescent probes would enable a multicolor imaging approach to determine their spatiotemporal relationship. Our initial attempts at integrating ZIMIR and Fura-2 imaging revealed heterogeneity in exocytotic activity when cells were depolarized to produce a fairly uniform [Ca2+]i rise (Fig. S11 and Movie S11), suggesting that more proximal events downstream of Ca2+ signaling may contribute to variations in insulin release between cells.

Finally, recent progress in ES cell research has generated new hope and excitement in engineering insulin-releasing β cells for cell replacement therapy in the case of diabetes (42). Because ZIMIR imaging can easily be applied to a population of primary β cells to screen their insulin release activity without the
requirement of cell transfection, it offers an efficient and convenient assay to screen for cell clones manifesting robust secretory response or to identify compounds or genes of therapeutic potential for treating diabetes using high-throughput platforms.

Materials and Methods

Details of synthesis and Zn\textsuperscript{2+} titration of ZIMIR, islet isolation, electrophysiology, and imaging are provided in SI Materials and Methods.

Cell Culture and ZIMIR Imaging by Wide-Field Fluorescence Microscopy. For cell imaging, we cultured cells in 35-mm Petri dishes with glass bottoms (MatTek). To label cells with ZIMIR, cells were washed with a secretion assay buffer (SAB, SI Materials and Methods). The DMSO stock solution of ZIMIR (1 – 2 μM) diluted in a small volume of SAB was added to cells to a final concentration of 1 μM. Cells were then incubated at −25 °C for 20 min and washed with SAB before imaging.

To image insulin/Zn\textsuperscript{2+} secretion, we routinely included 10 μM EDTA in SAB to chelate the residual Zn\textsuperscript{2+}. In addition, DPAS (2 mM) was added to SAB in most experiments to resolve the dynamics of oscillatory insulin release better.

To image [Ca\textsuperscript{2+}], and Zn\textsuperscript{2+} release concurrently, we loaded cells with both ZIMIR and Fura-2/AM (2 μM) in the presence of pluronic F-127 (1 g in 10 mL DMSO) (43). During loading, DMSO was kept below 0.5% and pluronic F-127 was less than 0.05%. Wide-field fluorescence microscopy was carried out on inverted fluorescence microscopes as described elsewhere (44).

Iset Labeling and ZIMIR Imaging by CLSM. Rodent islets were isolated from Sprague-Dawley rats or C57BL/6 mice after digesting exocrine tissues of pancreas using collagenase as described in SI Materials and Methods. Isolated islets were hand-picked and transferred to an imaging dish coated with polylysine (15–30 kDa, 25 μg/mL for 5 min) and cultured overnight in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated FBS. Cells were then incubated for another 2 h in serum-free medium and for an additional 2 h in serum-free and glucose-free medium. To image insulin release in islets, we labeled islets with ZIMIR (2 μM) in SAB buffer (3 mM glucose) for 20 min. Islets were then washed and imaged in SAB solution (with 10 μM forskolin) by CLSM using an LSM510 imaging system (Carl Zeiss) and a 40× oil immersion objective as described elsewhere (43).

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Experiments with rodents were carried out according to protocols approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center or by the UK Home Office Animal Scientific Procedure Act (1986). Rodent isolates were isolated from Sprague–Dawley rats or C57BL/6 mice after digesting exocrine tissues of pancreas using collagenase (5). Briefly, young adult animals 12–20 wk old were anesthetized by i.p. injection of Nembutal and killed by cervical dislocation. The internal organs were exposed after abdominal incision. After clamping the ampulla on the duodenum wall, we perfused the pancreas with ~4 mL of collagenase solution (Roche Collagenase P, catalog no. 11213857001; 1.4 mg/mL dissolved in HBSS). The pancreas was then digested in another 1 mL of collagenase solution at 37 °C for ~15 min. When the digestion suspension became homogeneous and appeared milky, we stopped the digestion by leaving the tube on ice and adding 14 mL of cold HBSS. Cells were centrifuged at 250 × g for 2 min. Supernatant was decanted, and cells were resuspended in 10 mL of HBSS. After repeating the process one more time, we hand-picked the resuspended isolates using a pipette under a dissection scope. Isolated rodent isolates were cultured at 37 °C with 5% (vol/vol) CO₂ in RPMI medium 1640 containing 10% (vol/vol) FBS.

Human isolates were obtained through the Integrated Islet Distribution Program sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases and the Juvenile Diabetes Research Foundation International, and they were shipped to us from the sponsored isolates’ isolation centers at the Scharp/Lacy Institute, University of Pennsylvania, University of Miami, University of Wisconsin, and Southern California Islet Consortium. Human isolates were cultured at low density (1,000 isolates eq/mL) in CMRL medium supplemented with 10% (vol/vol) FBS and glucose (final concentration of 10 mM) in 5% (vol/vol) CO₂ in RPMI medium 1640 containing 10% (vol/vol) FBS.
insulin (1:50 dilution of guinea pig anti-human insulin, catalog no. 4011-01F; Millipore) and glucagon (1:3,000 dilution of mouse anti-glucagon; catalog no. G2654; Sigma) at 4 °C overnight. The solution was decanted the following day, and the islets were washed three times (for 5 min each wash) with ice-cold PBS. We then added secondary antibodies (1:50 dilution of FITC-labeled anti-guinea pig, catalog no. 106-095-003 and 12,000 dilution of Cy3 labeled anti-mouse, catalog no. 111-165-144; both from Jackson) and incubated islets at RT for 1 h. After washing islets three times with ice-cold PBS, we acquired confocal images in both FITC and Cy3 channels in a 1-μm z-step using an LSM510 confocal laser scanning microscope (Carl Zeiss). During the entire procedure, care should be taken to minimize agitation and to avoid islets from detaching off the glass coverslip.

Concurrent Electrophysiological Recording and ZIMIR Imaging. Electrophysiological recordings and stimulation were done in standard whole-cell configuration using an EPC9 patch-clamp amplifier controlled by Pulse acquisition software (HEKA Elektronik). The pipette solution contained 10 mM NaCl, 125 mM Cs-glucamate, 10 mM KCl, 1 mM MgCl2, 5 mM HEPES (pH 7.15 with CsOH), 3 mM MgATP, and 0.1 mM CAMP. The extracellular bath solution contained 120 mM NaCl, 4.8 mM KC1, 24 mM NaHCO3 (saturated with CO2), 5 mM HEPES (pH 7.4 with NaOH), 0.5 mM NaH2PO4, 1.5 mM CaCl2, 0.5 mM MgSO4, 0.01 mM EDTA, and 3 mM glucose. The experiments were conducted at 33 °C using dissociated islet β cells from female CD-1 mice. After islet isolation and dissociation, islets were plated on glass coverslips and cultured for >24 h before the assay.

The imaging experiments were done on an Olympus IX-71 microscope with a UPlanFL N objective with a magnification of 40× (Olympus). For acquisition, an F-View-II camera and CT-20 excitation system equipped with an Hg arc lamp were used, under control of Cell^R software (all from Olympus). The dye was excited at 490 nm, and emission at 530 nm was recorded. Images were acquired at a frequency of 0.5 Hz with typical excitation times of 10 ms. The acquisition of the fluorescence and electrophysiological data was synchronized using the transistor–transistor logic (TTL) pulse. After the stabilizing standard whole-cell configuration, series resistance and cell capacitance were compensated for automatically by the acquisition software. Recordings, triggered by the TTL pulse from the imaging software, were started in current-clamp mode, after which the cell was voltage-clamped at −70 mV to allow the equilibration of the solution between the cell and the pipette. The depolarization-induced changes in the cell capacitance were then recorded using the “Sine + DC” method implemented in the acquisition software. The cell was held at −70 mV and subjected to 10 depolarizing 2.5-s pulses to 0 mV following at a 0.5-s interval during which a 500-Hz, 30-mV sine wave was applied (as shown in Fig. S2). Electrophysiological data were filtered at 10 kHz, digitized at 20 kHz, and further combined with imaging data and analyzed using Igor Pro software (WaveMetrics).

Synthesis. All reagents were purchased from Aldrich or VWR. Anhydrous solvents were stored over activated molecular sieves (3 Å or 4 Å). TLC was performed on precoated silica gel 60F-254 glass plates (EM Science). Reaction products were purified by low-pressure flash chromatography (FC) using silica gel 60 (63–200 μm; EM Science). 1H-NMR spectra were acquired on a Varian 400-MHz or 500-MHz spectrometer. Chemical shifts (δ, ppm) were reported against tetramethylsilane (0 ppm). MALDI-TOF MS were performed on a Voyager-DE PRO biospectrometry workstation (Applied Biosystems) using 2,5-dihydroxy benzoic acid as the matrix.

Preparation of 6-Nitro-4′,5′-Dimethylfluorescein Dipivaloyl Ester (Compound 1). 4-Nitrophthalic acid anhydride (0.97 g, 5.0 mmol) and 2-methyl resorcinol (1.30 g, 10.5 mmol) were suspended in 100 mL of methanesulfonic acid. The mixture was stirred at 80 °C for 8 h. After cooling, the reaction was quenched in 100 mL of ice water and the mixture was passed through a sintered glass filter. The retentate was dried under vacuum at 50 °C for 8 h. The resulting dark red solid was then added to a suspension of Cs2CO3 (3.58 g, 11 mmol) in dimethylformamide (DMF, 20 mL). To this solution was added 2.24 mL of pivalic anhydride. Two hours later, the reaction mixture was filtered and the residue was washed with MeOH (20 mL). The filtrate was evaporated, and the resulting residue was extracted with CHCl3 (3 × 50 mL) and saturated brine. The organic layer was dried over Na2SO4, concentrated, and purified by FC (hexane/EtOAc, 20:1–4:1) to provide the product (5-nitro isomer; 0.607 g, 21.2%), the 6-nitro isomer (0.780 g, 27.2%), and the mixture of both isomers (1.338 g, 46.7%) as white solids.

Preparation of 6-Nitro-4′,5′-dimethylfluorescein dipivaloyl ester: 1H NMR (CDCl3, 400 MHz): δ 8.86 [doublet (d), J = 2.0 Hz, 1H], 8.52 [doublet of doublet (dd), J = 2.0, 8.4 Hz, 1H], 7.37 (d, J = 8.4 Hz, 1H), 6.76 (d, J = 8.8 Hz, 2H), 6.64 (d, J = 8.8 Hz, 2H), 2.34 [singlet (s), 6H], 1.40 (s, 18H). MS was performed: 573.20 calculated for C39H32N2O6; observed: 574.57 (M + H)+, 596.52 (M + Na)+.

Preparation of 6-Nitro-4′,5′-Dibromomethylfluorescein Dipivaloyl Ester (Compound 2). Compound 1 (57.4 mg, 0.10 mmol) mixed with N-bromosuccinimide (55.2 mg, 0.31 mmol) and benzoyl peroxide (10 mg) in CCl4 (10 mL) was refluxed for 4 h. The mixture was cooled and filtered, and the solid residue was washed with EtO. The filtrate was concentrated and purified by FC (hexane/EtOAc, 10:1–8:1) to afford compound 2 (72 mg, 98.4%): 1H NMR (CDCl3, 400 MHz): δ 8.82 (dd, J = 2.0, 8.4 Hz, 1H), 8.24 (d, J = 8.4 Hz, 1H), 8.07 (d, J = 2.0 Hz, 1H), 6.76 (d, J = 8.8 Hz, 2H), 6.65 (d, J = 8.8 Hz, 2H), 2.35 (s, 6H), 1.40 (s, 18H). MS was performed: 573.20 calculated for C39H32N2O6; observed: 574.57 (M + H)+, 596.52 (M + Na)+.

Preparation of 2,2-Dimethoxy-N-[2-(2-pyridinylmethyl)-N-(2-pyridinylmethyl)-Aminoethane (6 and 7). These compounds were prepared based on a known procedure (9). Compound 5 (0.646 g, 3.03 mmol) (7) was mixed with 2-bromo-1,1-dimethoxyethane (1.44 mL, 12.12 mmol), Na2CO3 (3.21 g, 30.3 mmol), and potassium fluoride/celite (1:1, 240 mg) in 18 mL of acetonitrile. The mixture was refluxed for 3 d under argon, cooled, and filtered. The solid residue was washed with CH2CN. The filtrate was concentrated and purified by FC (dichloromethane/MeOH, 50:1–20:1) to afford compound 6 (0.542 g, 59%) as a yellow oil: 1H NMR (CDCl3, 400 MHz): δ 8.51 (m, 2H), 7.60 (dd, J = 7.6, 2.0 Hz, 2H), 7.37 (d, J = 7.6 Hz, 1H), 7.11 (m, 3H), 4.46 [triplet (t), J = 5.2 Hz, 1H], 3.93 (s, 2H), 3.32 (s, 6H), 3.05 [4H, multiplet (m)], 2.80 (2H, d, J = 5.6 Hz). MS was performed: 301.18 calculated for C17H23NO2: observed: 302.46 (M + H)+.

The above intermediate 6 (0.344 g, 1.14 mmol) was suspended in an aqueous 1-N HCl solution (16 mL) and stirred at RT for 6 h. The solvent was removed under vacuum, and the residue was
dried under a high vacuum overnight to provide the product (compound 7) as a yellow solid (0.482 g). 

1H NMR (D$_2$-MeOH, 400 MHz): 8.89 (d, J = 6.0 Hz, 1H), 8.67 (dd, J = 6.0, 0.8 Hz, 1H), 8.05 (d, J = 8.4 Hz, 1H), 7.96 (t, J = 6.8 Hz, 1H), 7.88 (m, 2H), 6.05 (t, J = 4.4 Hz, 1H), 4.36–3.37 (m, 4H), 3.18 (m, 2H). MS was performed: 256.14 calculated for C$_{15}$H$_{13}$N$_3$O$_3$$^+$; observed: 256.34 M$^+$. 

Preparation of Compound 3. A mixture of compound 2 (33.6 mg, 46 μmol), NaI (13.8 mg, 92 μmol), N-ethyl glycine ethyl ester (184 μmol), and proton sponge (20 mg, 92 μmol) in anhydrous acetonitrile (1.0 mL) was flushed overnight. The solution was concentrated, and the residue was purified by FC (hexane/EtOAc, 20:1–2:1) to yield the product compound 3a as a yellow oil (96%): 

1H NMR (CDCl$_3$, 400 MHz): 8.46 (dd, J = 1.8, 8.4 Hz, 1H), 8.03 (d, J = 2.0 Hz, 1H), 6.75 (s, 4H), 4.18 (m, 4H), 4.04 (m, 4H), 3.46 (s, 4H), 2.74 (t, J = 7.4 Hz, 4H), 1.38 (s, 18H), 1.02 (t, J = 7.2 Hz, 6H). Compound 3b was prepared similarly from compound 2 and N-dodecyl glycine ethyl ester in 78% yield: 

1H NMR (CDCl$_3$, 400 MHz): 8.46 (dd, J = 2.0, 8.4 Hz, 1H), 8.17 (d, J = 8.4 Hz, 1H), 8.03 (d, J = 2.0 Hz, 1H), 6.75 (s, 4H), 4.18 (m, 4H), 4.04 (m, 4H), 3.46 (s, 4H), 2.74 (t, J = 7.4 Hz, 4H), 1.38 (s, 18H), 1.02 (t, J = 7.2 Hz, 6H). 

Preparation of Compound 4. To a solution of compound 3a (84 μmol) in MeOH/THF/H$_2$O (4:1, 6 mL) was added sodium hydrosulfide hydrate (400 mg, 68%). The mixture was refluxed for 1.5 h. The solvent was concentrated under vacuum, and the residue was purified by reversed phase column chromatography (LiChroprep RP-18). 

To measure the Zn$^{2+}$ affinity of ZIMIR, we performed Zn$^{2+}$ titration of ZIMIR-C$_2$ (0.4 μM) using a Zn$^{2+}$ buffering system based on nitrilotriacetic acid (NTA) (7). The buffer contained 100 mM Heps (pH 7.5), 10 mM NTA, and varying concentrations of ZnSO$_4$ (0–9 nM) to reach a free Zn$^{2+}$ concentration between 0.1 nM and 43 nM. Zn$^{2+}$ concentrations above 43 nM were not buffered. However, because the commercial Heps always contains a trace amount of divalent metal (≤5 ppm), it was necessary to add a minimum amount of NTA to chelate the residual Zn$^{2+}$ present in the Heps solution. We found that adding ~0.5 μM NTA to 100 mM Heps solution was sufficient to reduce the fluorescence of ZIMIR-C$_2$ to the same level as seen in 43 nM (buffered) free Zn$^{2+}$. From that point, an increasing amount of ZnSO$_4$ was added to reach higher Zn$^{2+}$ concentrations. Two independent Zn$^{2+}$ titrations provided $K_d$(Zn$^{2+}$) values of 433 nM and 467 nM, with an average $K_d$(Zn$^{2+}$) of 450 nM. To examine the Zn$^{2+}$ binding selectivity of ZIMIR against Ca$^{2+}$ or Mg$^{2+}$, we measured the fluorescence intensity of ZIMIR-C$_2$ in a nominally Zn$^{2+}$-free solution containing 50 μM DPAS or in a solution containing 1 μM ZnCl$_2$ with or without Ca$^{2+}$/Mg$^{2+}$ (1 mM). 

Fig. S1. Synthesis of ZIMIRs. (a1) CH₃SO₃H, reflux, 8 h. (a2) Piv₂O, Cs₂CO₃, dimethylformamide, RT, 27% for two steps. (b) N-bromosuccinimide, benzoyl peroxide, CCl₄, reflux, 4 h, 98%. (c) NaI, proton sponge, N-ethyl glycine methyl ester (for 3a), or N-dodecyl glycine ethyl ester (for 3b), CH₃CN, reflux, 14 h, 78–96%. (d) NaHS, MeOH/THF/H₂O (10:3:3), reflux, 1 h, 81% for 4a and 74% for 4b. (e) 2-Bromo-1,1-dimethoxyethane, KF/celite (1:1), CH₃CN, reflux, 3 d, 60%. (f) 1 M HCl, 5 h, 99%. (g) NaBH₃CN, Na₂SO₄, RT, overnight, 36% for ZIMIR-C₂ and 18% for ZIMIR.

Fig. S2. ZIMIR labels plasma membranes of intact living cells rapidly, noninvasively, and stably. ZIMIR (1 μM) was added to MIN6 cells in HBS buffer (containing ~1 μM Zn²⁺ to enhance fluorescence intensity). Confocal images (Ex, 488 nm; Em, 510–550 nm) were subsequently acquired at 2 min (A), 5 min (B), 10 min (C), and 20 min (D). Cells were then washed with HBS buffer three times and imaged again at 21 min (E) and 140 min (F). Scale bar, 20 μm.

Fig. S3. Intracellular staining of rhodamine-transferrin (Rh-Tf) overlaps with that of ZIMIR. MIN6 cells were labeled with ZIMIR (1 μM) and Rh-Tf (20 μg/mL; Invitrogen) in serum-free DMEM at 37 °C for 30 min. Cells were then washed and imaged 15 min later. Scale bar, 10 μm.
Fig. S4. ZIMIR labeling had little effect on β cell growth, cell death, or insulin secretion. (A) Growth of ZIMIR-labeled MIN6 cells was identical to that of control unlabeled cells. Cells in a 35-mm dish were labeled with ZIMIR (1 μM) in SAB buffer for 30 min, washed, and cultured for the next 3 d. Cells were counted every 24 h. (B) Cell death after ZIMIR labeling showed little difference from that in control unlabeled cells. Cells were labeled with ZIMIR (1 μM) in SAB buffer for 30 min, washed, and incubated in the culture medium for another 2 h before apoptosis was assayed using the Cell Death Detection Elisa kit (Roche Applied Science) by measuring the absorption at 405 nm (Abs 405 nm). Positive controls for apoptosis included DNA topoisomerase inhibitor etoposide (0.5 mM, 2.5 h) and hypertonic shock [10 mM Tris (pH 7.4), 400 mM NaCl, 5 mM CaCl\(_2\), and 10 mM MgCl\(_2\) for 2 h]. Abs. (C) ZIMIR labeling did not affect insulin secretion in cultured MIN6 cells. MIN6 cells were stimulated with 40 mM KCl or 0.25 mM carbachol (CCH) for 30 min at 37 °C. The amount of released insulin (Insulin ELISA; Calbiotech) was normalized against control MIN cells bathed in SAB buffer with 3 mM glucose. (D) ZIMIR labeling did not affect insulin secretion in mouse islets. CD1 mouse islets in 12-well plates (6 islets per well) were incubated for 30 min in 0.5 mL of Krebs buffer with 3 mM glucose at 37 °C. Subsequently, islets were stimulated for a further 30 min with either 3 mM glucose (3G), 16.7 mM glucose (17G), or 30 mM KCl. Both the released insulin and the total insulin content were measured, and the percent of insulin secretion was calculated against total insulin content (% tot). n = 3 for all the measurements.

Fig. S5. Depolarization-induced changes in membrane capacitance coincide with increase in ZIMIR fluorescence. The cell was held at a plasma membrane potential (\(V_{m}\)) of −70 mV (black, Upper), and several trains of depolarizations were applied. Each train consisted of ten 2.5-s pulses to 0 mV separated by 0.5-s repolarizations to −70 mV (Upper). (Lower) Sinusoid (30 mV, 500 Hz) was recorded simultaneously and calibrated to extracellular Zn\(^{2+}\) concentration. The calibration was done by application of the extracellular solution with the Zn\(^{2+}\) concentration buffered by diluting 1 mM ZnCl\(_2\) stock in the extracellular solution containing 100 μM EDTA. The total Zn\(^{2+}\) required for 0.01 μM, 0.1 μM, 1 μM, and 10 μM free Zn\(^{2+}\) concentrations was calculated using Maxchelator (Chris Patton, Stanford University).
Fig. S6. ZIMIR signal remained elevated after termination of KCl stimulation. After KCl (40 mM) stimulation, MIN6 cells were washed with the SAB containing 5 mM KCl. EDTA (10 μM) was present throughout the experiment. F, fluorescence.

Fig. S7. DPAS chelates Zn$^{2+}$ much faster than EDTA. In this competition assay, fluorescence intensity (excitation, 490 nm; emission, 525 nm) of ZIMIR-C$_3$ (1 μM) in HBSS was recorded. The rate of fluorescence intensity decline after adding EDTA or DPAS reflects the kinetics of EDTA/Zn$^{2+}$ or DPAS/Zn$^{2+}$ complexation.

Fig. S8. ZIMIR rapidly labels cells of intact human islets. Representative CLSM images of a ZIMIR-labeled human islet at three focal planes 5 μm apart.
Fig. S9. Confocal ZIMIR imaging of insulin/Zn\(^{2+}\) release in a mouse islet bathed in SAB. Images from two example layers 15 μm apart before and 10 s after KCl (40 mM) stimulation are shown. The images correspond to Movie S7. Scale bar, 20 μm.

Fig. S10. ZIMIR imaging of GSIS in intact islets. (A) Confocal ZIMIR images of an islet before (Left) and after (Right) 20 mM glucose stimulation (corresponding to time point a in B). (B) Time courses of ZIMIR fluorescence of three example regions of interest (indicated in A). The images correspond to Movie S10. Scale bar, 20 μm.
Fig. S11. Concurrent imaging of insulin/Zn\textsuperscript{2+} release and [Ca\textsuperscript{2+}], in MIN6 β cells. Example ZIMIR fluorescence images (A) and Fura-2 pseudocolor ratio images (B) (340-nm vs. 380-nm excitation) corresponded to time points a and b marked on the time courses. ZIMIR intensity changes were plotted for three regions of interest, marked as dashed circles in the DIC image, and corresponded to the cell-cell contact of a cell doublet (1), the average of cell-cell contacts of a cell triplet (2), and along the plasma membrane of a singlet (3), respectively. Corresponding Fura-2 ratio changes were measured in the bulk cytosol of these three areas. F, fluorescence.

Movie S1. ZIMIR rapidly and noninvasively labels cell membranes of intact living cells. ZIMIR (1 μM) was added to MIN6 cells bathed in HBSS containing 20 mM Hepes (pH 7.3) and 5 mM glucose. Images were acquired every 30 s for ~20 min.
Movie S2. ZIMIR imaging of KCl (40 mM)-stimulated insulin/Zn^{2+} release in MIN6 cells. Time courses of membrane ZIMIR changes are shown in Fig. 3E.

Movie S3. ZIMIR imaging of insulin/Zn^{2+} release in primary β cells from a WT mouse (C57BL/6). Time courses of membrane ZIMIR changes are shown in Fig. 3F.

Movie S4. ZIMIR imaging of insulin/Zn^{2+} release in primary β cells from a Znt8 KO mouse (C57BL/6). Time courses of membrane ZIMIR changes are shown in Fig. 3G.
Movie S5.  ZIMIR imaging of oscillatory insulin/Zn\textsuperscript{2+} release in MIN6 cells. Time courses of membrane ZIMIR changes are shown in Fig. 4B.

Movie S6.  ZIMIR imaging of pulsatile insulin/Zn\textsuperscript{2+} release in primary β cells from human islets. Images were acquired every 4 s for ~12.5 min.
**Movie S7.** CLSM of insulin/Zn^{2+} release in a mouse islet on KCl stimulation. Images were acquired every 6 s for ~6 min. The two confocal layers were 15 μm apart.

**Movie S8.** CLSM of GSIS in a rat islet (Sprague–Dawley). Four clusters of β cells, highlighted by colored circles at the beginning and end of the movie, showed strong ZIMIR responses at different times after glucose challenge. Time courses of their corresponding ZIMIR fluorescence changes are shown as an animated plot to the right (also as in Fig. 6B).

**Movie S9.** CLSM of GSIS of the same rat islet shown in Movie S8. Images from three confocal layers 10 μm apart are shown. Images of middle layer are the same as in Movie S8.

**Movie S10.** CLSM of synchronized GSIS among several clusters of β cells in a rat islet (Sprague–Dawley). Time courses of the corresponding ZIMIR fluorescence changes are shown in Fig. S10.
**Movie S11.** Dual-color imaging of ZIMIR and Fura-2 in MIN6 cells. Time courses of ZIMIR and Fura-2 changes are plotted in Fig. S11.