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Glucagon-Secreting Alpha Cell Selective Two-Photon Fluorescent Probe TP-α: for Live Pancreatic Islet Imaging.

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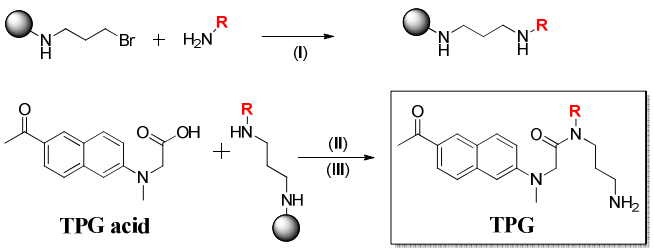
Supporting Information Placeholder

ABSTRACT: Two-photon (TP) microscopy has an advantage for live tissue imaging which allows a deeper tissue penetration up to one millimeter comparing to one-photon (OP) microscopy. While there are several OP fluorescence probes in use for pancreatic islet imaging, TP imaging of selective cells in live islet still remains a challenge. Herein, we report the discovery of first TP live pancreatic islet imaging probe; **TP-α** (Two Photon-alpha) which can selectively stain glucagon secreting alpha cells. Through fluorescent image based screening using three pancreatic cell lines, we discovered **TP-α** from a TP fluorescent dye library TPG (TP-Green). *In vitro* fluorescence test showed that, **TP-α** have direct interaction with glucagon with a significant fluorescence increase, but not with insulin or other hormones/ analytes. Finally, **TP-α** was successfully applied for 3D imaging of live islets by staining alpha cell directly. The newly developed **TP-α** can be a practical tool to evaluate and identify live alpha cells in terms of localization, distribution and availability in the intact islets.

INTRODUCTION

Pancreatic alpha cells secrete glucagon in response to low blood glucose level. Glucagon counter-regulates the hypoglycemic effect of insulin by glycogenolysis and gluconeogenesis.[1] Studies over the last decade showed that the human islets not only have architectural differences with the well-studied rodent islets, but also varied endocrine cell population.[2] These studies have revealed that the proportion of beta cells in human islet is much less than the murine islets (approximately 55% versus 77%). On the contrary, the fraction of alpha cells is greater in human islet than murine islet (approximately 35% versus 15%).[2] Together, the higher alpha cell proportion and the unique association of alpha and beta cells in human islets have drawn more research interests to study pancreatic alpha cells for understanding their function and influence on diabetes. Although several beta cell imaging probes are in use,[3-5] imaging of alpha cells is still in its infancy. The conventional way of alpha cell imaging is the use of glucagon antibody for immunostaining or transgenic mouse models which express a fluorescent reporter gene linked to glucagon promoter.[6] Furthermore, the metabolic status of cells has been utilized for pancreatic islets studies by NADPH auto-fluorescence imaging[7] or cytosolic calcium imaging.[8] Previously, our group reported an OP small molecule probe; Glucagon Yellow (GY: Figure S2b) which stains alpha cells selectively.[9] Despite the fact that GY has alpha cell selectivity, the negligible TP optical property (Figure S2C) has limited its application as a TP imaging probe. As such TP microscopy offers benefit over OP microscopy for deeper tissue imaging due to the utilization of high power pulsed near-infra-red excitation laser light.[10] GY showed high photo bleaching (Figure S2D), which further limits its suitability as a TP imaging probe. Even though a few small molecule one photon fluorescent probes has been published, till date no two-photon alpha cell imaging probe has been reported for live islet imaging.



Scheme 1. Solid-phase synthesis of TPG library. (I) 80 different aromatic and aliphatic amines (Table S1, Supporting Information) loaded on to the resin: DIEA, NMP, 70° C, 12 h. (II) Acid-amine coupling: HATU, DIEA, DMF/DCM, RT, 24 h. (III) Cleavage of final compound: 5% TFA in DCM in RT for 1 h gives the final TPG library compounds.

OP microscopy requires shorter excitation wavelength which limits its application in live deep tissue imaging due to shallow penetration depth of excitation light. On the contra-

ry, TP microscopy utilizes two near-infrared photons as source of excitation and offers superior advantages for live tissue imaging by means of a higher depth of penetration, localized area of excitation and long observation of items.[10] TP imaging technique has been assessed for pancreatic islets either with auto-fluorescence NADPH imaging[7] or with various hydrophilic and hydrophobic agents.[11] Development of more novel two-photon probes suitable for live islet imaging will therefore be of great value to future diabetic research.

In this study, we report the first TP fluorescent probe for islet imaging which has a selectivity of alpha cell and it is denoted by **TP-α** (Two Photon Alpha). Through a fluorescent cell based screening of TP fluorescent dye library TPG, we found **TP-α** which can selectively stains alpha TC1 cells against beta TC6 and acinar cell.

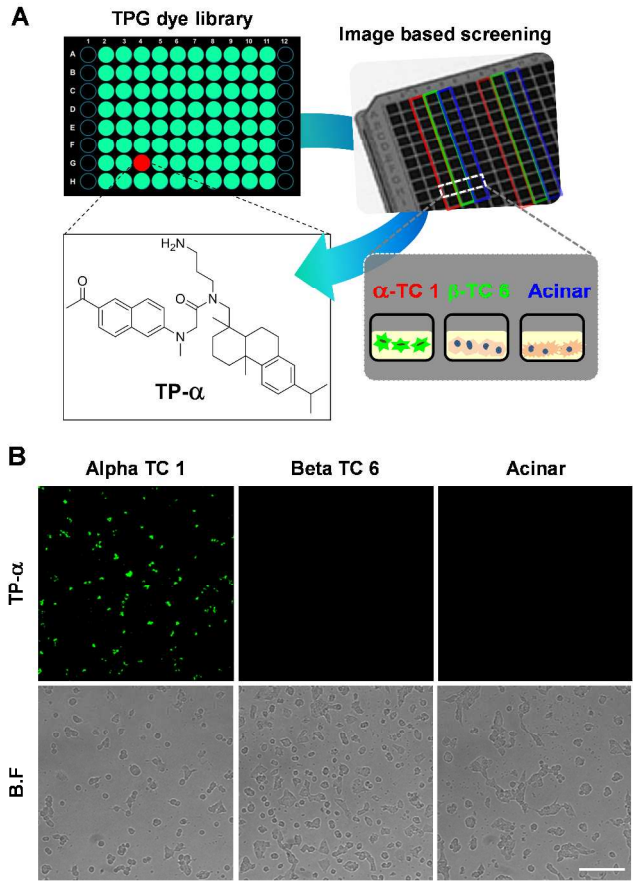


Figure 1. (A) Overview of image based screening for TPG library compounds. Three pancreatic cell lines alpha TC1, beta TC6 and acinar 266-6 cells were used for discovery of pancreatic two photon imaging probe with 80 TPG fluorescent dyes, lead to the discovery of alpha TC1 cell selective probe: **TP-α.** (B) Selective staining of alpha TC1 cell by **TP-α**, in comparison with beta TC6 and acinar cells. Live cells were stained with 1 μM **TP-α** and incubated at 37° C for 1 h before imaging. Images were captured with Image Xpress Micro system after PBS wash. Fluorescence data collected at 450-500 nm upon excitation at 370 nm. Scale bar 50 μm.

RESULTS

Development of Two-photon fluorescent dye library. Herein, we report the synthesis of two-photon fluorescent dye library by solid phase combinatorial synthesis using 2-acetyl-6-(methyl-amino) naphthalene (ACEMAN) core (Scheme S1 and S2). We chose the ACEMAN scaffold due to its well-known TP property (TP fluorescent action cross section is about 100 GM), reasonable OP fluorescent quantum yield (50%) and lower molecular mass (200 Da.).[12] The synthesis of TPG acid was done in solution phase based on previous report.[12] Diversity was introduced to the TPG scaffold (Scheme 1) by using 80 chemically different amine building blocks (Table S1). These diverse TP fluorescent dyes were generated without predesigned specificity towards any particular target. Hence, subsequent unbiased fluorescent image based screening with various panel of cell lines may lead to the discovery of totally novel TP fluorescent probes. We named this set of 80 compounds as Two-Photon-Green (TPG; Figure 1A and 2A) library because of their emission wavelength range ($\lambda_{abs}/\lambda_{em}$ = 370/510 nm, in PBS buffer). Detailed synthetic procedure and building block structures with characterization are available in Supporting Information.

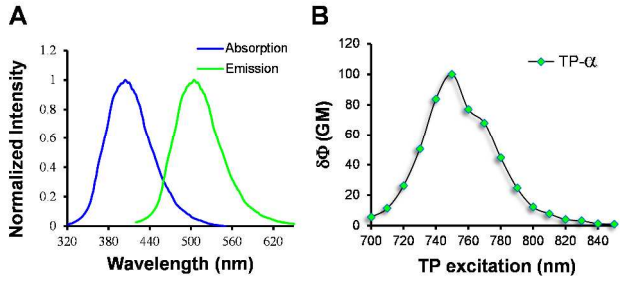


Figure 2. One-photon and two-photon optical property of **TP-α**. (A) One-photon absorption and emission spectra for **TP-α** 1 μM in PBS buffer (pH = 7.4). (B) Two-photon action cross section of **TP-α** measured in ethanol at 10 μM concentration.

Discovery of TP-α: an alpha TC1 selective probe. The cell-based screening with fluorescent dye library has led to the discovery of various cell-selective imaging probes.[13-18] To expedite the discovery of useful probes from the synthesized TPG library, we setup a cell-based screening system with three commercially available pancreatic cell lines; glucagon secreting alpha TC1 cells, insulin secreting beta TC6 cells and exocrine acinar 266-6 cells as a model system (Figure 1A). All three cell lines were seeded into 384 well plates and allowed to settle overnight before use. These cells were incubated with 1 μM of TPG library-80 compounds for 1 h before image acquisition. Images were analyzed based on fluorescence intensity to seek out the alpha cell selective probe. Primary image analysis based on relative fluorescent intensity revealed that one TPG compound shows higher fluorescent signal with alpha TC1 cells (glucagon secreting) in comparison to beta TC6 (insulin secreting) and exocrine acinar cells.

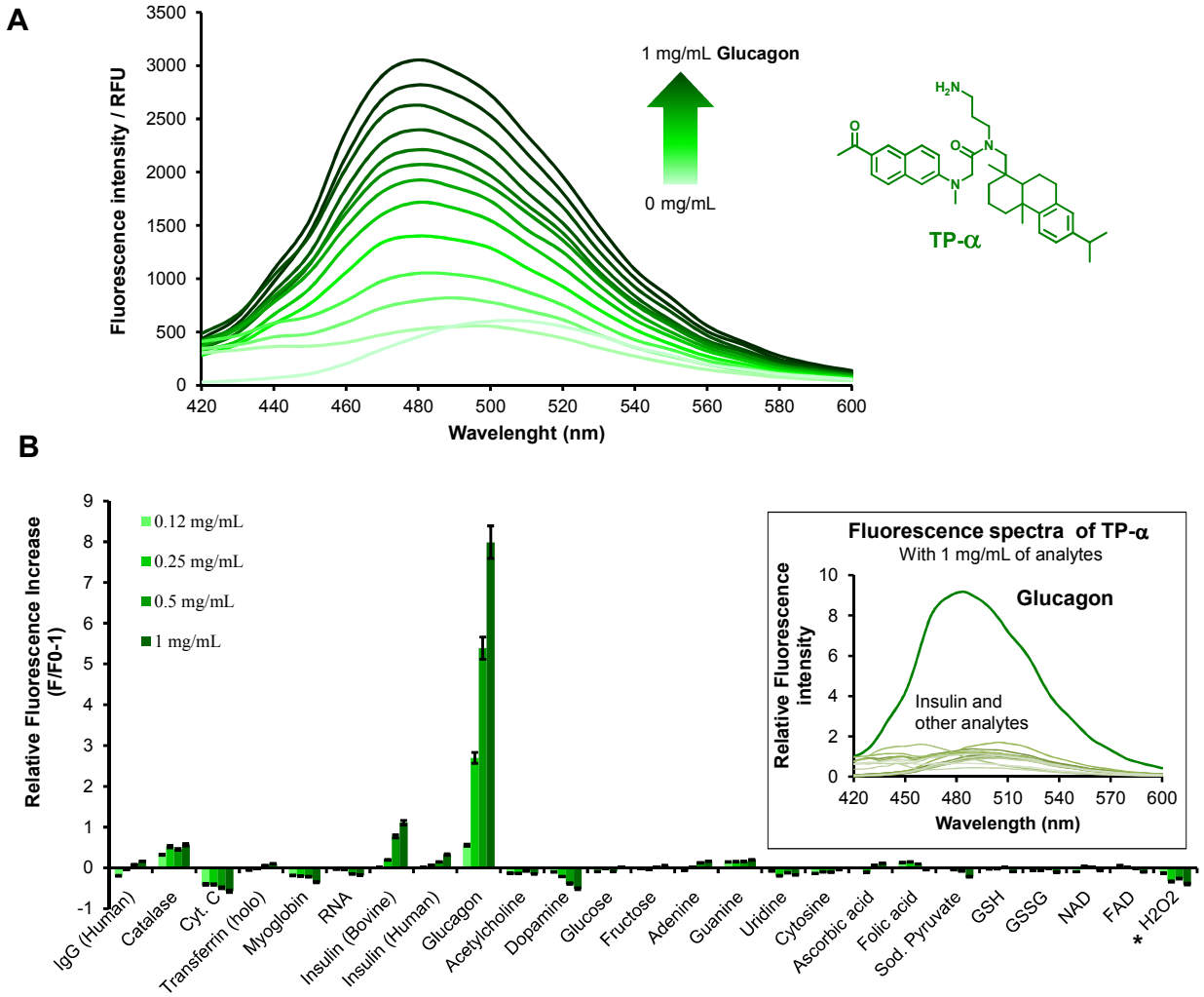


Figure 3. *In vitro* fluorescence response of **TP-α.** (A) Fluorescence spectra of **TP-α** (10 μM) upon incubation with different concentration of glucagon (from 0 mg/mL to 1 mg/mL) in 10 mM phosphate buffer (1% DMSO, pH 7.4) under excitation of 370 nm light. Insert is the structure of **TP-α.** (B) Selectivity of **TP-α** (10 μM) for glucagon in comparison with insulin, other proteins and small molecule analytes at 1 mg/mL concentration. Macromolecules: IgG (human), Catalase, Cytochrome C, Transferrin (holo), Myoglobin, RNA, Insulin (bovine), Insulin (human), Glucagon and small molecule analytes; Acetylcholine, Dopamine, Glucose, Fructose, Adenine, Guanine, Uridine, Cytosine, Ascorbic acid, Folic acid, Sod. Pyruvate, Glutathione, Glutathione reduced, β-Nicotinamide adenine dinucleotide (NAD), Flavin adenine dinucleotide (FAD), and H_2O_2 (*Hydrogen peroxide 30% solution in water). Insert shows the selective increase in fluorescent emission of 10 μM **TP-α** with 1 mg/mL glucagon in comparison to other analytes at 1 mg/mL concentration in PBS buffer. Values are represented as mean and error bars are standard deviation. (n=3).

After secondary and tertiary imaging tests (see Figure S1) the compound, **TPG-456** ($\lambda_{abs}/\lambda_{em}$=370/510 nm, TP fluorescence action cross section = 107 GM, OP quantum yield Φ = 0.69) was selected to be the final hit for glucagon secreting alpha TC1 cells (designated by **TP-α**: Figure 1A, 1B, and 2 in the manuscript, Figure S1, S2, 1H, 13C NMR and HRMS spectra in the Supporting Information). The MTT assay using alpha TC1 and beta TC6 cells revealed that the **TP-α** staining either at working (1 μM) or higher concentrations (2 μM) had minimal interference with the cell viability even up to 24 h (Figure S3). With these experiments, we confirmed the selectivity and non-toxicity of **TP-α** in alpha TC1 and beta TC6 cells in the working concentration range of the probe.

***In-vitro* fluorescence response of TP-α.** To elucidate the selectivity of **TP-α** towards glucagon secreting alpha TC1 cells, we performed an *in-vitro* fluorescence response test with glucagon (Figure 3A). **TP-α** showed concentration dependent increase in fluorescence emission with glucagon (up to 9 fold at 1 mg/mL, (Figure 3A, 3B) with a K_D of 65 μM (Figure S10). The selectivity test revealed that **TP-α** has higher fluorescence response with glucagon in comparison to insulin (Figure 3B). Similar *in-vitro* fluorescence test with various proteins, neurotransmitters, and analytes also shows that, **TP-α** has notable selectivity towards glucagon (Figure 3B). The glucagon selectivity of **TP-α** further supports the selectivity of the probe towards glucagon secreting alpha TC1 cells.

TP-α stains primary alpha cell selectively. The glucagon and alpha TC1 selectivity of **TP-α** further inspire us to evaluate the probe in mouse primary islet cells. Alpha cells are an integral component of the pancreatic islet of Langerhans. Islet tissues are scarce, and represent only about 1-2% of the whole pancreas.[19,20] The scarceness of these cells in

mice pancreatic islet makes selective alpha cell imaging a more challenging task. To avoid practical troubles of isolating alpha cells from mouse pancreatic islets, we decided to use whole islets for further studies. As the attachment of islets to the culture dish is known to be difficult, partially dissociated islet culture technique was adopted for immunostaining purpose.[21] The partially dissociated islets took over a week time in culture to spread out as monolayer. Staining with **TP-α** showed brightly stained cells at the edge of these cultured dissociated islets (Figure 4A, 5A-B). The glucagon antibody staining confirmed that **TP-α** bright cells are glucagon positive alpha cells (Figure 4B).

The flow-cytometry analysis of **TP-α** stained islet cells showed 10~25 % brighter sub-populations (Figure S7, S8) which is pertinent to the reported alpha cell population.[2,19] For quantitative analysis, we performed flow cytometry experiments of isolated mouse islet cells after double staining of **TP-α** with either glucagon or insulin antibody. The majority of **TP-α** staining cells are overlapped with glucagon antibody positive cells with an accuracy of 96% (Figure 4B) confirming that **TP-α** stains glucagon secreting alpha cells. Whereas, 99% of **TP-α** negative cells are glucagon antibody negative.

Another mouse pancreatic alpha cell marker $GABA_A$ receptor antibody[22] showed a similar result with glucagon antibody (Figure 9A). When tested with insulin antibody, less than 1% of the **TP-α** positive cells were positive to insulin antibody, demonstrating the high selectivity of **TP-α** for glucagon producing alpha cells over other islet cells. The double negative sub-populations may represent non-alpha, non-beta cell populations such as delta cells (Figure S9B).

TP-α stains alpha cells in live fresh islet. Finally, we applied **TP-α** staining for alpha cell imaging on freshly isolated pancreatic islet. **TP-α** stained pancreatic islets (Figure 5C-F) showed three-dimensional distribution of brightly stained alpha cells on the surface of mice pancreatic islets in live condition (Figure 5G, H). We found that, 1 μM **TP-α** staining for 1 h was the optimal staining for both OP and TP imaging (Figure 5, Figure S4). By evaluating the optical property of **TP-α** using TP spectral images, we found 750 nm and 430-530 nm to be the most suitable excitation light and emission channels respectively (Figure S5 and S6) for TP-α imaging. Likewise, TP optical sections of **TP-α** stained live islet even at the middle plane (~50 μm depth from the bottom of islet on the coverslip) showed good contrast between bright and dim cells (Figure 5C-5H). The discontinuous distribution of **TP-α** stained alpha cells at the mantle position on the islets displayed the native distribution of alpha cells (Figure 5C-5H) and matches with previous report.[23]

The surface localization of alpha cells makes them susceptible to fall off during the enzymatic (collagenase p) tissue digestion during islet isolation from the mouse pancreatic tissue.[24] When the isolated islets were stained with **TP-α**, we found that the over digested islets do not have **TP-α** bright cells on its surface. The simple **TP-α** staining can be a useful tool to examine the presence or absence of alpha cells on the isolated islets.

In order to assure that this mantle staining is due to the selective staining of alpha cells and not due to impermeability of the fluorescent probe, we stained both intact islets and dissociated islet cells with **TP-α** and confirmed with flow cytometry. For both intact and dissociated islet staining conditions, we found similar **TP-α** stains similar sub-population of 18 ± 5%. This revealed that, **TP-α** staining of these cells is because of its intrinsic selectivity towards glucagon secreting alpha cells and not due to surface localization of these cells. By far, this is the first demonstration where an alpha cell selective small molecule fluorescent probe was used for two-photon imaging of alpha cells in live pancreatic islet (Figure 5G, 5H).

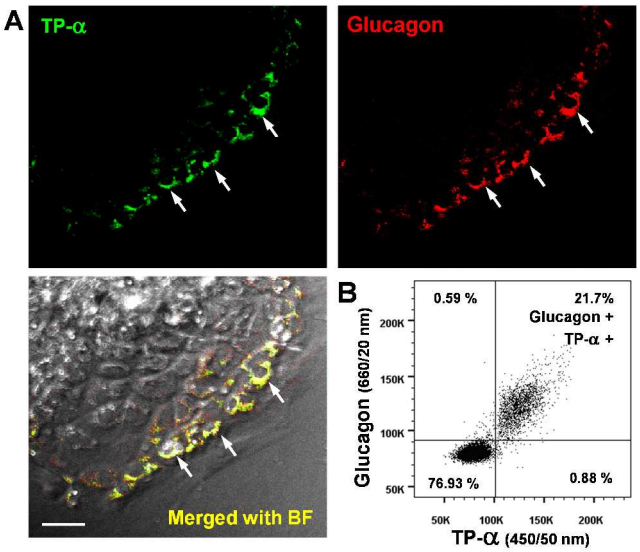


Figure 4. Antibody confirmation of **TP-α** selectivity by imaging and flow-cytometry. (A) Cultured dissociated islets stained with 1 μM **TP-α** (in green) followed by immunostaining with glucagon antibody (in red). Merged image demonstrates the **TP-α** stained cells to be glucagon positive alpha cells (white arrow). Scale bar is 50 μm. (B) Dual parametric dot plot for **TP-α** and glucagon antibody. About 17,000 islet cell counted.

DISCUSSION

New technological advancement in the TP microscopic techniques has created a high demand for the development of novel TP bio-imaging probes.[10,24] Although the fluorescent proteins have TP fluorescent excitation property,[25] their expression in target cells is expensive and tedious. Hence, small molecule fluorescent probes offer a better alternative. Cell based screening of fluorescent compound libraries has resulted in the discovery of various useful bio-imaging probes.[13-18] In the present study, we report the development of ACEMAN based TP fluorescent dye library and the discovery of a novel alpha cell selective probe, **TP-α**. The two-photon alpha cell probe (**TP-α**) labels alpha cells quickly and effectively in live conditions and offers significant advantages over conventional glucagon antibody labeling which requires fixations and permeabilization.

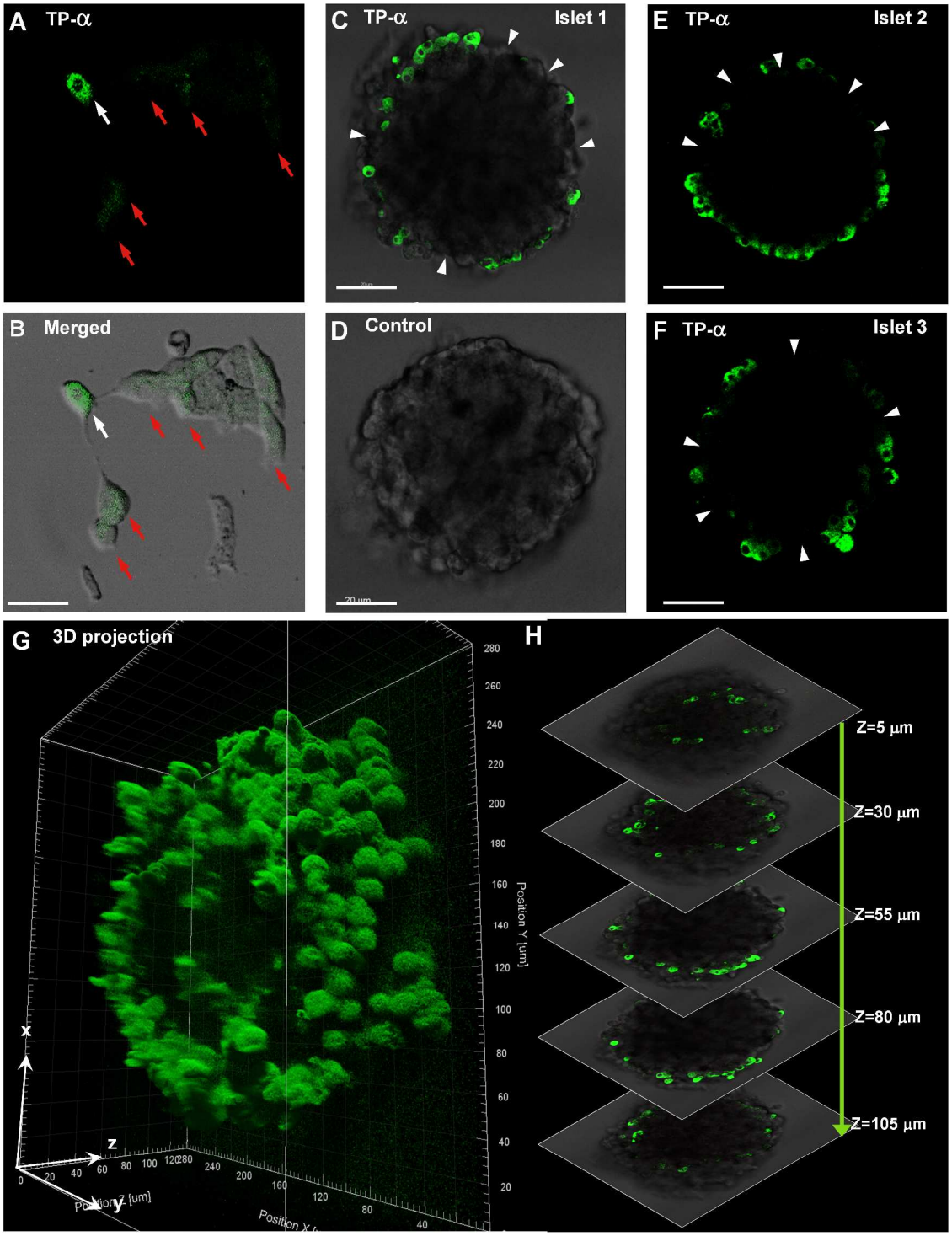


Figure 5. TP-α selectively satins mouse pancreatic alpha cells in live islet. (A) Selective staining of alpha cell by **TP-α** in the cultured dissociated islet cells. (B) Merged image with bright field showing both the **TP-α** bright alpha cell (white arrow) and **TP-α** dim other islet cells (red arrow). Scale bar 15 μm. (C) Overlay of bright field and TP fluorescence image of **TP-α** stained islets. Shows distribution of **TP-α** brightly stained alpha cells on the edge of the islet. Arrow head indicates absence of alpha cells in the mantle position. (~50 μm depths representative images). (D) For control DMSO was used. Scale bar 50 μm. (E) and (F) TP fluorescent optical section images of individual **TP-α** stained live islets. (G) 3D Surface projection of the two-photon z-stack images for **TP-α** stained live islet (step size, 0.17 μm) using image processing software IMARIS (Supporting Video S1). (H) Representative sectional overlaid TPM images of **TP-α** stained live islet at the depth range of 5 μm to 105 μm. It reveals the natural distribution of alpha cells in the mouse pancreatic islet in live condition. Two-photon imaging was performed on 16 weeks old mouse pancreatic islet. All images were taken with 40X water immersion objective using Leica TCS SP5X MP, Ex: 750 nm femtosecond (fs) laser light, Em: 430-530 nm (images are represented from n = 30 islets).

The two-photon alpha cell probe (**TP-α**) labels alpha cells quickly and effectively in live conditions and offers significant advantages over conventional glucagon antibody labeling which requires fixations and permeabilization. **TP-α** enables real-time staining and observation of alpha cells in their inherent condition in live intact islet with TP imaging. Since **TP-α** have emission ranging from 430 nm to 530 nm, it can be combined with other fluorescent proteins with yellow or red emission in islet for dual color imaging.

TP-α was discovered from image based screening of fluorescent dye library: TPG (containing 80 compounds) with glucagon secreting alpha TC1, insulin secreting beta TC6 and exocrine acinar 266-6 cell lines, based on relative fluorescent intensity. **TP-α** stained glucagon secreting alpha TC1 cells, showed significantly stronger fluorescent signal compared to other cells. From the *in-vitro* fluorescence test we found that, **TP-α** has more than 8 fold fluorescence intensity with Glucagon in comparison with Insulin and other pancreatic signaling molecules like acetylcholine,[26] dopamine (Figure 3B). This *in vitro* fluorescence study with total 23 diverse biological analytes revealed excellent glucagon selectivity of **TP-α** (Figure 3B). To demonstrate the suitable application for the glucagon selective **TP-α**, we envisioned to achieve selective staining of glucagon producing alpha cells. The pancreatic alpha cells are an integral component of islets and constitute a scarce sub-population of it (10-25%). Thus, we decided to isolate the pancreatic islet and stain them with **TP-α** directly. We found that 1 h incubation with 1 μM **TP-α** gives the best alpha cell selective staining in live fresh islets (confirmed by immunostaining, Figure 4). As anticipated, the TP imaging of **TP-α** stained islets has revealed the three dimensional distribution of alpha cells in the live islets.

Till date, the reported methodology for TP islet imaging is based on metabolic imaging (with glucose or other stimulation conditions).[7,8] Piston DW *et al.* has reported the TP imaging of islet for increased NADPH monitoring in beta cells with glucose stimulation.[7,27-29] However the NADPH TP imaging of islets can help visualize beta cells, but not alpha cells. Whereas, cytosolic calcium imaging of islets with Indo-1 staining followed by glucose or amino acid stimulus conditions can differentiate the alpha and beta cells.[8] But the calcium indicator Indo-1 has poor photo-stability[30] and the photo degradation of Indo-1 leads to the generation of a fluorescent but calcium insensitive probe.[30] Additionally Indo-1 stains all the islet cells and needs a different stimulus condition to differentiate alpha and beta cells.[8] However, **TP-α** can directly and selectively stain only the alpha cells in live islets. Also the selective staining of alpha cells with **TP-α** does not require any glucose or other stimulation. Glucagon yellow is another alpha cell selective one-photon fluorescent probe published earlier from our group. However, while evaluating the optical property we found that, GY have negligible TP action cross-section in comparison to **TP-α** (Figure S2A). The excellent photo stability of **TP-α** in contrast to GY (Figure S2D) makes **TP-α** a more valuable TP alpha cell imaging probe.

CONCLUSION

In conclusion, we have synthesized a TP fluorescent dye library TPG-80 compounds using solid support. The image-based screening of this library led to the discovery of a novel TP imaging probe **TP-α** for live alpha cell imaging in intact mice islets. **TP-α** is suitable for both TP islet imaging as well as the flow cytometry study of scarce primary alpha cells from mice pancreatic islet tissue. Furthermore, *in vitro* fluorescence response test of **TP-α** with 23 relevant biological analytes displayed an excellent selectivity towards glucagon. The glucagon and alpha cell selective **TP-α** may serve as a useful tool in pancreatic islet research for monitoring the survival or distribution of alpha cells during islet isolation and transplantation procedure. To facilitate dual color two-photon imaging of pancreatic islets more strategic designs are underway in our group to develop two-photon beta cell imaging probe.

MATERIALS AND METHODS

Characterization of TP-α. ^{1}H NMR TP-α (TPG-456) (300 MHz, CD_3OD), δ = 8.33 (dd, *J*=18, 1.8 Hz, 1H), 7.84 (dd, *J*= 10.5, 1.8 Hz, 1H), 7.80 (s, 1H), 7.56 (dd, *J*=8.4, 1.2 Hz, 1H), 7.05 (dd, *J*=9, 2.1 Hz, 1H), 6.95 (m, 1H), 6.85 (d, *J*=1.5 Hz, 1H), 6.74 (d, *J*=1.5 Hz, 1H), 4.44 (s, 2H), 3.16 (s, 3H), 3.08 (s, 5H), 2.64 (s, 3H), 2.11 (m, 4H), 1.26 (s, 6H), 1.17 (m, 14H), 1.11 (s, 3H), 0.966 (s, 3H). ^{13}C NMR TPG-M456 (75 MHz, CD_3OD), δ = 198.22, 170.60, 161.26, 160.81, 149.03, 146.33, 145.63, 144.95, 133.15, 129.82, 125.57, 125.19, 124.43, 122.86, 117.86, 114.97, 114.00, 104.52, 59.18, 39.95, 39.22, 38.97, 37.24, 36.95, 36.76, 36.47, 36.19, 35.24, 34.54, 32.64, 29.09, 28.60, 28.41, 24.48, 23.47, 22.45, 18.49, 16.09. ESI-MS m/z (M+H) calculated: 582.39, found 582.20. ESI-HRMS of TP-αm/z (M+H) calculated: 582.4060, and found 582.4062 (ESI HRMS Supporting Information).

Quantum yield measurements. Quantum yield were calculated by measuring the integrated area of emission spectra for TPG library compounds (including **TP-α**) in comparison to the same measurement for Coumarin-1 (ϕ=0.58) in DMSO as reference compound.[31] Both TPG library compounds and Coumarin 1 were excited at 370 nm and emission spectra were collected from 420-600 nm. Quantum yields were calculated using equation -1, where Φ is the quantum yield, *F* is the area of fluorescent emission, η is reflective index of the solvent, and Abs is absorbance at excitation wavelength selected for standards and samples. Emission was integrated between 420 to 600 nm.

$$\Phi_{(sample)} = \Phi_{(ref)} \frac{F_{(sample)}\,\eta_{(sample)}\,Abs_{(ref)}}{F_{(ref)}\,\eta_{(ref)}\,Abs_{(sample)}} \qquad (1)$$

***In vitro* fluorescence screening.** **TP-α** (10 μM) was used for fluorescence property measurement and *in vitro* screening in 10 mM phosphate buffer (pH 7.3, 1% DMSO). Fluorescence intensity was measured using Spectra MaxM2 plate reader in 96 well plate. We used 370 nm wavelength light for excita-

tion and emission was observed from 420 nm to 600 nm. All analytes were tested in four concentrations with serial dilution ranging from 1 mg/mL to 0.12 mg/mL. We used both macromolecules: IgG (human), Catalase, Cytochrome C, Transferrin (holo), Myoglobin, RNA, Insulin (bovine), Insulin (human), Glucagon and small molecule analytes: Acetylcholine, Dopamine, Glucose, Fructose, Adenine, Guanine, Uridine, Cytosine, Ascorbic acid, Folic acid, Sod. Pyruvate, Glutathione, Glutathione reduced, β-Nicotinamide adenine dinucleotide (NAD), Flavin adenine dinucleotide (FAD), and 30% Hydrogen peroxide in the selectivity test. After the addition of dye the 96 well plates were incubated on ice for 30 min before fluorescence measurement.

Two-photon absorption cross sections measurement. The TP emission spectra of TPG library compounds were determined over a broad spectral region by the two-photon induced fluorescence (TPF) method with Rhodamine B in methanol as a reference. A PTI, Quanta Master™ spectro fluorimeter and femtosecond (fs) Ti: Sapphire laser (Mira 900F, 220 fs pulse width, 76 MHz repetition rate, tuning range 740 - 840 nm, Coherent (USA) were used. Two-photon fluorescence measurements were performed in 10 mm fluorometric quartz cuvettes with 10 μM **TP-α** in Methanol, and 10 μM Rhodamine B as reference in same solvent. The experimental fluorescence excitation and detection conditions were conducted with negligible reabsorption processes which can affect TPA measurements. The two-photon absorption cross section of the probes was calculated at each wavelength according to equation 2.

$$\delta_{sample} = \delta_{reference} \frac{\Phi_{(ref)} I_{(sample)} C_{(ref)} \eta_{(sample)}^2 P_{(ref)}^2}{\Phi_{(sample)} I_{(ref)} C_{(sample)} \eta_{(ref)}^2 P_{(sample)}^2} \quad (2)$$

Where I is the integrated fluorescence intensity, C is the concentration, η is the refractive index, Φ is the quantum yield, and P is the incident power on the sample, subscript 'ref' stands for reference samples, 'sample' stands for samples. The uncertainty in the measured cross sections was about ± 10%.

Cell culture condition for screening. Insulin producing beta-TC6 (ATCC® CRL-11506™), glucagon producing alpha TC1 clone 6 (ATCC® CRL-2934™) and exocrine acinar cells 266-6 (ATCC® CRL-2151™) were obtained from the American Type Culture Collection (ATCC) and maintained according to ATCC protocols. Beta and acinar cells were cultured using Dulbecco's Modified Eagle's Medium (DMEM) with 4500 mg/L D-glucose, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Alpha TC1 cells were cultured in DMEM with 1000 mg/L D-glucose, supplemented with 15% heat inactivated FBS, 2.0 g/L D-glucose, 15 mM HEPES, and 0.1 mM non-essential amino acids (GIBCO, Life Technologies, Carlsbad, CA, United States).

Image-based screening and hit selection. A primary screening platform was established with the alpha TC1, beta-TC6 and acinar 266-6 cells. All three cell lines were seeded into 384 well plates and allowed to settle overnight before use. The cells were incubated with 0.5 and 1 μM of TPG library-80 compounds for 1 h. Wells were washed one time with fresh media to reduce the background before image acquisition. Automated imaging system ImageXpress® Micro with 10X objectives was used to capture the images. Images were analyzed based on fluorescence intensity to seek out the alpha cell selective probe. An alpha cell selective fluorescent probe will show higher fluorescent signal in the alpha TC1 cells in comparison to beta TC6 and acinar cells. TPG-456 (**TP-α**) showed consistently higher fluorescent signal in alphaTC1 cells then the beta and acinar cells in the secondary and tertiary tests (see Figure S1).

OP and TP imaging. All confocal and TP imaging was done using Leica TCS SP5X MP (Leica Microsystems CMS GmbH, Mannheim, Germany) with 405 nm laser light for OP excitation and 750 nm femtosecond (fs) pulsed laser for TP excitation for **TP-α**. Images were taken using 10X dry, 40X water and 100X oil objectives. The TP excitation of **TP-α** was achieved by titanium-sapphire laser light set at wavelength 750 nm and an output power of 2710 mW. To obtain the probe signal, internal photomultiplier tube was set at 430 to 530 nm. Image analysis and intensity measurements were carried out by Leica Application Suite Advanced Fluorescence (LAS AF). 3D projection of Z-stack TP images were processed with IMARIS software (Bitplane AG, Switzerland) and the intensity is depicted as false color.

Islet isolation and imaging. Primary pancreatic islets were isolated from the 16 week old C57BL/6 (WT) male mice. The animal handling and tissue harvesting is in accordance with the Institutional Animal Care and Use Committee of Singapore Bio-imaging Consortium (Agency of Science, Technology and Research, Singapore). Pancreatic islets were isolated by Collagenase P digestion and islet picking methods as described earlier.[21] Briefly, fresh pancreas was cut into small pieces and digested with 1 mg/mL collagenase P (Roche, Indianapolis, IN, USA) solution in Hank's Balanced Salt Solution (HBSS; Invitrogen) for 10 minutes at 37° C on a shaker, followed by 4 times buffer washing to remove the digested exocrine tissue. Manual picking of pancreatic islets was carried out using Zeiss Stemi DV4 stereomicroscope. Islets were maintained in DMEM with 4500 mg/L glucose + 10% FBS and 1% penicillin-streptomycin (GIBCO, Life Technologies, Carlsbad, CA, United States) for 24 h before TP imaging. Islets were stained with 1 μM **TP-α** for 1 h at 37° C and were transferred to fresh media for TP image acquisition.

Dissociation and culture of islet. Primary pancreatic islet cells were dissociated by trypsinization and trituration method.[21] Approximately 50 - 100 islets were dissociated by incubating with 0.25% Trypsin-EDTA (1X), phenol red (GIBCO) at 37° C for 2 minutes followed by trituration with repeated pipetting. Finally, the dissociated islet cells were transferred to the 4500 mg/L D-glucose DMEM with 10% FBS and 1% penicillin-streptomycin (GIBCO) for culture. Dissociated cells were cultured for 1 week with media change every 2-3 days. The cells were stained with 1 μM **TP-α** for 1 h at 37° C and were transferred to fresh media before TP image acquisition.

Immunostaining. Primary cells and islets were fixed in 4% Paraformaldehyde and permeabilized with 0.1% Triton-X 100. Fixed Cells were identified by primary antibodies for overnight at 4° C at the respective dilutions. For alpha cell - Glucagon antibody (Monoclonal Anti-Glucagon antibody produced in mouse, G2654, Sigma-Aldrich 3050 Spruce St. St. Louis, USA) 1:2000. Another mice pancreatic alpha cell marker $GABA_A$ receptor antibody (Anti-GAB_A A Receptor β 2,3 Chain Antibody, clone BD17 produced in mouse: MAB341, Merck Millipore,) 1:200. For Insulin (Insulin [L6B10] Mouse mAb, Cell Signaling #8138) 1:200. For secondary antibody staining, Cy5® goat anti-mouse IgG (Invitrogen, Molecular Probes Inc, USA) was used for all three primary antibodies. Flow-cytometry analysis was done by BD LSR II Flow Cytometer.

Cytotoxicity assays. Cytotoxicity assays were carried out using the MTS reagent kit (Promega) for 1-24 h, with 1 and 2 μM **TP-α** treated alpha and beta cells in accordance with the manufacturer's instructions.

Flow cytometry. Primary islet cell cultures were generated from the islets of C57BL/6 (WT) male mice as described above. Cells were stained with 1 μM of **TP-α** for 1 h at 37° C and washed with Phosphate Buffer Saline (PBS) before acquisition on the BD LSR II analyzer. Cells were acquired using the appropriate filters for **TP-α** (DAPI; excitation laser light 355 nm and emission at 450/50 nm, Long pass filter 410 nm), and secondary antibody Cy5; excitation laser light 640 nm and emission at 660/20 nm.

ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedure and building block structures with characterization are available in Supporting Information. "This material is available free of charge via the Internet at http://pubs.acs.org."

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Notes

The authors declare no competing financial interests.

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Table of Content (Graphics)