Real-time detection of acetylcholine release from the human endocrine pancreas

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Neurons, sensory cells and endocrine cells secrete neurotransmitters and hormones to communicate with other cells and to coordinate organ and system function. Validation that a substance is used as an extracellular signaling molecule by a given cell requires a direct demonstration of its secretion. In this protocol we describe the use of biosensor cells to detect neurotransmitter release from endocrine cells in real-time. Chinese hamster ovary cells expressing the muscarinic acetylcholine (ACh) receptor M3 were used as ACh biosensors to record ACh release from human pancreatic islets. We show how ACh biosensors loaded with the Ca^{2+} indicator Fura-2 and pressed against isolated human pancreatic islets allow the detection of ACh release. The biosensor approach is simple; the Ca^{2+} signal generated in the biosensor cell reflects the presence (release) of a neurotransmitter. The technique is versatile because biosensor cells expressing a variety of receptors can be used in many applications. The protocol takes ~3 h.

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INTRODUCTION

Increases (hyperglycemia) and decreases (hypoglycemia) in blood glucose concentration can be fatal and are efficiently prevented by the secretion of pancreatic islet hormones. The concerted output of insulin and glucagon from the endocrine cells in the human pancreas produces a dynamic hormonal balance that counteracts blood glucose fluctuations. As a result, blood glucose levels are maintained at a concentration of ~5 mM. The hormonal output from the islet is orchestrated by a combination of factors, such as nutrients, incretins, nervous input and paracrine signaling between islet cells. For instance, certain neurotransmitters, including ACh, γ-aminobutyric acid (GABA), ATP, noradrenalin and dopamine, have been shown to modulate insulin and glucagon secretion and thus have been proposed to have an important paracrine signaling role in islet cell function.

To establish unambiguously that a substance is a neurotransmitter in a given tissue, however, one needs to show that (i) the substance is present within the releasing cell, (ii) the substance is secreted in response to adequate stimulation and (iii) specific receptors for the substance are present on target cells[1]. Meeting these criteria in the human endocrine pancreas is technically challenging, particularly because genetic manipulation of the different signaling components is not possible. A rigorous demonstration that any given neurotransmitter candidate is involved in paracrine signaling in the islet requires showing that the transmitter is present in pancreatic endocrine cells, that it is released in response to stimuli (e.g., changes in glucose levels), and that the transmitter affects other islet cells. Here we present a strategy for validating ACh as a paracrine signal in human pancreatic islets, which can be adapted to test other neurotransmitter systems.

Current methods

A first examination of paracrine signaling generally involves detecting receptors on target cells. Receptor-mediated responses to the application of candidate substances can be readily measured in endocrine cells by determining changes in hormone secretion, increases in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) or changes in electrical activity. When changes in target cell activity are monitored while the extracellular concentration of the candidate substance is manipulated (i.e., by diminishing its degradation), the presence and efficacy of endogenous levels of this substance in the tissue can be demonstrated indirectly. This approach has been used to infer the roles of ATP and ACh as autocrine/paracrine signals in human pancreatic islets[2,3]. Another strategy is to detect different components of the machinery needed for paracrine signaling using immunohistochemistry or reverse transcription–PCR. Several signaling molecules as well as molecules associated with their synthesis and transport have been localized to endocrine cells using this technique[3–5].

Directly observing the release of a transmitter candidate in the appropriate physiological context is likely to represent the most stringent demonstration of its involvement in paracrine signaling. After stimulating islets *in vitro* with elevated (or reduced) glucose, one can test for transmitter secretion by assaying the bathing medium with techniques such as HPLC or ELISA. To avoid adverse effects caused by the accumulation of hormones and neurotransmitters in the bath, perfusion assays to monitor hormone secretion have been developed[6]. Here the temporal resolution is determined by the sampling frequency, which in turn is restricted by the detection limits of the assays used to detect the neurotransmitter or hormone. The dynamics of neurotransmitter release can be recorded with superior temporal resolution and in real time using electrochemical detection, but only a few neurotransmitters can be detected by direct redox activity at an electrode[7]. Moreover, electrochemical recordings are affected by interference from other electroactive neurotransmitters or from high concentrations of electroactive metabolites. Therefore, current methods have limited

temporal resolution, cannot be performed in real time, are restricted to assaying a few neurotransmitters, or require specialized equipment and expertise. A method that is simple and suited for real-time detection of hormone or neurotransmitter release, however, is the use of biosensor cells.

Real-time detection of hormone and neurotransmitter release with biosensor cells

Biosensor cells are cells that express high levels of endogenous receptors for a transmitter/hormone or are genetically engineered to overexpress these receptors. Depending on the downstream signaling pathways, receptor activation in biosensor cells can be measured either with $[Ca^{2+}]_i$ imaging or with electrophysiological methods. Well-designed biosensor cells will reliably respond to low concentrations of a specified neurotransmitter and not to other transmitters or to other agents such as test stimuli or pharmacological antagonists used in the experiments. For example, Chinese hamster ovary (CHO) cells expressing 5-HT2c receptors have shown that certain taste bud cells secrete serotonin[8], or pancreatic beta cells overexpressing P2X receptors have been used to detect their own ATP secretion via small transient kiss-and-run fusion pores[9]. Furthermore, biosensor cells can be designed to express a combination of receptors to demonstrate the co-release of neurotransmitters[10].

The concept of using cells expressing high-affinity receptors to identify neurotransmitters secreted by cells and tissues is not new. Sensory cells in the cochlea, retina and taste buds have been probed with biosensors[8,11,12]. Several studies have used biosensor cells to detect the release of neurotransmitters from pancreatic islet cells. Rorsman and colleagues have made extensive use of this technique to detect GABA and ATP release from beta cells[9,13–15]. Similar to studies in chromaffin cells[16,17], their approach consists of infecting beta cells with $GABA_A$ or P2X receptors to create 'autosynapses' and record the beta cell's own GABA and ATP secretion with patch-clamp electrophysiology. Glucagon secretion from human islets has also been monitored with biosensor cells. This was made possible using an approach in which islets are placed on a layer of HEK293 cells expressing glucagon receptors and $[Ca^{2+}]_i$ is measured in the biosensor cells while islets are stimulated[18]. These studies support the importance and utility of using cellular biosensors to record neurotransmitter and hormone secretion from tissues of interest.

We have recently used the protocol described here to measure ACh secretion from human pancreatic islets[3]. In experiments designed to explore parasympathetic innervation in human islets, we found that human islets lacked prominent cholinergic innervation. Instead, alpha cells themselves express high levels of the vesicular ACh transporter, suggesting that ACh is packaged in secretory vesicles for exocytotic release from cells intrinsic to the islet. We therefore used the biosensor approach to examine ACh release from human islet endocrine cells. CHO cells expressing the muscarinic receptor M3 were loaded with the Ca^{2+} indicator Fura-2 and pressed against isolated human islets. We found that $[Ca^{2+}]_i$ responses could be evoked under conditions that stimulate human alpha cells but not beta cells. By contrast, we could not measure ACh secretion from mouse islets. This allowed us to conclude that human alpha cells store and secrete ACh.

Advantages and applications of the technique

The biosensor cell technique enables one to assess neurotransmitter secretion in real time. It has great sensitivity because it detects the neurotransmitter near to its release site. Biosensor cells can be used to test for transmitter release from individual isolated cells or from intact tissues (e.g., islets). To determine whether test stimuli elicit release directly, biosensor cells can be used to measure transmitter secretion from single cells. When the same cells are investigated in the intact tissue it is feasible to study paracrine interactions by measuring how the secretory profile (i.e., biosensor cell response) changes in the presence of receptor agonists or antagonists for the putative paracrine signals. When coupled with $[Ca^{2+}]_i$ imaging, the biosensor approach is straightforward. $[Ca^{2+}]_i$ signals generated in the biosensor cell reflect the presence (release) of a neurotransmitter. Biosensor cells sensitive to different neurotransmitters can be used to measure the secretion of two neurotransmitters concurrently. For instance, GABA and ACh secretion could be monitored simultaneously by using biosensor cells expressing ionotropic $GABA_A$ receptors and metabotropic muscarinic receptors and recording Cl currents and Ca^{2+} mobilization from intracellular stores. Furthermore, it is feasible to record responses in the secreting cell and in the biosensor cell simultaneously, which allows stimulus-secretion coupling to be studied.

In the particular case of the pancreatic islet, the use of biosensor cells is likely to advance our understanding of paracrine regulation of hormone secretion. Several neurotransmitters have been proposed as paracrine/autocrine signals in the pancreatic islet. For most of these substances, however, there is no direct evidence that they are being released from islet cells. A systematic approach using a battery of biosensor cells (**Table 1**) could determine which neurotransmitters are secreted within the islet and define under which circumstances they are released. For instance, although beta cells secrete serotonin to increase beta cell proliferation during pregnancy[5], it is unclear whether serotonin has a role in normal islet physiology. Furthermore, different islet cells express dopamine receptors[19], but it is not known whether dopamine is released as a paracrine signal. In view of the current increased availability of human islets for research, this approach can be applied to assess paracrine signaling in the human islet, thereby providing results of relevance to human health with the possibility of translation into the clinic.

Biosensor cells are being successfully used to detect neurotransmitter release in situations in which conventional assays may fail. Direct detection of ACh by current analytical methods, for instance, requires a sensing scheme in which acetylcholinesterase converts ACh to choline and choline is oxidized by choline oxidase to produce detectable hydrogen peroxide[7]. The ACh electrodes need to be chemically modified to be selective and are fragile because detection depends on the activity of the coimmobilized enzymes acetylcholinesterase and choline oxidase. The sensitivity of these electrodes for ACh (limit of detection: 10 nM–0.1 mM; ref. 7) is lower than that of the biosensor cells (approximately 1–10 nM threshold concentration for Ca^{2+} responses). Although analytical methods may provide quantitative results, the biosensor cells are exceptionally hardy and reliable, making it easy to carry out studies of ACh secretion.

Biosensor cells are also quite versatile and readily available. For instance, CHO cells stably expressing a wide variety of different transmitter receptors are commercially available (**Table 1**) and are easily maintained in the laboratory, making these cells biosensors of choice for many applications. The broad availability of CHO biosensors allows a systematic approach to exploring a wide variety

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TABLE 1 | Biosensors to be used for identifying islet transmitters and hormones, and their sources.

Biosensor	Receptor(s)	Source
ACh	Rat M3	ATCC, CRL-1981
ATP	P2Y1/P2Y2	CHO-K1,-1C19 cell lines
5-HT	5-HT2c	K.A. Berg, University of Texas Health Science Center, San Antonio, Texas
Glutamate	(a) Mouse NR1/2a	F. Nyberg, Uppsala University, Sweden
	(b) Mouse NR1/2b	
GABA	$GABA_B$ receptor	K. Kaupmann, Novartis, Basel, Switzerland
Adrenaline, noradrenaline	Mouse α1A	W.B. Jeffries, Creighton University, Omaha, Nebraska
Adrenaline, noradrenaline	β3 Adrenergic receptor	Millipore Ready-To-Assay Ca^{2+}-optimized cells[a]
Dopamine	D_5 dopamine receptor	Millipore Ready-To-Assay Ca^{2+}-optimized cells[a]
Glucagon	Human GCGR	Millipore Ready-To-Assay Ca^{2+}-optimized cells[a]
Somatostatin	Sst3 somatostatin receptor	Millipore Ready-To-Assay Ca^{2+}-optimized cells[a]
Ghrelin	Ghrelin receptor	Millipore Ready-To-Assay Ca^{2+}-optimized cells[a]
GLP-1	Human GLP-1R	D.M. Ignar, Glaxo Wellcome, Research Triangle Parc, North Carolina

All the expressed receptors in these cell lines couple to Ca^{2+} mobilization either through endogenous G proteins or through promiscuous Gα proteins (e.g., Gα15). These cells can be used with $[Ca^{2+}]_i$ imaging as the readout.

[a]Millipore ChemiScreen and Ready-To-Assay cells are generated from proprietary host cell lines expressing the promiscuous G protein Gα15.

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of transmitters. We have used biosensor cells to detect ATP, serotonin, ACh, glucagon, GABA and noradrenaline secretion from taste buds, pancreatic islets or single taste and islet cells with simple Ca^{2+}-imaging equipment[3,8,10,18,20,21]. Most modern laboratories already have the equipment and expertise available, thus making this a low-cost approach to solving important research questions. The measurements are rapid, robust (there is a high signal-to-noise ratio), reliable and reproducible from sample to sample, from day to day and from experimenter to experimenter.

The biosensor cell approach can also be adapted for detection of neurotransmitter secretion *in vivo*. A recent study used chronically implanted biosensor cells ('cell-based neurotransmitter fluorescent engineered reporters' or 'CNiFERs') to monitor ACh release in the frontal cortex in living rats[22]. The temporal resolution was comparable to that of electrochemistry and ~100-fold faster than microdialysis. A similar approach may not be feasible for studying islets in the pancreas, but biosensor cells expressing genetically encoded Ca^{2+} indicators could be transplanted together with islets into the anterior chamber of the mouse eye, where islets and biosensor cells can be monitored noninvasively[23,24]. In this transplantation site, neurotransmitter secretion from vascularized and innervated islet grafts could be studied *in vivo*.

Limitations

A limitation of the biosensor technique is that it is mainly qualitative. The responses in the biosensor cell not only reflect the extracellular concentration of the neurotransmitter but are also shaped by the cell's own signaling components, which may be prone to desensitization or, when receptor activation leads to intracellular Ca^{2+} release, to Ca^{2+} depletion. Because the approach relies on Ca^{2+} signals, events cannot be measured on a millisecond timescale. Moreover, individual biosensor cells may have different sensitivities to the neurotransmitter. Therefore, results from different experiments cannot be compared quantitatively. A way around this limitation is to use internal reference stimuli or to calibrate the biosensor cells with direct application of known concentrations of the neurotransmitter. As with all microscopic approaches, the biosensor technique is also affected by spatial constraints such as the distance and access to the release sites. This also precludes scaling up the technique for high-throughput analyses. With the appropriate controls (see below), however, the biosensor cell technique can provide information that can define the secretory phenotype of a cell.

Experimental design

To detect ACh release, we use CHO K1 cells stably expressing mouse M3 muscarinic ACh receptors (hereafter 'ACh biosensors'). Biosensor cells are CHO K1 cells stably expressing ACh biosensors purchased from American Type Culture Collection (ATCC). The M3 receptor is Gq-coupled and mediates an increase in intracellular Ca^{2+} through the IP3 and PLCβ2 signaling cascade. Biosensor cells maintain Ca^{2+} mobilization from intracellular stores in response to ACh in the absence of extracellular $[Ca^{2+}]$. They can therefore be used to test for Ca^{2+} influx–dependent exocytosis by measuring how secretion changes in the nominal absence of extracellular $[Ca^{2+}]$. ACh biosensor responses can therefore be measured using $[Ca^{2+}]_i$ imaging. ACh biosensors reliably respond to low concentrations of ACh ($EC_{50} \approx 50$ nM), making them highly sensitive ACh detectors. In this protocol, we load ACh biosensors with the $[Ca^{2+}]_i$ indicator Fura-2 and manipulate them toward and abut

them against isolated human islets using a fire-polished glass micropipette. We hold the biosensor onto the micropipette using gentle suction and bath-apply stimuli (changes in glucose concentration) to activate pancreatic endocrine cells. Glucose stimulation does not elicit increases in $[Ca^{2+}]_i$ in ACh biosensors alone (i.e., in the absence of an islet). However, when positioned next to an islet, ACh biosensors respond briskly (i.e., $\Delta[Ca^{2+}]_i$) when the islet is stimulated with a decrease in glucose concentration, indicating that the islet has released ACh. We can block ACh biosensor signals with the muscarinic antagonist atropine, confirming that responses in the biosensors are indeed mediated by ACh and its activation of the transfected M3 receptors.

We designed our stimulation protocol to evoke responses in different cell populations. For instance, we use KCl depolarization to distinguish endocrine from exocrine cells. Only endocrine cells respond to KCl depolarization, that is, they express voltage-dependent Na^+ and Ca^{2+} channels. A possible contribution of transmitter release from axons, which would also be depolarized by KCl, can be ruled out because neuronal elements degenerate from islet preparations after 2 or more days in culture[18,25]. A decrease in glucose concentration or stimulation with kainate specifically activates the glucagon-secreting alpha cells[18]. KCl depolarization, glucose decrease or kainate stimulation all evoke robust ACh release, as measured by large $[Ca^{2+}]_i$ responses in ACh biosensors. In contrast, raising the glucose concentration stimulates beta and delta cells, but this stimulus does not evoke ACh release. This stimulation protocol allowed us to conclude that ACh was released from alpha cells.

Islet viability. When using human islets it is important to bear in mind that the quality of islet preparations is highly variable. A larger number of experiments may be required to produce satisfactory results. For instance, we tested 75 human islets and detected ACh secretion from 17 islets[3]. Although this response incidence was low, it was still significantly higher ($P < 0.01$; Fisher exact test) than the one we obtained with mouse islets, as we could not record ACh secretion from mouse islets (none of the 26 tested mouse islets responded). To ensure that mouse islets were healthy, we simultaneously monitored Ca^{2+} responses in islets and biosensor cells. KCl depolarization induced responses in islets but not in biosensor cells apposed to the islet, indicating that mouse islet cells were viable but did not secrete ACh. This control for viability of the secreting cells should be performed when release cannot be detected. It is also important to rule out false-negative results by confirming that biosensor cells remain fully responsive to their designated neurotransmitter or hormone at the end of the recording session.

Neurotransmitter secretion. Secretion of different neurotransmitters may be more or less robust. Moving the biosensor cell to search for release sites may be needed if the transmitter is secreted locally by few cells in the tissue. Inhibiting the degradation of the transmitter may also help in improving its detection. In contrast, massive neurotransmitter release may produce saturating responses in biosensor cells that do not allow the temporal pattern of release to be followed. To obtain a faithful representation of secretory dynamics (e.g., pulsatility), biosensor cells can be moved away from the transmitter source or less-sensitive biosensor cells can be used.

Controls. An essential aspect of the analysis of neurotransmitter release using biosensors is that adequate controls must be established before any conclusions can be drawn. Specifically, it is important to establish that the biosensor cell responds only to the designated transmitter and not to other substances. For ACh biosensors, this means that only ACh should activate the biosensor. One approach is to test every candidate substance that may be released by the target tissue (in this case, the pancreatic islet). Although this is possible in theory, there may be a number of unknown substances released by the target sample. A more practical approach is to test whether an antagonist of the cognate receptor for the neurotransmitter being studied (e.g., here, ACh) blocks stimulus-evoked biosensor responses. In our study, atropine (5 μM) completely blocked muscarinic receptors on the ACh biosensors and eliminated transmitter-evoked responses generated by islet stimulation.

Parenthetically, CHO cells express endogenous transmitter receptors (see http://www.tumor-gene.org/cgi-bin/GPCR/by_cell_line.cgi). Particularly troublesome are endogenous P2Y receptors. Biosensors engineered from CHO cells may thus respond to the designated neurotransmitter as well as to ATP if it is also released from the target. A simple and effective method to overcome this complication is to desensitize the endogenous P2Y receptors before the experiment by incubating biosensor cells in 500 μM ATP for 60 min while loading. This procedure effectively eliminates P2Y-mediated responses for several hours yet does not affect the sensitivity of the transfected biosensor to its designated transmitter[26].

Other important controls include testing that any stimuli or pharmacological agents (agonists, antagonists) used in a study do not themselves either elicit biosensor responses (as mentioned above) or alter the ability of biosensors to respond to their designated transmitter. These controls can be readily and conveniently conducted by Ca^{2+} imaging of biosensor cells plated at low density and in the absence of target tissues or cells.

MATERIALS

REAGENTS

Biosensor cell line

- CHO K1 cells stably expressing mouse M3 muscarinic ACh receptors (ATCC, cat. no. CRL-1981)

Islets

- Human islets from multiorgan donors (Integrated Islet Distribution Program, coordinated by City of Hope and sponsored by the US National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases and JDRF) or from the Human Islet Cell Processing Facility at the Diabetes Research Institute, University of Miami Miller School of Medicine

! CAUTION Appropriate institutional regulatory board guidelines must be followed and informed consent should be obtained for the use of human samples.

- CMRL 1066, CIT culture medium (Cellgro, Mediatech, cat. no. 98-304-CV)
- Ham's F-12 medium (Invitrogen, cat. no. 10131027)
- Hanks' buffered salt solution (Invitrogen, cat. no.14065056)
- Penicillin-streptomycin (Invitrogen, cat. no. 15140148)
- Geneticin (50 mg ml^{-1}; Invitrogen, cat. no. 10131027)
- BSA (Invitrogen, cat. no. 15561020)
- MEM Na pyruvate (Invitrogen, cat. no. 11360-070)
- HEPES (Sigma, cat. no. H0887)
- Fura-2AM special packaging (Invitrogen, cat. no. F1221)
- DMSO (Sigma, cat. no. D5879) **▲ CRITICAL** The solution is light sensitive and should be stored in a dark glass bottle. Do not store it in a plastic container.

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- Kainic acid (Kainate; Tocris, cat. no. 0222) ! CAUTION It is a potent excitant and neurotoxic solution. Handle it wearing gloves.
- ACh chloride (Sigma, cat. no. A6625)
- ATP disodium salt (Sigma, cat. no. A2383) ▲ CRITICAL ATP is rapidly degraded. Use within 1 h of preparation.
- Atropine sulfate salt monohydrate (Sigma, cat. no. A0257)
- Fetal bovine serum dialyzed (Gibco, cat. no. 26400044)
- Trypsin (0.25% (wt/vol); Invitrogen, cat. no. 15050-065)
- NaCl
- KCl
- $CaCl_2$
- $MgCl_2$
- NaOH
- Nanopure UV water (Thermo Scientific)

EQUIPMENT

- Olympus IX71 inverted fluorescence microscope (Olympus Optical)
- Olympus UApo/340 ×20/0.70 NA water-immersion objective (Olympus Optical)
- Andor iXon+ EM camera (Andor Technology)
- Gravity-driven six channel perfusion valve control system VC-6 (Warner Instruments)
- Lambda LS xenon lamp controlled by a Lambda 10-3 controller (Sutter Instruments)
- PC running Indec Biosystems Imaging Workbench 6.0.25 (Indec Biosystems)
- TMC floating table
- Capillary holder (custom made)
- Microsyringe (3 ml, filled with liquid paraffin, affixed to Baltimore Instruments micrometer)
- Borosilicate glass capillary tubes (outer diameter (o.d.) 1.2 mm, inner diameter (i.d.) 0.6 mm) with filament (Sutter Instruments)
- P-87 Flaming/Brown micropipette puller (Sutter Instruments)
- Microforge for fire polishing with tungsten filament, electrically heated (custom made)
- Narishige MO-103 hydraulic manipulator (×2; Narishige Group)
- Tygon tubing (i.d. 1/16 inch; Warner Instruments)
- Warner Instruments RC-25 recording/perfusion chamber (Warner Instruments)
- VWR 2400 CO2 incubator (VWR)
- Eppendorf 5804 centrifuge (Eppendorf)
- Dismic (3cp cellulose acetate 0.45-µm hydrophilic syringe filters; Advantec)
- Shaker (American Dade, cat. no. R4140)
- Gilson pipettors (VWR 200-µl and 1-ml tips; Gilson)
- Eppendorf centrifuge tubes (1.5 ml; Eppendorf)
- BD 1-ml syringes (Becton Dickinson)
- Plexiglas sample container
- Bio-Plex protein array system (Bio-Rad)
- Culture dishes

REAGENT SETUP

Islet culture medium Islet culture medium is prepared by mixing 500 ml of CMRL 1066 medium, 50 ml of fetal bovine serum and 5 ml of 100× penicillin-streptomycin. Store at 4 °C for up to 1 week.

Biosensor culture medium Biosensor culture medium is prepared by mixing 440 ml of Ham's F-12 medium supplemented with 50 ml of fetal bovine serum and 10 ml of geneticin (100 µg ml^{-1}). Store at 4 °C for up to 1 month.

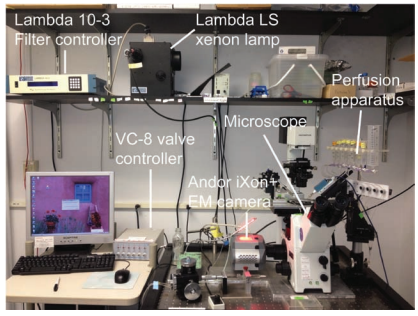

Figure 1 | Imaging setup for real-time imaging of neurotransmitter release with biosensor cells. The photograph shows the inverted microscope equipped with fluorescence lamp, camera and perfusion apparatus.

Extracellular solution Combine 25 ml of 5 M NaCl, 5.9 ml of 1 M KCl, 2.56 ml of 1 M $CaCl_2$, 1.2 ml of 1 M $MgCl_2$, 25 ml of 1 M HEPES, 1 g of BSA and Nanopure UV water to complete 1 liter. Adjust the pH to 7.4 with NaOH. Store at 4 °C for up to 1 month. Add 25, 75, 275 and 400 µl of 2 M glucose to 50 ml of extracellular solution to obtain a 1 mM, 3 mM, 11 mM and 16 mM glucose concentration, respectively. Add 402 µl of 3 M KCl to 50 ml of extracellular solution to obtain an extracellular solution containing 30 mM KCl.

HHP solution HHP solution is prepared by mixing 49 ml of 10× Hanks' buffered salt solution, 5 ml of 1 M HEPES, 5 ml of 100 mM MEM Na pyruvate and 441 ml of Nanopure UV water. Store at 4 °C for up to 1 month.

Fura-2 solution Add 100 µl of DMSO to a vial of pre-packaged Fura-2AM (50 µg) to prepare a 5 mM stock solution (store for 6 months at −20 °C). Add 1 µl of this stock solution and 20 µl of FBS to 1 ml of HHP solution to get a final concentration of 5 µM Fura-2AM and 2% (wt/vol) FBS. Use within 6 h.

EQUIPMENT SETUP

Ca^{2+}-imaging setup We acquire images ratiometrically using Fura-2 optics, with an Olympus IX71 inverted fluorescence microscope and a ×20 UAPO water-immersion objective (NA = 0.70) optimized for UV excitation (**Fig. 1**). A Lambda LS xenon lamp controlled by a Lambda 10-3 controller provides fluorescence excitation at 340 and 380 nm. We acquire fluorescence using an Andor iXon EM CCD camera. We carry out imaging analysis using Imaging Workbench 6.0.25, with Fura-2 emission ratio (340/380) taken as representative of $[Ca^{2+}]_i$.

Preparation of micropipettes Pull micropipettes from microcapillary glass. Break the tips and re-forge them with the fire polishing microforge such that they are large and flat fronted. The desired final tip i.d. is 2 µm, the o.d. is 5 µm. Micropipettes can be stored in a sealed container for 2 months.

PROCEDURE

Islet culture ● TIMING 48–120 h

1| Culture islets and islets cells (37 °C, 5% CO_2) in islet culture medium for 48–120 h.

▲ CRITICAL STEP Culturing islets depends on study parameters. For instance, culturing islets for more than 2 d will eliminate neuronal elements[18,25].

Culture of biosensor cells ● TIMING 48–72 h

2| Maintain biosensor cells in biosensor culture medium at 37 °C and 5% CO_2 in a 35-mm culture dish until they are used for imaging. Split cells every 2–3 d, and maintain them at a confluence of around 70%.

! CAUTION Receptor expression efficiency is reduced after passage 200 or if cells are cultured at a confluence >70%.

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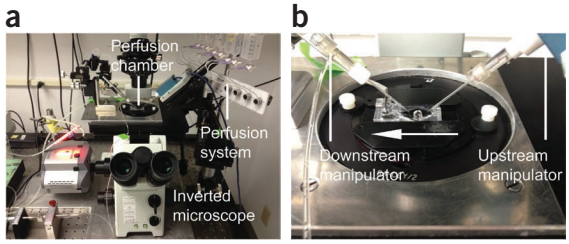

Figure 2 | Perfusion setup to detect neurotransmitter release from human islets with biosensor cells. (**a**) Experiments are performed in a perfusion chamber placed on an inverted microscope. Solutions are delivered using a gravity-fed perfusion system. (**b**) Two hydraulic manipulators are used to abut the biosensor cells to the human islet within the perfusion chamber; an upstream manipulator holds the human islet in place and the downstream manipulator presses the biosensor cell against the islet. The arrow indicates the flow direction.

Imaging neurotransmitter release ● TIMING ~3 h

3| Treat biosensors with 0.5 ml of trypsin (0.25% (wt/vol)) for 1 min, wash them from the dish with biosensor culture medium and centrifuge them for 3 min at 65*g* (22 °C); remove the medium and discard.

4| Filter 1 ml of 5 µM Fura-2 solution with 500 µM ATP with an 0.45-µm syringe filter, add it to the pellet and agitate to separate the biosensors. Load the biosensors for 60 min on a shaker platform at 60 r.p.m.

5| Centrifuge the biosensors at 65*g* for 3 min (22 °C), remove the Fura-2 solution and resuspend the cells in 300 µl of extracellular solution.

■ **PAUSE POINT** Biosensor cells will remain healthy for several hours in extracellular solution at 22 °C.

6| Collect 10–20 islets from Step 1 with a pipette and deposit them into the upstream section of the perfusion chamber (**Fig. 2**).

7| Pick up an islet with the upstream manipulator. Islets are more resilient to physical damage than biosensors (see below).

8| Deposit 5 µl of suspended biosensors (from Step 5) downstream of the islets and wait 5 min for the biosensors to lightly secure to the dish.

▲ **CRITICAL STEP** After 5 min, the biosensors are beginning to weakly attach to the chamber. On starting the perfusion, a few will be swept away but many will stick.

9| To identify sensitive biosensor cells, screen with a low concentration of the target neurotransmitter (in this case, 100 nM ACh). Apply 100 nM ACh in extracellular solution (22 °C) using the gravity-driven perfusion system, and then acquire images with the Ca^{2+}-imaging setup every 5 s. Monitor the Fura-2 emission ratio (340/380) on the screen and record changes in the Fura-2 emission ratio in regions of interest drawn around biosensor cells in the visualized field. The most sensitive cells will have the largest increases in the Fura-2 emission ratio (i.e., the largest increases in $[Ca^{2+}]_i$) in response to the application of ACh. Very gently pick up sensitive biosensors with the downstream manipulator (**Fig. 3**).

! **CAUTION** The window for the viable collection of a sensitive biosensor cell is only from 5 to 15 min after deposition. Collect too soon and the biosensor will wash from the surface and be lost when screening; collect too late and the biosensor will be too firmly attached to surface, resulting in damage when collecting.

▲ **CRITICAL STEP** Concentration-response curves determined separately show an $EC_{50} \approx 50$ nM (**Fig. 3**).

▲ **CRITICAL STEP** It is necessary to be extremely gentle, as biosensor cells are very easily damaged either by the pipette or by the suction from the pipette on collection. If you think there is a possibility that the cell is damaged, it is more than likely that it is. In this case, it is better to start again. The biosensor-holding pipette must be correctly designed. If the biosensor micropipette is too sharp, a firm hold on the biosensor cannot be established without damage. If it is too flat, too much of the surface area of the biosensor will be in contact with the glass, thereby limiting the access of the neurotransmitter to receptors on the biosensor cell.

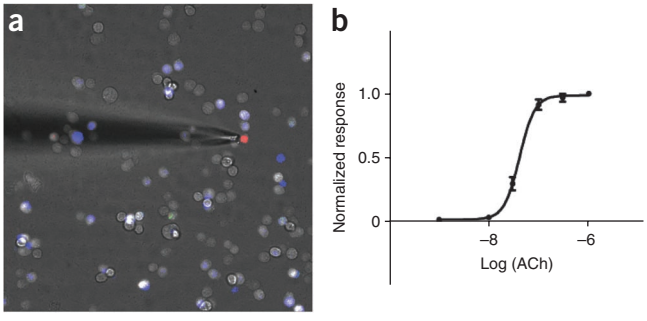

Figure 3 | Screening of biosensor cells with acetylcholine (ACh). (**a**) Biosensor cells show different sensitivities to ACh. The magnitude of the Ca^{2+} response to ACh is represented in pseudocolor scale and superimposed on a transmitted light image. The cell approached by pipette shows the largest response (red) and is selected for the experiment. (**b**) Concentration-response relationship for ACh in ACh biosensor cells ($EC_{50} \approx 50$ nM).

10| Image the secured biosensor for 5 min to ensure stability. Monitor the Fura-2 emission ratio and confirm that it stays constant for 5 min (that is, that the Ca^{2+} levels

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return to and remain at the baseline). Rescreen with 100 nM ACh to confirm sensitivity (as in Step 9); the signal must be highly repeatable and highly similar in magnitude.
? TROUBLESHOOTING

11| Bring the biosensor cell into close apposition with the captured islet (from Step 7) (**Fig. 4**) and start perfusion of the selected solution (in this case, extracellular solution). The perfusion system electronically opens and closes pinch valves, controlling the flow from up to six solution reservoirs. The system allows switching between solution reservoirs. Ensure that there is no movement of the target cell because of perfusion.

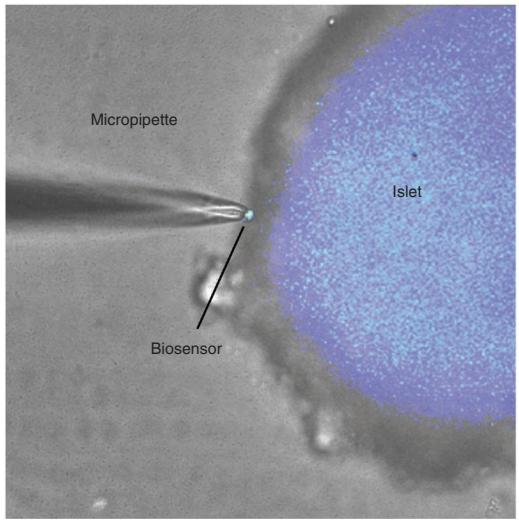

Figure 4 | Biosensor cell approach. A biosensor cell is brought next to a human islet with the aid of a micropipette and a micromanipulator.

12| Stimulate islets by switching the perfusion system to extracellular solutions containing KCl or different glucose concentrations.

13| Shortly before (2 min), during (up to 10 min) and after stimulation (until the response subsides), acquire images with the Ca^{2+}-imaging setup every 5 s and monitor the Fura-2 emission ratio (340/380) in the biosensor cell. The Fura-2 emission ratio increases when biosensor cells sense ACh. We refer to the peak change in the Fura-2 emission ratio as the amplitude of the response (Δ 340/380). Use these amplitudes to compare the effects of stimulation under different conditions. If the biosensor cell senses ACh in response to stimulation of the islet, repeat Step 12 in the presence of the muscarinic ACh receptor antagonist atropine to confirm that ACh was released.
▲ CRITICAL STEP Neurotransmitter release from whole islets may show spatial variability and therefore many potential release sites around the islet may need to be tested before success is achieved.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
10	Erroneous Ca^{2+} signals from the biosensor in the absence of stimulation	Damage to the biosensor on collection	Deposit more biosensors in the chamber, rescreen and start imaging again
		Movement of the islet induces mechanical stress	Brace the islet against the chamber floor with a micropipette to ensure minimal movement
13	Signals in the biosensor are reducing over time	Pipette suction is too great	Reduce the suction pressure from the microsyringe
		Photobleaching of Fura-2 within the biosensor cell	Reduce the exposure time or increase the time between scans to minimize photobleaching
		Natural rundown of the living biosensor cell	Deposit more biosensors in the chamber, rescreen and start imaging again
	No signal is recorded from the biosensor upon stimulation of the islet	The biosensor may have deteriorated	Rescreen the biosensor distant to the islet to ensure sensitivity
		Islet is unhealthy	Deposit, collect and screen additional islets from stock; if you are still unsuccessful, the culture may be bad

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● TIMING

Step 1, islet culture: 48–120 h
Step 2, culture of biosensor cells: 48–72 h
Step 3, prepare biosensors: ~10 min
Steps 4 and 5, load and resuspend biosensors: ~70 min
Steps 6 and 7, prepare islets: ~20 min
Steps 8–11, screen, retest and position biosensor: ~30 min
Steps 12 and 13, simulate and measure islets: ~30–60 min

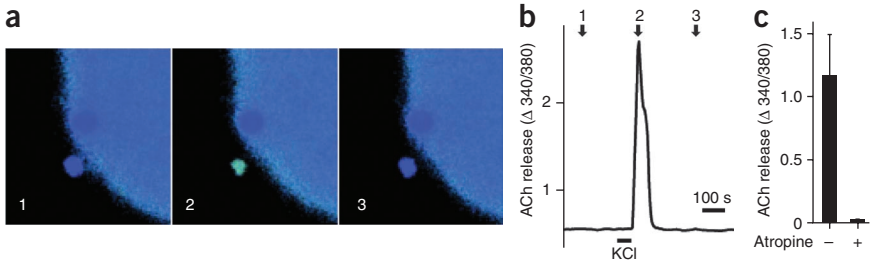

Figure 5 | Real-time detection of ACh release. (**a**) Three sequential images (1–3) showing $[Ca^{2+}]_i$ responses in an ACh biosensor cell apposed to a human islet. The biosensor cell shows an increase in $[Ca^{2+}]_i$ in response to islet stimulation with KCl depolarization (middle, increase in $[Ca^{2+}]_i$ appears green in pseudocolor scale). Control experiments show that KCl depolarization (30 mM) does not directly stimulate biosensor cells. (**b**) Trace of biosensor $[Ca^{2+}]_i$ response with arrows indicating the time points shown in **a**. (**c**) The biosensor $[Ca^{2+}]_i$ response to KCl is abolished in the presence of the muscarinic antagonist atropine, indicating that ACh was detected (n = 8 experiments). Error bars indicate s.e.m.

ANTICIPATED RESULTS

The protocol above has been used to detect neurotransmitter release in real time (**Fig. 5**). Following this protocol, semiquantitative assessment can be made of neurotransmitter release from a variety of tissues. With this protocol we can identify neurotransmitter sources and describe their secretory patterns to define the roles of many of the neuroactive substances that have been suggested to be paracrine signals in the islet.

We record $[Ca^{2+}]_i$ responses in ACh biosensor cells while the pancreatic islets are stimulated. During a typical experiment, we monitor ACh release by looking at changes in Fura-2 fluorescence in the microscopic image of the biosensor cell (**Fig. 5a**) and by looking at traces of the Fura-2 340/380 ratio in a region of interest drawn around the biosensor cell (**Fig. 5b**). This online monitoring allows early decisions to be made about whether or not it is worth continuing the experiment. Many neurotransmitters are secreted in response to cell membrane depolarization, and therefore depolarization with KCl (30 mM) should elicit neurotransmitter release. An ACh biosensor cell placed against a human islet should readily sense ACh release in response to KCl depolarization, as indicated by a robust $[Ca^{2+}]_i$ response (**Fig. 5**). If ACh release in response to KCl is weak, the human islet preparation is not of sufficient quality or the position of the biosensor cell is not adequate. Additional stimulation with changes in glucose or drugs should only be carried out if the responses to KCl are robust.
We recommend performing in the same recording the control experiment to show that ACh mediates the response. Thus, blocking muscarinic receptors with atropine (5 μM) should abolish the $[Ca^{2+}]_i$ response in the ACh biosensor cell (**Fig. 5c**).
We use the amplitude of the $[Ca^{2+}]_i$ response (Δ 340/380) for quantification and comparisons between experimental groups.

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