TECHNOLOGY

Two-photon imaging of pancreatic beta cells in real time *in vivo*

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Here we present the first generation of two-photon beta cell specific *in vivo* imaging probes based on GLP1R targeting peptides. Among the three compounds of potential interest, we found quite unexpectedly that a squarine-rotaxane conjugate (2PEx-647) had near ideal *in vivo* imaging characteristics.

***Keywords*: Glucagon-Like Peptide-1; Pancreatic Beta Cells; Multiphoton Imaging; Diabetes Research; Targeted Labelling.**

INNOVATION

Glucagon-like peptide-1 receptor (GLP1R) targeted affinity ligands based on exendin-4 peptides have emerged as powerful therapeutic and diagnostic tools to study pancreatic beta cells. Unfortunately, minor molecular modifications to the peptide sequence can have major effects on receptor affinity and pharmacokinetics. In order to image GLP1R and its inhibition in pancreatic islets at subcellular resolutions *in vivo* it would be highly desirable to develop optimized two photon imaging probes, capable of imaging at greater depths than possible by confocal imaging. In our manuscript we detail the synthesis and usage of a series of novel two-photon imaging probes for intravital imaging of pancreatic beta cells.

NARRATIVE

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by the destruction of beta cells ultimately leading to hypoinsulinemia and hyperglycemia. A variety of imaging approaches have been proposed to image beta cell mass[1,2] with many of them converging on modified exendins for MRI[3], SPECT[4], PET[5,6] or fluorescence imaging[6–8]. Over the years we have developed a series of pharmacokinetically optimized exendins, primarily for one-photon excitation confocal imaging. Interestingly, by using a library approach we previously found that fluorochrome attachments can play a major role in the *in vivo* properties of modified exendins[8]. For example, a R12 modified with the near infrared fluorochrome VT750 S had a IC_{50} of 2.6 ± 1.1 nM whereas a VT680-XL version (exhibiting lower fluorochrome quenching) had a ~50 fold lower affinity (115 ± 1.2 nM)[8]. The design criteria were not entirely foreseeable by crystallographic predictions[9]. Indeed, the latter anticipated that ideal version would have to be modified at the 40 position whereas experiments showed that 12 modifications were equally well tolerated[9].

In parallel to the above developments, the technology for multiphoton imaging of organs in live mice has advanced rapidly over the last few years[10,11]. Recent progresses in the field have included better optics and hardware, pre-chirped femtosecond lasers, and motion suppression techniques[12], which are essential for the implementation of two-photon imaging in the pancreas of live mice. Two-photon imaging is critical for imaging at higher spatial resolution, at greater depths, and to prevent photo bleaching and phototoxicity during prolonged imaging sessions[13]. However, to the best of our knowledge, beta cell specific multiphoton imaging probes that work well in live animals have not been described to date. Here we report the preparation of a series of two-photon capable fluorescent exendin (2PEx) probes **(Fig. 1)** for *in vivo* beta cell imaging in mice that are highly specific and stable over extended period of time. The utility of 2PEx probes is demonstrated in intravital imaging of a transgenic diabetic mouse model.

We started the synthesis with a previously validated exendin-4 peptide sequence ($E4_{Pra40}$)[8], but exchanged the propargylglycine at the position 40 to a cysteine. Using a maleimide labeling strategy, we prepared three different fluorescent compounds **(Fig. 1)**. This was achieved by attaching Alexa Fluor 488 (**2PEx-488**), Alexa Fluor 568 (**2PEx-568**) or SeTau-647 (**2PEx-647**). These putative fluorochromes were chosen because of their reported stability, strong excitation in the near infrared window and two-photon properties[14]. The squarine-rotaxane, SeTau-647, has a large two-photon cross-section in the near infrared window with a maximum of 3000 GM at 920 nm, while exceeding 6000 GM at 740 nm. The presence of such a strongly blue-shifted two-photon cross-section, with respect to twice the wavelength of the one-photon absorption[14,15] allows imaging at lower excitation wavelength, supporting co-excitation of spectrally distinct fluorochromes and improving resolution. All coupling reactions were completed within 2 hours at RT and compounds were purified by size exclusion chromatography. The synthesis and purification resulted in a ~70% yield of the desired products (**2PEx-488**, **2PEx-568** and **2PEx-647**).

Exendin-4 is a high affinity agonist of the GLP-1 receptor and is rapidly internalized within minutes of docking to the receptor[16]. An ideal 2PEx probe should maintain high binding affinity and perhaps selective internalization in order to exhibit high fluorescent signal *in vivo*[8]. The binding affinities of the 2PEx probes were initially determined using a

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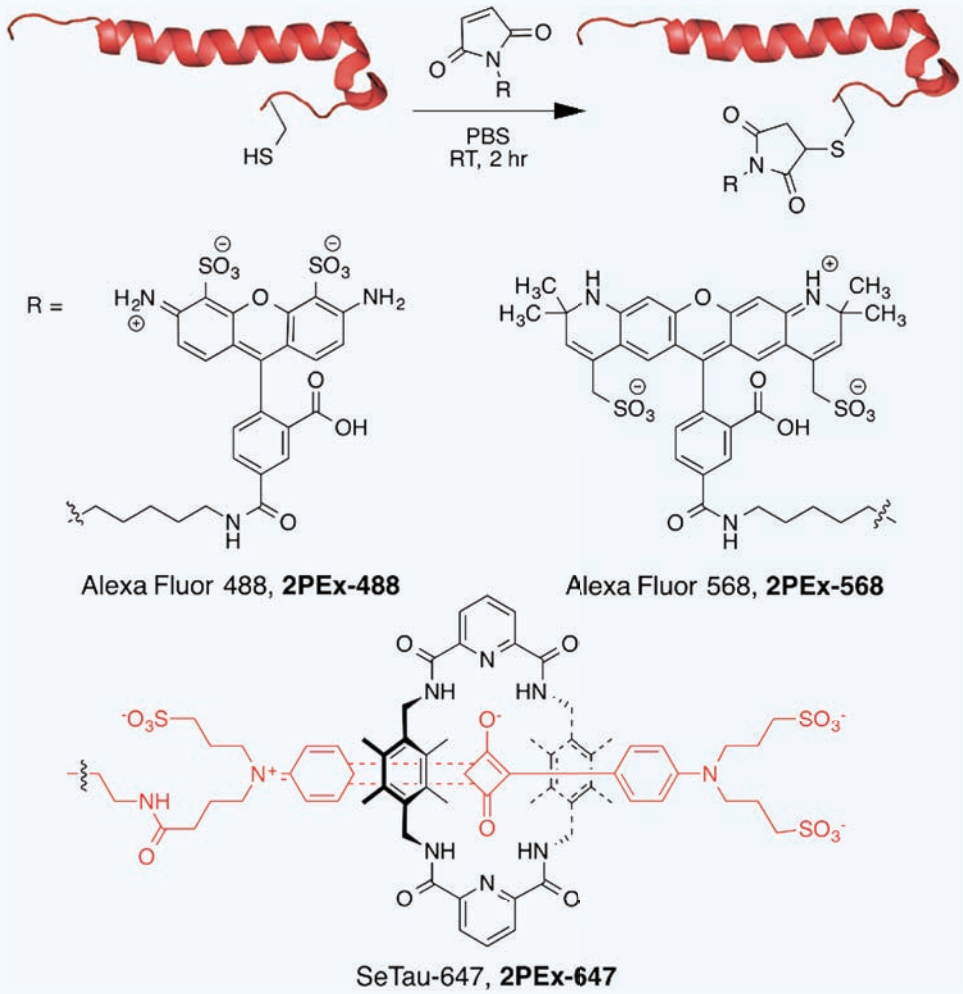

Figure 1 Synthesis of the two-photon exendin-4 conjugates **2PEx-488**, **2PEx-568**, and **2PEx-647**.

competitive binding assay with ^{125}I exendin (9-39) in human embryonic kidney cells stably transfected with the human GLP-1 receptor (HEK-hGLP1R). All three of the 2PEx probes maintained high binding affinity for the GLP-1 receptor in the nM range **(Fig. 2a)** and showed binding and internalization by beta cells in murine pancreata after IV injection of the probes via tail-vein **(Fig. 2b)**. **2PEx-647** routinely resulted in the highest signal-to-background ratio, resulting in its deeper analysis **(Fig. 2c)**.

To allow for *in vivo* two-photon imaging of pancreatic islets, a probe should accumulate specifically in beta cells with minimum distribution to other cells following systemic injection, exhibit high fluorescence signal, and be resistant to photobleaching. Since it can be difficult to identify islets at low magnification in live pancreas, ideal probes should impart high signal-to-background noise. **Figure 3a** demonstrates the ease at which islets can be identified in a pancreas by confocal imaging *in vivo* or *ex vivo* at 4X magnification, and the high resolution obtained by imaging **2PEx-647** at higher magnification.

To evaluate the *in vivo* performance of **2PEx-647** we performed intravital imaging using a similar set-up as previously described but in two-photon mode[7]. Lectin-FITC was injected to identify the vasculature **(Fig. 3b, green)**, prior to injecting 200 pmole of **2PEx-647**. The compound accumulated rapidly and by 10 minutes after injection, the islet signal had increased 12-fold over background **(Fig. 2c)** and allowed for single cell two-photon imaging for several hours **(Fig. 3b)**.

To confirm the beta cells specificity of **2PEx-647** following *in vivo* injection, we performed immunofluorescence microscopy staining for the predominate hormones used to identify each of the islet cells (insulin for beta cells; glucagon for alpha; somatostatin for delta). The **2PEx-647** signal was clearly localized to beta cells as demonstrated by insulin staining **(Supplementary Fig. 1a)**. Conversely, the alpha and delta cells did not internalize the probe (**Supplementary Fig. 1b,c**).

We next compared the imaging properties of **2PEx-647** by confocal and two-photon microscopy. Not surprisingly, two-photon microscopy allowed for imaging of islets deeper in the pancreas **(Fig. 4a)**. These results and concomitant higher SBR demonstrate the advantage of using **2PEx-647** with two-photon microscopy to image beta cells deep in the pancreas.

The destruction of beta cell is a complex immunological process involving many different cell types and processes[17]. To understand the interactions of some of these players in detail, it will ultimately be necessary to image more than one target *in vivo*. To demonstrate proof of principle we utilized NOD FoxP3-I-GFP mice to image regulatory T cells (Treg) in **2PEx-647** stained islets. As is shown in **Fig. 4b,c**, *in vivo* multiphoton imaging allowed to resolve Tregs within and around islets.

In conclusion, we have synthesized, characterized and tested three two-photon compatible imaging probes to visualize pancreatic beta cells by intravital microscopy. The three conjugates presented in the paper were the best performing of a series of exendin-4-fluorophore conjugates. We determined that **2PEx-647** had the highest signal-to-background ratio and best *in vivo* imaging properties. The probe, containing a squarine-rotaxanes dye, is selective for beta cells, with nanomolar binding affinity for the GLP-1 receptor. Also the probe allows for high resolution and deeper imaging compared to confocal probes while minimizing photo-bleaching. The ability to easily identify the islets and to image at single cell resolution will facilitates future studies to better understand the etiology of type 1 diabetes or to image receptor overexpression in insulinomas.

METHODS

Material and methods

All reagents were purchased from commercial sources and used as received. Exendin-4 (1-39) amide was obtained from Amylin/Eli Lilly (San Diego, CA). Exendin-4 (40C) (molecular weight: 4290, HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSC-amide) was custom-synthesized at 25 mg scale by CSBio (Menlo Park, CA). Fluorochromes Alexa Fluor 488 and Alexa Fluor 568 were purchased as the maleimide from Invitrogen. SeTau-647 (K9-4142) was purchased as

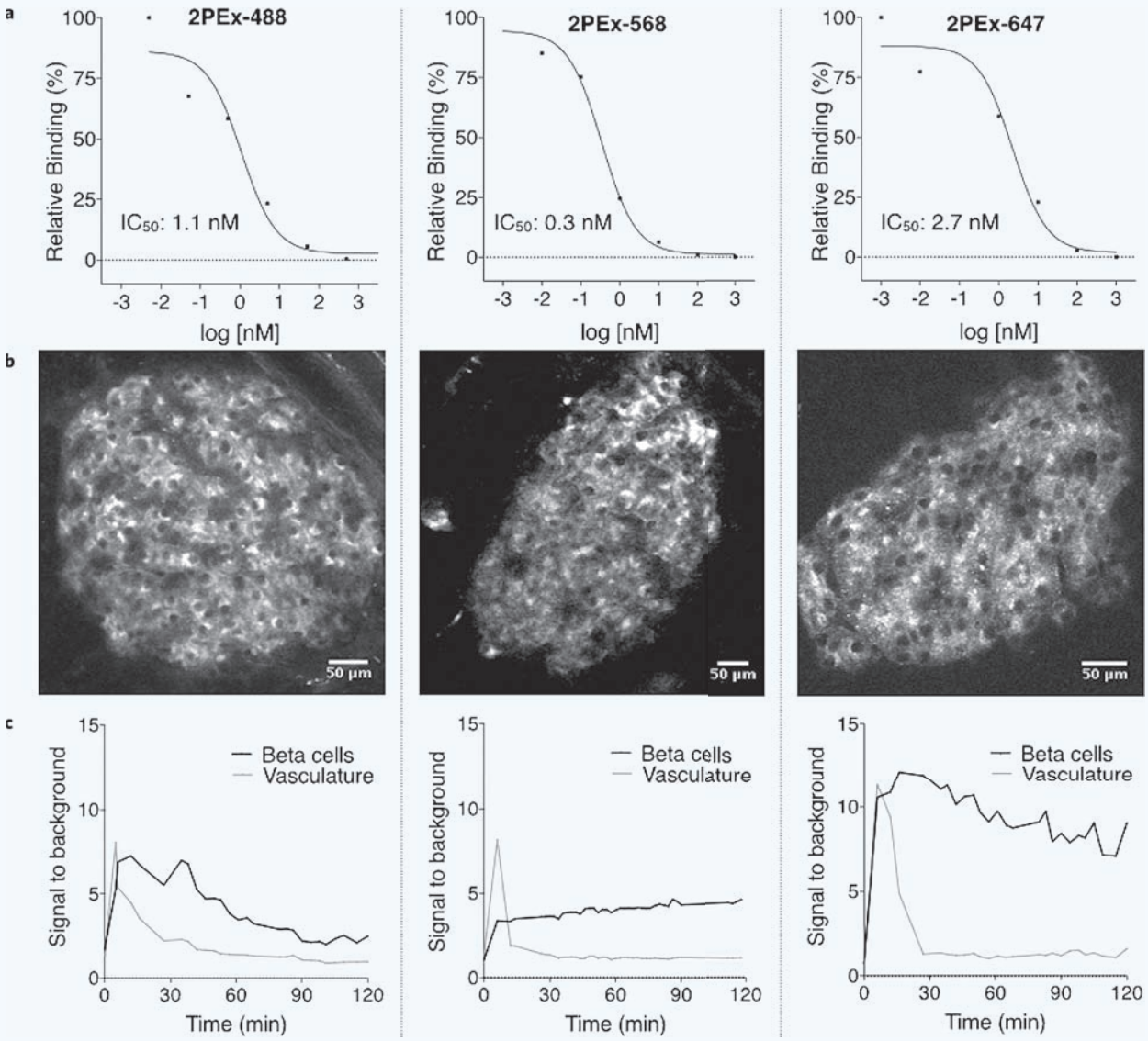

Figure 2 Binding, internalization and pharmacokinetics studies of **2PEx-488** (left), **2PEx-568** (middle), **2PEx-647** (right). (**a**) Determining the IC_{50} *in vitro* with an ^{125}I-exendin(9-39) competitive binding assay in HEK-hGLP1R cells. (**b**) Two-photon images of the probes' internalization in *ex vivo* mouse pancreatic islets. (**c**) Blood half-life measurement (grey) and increase in beta cells signal (black) following a dose of systemic 2PExs probes injection in a C57BL/6 mouse. Two-photon excitation was performed at 960 nm, 800 nm, and 910 nm for **2PEx-488**, **2PEx-568**, and **2PEx-647** respectively. Images in 2b are 512 × 512 in size and were acquired with an integration time of 20 µs/pixel.

the maleimide from SETA BioMedicals (Urbana, IL). LC-ESI-MS analysis was performed on a Waters (Milford, MA) LC-MS system. For LC-ESI-MS analyses, a Waters Xterra C18 5 um column was used.

Preparation of 2PEx-488, -568 and -647

To a solution of 58 µL (0.5 µg) Exendin-4 in 392 µL of 1X PBS, 0.5 mg of maleimide functionalized dye was added dropwise. The reaction mixture was stirred at room temperature in the dark for 2 hours. The mixture was applied to a NAP-5 column and eluted with 1X PBS. Six 500 µL fractions were isolated. The first three fractions were concentrated and washed 3 times using a 3kDa spin filter (14,000 g, 10 minutes). Nano drop and a bicinchoninic acid assay were performed to determine yield. Yielding 60–70% of the title compounds. LC-ESI-MS m/z : **2PEx-488**: 1248 $M+4H^{+4}$ **2PEx-568**: 1288 $M+4H^{+4}$ **2PEx-647**: 1444 $M+4H^{+4}$.

Cell culture

Human embryonic kidney (HEK-293) cells stably expressing the human GLP-1 receptor were grown in high-glucose DMEM containing 10% heat-inactivated FBS, 50 U/mL penicillin, 10 µg/ml streptomycin, 1 mM sodium pyruvate and 150 µg/mL G418 (Geneticin).

Receptor binding assay

The receptor binding affinities of the fluorescent Exendin-4 were determined in HEK-hGLP1R using a slight modification of a previously described method[8]. Briefly, cells were seeded in a 96 well plate at 5.5×10^4 cells per well, grown for 48 hours, washed twice with binding buffer (120 mM NaCl, 1.2 mM $MgSO_4$, 13 mM sodium acetate, 5 mM KCl, 1.2 g/L Tris, 2 g/L bovine serum albumin (BSA), and 1.8 g/L glucose, pH 7.6) and co-treated with 30 pM of ^{125}I-exendin-4 (9-39, PerkinElmer, Boston,

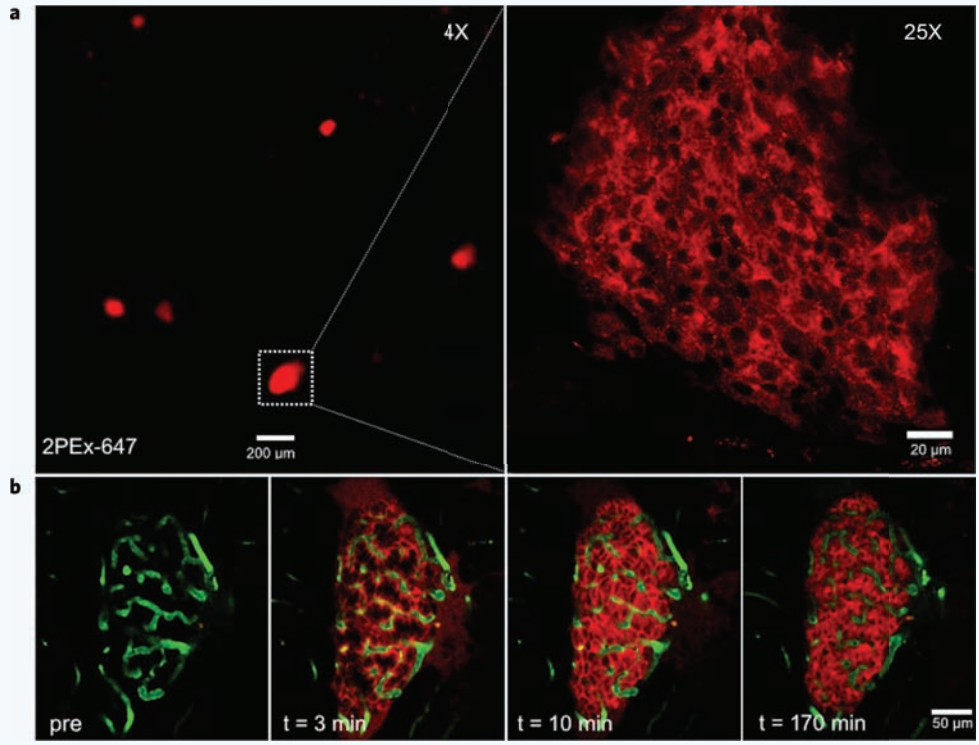

Figure 3 Confocal and two-photon intravital imaging of **2PEx-647** in a C57B/L mouse. (**a**) The ease of identification of islets at 4× as acquired in confocal mode is demonstrated. Image size, 1024 × 1024 pixels. Integration time, 20 µs/pixel. Excitation wavelength, 633 nm. High resolution image of the same islet at 25×, as acquired in two-photon mode. Image size, 1024 × 1024 pixels. Integration time, 10 µs/pixel. Excitation wavelength, 910 nm. (**b**) Time course of **2PEx-647** accumulation in single cells as measured by two-photon intravital imaging (green: vasculature dye; red: **2PEx-647**). Images' size, 512 × 512 pixels. Images correspond to the maximum intensity projections of a z-stacks consisting of 15 slices spaced by 4 microns in depth. All acquisitions were performed with an integration time of 10 µs/pixel. Excitation wavelength, 910 nm.

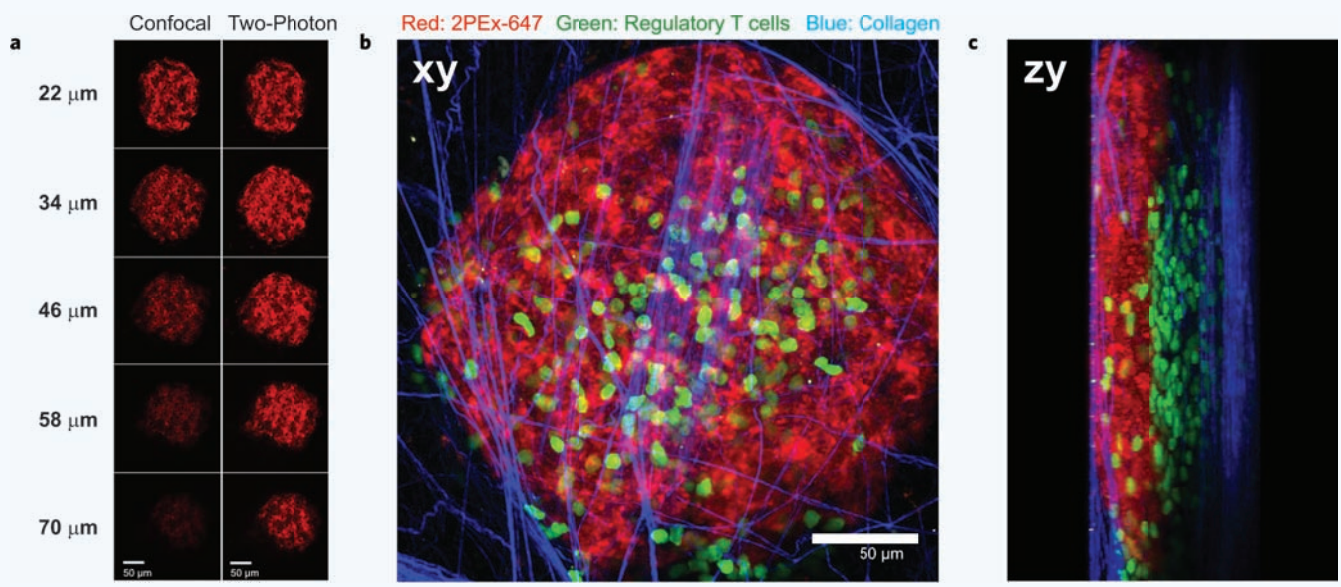

Figure 4 Depth and multiplex imaging. (**a**) Depth comparison of the signal detection of **2PEx-647** in the islet of a live C57B/L mouse imaged with confocal and two-photon imaging. Single plane images, 512 × 512 pixels. Integration time, 10 µs/pixel. Confocal, excitation at 473 nm and 635 nm. Two-photon, excitation at 910 nm. (**b,c**) Multi-color two-photon imaging of a NOD FoxP3-GFP mouse (red: **2PEx-647**, green: GFP expressed in regulatory T cells, blue: collagen). Image size, 1024 x 1024 pixels. Images correspond to the maximum intensity projection (xy view) consisting of 80 slices spaced by 2 µm in depth. C, maximum intensity projection (zy view). Integration time 10 µs/pixel. Excitation wavelength, 910 nm.

MA) and unlabeled exendin-4 or fluorescent exendin-4 analogues (final concentration range: 10^{-12}-10^{-6} M). After incubating for 2 hours at 37°C, the cells were washed three times with chilled PBS containing 1 mg/mL BSA, lysed (RIPA 1× buffer, 15 minutes) and ^{125}I contents were measured using a Wallac Wizard 3″ 1480 Automatic Gamma Counter.

Immunohistochemistry

Immunofluorescence labeling of the pancreas from C57BL/6J was performed to visualize co-localization of the probe in insulin positive glucagon and somatostatin negative cells. **2PEx-647** was injected 1 hour prior to euthanization. The pancreas was extracted and embedded in

OCT compound (Sakura Finetek) and flash-frozen in an isopentane bath of dry ice. The frozen tissues were sectioned (5-µm thickness), mounted on microscope slides, and stored at –80°C. Slides were then incubated at 4°C o/n with primary antibodies diluted with 4% goat normal serum in PBS solution. The following primary antibodies were used for immunostaining: rabbit anti-insulin (1:25, Santa Cruz), rabbit anti-glucagon (1:1000, Millipore), rabbit anti-somatostatin (1:250, Dako) and rabbit anti-c-peptide (1:100, Cell Signaling). A specie-matched secondary antibody was used for immune detection (Alexa Fluor 488 conjugated goat anti-rabbit, 1:100, Life Technology). Immunofluorescence images were acquired with an epifluorescence microscope, BX63 (Olympus), with a Neo sCMOS Monochrome camera (ANDOR).

Intravital imaging

Imaging was performed using a custom made dual confocal and two-photon Olympus FV1000 imaging system (Olympus, America) with a Mai-Tai deep-see laser (Spectra Physics, CA, USA). Two-photon imaging was done with a 25× (XLPLN25×, N.A. 1.05, Olympus) water immersion multi-photon objective and confocal imaging with a 4× air objective (UPlanSApo, N.A. 0.16, Olympus), 20× (XLUMPLFLN, N.A. 1.00, Olympus) water immersion objectives. Confocal imaging of **2PEx-647** was performed by exciting with a 633 nm diode laser and collection of the light using dichroic beam splitters DM405/488/559/635 and SDM640 combined with a BA655-755 band-pass filter.

For two-photon imaging samples were excited at 960 nm, 800 nm, or 910 nm depending on the imaging probe. Emission light was collected using dichroic mirrors 685LP nm, 570 LP, and 485 LP, combined with emission filters BA495-540 HQ (**2PEx-488**, FITC-lectin), BA420-460 (second harmonic generation), BA575-630 (**2PEx-568**), and BA630/80 (**2PEx-647**).

Images, stacks and movies were collected before and after i.v. tail vein injection of 200 pmoles of **2PEx-647** in 100 µl PBS. This optimal dose as well as the laser settings that provided good signal to noise but avoided photobleaching or damage to cells were determined by *in vivo* imaging of doses from 2 nmol to 20 pmol.

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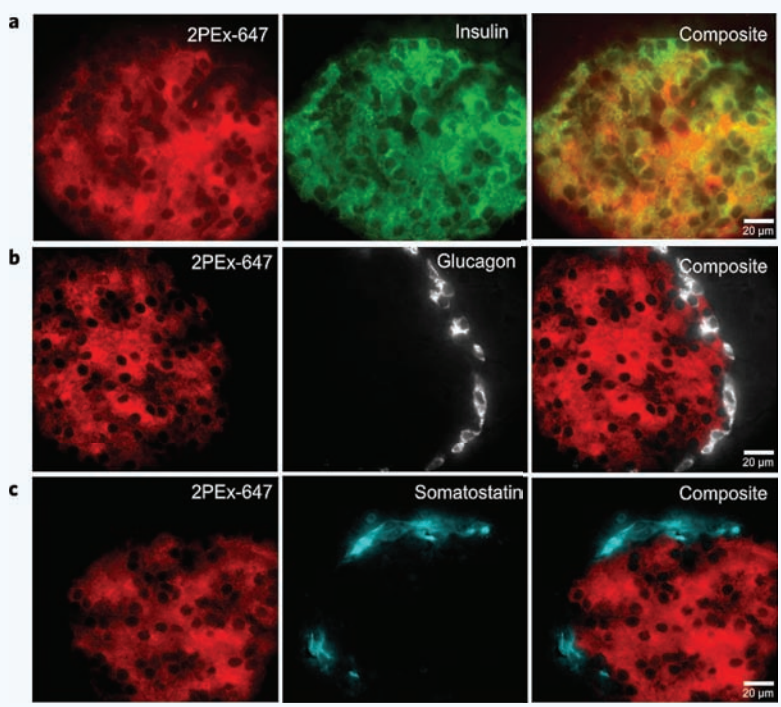
SUPPLEMENTARY INFORMATION



Supplementary Figure 1 Fluorescence histology of pancreas sections from a C57BL/6 mouse injected with **2PEx-647**. Left column: islet stained with **2PEx-647** probe, middle column: same islet stained with either (**a**) anti-insulin, (**b**) anti-glucagon or (**c**) somatostatin, right: merge.