A High-content *In Vitro* Pancreatic Islet β -cell Replication Discovery Platform

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Abstract

Loss of insulin-producing β -cells is a central feature of diabetes. While a variety of potential replacement therapies are being explored, expansion of endogenous insulin-producing pancreatic islet β -cells remains an attractive strategy. β -cells have limited spontaneous regenerative activity; consequently, a crucial research effort is to develop a precise understanding of the molecular pathways that restrain β -cell growth and to identify drugs capable of overcoming these restraints. Herein an automated high-content image-based primary-cell screening method to identify β -cell replication-promoting small molecules is presented. Several, limitations of prior methodologies are surmounted. First, use of primary islet cells rather than an immortalized cell-line maximizes retention of *in vivo* growth restraints. Second, use of mixed-composition islet-cell cultures rather than a β -cell-line allows identification of both lineage-restricted and general growth stimulators. Third, the technique makes practical the use of primary islets, a limiting resource, through use of a 384-well format. Fourth, detrimental experimental variability associated with erratic islet culture quality is overcome through optimization of isolation, dispersion, plating and culture parameters. Fifth, the difficulties of accurately and consistently measuring the low basal replication rate of islet endocrine-cells are surmounted with optimized immunostaining parameters, automated data acquisition and data analysis; automation simultaneously enhances throughput and limits experimenter bias. Notable limitations of this assay are the use of dispersed islet cultures which disrupts islet architecture, the use of rodent rather than human islets and the inherent limitations of throughput and cost associated with the use of primary cells. Importantly, the strategy is easily adapted for human islet replication studies. This assay is well suited for investigating the mitogenic effect of substances on β -cells and the molecular mechanisms that regulate β -cell growth.

Keywords

Medicine; Issue 113; Rat islet; isolation; β -cell; diabetes; Ki-67; PDX-1; insulin; regeneration; replication

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Video Link

The video component of this article can be found at <https://www.jove.com/video/54298/>

Disclosures

The authors declare that they have no competing financial interests.

Introduction

Diabetes encompasses a collection of disorders sharing the common end-point of disrupted glucose homeostasis. Although the pathogenic mechanisms of diabetes subtypes are distinct, they share the consequence of decreased β -cell mass, *i.e.*, loss of insulin production capacity^{1,2}. Presently, diabetes treatment strategies rely upon chronic administration of exogenous insulin, pharmacologic stimulation of insulin production or enhancement of insulin sensitivity, and rarely, the transplantation of pancreatic islets or whole pancreas^{3,4}. Regrettably, the success of these strategies is short-lived and/or fails to sufficiently recapitulate the function of endogenous insulin production. Despite the utility of developing a method to stimulate β -cell regeneration, no such approach exists. Consequently, a major diabetes research goal is to develop methods to generate new β -cells or to expand endogenous β -cell mass⁵. Although β -cell regeneration from renewable sources such as embryonic stem cells is advancing, safety and efficiency concerns male the pursuit of alternative strategies, including expansion of mature β -cells, a priority^{6,7}. Importantly, the predominant source of new β -cells *in vivo* is pre-existing β -cells rather than specialized progenitor cells^{8,9}. Although β -cells appear to have limited replication capacity, a small increase in β -cell mass (~ 30%) may be sufficient to restore glucose homeostasis in many diabetics. Furthermore, *in situ* pharmacologic stimulation of β -cell mass is a potentially inexpensive and scalable treatment strategy. Herein a high-content screening method for identifying and characterizing small molecules that stimulate β -cell growth is presented.

A variety of *in vitro* experimental methods may be used to identify gene products and/or molecules that promote primary β -cell replication. Early efforts for measuring β -cell replication induction used fetal rodent pancreata culture or intact Isolated islet cultures to measure [³H] thymidine incorporation, BrdU incorporation or mitotic bodies within the aldehyde-thionine or insulin stained population in response to specific treatment conditions^{10,11}. These *in vitro* approaches and close variants thereof have several limitations. Prominent deficiencies include (1) the use of fetal cells which, unlike mature β -cells, display a high basal β -cell replication rate and are growth regulated in a distinct manner¹²; (2) the subjective nature of experimenter-dependent adjudication of β -cell replication events; (3) the labor and time intensive nature of experimenter-dependent counting of β -cell replication events retards experimental throughput; (4) the use of nuclear incorporation/stain/appearance to identify replication events and a non-overlapping cytoplasmic stain to identify β -cells leads to misattribution of proximate non- β -cell replication events to β -cells.

More recently mature primary β -cells have been used to assess the impact of transgene over-expression as well as gene product or compound treatment on β -cell replication^{13–16}. However, these studies have also relied upon subjective counting of replication events, cytoplasmic staining-or non-specific methods for β -cell identification and/or labor-intensive steps that limit throughput, *e.g.*, individual slide-well plating of cells or intact islet paraffin embedding and processings¹⁷. Notably, an image-based human β -cell replication screening methodology, similar to the one presented herein, has been published¹⁸; however, successful use of this assay has not been demonstrated and the use of human islets for primary screening may not be broadly feasible.

An alternative strategy for identifying replication-promoting substances is to assess growth induction of β -cell lines. Initial efforts used transformed β -cell-lines such as min6 cells or INS 832/13-cells^{14,19–21}. However, these cell-lines demonstrate unrestrained growth and bear little resemblance to well-differentiated β -cells²². Consequently, growth-induction capacity is minimal, of unclear relevance and sometimes difficult to recapitulate. An Improved strategy for cell-line based screening utilizes “reversibly transformed” cells that are growth arrested in the absence of tetracycline (doxycycline)-dependent SV40 T antigen expression^{23,24}. However, it is unclear whether these cells revert to a “normal” β -cell-like state upon doxycycline removal. Unfortunately, use of these cells has yielded generalized growth-promoting compounds that don’t appear to have Immediate utility²⁴. Overall, the use of cell-lines to study growth regulation of a cell-type displaying minimal spontaneous replication activity may have limited applicability.

The β -cell replication screening platform presented herein utilizes mature primary rat β -cells to retain *in vivo* growth regulation to the extent possible, islet-cell cultures of mixed cell-type composition to enable identification of lineage-restricted growth-promoting activities, multi-well formatting to maximize throughput and automated analysis to eliminate bias and facilitate throughput. Successful use of this platform has enabled identification of several compounds that promote β -cell replication^{25,26}. Additionally, the assay has been used for structure-activity relationship studies and chemical epistasis experiments to provide mechanistic insights into the molecular regulation of β -cell replication. The presented platform was successfully adapted for lentiviral RNAi-based investigation of β -cell replication pathways²⁵. Limitations of the assay include restricted scalability (use of primary cells), use of rodent rather than human islet-cells (though the assay may be adapted for human islet studies), expenses associated with antibody-based imaging and primary islet use, the use of dispersed islets (disrupted islet architecture) to facilitate automated image acquisition and dependence upon the availability of an automated microscope with image acquisition and analysis capability. Although a facile *in vivo* screening methodology for identifying gene products or compounds that stimulate β -cell regeneration *in situ* would be Ideal, such a platform is not yet available²⁷. Consequently, the described platform is appropriate for researcher interested in investigating most aspects of β -cell replication.

Protocol

This protocol was carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) of Stanford University School of Medicine. The described protocol is scaled for islet isolation from six 250 – 300 g (8 – 9 weeks old) male Sprague Dawley rats, which is sufficient to generate 228 wells of a 384-well plate for islet-cell replication assessment.

1. Material Preparation

1. Prepare *coating media* prior to initiating islet isolation by collecting the conditioned media of 804G rat bladder carcinoma cells maintained at confluence for 3 days (20 ml of RPMI1640) in a 15 cm tissue culture dish²⁸. Sterile filter and store (–20 °C) the conditioned media for later use.

2. Sterilize surgical tools (one 12 cm toothed tissue forceps, one 11.5 cm fine scissors, one 14.5 cm surgical scissors, two 16 cm curved forceps, one 12 cm curved hemostat, one 12 cm scalpel handle) and one 30 mesh tissue sieve prior to initiating islet isolation.
3. Prepare *pancreatic digestion solution* by dissolving a 60%:40% mixture of purified class I:class II collagenases (total collagenase activity of 300,000 – 400,000 units) in 60 ml of 1× Hanks' Balanced Salt Solution (HBSS) supplemented with calcium and magnesium. Load 10 ml of pancreatic digestion buffer into 10 ml syringes (six) with a 1" 22 G needle and place on ice.

Note: 10 ml of digestion solution is injected per rat. Scale accordingly.
4. Prepare 4.5 ml of anesthetic cocktail in a ventilated hood by mixing 1.3 ml of ketamine (100 mg/ml), 0.2 ml of xylazine (100 mg/ml) and 3 ml of PBS. Prepare the anesthetic cocktail within 1 hr of initiating islet isolation.
5. Prepare *wash buffer* by adding 25 ml of newborn calf serum to 500 ml of HBSS. Place wash buffer on ice for later use.
6. Prepare 1 bottle of *islet medium* which is 500 ml of low glucose Dulbecco's Modified Eagle Medium (DMEM), 50 ml fetal bovine serum (FBS), 5,000 units/ml of penicillin and 5,000 µ/ml of streptomycin.
7. Prepare *islet function medium* from functionality/viability solution (500 ml) with 2% FBS, 2 mM glutamine, 5 mM glucose, 5,000 units/ml of penicillin and 5,000 µg/ml of streptomycin. Store the *islet function media* (4 °C) for later use.
8. Prepare 40 ml blocking buffer by adding 2.5 ml donkey serum and 0.12 ml triton X-100 to 37.38 ml of 1× phosphate buffered saline (PBS). Store blocking buffer (4 °C) for later use.
9. Obtain the required antibodies prior to Initiating the protocol.

Note: PDX-1 (TRITC) and Ki-67 (FITC) co-staining is used for primary β-cell replication screening. For lineage-specific replication analysis, perform immunofluorescence staining for additional cell identity markers (insulin, glucagon, vimentin or somatostatin; TRITC) along with nuclear (DAPI), PDX (Cy5) and Ki-67 (FITC) staining. Alternative replication markers, *e.g.*, PCNA, may also be used.
10. Prepare a 228-well treatment plan with each treatment condition performed in quadruplicate. Include positive- (5-Iodotubercidin [1 µM] or dipyridamole [15 µM]) and vehicle-control (DMSO 1:666 dilution) wells.

2. Perfusion of the Pancreas

1. Anesthetize rats by i.p. Injection of diluted anesthetic cocktail solution (0.30 ml/100 g body weight). Once the rat is fully anesthetized and unresponsive to noxious stimuli, perform cervical dislocation.

2. Place the euthanized rat in supine position (cranial-caudal orientation) on a stack of paper towel and spray the abdominal area with 70% ethanol.
3. Open the abdominal cavity with a U-incision (base at pubic region) and retract skin in the rostral direction over chest to expose the abdominal cavity.
4. Carefully grab the duodenum using two pairs of curved forceps, locate the duodenal entry of the common bile duct (sphincter of Oddi) and clamp the duodenal opening at the papilla with a curved hemostat.
5. Lift the curved hemostat used to clamp the common bile duct papilla and grab the bile duct close to liver using a curved forceps. Use blunt dissection with the curved forceps to isolate and expose the bile duct.
6. Reposition the rat with the head proximal and the feet distal.
7. Cannulate the common bile duct (CBD) at or just distal to the junction of the cystic and common hepatic ducts with a *pancreatic digestion solution*-loaded 10 ml syringe and slowly inject 10 ml of the *pancreatic digestion solution*. While injecting, support the CBD with a scalpel handle. Reposition the needle if the duct and pancreas fail to inflate.
8. Remove the inflated pancreas using the curved forceps and fine scissors to separate it from the descending colon, intestines, stomach and spleen. Place the inflated pancreas on ice in a 50 ml tube containing 5 ml of *pancreatic digestion solution*.

Note: For maximum islet yield, collect all pancreata within 30 min and place only 1 or 2 pancreata in each 50 ml digestion tube.

3. Dissociation of the Pancreas

1. Once all pancreata are collected, place the digestion tubes into a 37 °C water bath for 15 min. Gently swirl the tubes every 3 min.
2. After 15 min, vigorously shake (vertically) the tubes 10 times and add 10 ml of cold *wash buffer*. Vigorously shake the tubes an additional 5 times and place on ice.
3. Fill the digestion tubes to 50 ml with cold *wash buffer* and mix by inverting tubes 5 times.
4. Centrifuge the tubes at $97 \times g$ at 4 °C for 1 min and pour off the supernatant. Be careful not to dislodge the pelleted tissue.
5. Add 25 ml *wash buffer* and re-suspend the tissue by gently vortexing. Repeat steps 3.4 and 3.5 twice more.
6. Place a 30 mesh tissue sieve over a sterile 250 ml beaker and pour the tissue suspension onto the sieve. Rinse the digestion tube with an additional 20 ml of *wash buffer* and pour onto the mesh. The undigested pancreatic and fat tissues are removed at this step.

7. Pour the filtered material into two fresh 50 ml tubes and pellet the tissue by spinning at $97 \times g$ for 1 min at 4 °C. Decant supernatant and invert tubes to drain excess buffer.

4. Purification of Islets

1. Add 20 ml of cold polysucrose/sodium diatrizoate solution (1.119 g/ml density) to the pelleted pancreatic tissue. Homogenously re-suspend the contents of each tube by gently pipetting up and down five times.
2. Gently overlay the polysucrose/sodium diatrizoate solution with 10 ml HBSS. Maintain a sharp liquid interface by slowly adding the HBSS along the tube wall.
3. Centrifuge the digested pancreata at $560 \times g$ for 15 min (4 °C) with slow acceleration and no brake.
4. Collect the islet layer from the interface using a 10 ml pipette and place islets in fresh 50 ml tubes. Do not pool islets at this stage.
5. Add 40 ml of *wash buffer* to each 50 ml tube and spin for 1 min at $140 \times g$ at 4 °C.
6. Pour off the supernatant and re-suspend pooled islets in 50 ml of *wash buffer* by inverting the tubes several times. Centrifuge the tubes for 1 min at $97 \times g$ at 4 °C to collect islets in a pellet.
7. Pour off the *wash buffer* and repeat the wash. Bring islets into the tissue culture hood and aspirate the supernatant.
8. Re-suspend each tube of islets in 6 ml of *islet medium*.
9. Transfer the islets from each 50 ml tube into a well of six-well tissue culture dish. Swirl the 6-well dish to collect islets into the middle of the well and observe islet quality and purity under a microscope.
10. Manually collect islets using a 1 ml pipette and transfer the picked islets into the adjacent well containing 6 ml of islet media. Repeat islet swirling and picking until > 90% purity is achieved.
11. Transfer the purified islets to a 10 cm tissue culture dish containing 20 ml of *islet medium*.
12. Place islets in a tissue culture incubator (37 °C, 5% CO₂) overnight (Figure 1A).
13. In preparation of plating islet cells, coat the wells of a 384-well plate with 40 µl of 804G conditioned media per well. Incubate overnight in a tissue culture incubator.

5. Dispersion and Plating of Islet Cells

1. The following day, gently detach islets from the 10 cm dish with a cell scraper and transfer the islets into a 50 ml tube.

2. Pellet the islets by centrifugation for 1 min at $22 \times g$ at room temperature. Aspirate the supernatant.
3. Wash the islets with 20 ml of warm PBS and decant as above.
4. Re-suspend islets in 0.25 % trypsin (150 μ l per rat pancreas) and incubate at 37 °C for 10 min. Pipette islets up and down 10 times using 1 ml pipette after 5 and 10 min of incubation.
5. After 10 min, evaluate a 20 μ l sample of the trypsinized islets under the microscope to ensure complete digestion and to count the islet cells using a hemocytometer. If undigested islets remain, continue incubation for an additional 3 – 5 min and pipette up and down 10 more times. Repeat as necessary.

Note: The typical yield is ~ 125,000 – 150,000 islet-cells per rat.
6. Remove 804G conditioned media-coated 384-well plate from the incubator and remove media using a 12-well manifold aspirator
7. Suspend islet cells at a density of 45,000 cells per ml in *Islet function medium* and plate 70 μ l (3,150 cells) per well with a multichannel pipette (Figure 1B).

Note: Because β -cells located in the outer wells tend to migrate towards the perimeter of the plate use rows C to N and columns 4 to 21 to plate 228 wells with the islet-cells isolated from 6 rats.
8. Place the plate in the tissue culture incubator for 48 hr to allow cell adhesion.

6. Islet-cell Culture Treatment, Fixation and Staining

1. Prepare 200 μ l of vehicle and compounds (treatment conditions are performed in quadruplicate) at a $2\times$ concentration in *Islet function medium*. Arrange compounds in a sterile 96-well plate to facilitate multi-well pipetting.
2. Carefully remove Islet medium with a 12-well manifold aspirator and replace with Islet function medium (35 μ /well) using a 12-well pipette. Remove and replace media from one row at a time to limit risk of drying (Figure 1C).
3. Initiate treatment by transferring 35 μ l/well of the $2\times$ compound solutions. Return plate to the incubator for the 48 hr treatment duration.
4. After 48 hr of treatment, decant the treatment media by inverting the plate.
5. Gently wash the islet cells with 50 μ l per well of $1\times$ PBS (1 min). Add the PBS using a multi-channel pipette.
6. Decant the PBS and fix the cells by adding 50 μ l per well of cold 4% paraformaldehyde (PFA) diluted in $1\times$ PBS. Incubate cells for 15 min at 4 °C.
7. Wash the cells three times (5 min each) by inverting the plate to remove the PFA and gently adding 60 μ l of PBS per well as above.

8. Prepare 15 ml antigen retrieval solution by mixing 14.25 ml formamide and 0.75 ml 0.15 M sodium citrate (pH 6). Make the antigen retrieval solution immediately before use.
9. Remove PBS from wells by inverting the plate and add 60 μ l antigen retrieval solution to each well. Heat the plate in 70 °C water bath for 45 min. Place a heating block on top of the plate during this incubation to prevent warping of the multi-well plate.

Note: The water should be half-way up the plate skirt; the plate should not be submerged.
10. Remove the plate from water bath and allow it to cool for 20 min at room temperature. Ensure that the plate is not warped by visual inspection.
11. Wash the cells with 60 μ l per well of 1 $\times$ PBS three times (5 min each). Add PBS with a multi-channel pipette and decant PBS by inverting the plate.
12. Use a multi-channel pipette to add 40 μ l per well of blocking buffer and incubate for 30 min at room temperature. Remove the blocking buffer by inverting the plate and decanting.
13. Add 40 μ l of mouse anti-human Ki-67 (1:200) and goat anti-human PDX-1 (1:100) antibodies diluted in blocking buffer to each well and incubate at 4 °C overnight.
14. The next day, decant the anti-body solution and wash the cells with 1 $\times$ PBS three times (5 min each) as described above. Decant the PBS.
15. Add 40 μ l per well of diluted (1:200) biotinylated donkey anti-mouse IgG and rhodamine-conjugated donkey anti-goat IgG in blocking buffer. Incubate for 1 hr at room temperature.
16. Wash the cells with 1 $\times$ PBS three times, 5 min each. Add 40 μ l of diluted (1:400 in blocking buffer) fluorescein-conjugated streptavidin to each well. Incubate 30 min at room temperature.
17. Wash the cells with 1 $\times$ PBS three times, 5 min each. Add 40 μ l per well of DAPI (300 nM in blocking buffer) and incubate for 15 min at room temperature.
18. Wash cells with 1 $\times$ PBS two times and leave cells in 40 μ l 1 $\times$ PBS. The plate is now ready to be analyzed.

7. β -Cell Replication Analysis

Note: An assay protocol must be established for the high content screening microscope used to measure β -cell replication. In its simplest composition, this protocol is a two-color assay where an identity marker is used to define β -cells (PDX-1⁺-cells) and a replication marker (Ki-67) is used to define cell division events.

1. Identify objects (β -cells) based upon the average fluorescence intensity of PDX-1 staining (549 nm filter) within an approximate circle (length:width < 1.6) within the expected pixel area of a β -cell nucleus (Figure 2A).

2. Next, establish settings to identify replication events (Ki-67-positivity) based upon the average fluorescence intensity (485 nm filter) within the PDX-1⁺ area (Figure 2B).

Note: Exposure times must be fixed to allow pixel intensity comparisons across images. Assay protocol parameters are adjusted such that machine calls consistently exclude nonspecific staining, debris and cell aggregates that could adversely affect data quality.

3. Analyze a minimum of 500 β -cells per well to maximize accuracy and limit variability.

Note: The basal β -cell replication index in rat islet cultures is expected to be 0.5 – 3%. Typically, 40 images per well is more than sufficient to identify 500 β -cells.

4. Prior to analyzing the entire plate, analyze selected negative- (DMSO-treated) and positive-control (5-iodotubercidin [1 μ M] or dipyrindamole [15 μ M] -treated wells to assess the validity of the assay protocol. When the assay is established correctly, positive control compounds induce at least a two-fold increase in the percentage of replicating β -cells.

5. Read the entire plate once the assay protocol is validated.

Representative Results

To assess β -cell or α -cell replication, a four-color assay protocol is required. First, objects are identified by DAPI staining (Channel 1, 386 nm). Next, β -cells (event 1) are counted: objects that co-express PDX-1⁺ (channel 2, 650 nm) and peri-nuclear insulin (channel 3, 549 nm). Subsequently, replicating β -cells (event 2) are counted: β -cells (event 1) that co-express Ki-67 (channel 4, 485 nm) (Figure 3). The percentage of replicating β -cells is calculated: (event 2/event 1) $\times$ 100. To quantify α -cell replication, total objects are identified by DAPI staining (channel 1) and then α -cells (event 1) are enumerated by the absence of PDX-1 staining (channel 2) and the presence of perinuclear glucagon staining (channel 3). Replicating α -cells (event 2) are the number of α -cells that co-express Ki-67 (channel 4) (Figure 4). The percentage of replicating α -cells is calculated: (event 2/event 1) $\times$ 100.

Before initiating the experiment, a compound treatment plan is made (Table 1A). Here, a small scale assay was run using negative control (DMSO-treated) and positive control (dipyrindamole-treated [15 μ M]) conditions to assess PDX-1, β -cell and α -cell replication in a 384-well format (49 images per well). Experimental data demonstrate dipyrindamole to selectively promote PDX-1⁺-cell and β -cell replication but not α -cell replication. The average number of identified PDX-1⁺-cells per well was $5,541 \pm 555$ for DMSO-treated and $6,439 \pm 363$ for dipyrindamole-treated conditions ($p < 0.01$); demonstrating a dipyrindamole-dependent increase in PDX-1 cell number (Table 1B, *top*). The average percent PDX-1⁺-cell replication (DMSO⁺PDX1⁺Ki-67⁺/DMSO⁺PDX-1⁺) is $3.35 \pm 0.50\%$ for DMSO-treated and $10.10 \pm 0.98\%$ for dipyrindamole-treated conditions ($p < 0.01$) (Table 1B, *bottom*). Importantly, there are similar PDX-1⁺-cell replication rates in wells subsequently analyzed

for β -cell (rows C–F) and α -cell replication (rows G–J): DMSO= 3.3 ± 0.66 (rows C–F) and 3.4 ± 0.23 (rows G–J) ($p = 0.80$); dipyrindamole= 9.8 ± 0.86 (rows C–F) and 10.4 ± 0.11 (rows G–J) ($p = 0.43$). Hence, these wells, although stained for different cell-lineage markers (insulin and glucagon), responded similarly to drug treatment.

Next the replication rates of β -cells and α -cells were calculated from the raw data (Table 2). The average number of β -cells (event 1) was increased in response to dipyrindamole treatment: DMSO $3,930 \pm 488$ vs. dipyrindamole $4,589 \pm 218$ ($p < 0.05$). Notably, there is a significant reduction in the number of β -cells ($3,930 \pm 488$) compared to PDX-1 cells ($5,541 \pm 555$) ($p < 0.01$). This is a result of excluding PDX-1⁺ -cells that are insulin[−] from the β -cell count, *e.g.*, δ -cells or β -cell progenitor cells, and the additional stringency of requiring β -cells to be insulin⁺. Notably, the number of α -cells (event 1) did not increase with dipyrindamole treatment (DMSO $1,465 \pm 123$ vs. dipyrindamole $1,467 \pm 74.7$; $p = 0.93$). Hence, dipyrindamole selectively increases β -cell number. The number of replicating β -cells (event 2) is increased in response to dipyrindamole treatment (DMSO 131 ± 31 vs. dipyrindamole 476.5 ± 39.6 ; $p < 0.01$) but the number of replicating α -cells (event 2) is not (DMSO 10.25 ± 3 vs. dipyrindamole 9.0 ± 1.6 ; $p = 0.5$). Finally the percentage of replicating β -cells and α -cells is calculated ((event 2/event 1) $\times 100$). Indeed, dipyrindamole increases the percentage of replicating β -cells but not α -cells (summarized in Figure 5). Importantly, the basal β -cell replication rate of a healthy culture varies between experiments (0.5 – 3%) but should be highly consistent across wells within an experiment. Because the basal replication is variable, the compound-induced β -cell replication rate is also variable across experiments; however, the fold-induction of β -cell replication is consistent across experiments and allows compound efficacy to be reliably compared across experiments. Notably, the α -cell replication rate is ~ 5 times lower than the β -cell replication rate and cultures contain $\sim 1/3$ as many α -cells as β -cells; consequently, the α -cell replication index displays increased variability compared to the β -cell replication index. To limit variability, the images per well is increased from 49 (used here) to a maximum of 81.

Discussion

Experimental methods for studying the molecular pathways that control β -cell growth and regeneration are important tools for diabetes researchers. Herein, a rat-islet-based screening platform to identify and characterize small-molecule stimulators of β -cell replication is presented.

While most aspects of this protocol are easily performed by experienced researchers, a few steps require particular technique. First, during islet isolation, cannulation of the bile-duct without disrupting its integrity requires practice. A helpful strategy is to minimally inflate the bile duct to ensure proper positioning of needle before fully dispensing the pancreatic digestion solution. Second, efficient islet isolation from exocrine tissue requires close attention to digestion duration and appropriate agitation. Care should be taken to ensure even heating throughout the digest through intermittent swirling. Third, experimental success depends upon proficient discrimination between islets and exocrine debris during manual islet picking; this skill improves with practice. Fourth, islet dispersion and plating is done such that all islets are dispersed into a mixture of single-cells and small clusters of two or

three cells. Over- and under-digestion of islets prior to plating interfere with experimental performance. Freshly plated islet-cells should be approximately 50% confluent before spreading and allowed to attach over the 48 hr without being disturbed. Figure 1 shows the appearance of successful islet cell cultures at various times after plating. Fifth, media changes for islet-cell cultures are done carefully to avoid disruption of weakly adherent cells. Sixth, antigen retrieval must be done cautiously to avoid warping of the multi-well plate but sufficiently to enable successful Ki-67 staining which is sensitive to both under- and over-exposure to temperature elevation; warped plates cannot be used for automated image acquisition. Note, excessive pancreas digestion, exposure to exocrine tissue debris and/or trypsinization are common causes for islet-cell culture failure.

One limitation of this protocol is the reliance upon specific expression markers for β -cell identity and replication events. Use of single markers has the potential to be misleading. For instance, PDX-1 is expressed by β -cells, a subset of somatostatin-expressing δ -cells and β -cell progenitors^{29,30}; hence, compounds which increase PDX-1-cell replication might be specifically acting on a non- β -cell population. Consequently, confirmatory studies that use alternative β -cell markers are necessary. Similarly, Ki-67 expression is not a perfect marker for replication and additional indicators such as BrdU and phospho-histone 3 should be used³¹. A second limitation of this assay is that compounds which induce β -cell replication may simultaneously increase β -cell apoptosis or de-differentiation. Hence, it is important to evaluate whether a compound induces an absolute increase in β -cell number, and/or induces β -cell apoptosis and whether compound-treated β -cells retain normal function (glucose-stimulated insulin secretion)³². A third limitation of this assay is the modest throughput associated with use of primary islet-cells; islets from six rats generates 228 experimental wells which allows ~ 55 compounds to be screened in quadruplicate (plus positive and negative controls). An additional limitation of the described β -cell replication screening platform is the use of rat islets rather than human islets. Differences between human and rat islets are sufficient to require that all replication-promoting compounds identified using rat islet cultures are confirmed using human islet cultures. However, given the limited availability, and high cost of human islets, primary screening with human islets is impractical for most researchers.

The primary advantages of this protocol are: 1) the use of freshly isolated primary islets to maintain normal growth restraints to the greatest extent possible, 2) the use of a 384-well format to allow moderate throughput screening (typically islets isolated from six rats are sufficient for 228 wells) and 3) the use of automated Image acquisition and analysis to accelerate the pace of experimentation and to limit bias. By contrast, commonly used alternative approaches rely upon manual image acquisition and subjective assessment of β -cell replication events or whole islet incorporation of radioactive thymidine which does not discriminate between cell-lineages, example fibroblast versus β -cell replication^{15,17}. Hence, the presented protocol represents an important and improved tool for investigating the growth-regulation of β -cells.

We envision a variety of adapted uses for this screening platform. First, an absolute increase in β -cell number can be measured by counting the number of PDX-1⁺- or insulin⁺-cells. To account for variability in the number of cells plated per well a higher number of replicates

per condition may be included (typically $n=8$). Second, the platform may be adapted for lentivirus-based overexpression or knock-out/down experiments to identify pathways involved in β -cell replication. In summary, the described experimental technique is broadly useful for identifying compounds and the molecular pathways that regulate β -cell replication.

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Figure 1. Isolated and Dispersed Rat Islets

(**A**) A micrograph of healthy Isolated rat islets; 100 µm scale bar shown. (**B**) Micrographs (5× and 10× objectives) of dispersed rat islets 1 hr after plating; 100 µm (left) and 200 µm (right) scale bars shown. (**C**) Micrographs (5× and 10× objectives) of dispersed rat islets 48 hr after plating; 100 µm (left) and 200 µm (right) scale bars shown. Please click here to view a larger version of this figure.

Figure 2. Automated Identification of PDX-1⁺ and Ki-67⁺ islet Cells

(A) Compound-treated dispersed rat islet cells stained for PDX-1. PDX-1⁺ objects are circled in blue; excluded objects are circled in orange. Scale bar of 150 μm shown. (B) The same field of islet-cells stained for Ki-67. PDX-1⁺ ki-67⁺ double-positive cells are circled in green. Scale bar of 150 μm shown. [Please click here to view a larger version of this figure.](#)

Figure 3. Automated identification of Replicating β -cells

Replicating β -cells are Identified by DAPI (left-upper corner, blue circles), PDX-1 (right-upper corner, green circles), insulin (left-lower corner, magenta circles) and Ki-67 (right-lower corner, red circles) co-expression. The merged image (right) shows several replicating β -cells. Scale bar of 150 μ m shown. Please click [here](#) to view a larger version of this figure.

Figure 4. Automated Identification of Replicating α -cells

Replicating α -cells are identified by DAPI (left-upper corner, blue circles), the absence of PDX-1 (right-upper corner, green circles), glucagon (left-lower corner, magenta circles) and Ki-67 (right-lower corner, red circle) co-expression. The merged image (right) shows a rare replicating doublet of α -cells (yellow arrow). Scale bar of 150 μ m shown. [Please click here to view a larger version of this figure.](#)

Figure 5. Dipyridamole Induces PDX-1⁺ and β-cell Replication but Not α-cell Replication

Graphical representation of PDX-1-cell (top left), β-cell (top right) and α-cell (bottom left) replication in DMSO- and dipyridamole-treated wells are shown. Bars represent the mean of independently treated wells (PDX-1 replication, n= 8; β-cell and α-cell replication, n= 4). Error bars indicate the standard deviation (* p< 0.01). Please click [here](#) to view a larger version of this figure.

Table 1**Representative Experimental Plan and PDX-1⁺-Cell Replication Data. (A)**

An outline of the treatment plan is shown. Wells were treated with DMSO (column 4) or dipyrindamole (column 5). Staining was performed with DAPI, anti-Pdx-1, anti-insulin (normal lettering) or anti-glucagon (italic lettering) and Ki-67 (**B**) Total number of PDX-1⁺ cells (top, DAPI⁺PDX-1⁺) per well and the percentage of replicating PDX-1 cells (bottom, DAPI⁺PDX-1⁺Ki-67⁺/DAPI⁺PDX-1⁺ × 100) per well are shown.

A.	Treatment:		Staining:
	4	5	
C	DMSO	DIPYRIDAMOLE	Insulin, DAPI, PDX-1, Ki-67
D	DMSO	DIPYRIDAMOLE	Insulin, DAPI, PDX-1, Ki-67
E	DMSO	DIPYRIDAMOLE	Insulin, DAPI, PDX-1, Ki-67
F	DMSO	DIPYRIDAMOLE	Insulin, DAPI, PDX-1, Ki-67
G	<i>DMSO</i>	<i>DIPYRIDAMOLE</i>	<i>Glucagon, DAPI, PDX-1, Ki-67</i>
H	<i>DMSO</i>	<i>DIPYRIDAMOLE</i>	<i>Glucagon, DAPI, PDX-1, Ki-67</i>
I	<i>DMSO</i>	<i>DIPYRIDAMOLE</i>	<i>Glucagon, DAPI, PDX-1, Ki-67</i>
J	<i>DMSO</i>	<i>DIPYRIDAMOLE</i>	<i>Glucagon, DAPI, PDX-1, Ki-67</i>
B. Number of PDX-1⁺ Cells			
	DMSO	DIPYRIDAMOLE	
	4	5	
C	5158	6249	
D	6365	6795	
E	4830	6974	
F	5988	6408	
G	<i>5574</i>	<i>6532</i>	
H	<i>5939</i>	<i>6411</i>	
I	<i>5612</i>	<i>6387</i>	
J	<i>4862</i>	<i>5759</i>	
Percent of PDX-1⁺ Ki-67⁺ Cells			
	DMSO	DIPYRIDAMOLE	
	4	5	
C	3.04	9.99	
D	3.91	10.57	
E	2.51	8.58	
F	3.77	10.14	
G	<i>3.44</i>	<i>10.1</i>	
H	<i>3.06</i>	<i>10.69</i>	
I	<i>3.58</i>	<i>9.07</i>	
J	<i>3.52</i>	<i>11.74</i>	

Table 2
Representative β -cell and α -cell Replication Data

The total number of β -cells (Event 1: DAPI⁺PDX-1⁺insulin⁺; top table rows **C–F**, normal lettering)), α -cells (Event 1: DAPI⁺PDX-1⁺glucagon⁺; top table rows **G–H**, italic lettering), replicating β -cells (Event 2: DAPI⁺PDX-1⁺insulin⁺Ki-67⁺; middle table rows **C–F**, normal lettering) and α -cells (Event 2: DAPI⁺PDX-1⁺glucagon⁺Ki-67⁺; middle table rows **G–H**, italic lettering) per well are shown. Additionally, the percentage of replicating β -cells(DAPI⁺PDX-1⁺insulin⁺Ki-67⁺/DAPI⁺PDX-1⁺insulin⁺ $\times$ 100; bottom table rows **C–F**, normal lettering) and α -cells (DAPI⁺PDX-1⁺glucagon⁺Ki-67⁺/DAPI⁺PDX-1⁺glucagon⁺ $\times$ 100; bottom table rows **G–J**, italic lettering) per well are shown.

Event 1:			
	DMSO	DIPYRIDAMOLE	
	4	5	
C	3557	4352	# DAPI(+) PDX-1(+) Insulin(+)
D	4387	4542	# DAPI(+) PDX-1(+) Insulin(+)
E	3462	4879	# DAPI(+) PDX-1(+) Insulin(+)
F	4315	4585	# DAPI(+) PDX-1(+) Insulin(+)
G	<i>1594</i>	<i>1567</i>	# DAPI(+) PDX-1(+) Glucagon(+)
H	<i>1477</i>	<i>1439</i>	# DAPI(+) PDX-1(+) Glucagon(+)
I	<i>1491</i>	<i>1390</i>	# DAPI(+) PDX-1(+) Glucagon(+)
J	<i>1298</i>	<i>1474</i>	# DAPI(+) PDX-1(+) Glucagon(+)
Event 2:			
	DMSO	DIPYRIDAMOLE	
	4	5	
C	113	425	# DAPI(+) PDX-1(+) Insulin(+) Ki-67(+)
D	152	511	# DAPI(+) PDX-1(+) Insulin(+) Ki-67(+)
E	97	466	# DAPI(+) PDX-1(+) Insulin(+) Ki-67(+)
F	163	504	# DAPI(+) PDX-1(+) Insulin(+) Ki-67(+)
G	<i>13</i>	<i>9</i>	# DAPI(+) PDX-1(+) Glucagon(+) Ki-67(+)
H	<i>10</i>	<i>9</i>	# DAPI(+) PDX-1(+) Glucagon(+) Ki-67(+)
I	<i>6</i>	<i>11</i>	# DAPI(+) PDX-1(+) Glucagon(+) Ki-67(+)
J	<i>12</i>	<i>7</i>	# DAPI(+) PDX-1(+) Glucagon(+) Ki-67(+)
% Replication:			
	DMSO	DIPYRIDAMOLE	
	4	5	
C	3.18	9.77	
D	3.47	11.25	
E	2.8	9.55	
F	3.78	10.99	
G	<i>0.82</i>	<i>0.57</i>	

Event 1:			
H	0.68	0.63	
I	0.4	0.79	
J	0.92	0.48	

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