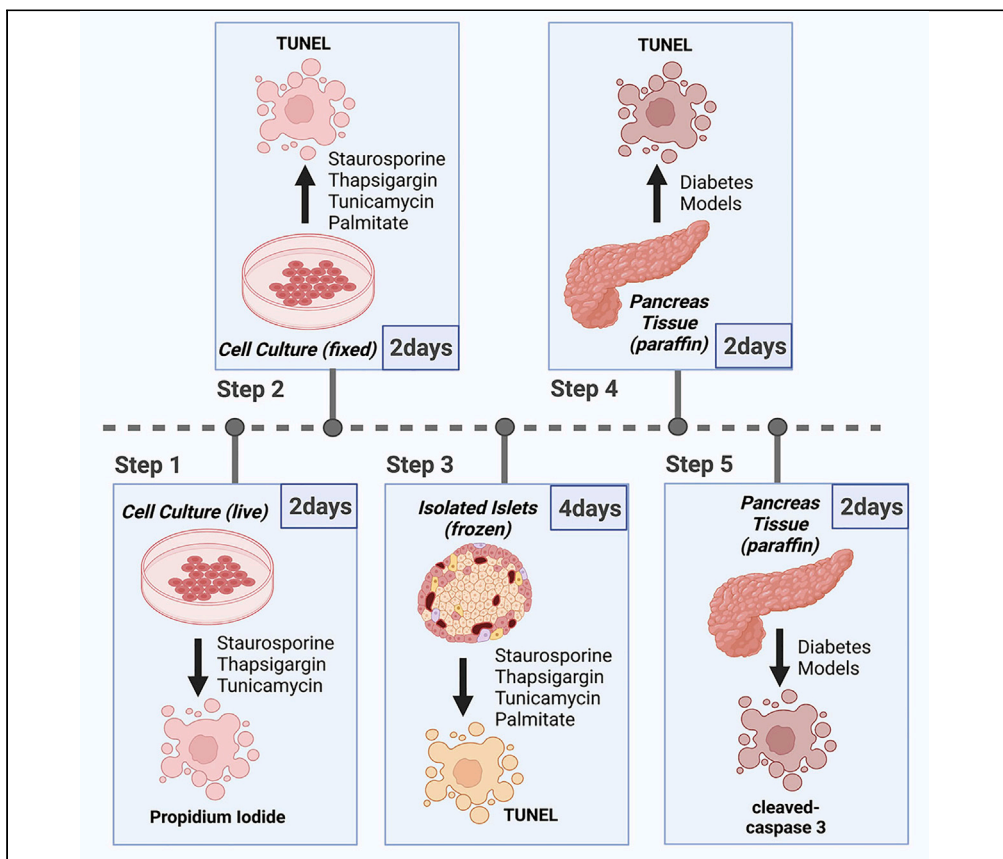


Protocol

Inducing and measuring apoptotic cell death in mouse pancreatic β -cells and in isolated islets



Type 2 diabetes is mediated by insulin resistance and pancreatic β -cell failure, the latter reflecting a combination of β -cell dysfunction, dedifferentiation, and apoptosis. Quantification of β -cell apoptosis in diabetes can be challenging both with respect to methodology and selection of clinically relevant inducers and readouts. This protocol describes approaches to measure cell death in immortalized β -cells, primary mouse islet preparations, and pancreatic tissue. The resulting information may be useful for mechanistic studies and assessment of the contribution of β -cell death to pathogenesis.

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Highlights
Modeling β -cell
death using
physiological and
pharmacological ER
stressors

Measurement of total
cell death in live cells
with propidium
iodide entry

Apoptosis in fixed
samples with TUNEL
and active caspase 3
immunostaining

These approaches
can be adapted to
other tissues and cells

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Protocol

Inducing and measuring apoptotic cell death in mouse pancreatic β -cells and in isolated isletsWendy M. McKimpson^{1,3,4,*} and Richard N. Kitsis²¹Department of Medicine (Endocrinology), Columbia University, New York, NY 10032, USA²Departments of Medicine and Cell Biology, Wilf Family Cardiovascular Research Institute, Einstein-Mount Sinai Diabetes Research Center, and Albert Einstein Cancer Center, Albert Einstein College of Medicine, Bronx, NY 10461, USA³Technical contact⁴Lead contact*Correspondence: wm2347@cumc.columbia.edu
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SUMMARY

Type 2 diabetes is mediated by insulin resistance and pancreatic β -cell failure, the latter reflecting a combination of β -cell dysfunction, dedifferentiation, and apoptosis. Quantification of β -cell apoptosis in diabetes can be challenging both with respect to methodology and selection of clinically relevant inducers and readouts. This protocol describes approaches to measure cell death in immortalized β -cells, primary mouse islet preparations, and pancreatic tissue. The resulting information may be useful for mechanistic studies and assessment of the contribution of β -cell death to pathogenesis.

For complete details on the use and execution of this protocol, please refer to McKimpson et al. (2021).

BEFORE YOU BEGIN

An important driver of type 2 diabetes is the “failure” of pancreatic β -cells, which occurs through β -cell dysfunction, dedifferentiation, and loss (Butler et al., 2003; Muoio and Newgard, 2008; Pick et al., 1998; Talchai et al., 2012). While multiple cell death programs have been described (Galluzzi et al., 2018), β -cell loss during diabetes appears to occur primarily through apoptosis (Johnson and Luciani, 2010). Apoptosis in type 2 diabetes is mediated both through the death receptor and mitochondrial pathways (Liadis et al., 2007; Zhou et al., 2000). Although the precise signaling is incompletely understood, we recently described one relevant mechanism (McKimpson et al., 2021).

The defining event in apoptosis is the activation of caspases, which are a subclass of cysteinyl proteases that cut peptide bonds following aspartic acid residues (Ramirez and Salvesen, 2018). Caspases are synthesized as pro-enzymes called procaspases that undergo activation in response to death signals. The most upstream procaspases are activated by forced proximity and subsequent *trans*-cleavage following recruitment into specific multiprotein complexes (Pop and Salvesen, 2009). Activated upstream caspases subsequently cleave and activate downstream procaspases-3, 6, and -7, which then cut multiple cellular proteins – structural and signaling – to bring about apoptosis.

We will employ two apoptosis assays in this protocol that reflect downstream caspase activation. The first involves immunostaining with an antibody specific for active (cleaved) caspase-3 (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995). This antibody recognizes a neo-epitope on caspase-3 that is revealed following procaspase-3 cleavage. The second assay assesses DNA fragmentation, a downstream consequence of active caspase-3. During apoptosis, DNA is cleaved in the linker regions between nucleosomes by caspase-activated DNase (CAD; also called DFF40) (Enari et al.,



1998; Liu et al., 1999). In healthy cells, CAD is inhibited by inhibitor of caspase-activated DNase (ICAD; also called DFF45). This inhibition is relieved during apoptosis when ICAD is cleaved and inactivated by caspase-3. The resulting DNA fragmentation can be quantified using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) that labels free 3'hydroxyl groups at double stranded DNA breaks (Gavrieli et al., 1992).

We also employ in cell culture studies a third commonly used assay that assesses cellular death based on loss of plasma membrane integrity. In our protocols, this was quantified based on the ability of the cell-impermeable chemical propidium iodide (PI) to gain entry into cells following which it intercalates into the DNA to provide a nuclear fluorescent signal. We use this as a general death readout. Although cells undergoing apoptosis *in vivo* maintain plasma membrane integrity until phagocytosed by macrophages, this clean-up mechanism is not present in cell culture. Thus, apoptotic cells in culture eventually lose plasma membrane integrity allowing their loss of viability to be scored by PI entry.

This protocol describes methods for inducing and measuring cell death in three systems relevant to type 2 diabetes: MIN6 cells (an immortalized mouse β -cell line), isolated mouse pancreatic islets, and mouse pancreatic tissue. Importantly, β -cell apoptosis occurs in response to pathophysiologically similar genetic or pharmacological stimuli in each model system. However, due to differences in the experimental details, distinct, but complementary, measurements of cell death are required. In the first section, model chemical inducers of ER stress and palmitate (which mimics lipotoxicity and induces ER stress) are used to stimulate apoptosis in MIN6 cells. Death is subsequently measured using live/dead staining with PI and TUNEL. The second section adopts the above stimuli for use in primary mouse islets. In the final part, an adapted TUNEL protocol and immunostaining for cleaved caspase-3 is described for pancreatic tissue isolated from mice with diabetes. While our methods are optimized for β -cells, these approaches can be adapted to other mouse and human cell types and tissues.

Institutional permissions

All studies were approved by the Institute for Animal Studies of the Albert Einstein College of Medicine. Readers are reminded that they must acquire prior approval from their institution for experiments on mice and must be performed in accordance with relevant institutional and national guidelines and regulations.

Media preparation for palmitate treatments

⌚ Timing: 18 h

1. Prepare palmitate media.
 - a. Prewarm complete cell or islet culture media (see “materials and equipment” section below for media recipes) to 37°C.
 - b. Prepare two aliquots of 40 mL of culture (cell or islet) media in a 50 mL conical tube. Add 1.6 g BSA (Fisher, cat# BP9704-100) to each tube for a final concentration of 600 μ M BSA in media (this concentration is similar to physiologic levels of BSA in the blood and is necessary to dissolve sodium palmitate). Scale up the amount of media prepared as needed for experiments.
 - c. Allow BSA to completely dissolve in media.
 - d. Set aside one of the two 40 mL aliquot of media to be used as control media during experimental treatments. Palmitate will be dissolved in the second aliquot.

⚠ **CRITICAL:** Make sure to use fatty acid free BSA to dissolve the palmitate.

- e. Measure out the desired amount of sodium palmitate. For a concentration of 1.2 mM palmitate, 13.4 mg Sodium Palmitate is needed for 40 mL media (culture media supplemented with BSA).
- f. Add palmitate to the second aliquot of media supplemented with BSA (prepared above in "d."). The other aliquot will be used for control treatments and/or diluting palmitate media to the desired concentration for experiments.
- g. To protect media from contamination, wrap conical tubes in parafilm and place in a clean plastic Ziploc bag.
- h. Secure tubes horizontally in incubator with shaking capabilities.
- i. To dissolve palmitate, incubate both aliquots of media (control and palmitate) in incubator set to 55°C overnight (~16 h) shaking at ~100 rpm.
- j. The next morning, filter sterilize the media.
- k. Allow media to acclimate to 37°C before using for treatments.

Note: Media prepared above (supplemented with BSA) is used for the experiments employing palmitate to induce cell death.

Isolate pancreatic islets

⌚ Timing: 22 h

Note: Pancreatic Islets should be isolated at least 1 day prior to experiment as it is necessary to let the islets recover overnight (~16 h) before the start of any death treatment.

2. Prepare necessary equipment and buffers for isolation and culture.
 - a. Cool large centrifuge (one that can accommodate 50 mL conical tubes) to 4°C.
 - b. Heat shaking water bath to 37°C.
 - c. Prechill M199 (Gibco, cat# 11150) media and Histopaque (Sigma, cat# 10771-100 mL) to 4°C.
 - d. Supplement a second bottle of M199 media with 10% FBS and prechill to 4°C.
 - e. Make Collagenase buffer (1 mg/mL) by adding 30 mg Collagenase P (Roche, cat# 11249002001) to 30 mL cold M199 Medium (without any FBS). This is enough buffer to isolate islets from approximately 8 mice.

⚠ **CRITICAL:** Collagenase buffer should be made fresh for each islet isolation and kept on ice throughout the procedure.

 - f. If not already done, make islet culture media by supplementing RPMI 1640 media with 10% FBS and prewarm to 37°C.
3. Pancreas perfusion.
 - a. Prepare syringe by bending a 30 gauge 1/2 inch needle to a 45° angle and placing 3 mL collagenase buffer into 10 mL syringe.
 - b. Sacrifice mouse according to mouse protocol and wet fur with 70% EtOH.
 - c. Open the abdomen revealing the internal organs. With the mouse's head facing toward the researcher, flip the liver down, and gently push the intestine towards the right to reveal the connection of the pancreas and intestine. Clamp the bile duct leading to the duodenal intersection (place clamp directly on top of the Peyer's patch) using a Surgical clip (FST cat # 18039-45). If clamp is improperly placed, collagenase buffer will flow into the intestine during perfusion. If this occurs, it is best to secure clamp by adding a second surgical clip on the intestine, NOT by removing and replacing the first clip.
 - d. Insert the needle (with the opening facing upwards) into the bile duct (from the side closest to the liver in the direction of the clamp at the intestine) and clamp the needle with forceps for

stabilization. Please see this recent protocol for a further description and images of this technique (Lee and Engin, 2020).

- e. Slowly inject collagenase buffer (about 3 mL), until the pancreas is fully extended towards the spleen. A correctly placed needle should result in pressure to the syringe during injection. A partial inflation of the pancreas will result in a low yield of islets. In the event of a partial inflation, the uninflated pancreas can be partially inflated by direct injection with an additional 1–2 mL collagenase.
 - f. Remove the pancreas by removing the attached intestines and the spleen and place it in a 50 mL conical tube on ice. Be careful to not let the pancreas sit on ice in collagenase buffer for more than 1 h.
4. Digesting the pancreas.
- a. After all animals are processed, incubate the tube containing the pancreata in 37°C water bath for 17–19 min. Digesting pancreas in a shaking water bath (set to 80 rpm) will increase islet yield. Duration of Collagenase P digestion has to be optimized for each new batch of enzyme.
 - b. At the end of the incubation, add 20 mL of cold M199 w/ 10% FBS to stop the digestion and handshake tube for 10 s to break up the pancreas.

△ CRITICAL: Do remaining steps on ice where possible to prevent insulin dumping from the islets.

- c. Pellet the digested tissues by brief spin at 250 g for 2 min at 4°C.
 - d. After the spin, decant supernatant by pouring off liquid, and wash the pellet by adding 25 mL M119 w/ 10% FBS and hand shake (or vortex) for 30 s.
 - e. Repeat centrifugation and wash (previous two steps) twice (three is also ok) for a total of three washes. After the last wash, spin at 250 g for 2 min at 4°C and decant supernatant.
 - f. Resuspend the pellet with 15 mL M199 w/ 10% FBS.
 - g. Filter suspension through 400 µm wire mesh by putting the mesh on top of a 50 mL conical tube. Rinse the tube and mesh with 5 mL M199 w/ 10% FBS for a final volume of 20 mL. At this point, it is OK to pick islets, but it will be fairly difficult.
5. Isolating islets using Histopaque.
- a. Centrifuge at 250 g for 2 min at 4°C to pellet and completely decant supernatant.

△ CRITICAL: Use a P200 pipet to completely get rid of the remaining media (any leftover media will change the density of the Histopaque).

- b. Add 5 mL of Histopaque-1077 to the pellet and loosen the pellet by shaking. Add another 5 mL of Histopaque to wash the walls of the conical tube (for a total volume of 10 mL) and mix well.
- c. Slowly (switch pipet-aid to low speed and add dropwise to side of 50 mL tube) add 10 mL of M199 (no FBS) on top of the Histopaque, making sure to keep the interphase sharp.
- d. Spin at 1,700 g for 20 min at 4°C. Tubes must be weighed in order to guarantee that the centrifuge is balanced. Note that both the acceleration and deceleration rates must be very slow, not to disturb the interface.
- e. After the spin, collect islets from the interface with a 10 mL pipet. The interface layer will be light pink with visible tissues. Collect this layer and some of the surrounding media for a total of 10 mL.
- f. Place islets in a new 50 mL conical tube and wash by adding 25 mL of M199 w/ FBS and spin at 250 g for 2 min at 4°C.
- g. In order to remove the toxic Histopaque from the cells, repeat the previous step (wash and spin) two more times, for a total of three washes. At this point, it is ok to pick islets.

Isolated wild type mouse islets

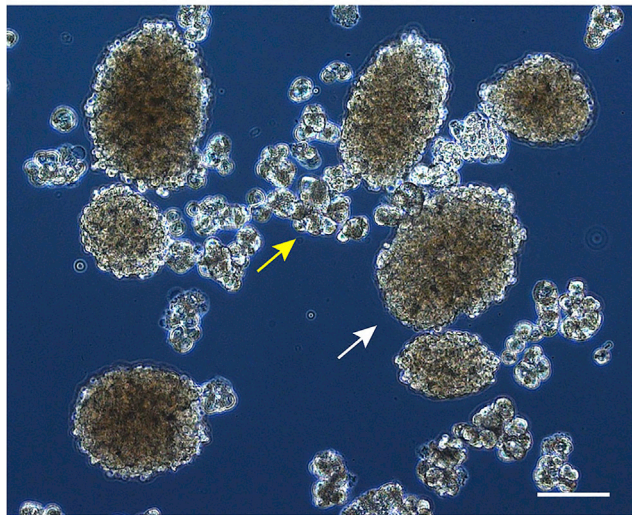


Figure 1. Bright field image of mouse pancreatic islets after isolation procedure
White arrow denotes pancreatic islet. Yellow arrow denotes exocrine cells. Scale bar is 100 μ m.

Optional: Gravity sedimentation can be used to make islet picking easier. This is especially helpful if there are a lot of exocrine cells in the isolation (Figure 1).

6. Further Purification of Islets using Gravity Sedimentation.
 - a. Resuspend pellet in 35 mL M199 w/ FBS and let sit for 4 min on ice. Gently remove the top 20 mL and add new 20 mL new media. Invert the tube to mix the islets and let sit for another 4 min. This may be repeated for a total of 4 cycles (or up to 6 if the preparation is dirty).
 - b. At the end of all the washing, get rid of the top media leaving only 10 mL in the conical tube. Put the top 5 mL in one plate, this will be mainly exocrine. The 5 mL left in the tube will contain small islets, and the bottom pellet should be mainly islets.
 - c. Resuspend islets in islet culture media and place in a sterile petri dish.

△ CRITICAL: It is important to place islets on a non-coated tissue culture plate so that they remain in suspension. Islets should recover overnight (~16 h) in cell culture incubator before the start of any treatments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Insulin (dilute 1:2000)	Dako	cat# A056401-2 (RRID: AB_2617169)
Cleaved caspase-3 (Asp175) (dilute 1:100)	Cell Signaling Technology	cat# 9661, (RRID: AB_2341188)
Alexa Fluor 488 (dilute 1:1000)	Invitrogen	cat# A-11034 (RRID: AB_2576217)
Alexa Fluor 568 (dilute 1:1000)	Invitrogen	cat# A-11075 (RRID: AB_141954)
Chemicals, peptides, and recombinant proteins		
Hoechst 33342	Invitrogen	cat# H3570
Propidium iodide	Sigma-Aldrich	cat# P4170-25MG
Staurosporine	Sigma-Aldrich	cat# S6942-200UL
Tunicamycin	Sigma-Aldrich	cat# T7765-1MG

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Thapsigargin	Fisher Scientific	cat# T7458
Palmitate	Sigma-Aldrich	cat# P9767-5G
BSA (fatty acid-free)	Fisher Scientific	cat# BP9704-100
Collagenase P	Roche	cat# 11249002001
z-VADfmk	R&D Systems	cat# FMK001
AAT	Sigma-Aldrich and purified in laboratory	cat# SRP6312-1MG
DNAse I	Roche	cat# 4716728001
Proteinase K	Millipore	cat #21627
16% Paraformaldehyde solution	Fisher Scientific (Electron Microscopy Sciences)	cat# 50-980-487 (EMS# 15710)
Antigen Unmasking Solution	Vector Laboratories	cat# H-3300
VECTASHIELD Vibrance Antifade Mounting Medium with DAPI	Vector Laboratories	cat# H-1800-10
Critical commercial assays		
Fluorescein In Situ Cell Death Detection Kit	Roche Applied Science	cat# 11684795910
Experimental models: Cell lines		
MIN6 cells (mouse pancreatic β -cell line derived from transgenic mouse expressing simian virus 40 T antigen)	Provided from Peter Arvan at the University of Michigan	N/A
Experimental models: Organisms/strains		
<i>ob/ob</i> mice (male <i>ob/ob</i> mice > 16 weeks provide a more consistent model of type 2 diabetes). <i>ob/+</i> mice (on a C57BL/6J background) purchased from Jackson Labs were expanded by crossing with wild type (C57BL/6J) mice from Jackson Laboratory. <i>ob/ob</i> mice for experiments were generated through <i>ob/+</i> \times <i>ob/+</i> breedings. Note: <i>ob/ob</i> mice are subfertile.	The Jackson Laboratory	cat# 000632 (RRID: IMSR_JAX:000632)
Wild type (C57BL/6J) mice (mice of either gender, age 10–12 weeks should be used for isolated islet experiments). Wild type mice for experiments were generated through <i>ob/+</i> \times <i>ob/+</i> breedings.	The Jackson Laboratory	cat# 000664 (RRID: IMSR_JAX:000664)
Software and algorithms		
GraphPad Prism 6	GraphPad Software	https://www.graphpad.com/
ImageJ	National Institutes of Health	https://imagej.nih.gov/

MATERIALS AND EQUIPMENT

Culture media for MIN6 cells

Reagent	Final concentration	Amount
DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning, cat# 10-013-CV)	n/a (entire bottle)	500 mL
Fetal bovine serum (Gibco, cat# 16000-044)	10%	50 mL
Penicillin streptomycin solution (Gibco, cat# 15140-122)	1%	5 mL
β -mercaptoethanol (Sigma-Aldrich, cat# M7522)	140 μ mol/L	5.4 μ L
Total	n/a	~555 mL

Culture media for primary islets

Reagent	Final concentration	Amount
RPMI 1640 media (Gibco, cat# 11875-093)	n/a (entire bottle)	500 mL
Fetal bovine serum (Gibco, cat# 16000-044)	10%	50 mL
Penicillin streptomycin solution (Gibco, cat# 15140-122)	1%	5 mL
Total	n/a	555 mL

Note: Culture media prepared above is used for the experiments employing staurosporine, thapsigargin, or tunicamycin to induce cell death.

1 × PBS-T (0.01% triton X-100 in 1 × PBS)

- 50 µL triton X-100.
- 500 mL 1 × PBS.

Cell permeabilization solution

- 100 µL triton X-100.
- 100 mL 1 × PBS.

Note: Triton X-100 is viscous and will require a wide-mouthed tip to pipette this solution. It will also take several minutes for the triton X-100 to fully dissolve.

4% paraformaldehyde (PFA)

- One aliquot (10 mL) of Electron Microscopy Sciences 16% PFA solution (cat# 15710).
- 30 mL 1 × PBS.

Note: Fresh PFA should be prepared for every experiment.

⚠ **CRITICAL:** PFA is toxic and should be prepared in a chemical hood.

STEP-BY-STEP METHOD DETAILS

Measuring death in MIN6 cells with live cell imaging

⌚ Timing: 2 days, 22 h

This step describes how to trigger apoptotic cell death in MIN6 cells using staurosporine, thapsigargin and tunicamycin. Cell death is measured using live cell imaging with propidium iodide (PI). In this context, cells become positive for PI when their plasma membrane becomes permeable, indicating that a death program is activated in these cells.

1. Cell preparation and treatment:
 - a. Plate MIN6 cells at a density of 5×10^4 cells/cm². For a 6-well tissue culture dish this is roughly equal to 500,000 cells / well. To adapt this protocol for other cells, this number will need to be optimized.
 - b. Allow cells to recover overnight (~16 h).
 - c. Begin death treatment in the late afternoon of the following day (Figure 2). Recommended death inducers and concentrations for β -cells include: the general inducer of cell death staurosporine (1 μ M; (McKimpson et al., 2013)) and the ER stress inducers thapsigargin (1 μ M) and tunicamycin (5 ng/mL). Although shorter treatments can be performed, we recommend overnight (16 h) treatments at these concentrations.

Note: If pretreatments are to be included as conditions in the experiment, begin pretreatment 2 h prior to adding death stimuli. Examples of pretreatments that would inhibit β -cell death include pretreatment with the pan-caspase inhibitor z-VADfmk (200 μ M) or the serine protease inhibitor AAT (0.5 mg/mL).

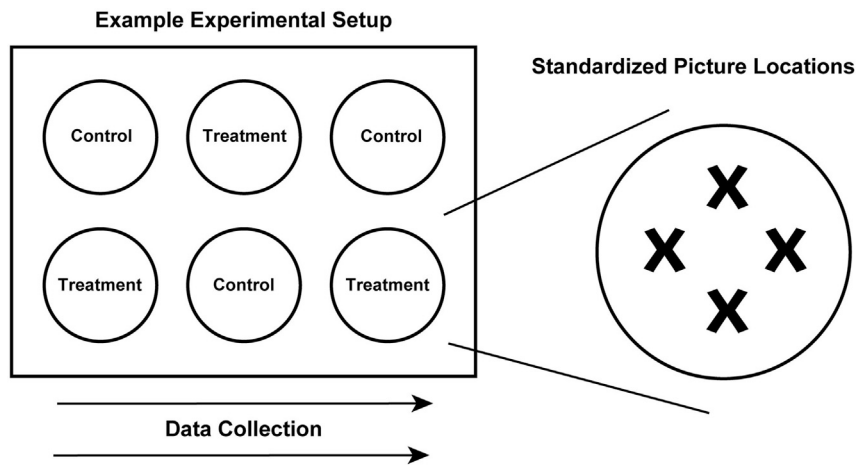


Figure 2. Suggested experimental setup and imaging locations

Left: To obtain the most reproducible results across multiple experiments, we recommend a setup in which individual control and experimental replicates are alternated on a plate. This permits simultaneous imaging of both control and experimental samples so as not to skew experimental results due to length of experimental treatment or time out of cell culture incubator. Right: For imaging technical replicates in the same well, we suggest taking pictures in standardized locations that avoid both the center and outside edges of the well.

2. Staining and imaging cells:

- a. When ready to perform live/dead imaging, add propidium iodide (PI) (dilute stock (1 mg/mL in H₂O) 1:1000) and Hoechst 33342 (dilute stock 1:1000) directly to media. Gently swirl plate to mix.

△ **CRITICAL:** Do not wash cells between the end of treatment and performing live/dead imaging. This could skew final results as cells undergoing apoptosis may only be loosely attached to the cell culture plate.

△ **CRITICAL:** Samples are light-sensitive after adding PI and Hoechst 33342.

- b. Incubate cells for 5 min in the dark at 37°C.
- c. Image cells as described in [Figure 2](#). Since the amount of death can vary based on the position in the well, we recommend imaging a minimum of 4 fields/well and averaging these values to minimize technical variation. Control wells should have few cells that stain positive for propidium iodide, whereas there should be a significant increase in PI+ cells in wells containing cells treated with a death inducer ([Figure 3](#)). Quantify dead cells by counting PI+, Hoechst+ cells and PI-, Hoechst+ cells. It is common for strongly PI+ cells to have weak Hoechst+ staining. Data can be presented as percentage PI+ cells / total (PI+, Hoechst+ and PI-, Hoechst+) cells. For sample quantification, please see Figures 2D and 2E of ([McKimpson et al., 2021](#)). [Troubleshooting 1](#) and [3](#).

△ **CRITICAL:** Cells must be imaged immediately after incubation as long-term exposure of Hoechst and PI is toxic to cells.

Note: Propidium iodide does not detect cell death optimally in response to palmitate. We suggest using TUNEL as an alternative.

Note: Propidium iodide will also label cells undergoing necrotic cell death. Since β-cells mainly undergo apoptosis, this is not a confounding factor here. However, if this is a concern, PI can be omitted, and condensed nuclei measured using Hoeschst staining alone.

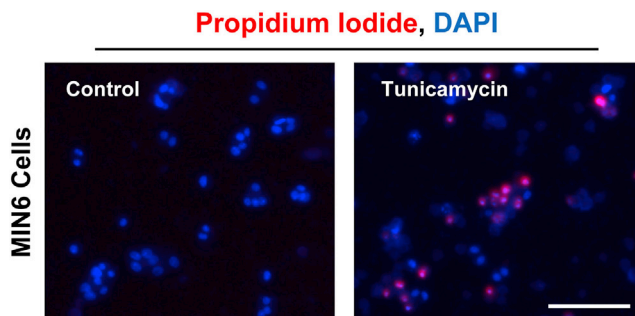


Figure 3. Live/dead imaging in MIN6 cells

To induce death, cells were treated for 16 h with 5 ng/mL Tunicamycin. Propidium Iodide demarcates cells that no longer have an intact plasma membrane and DAPI counterstains nuclei. Scale bar is 50 μ m.

TUNEL in MIN6 cells

⌚ Timing: 4 days, 22 h

This step describes how to measure cell death in fixed MIN6 cells by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). This method is a second way to measure activation of cell death machinery and measures DNA fragmentation. In addition, this method of detection can be used to measure death after treatment with palmitate.

3. Cell preparation.
 - a. Culture cells directly onto 6-well tissue culture plates (Fisher, cat# FB012927), without further coating of the plates or use of a coverslip. Begin with cells > 70% confluent as some cells will be lost during staining. Cells will be especially easy to detach after death treatment.
 - b. Begin death treatment as described above in step 1c of the Step-by-step method details.
4. Prepare staining reagents.
 - a. Prepare 4% paraformaldehyde (PFA) and cell permeabilization solution (see “Materials and equipment” section for recipes).
 - b. Using the Fluorescein In Situ Cell Death Detection Kit (Roche), make TUNEL reaction mixture as recommend by [manufacturer](#).
 - i. Specifically, remove 100 μ L of label solution (reaction mix) and place in separate tube. This will be used as a label-only negative control.
 - ii. Perform a quick spin with tube containing enzyme solution to get all the liquid contents to the bottom of the tube.
 - iii. Subsequently, remove 50 μ L of enzyme solution (entire contents) from its tube and add this to the remaining 400 μ L of label solution (entire contents) in the original label solution tube.

⚠ CRITICAL: TUNEL reaction mixture is light-sensitive and should be kept on ice in the dark.

5. Cell fixation and TUNEL staining.
 - a. After completion of cell treatment, carefully wash cells three times with 1 \times PBS.
 - b. Fix cells with 4% PFA for 10 min at 20°C.

Note: This step and the following wash/permeabilization should be performed in a chemical hood as PFA is toxic.

Note: PFA should be at 20°C when placed on cells.

- c. Wash cells with 1 \times PBS.

- d. Permeabilize cells with cell permeabilization solution for 5 min at 20°C.
- e. Add previously prepared TUNEL solution (label + enzyme) to sample making sure that the entire sample is covered with solution. If using a 6-well plate, add approximately 100 μ L the center of each well.
- f. Add label solution alone to at least 1 sample to include as a negative control.
- g. Treat at least one sample with DNase for the positive control.
- h. Incubate cells for 1 h in the dark at 37°C. To prevent drying, make sure that the samples are covered and/or in a hydrophobic chamber.

⚠ CRITICAL: Samples are light-sensitive after adding TUNEL solution.

6. Prepare cells for imaging and counterstain with DAPI.
 - a. Wash cells with 1 \times PBS for 5 min \times 2.
 - b. Wash cells with 1 \times PBS-T for 5 min, 1 time.
 - c. Coverslip samples using mounting media with DAPI (VECTASHIELD Vibrance Antifade Mounting Medium with DAPI, cat# H-1800-10) and glass coverslips (Corning, cat# 284518). For this, in the center of each well of the 6-well culture plate, first place a drop of mounting media directly on top of the fixed cells. Then, place a glass coverslip in the center of each well on top of the mounting media, being careful to avoid creating bubbles.
7. Follow the methods described above (Figure 2) to image and quantify samples for TUNEL staining. For sample quantification, please see Figure 2F of McKimpson et al. Dev Cell 2021. [Troubleshooting 1](#) and [7](#).

⏸ Pause point: If placed in the dark at 4°C, cells can be successfully imaged for up to 1 week before fluorescence signal fades. We recommend imaging 1 day after staining.

TUNEL in isolated pancreatic islets

⌚ Timing: 8 days, 22 h

This step describes how to adapt the TUNEL protocol described above for primary mouse islets. This includes treatment of islets with a death stimulus, fixing and embedding tissue for frozen sectioning, TUNEL staining of islet sections, and subsequent imaging.

8. Prepare and treat islets.
 - a. Hand pick islets using a P20 pipet with a P10 (barrier) tip (Figure 1). It is important to select for only the most pure, best looking islets. They are 50–250 μ m in diameter, but the islets \sim 150 μ m are the best to work with. The average islet yield from a 3–5 month old C57Bl/6 is about 100–200 islets/mouse.
 - b. Place 150–200 islets / well in a 12-well non-tissue culture treated plate.

Note: Islets will adhere to the bottom of plate if placed in a tissue culture treated dish.

- c. Resuspend islets in 1 mL culture media (or control/treatment media if performing palmitate treatment).
- d. Treat islets with death stimulus. Recommended death inducers and concentrations for isolated islets include the ER stressor thapsigargin (1 μ M; Fisher Scientific) for 24 h and 1.2 mM Palmitate (preparation described above) for 3 days.
9. Prepare islets for frozen sectioning.
 - a. Prepare 4% PFA (see [materials and equipment](#) section).
 - b. Upon completion of treatment, use a P1000 pipet to collect the contents of each well (islets/ media) in a separate 1.5 mL Eppendorf tube.
 - c. Centrifuge for 3 min at 100 g at 20°C to collect the islets at the bottom of the tube.

- d. Carefully discard supernatant and wash islets with 1 mL 1 × PBS.
- e. Fix islets by resuspending in 300 μL 4% PFA and rotate tubes for 10 min at 4°C.
- f. In chemical hood, add 1 mL 1 × PBS to each tube.
- g. Centrifuge tubes for 3 min at 100 g.
- h. In chemical hood, discard supernatant in PFA waste container.
- i. Wash islets with 1 mL 1 × PBS.
- j. Centrifuge for 3 min at 100 g at 20°C to collect the islets at the bottom of the tube.
- k. Carefully discard supernatant, leaving only ~50 μL of PBS in the tube.
- l. Using a P200 pipet, resuspend islets and transfer to the center of a cryomold.
- m. Wait for islets to sink to the bottom of the mold.
- n. Flash freeze on dry ice.
- o. While cryomold (with frozen islets) is placed on dry ice, slowly and sequentially add OCT to the mold, beginning on the outer edges, working in towards the islet.
- p. Once the mold is fully filled with OCT, allow OCT to freeze completely.
- q. Wrap block in foil and place in –80°C freezer.

▮▮ **Pause point:** As long as the block is protected, block may be placed in –80°C freezer prior to sectioning.

10. Using cryostat, prepare 5 μm tissue sections.

Note: Islets should be located very near the bottom of the block. Use a microscope while trimming frozen block to avoid losing the sample.

▮▮ **Pause point:** Completed slides can be placed at –80°C until ready to perform staining.

11. Perform TUNEL staining.

- a. Prepare TUNEL reagents (see step #4).
- b. Allow slides to acclimate to 20°C (from –80°C) for 10 min.
- c. Place slides in slide holder and submerge in 1 × PBS.
- d. Wash in 1 × PBS on shaker for 3 min.
- e. Place in new 1 × PBS and perform two more washes on shaker for 3 min each.
- f. Add previously prepared TUNEL solution (label + enzyme) to slide, by adding dropwise to each tissue section. For slides ~75 μL is needed for each slide.
 - i. Add label solution alone (no TdT) to at least 1 sample to include as a negative control in the experiment.
 - ii. Slides can be treated with DNaseI for positive control.
- g. To distribute TUNEL solution evenly, cover each slide with a rectangular coverslip (Corning, cat# 2975245).
- h. Place slides in hydrophobic chamber and incubate for 1 h in the dark at 37°C.

△ **CRITICAL:** Samples are light-sensitive after adding TUNEL solution.

- i. Gently remove coverslip by place slide vertical in container filled with 1 × PBS. Coverslip will come off by itself as the slide is pulled up. Allow gravity to remove coverslip. If the coverslip does not automatically slide off, let the slide sit in the 1 × PBS for 30 s and then attempt to remove the coverslip a second time. To aid in removal of the coverslip, it is ok to gently move the slide up and down in 1 × PBS.

△ **CRITICAL:** Do not force coverslips off by using hands to grab them as this may damage the tissue.

- j. Coverslipping with DAPI.

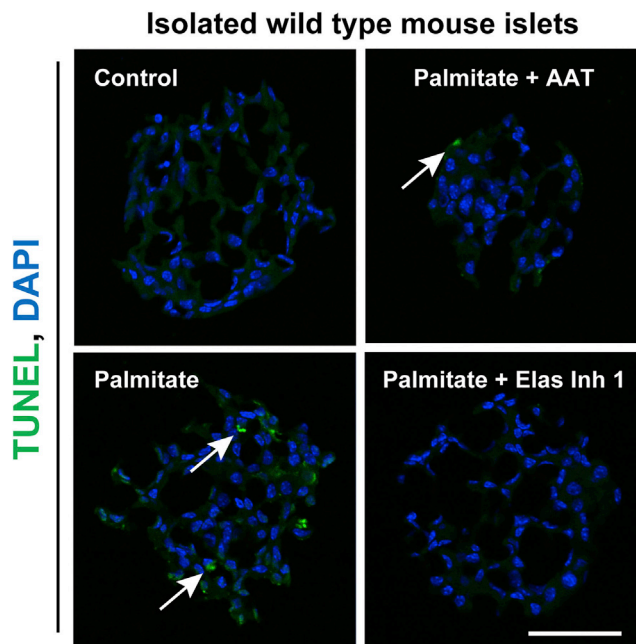


Figure 4. TUNEL staining in isolated islets

Isolated islets were treated 3d with 1.2 mM palmitate. To inhibit cell death, islets were pretreated with 0.5 mg/mL purified human AAT or 0.2 mg/mL elastase inhibitor (Elas Inh 1) 2 h prior to initiation of death stimulus. After completion of death treatment, islets were fixed, embedded in OCT, and sectioned. White arrow denotes TUNEL+ cell. DAPI counterstains nuclei. Scale bar is 50 μ m. For quantification of this experiment, please see Figure 5D of (McKimpson et al., 2021).

- i. Wash slides with 1 \times PBS for 5 min \times 2.
- ii. Wash slides with 1 \times PBS-T for 5 min, 1 time.
- iii. Coverslip slides with mounting media with DAPI (VECTASHIELD Vibrance Antifade Mounting Medium with DAPI, cat# H-1800-10) and rectangular coverslips.
- k. Image samples. An example experiment is shown below (Figure 4). For sample quantification please see Figure 5D of McKimpson et al. Dev Cell 2021. [Troubleshooting 1, 4, and 7.](#)

Measuring cell death in mouse pancreatic tissue with TUNEL

⌚ Timing: 10 days, 22 h

This step describes how to adapt the TUNEL protocol for pancreatic tissue prepared for paraffin sectioning. To adapt this protocol to frozen tissue sections, omit the deparaffinization, antigen retrieval, and proteinase K treatment steps. This protocol also includes a coimmunostain for insulin, a β -cell marker. Note that if staining with another cell marker is desired, it may be necessary to switch the order of immunostaining and TUNEL staining as some antibody staining may not be able to withstand the proteinase K treatment step.

12. Prepare pancreatic tissue from mice.
 - a. Dissect entire pancreas from desired mouse model using the spleen to grab the tissue so that the pancreas is not damaged.
 - b. Thoroughly wash pancreas with ice-cold 1 \times PBS.
 - c. To fix tissue, in chemical hood, place pancreas in 50 mL tube containing 5 mL 4% PFA.
 - d. Place tube containing tissue at 4°C for 16 h.
 - e. Discard PFA in properly marked waste container.

- f. Wash tissue with cold 1 × PBS.
- g. Discard 1 × PBS (containing some remnant PFA) in properly marked waste container.
- h. Dehydrate tissue by placing pancreatic tissue in 30% EtOH for 5 min. Discard liquid and re-submerge tissue with 5 mL 50% EtOH for 5 min. Discard liquid and resubmerge tissue with 5 mL 70% EtOH. Place tissue in paraffin cassette labeled with pencil and resubmerge in 70% EtOH.
- i. Process tissue for paraffin using program specific for pancreatic tissue.
- j. Embed pancreas in paraffin.
- k. Using microtome, prepare 5 μm tissue sections.

▮▮ Pause point: Completed slides can be placed at 20°C until ready to perform staining.

13. Prepare tissue sections for staining.
 - a. Bake slides at 65°C until paraffin has melted off (approximately 1–2 h).
 - b. Cool slides at 20°C for 1 h.
 - c. Heat water bath to 90°C.
 - d. Prepare antigen retrieval solution by diluting 2.5 mL Antigen unmasking solution (Vector Laboratories, cat# H-3300) in 250 mL of dH₂O (1:100 dilution).
 - e. Preheat antigen retrieval solution to 90°C in water bath.
 - f. Deparaffinize tissue sections by placing in Xylene (5 min × 3 times), 100% EtOH (1 min × 2), 95% EtOH (1 min × 2), 70% ETOH (1 min × 2) and then in 1 × PBS (1 min × 2).
 - g. Submerge slides in antigen retrieval solution at 90°C for 10 min.
 - h. Remove slide container from water bath and place entire container in ice to cool for 10 min.
 - i. Wash slides with 1 × PBS for 5 min.
 - j. Wash slides with 1 × PBS-T for 5 min.
14. Proteinase K pretreatment.
 - a. Prepare Enzyme Solution (Proteinase K from Millipore, cat# 21627, 200 μg/mL) by diluting 100 μL of proteinase K in 900 μL of DNase/RNase free distilled water (1:10 dilution).
 - b. Lay slides horizontal and incubate with enzyme solution for 15 min at 20°C.
 - c. Place slides in slide holder and submerge in 1 × PBS.
 - d. Wash in 1 × PBS on shaker for 3 min.
15. Perform TUNEL staining.
 - a. Prepare TUNEL reagents (see step #4).
 - b. Place in new 1 × PBS and perform two more washes on shaker for 3 min each.
 - c. Add previously prepared TUNEL solution (label + enzyme) to slide, by adding dropwise to each tissue section. For slides ~75 μL is needed for each slide.
 - i. Add label solution alone (no TdT) to at least 1 sample to include as a negative control in the experiment.
 - ii. Slides can be treated with DNaseI for positive control.
 - d. To distribute TUNEL solution evenly, cover each slide with a coverslip.
 - e. Place slides in hydrophobic chamber and incubate for 1 h in the dark at 37°C.

△ CRITICAL: Samples are light-sensitive after adding TUNEL solution.

- f. Gently remove coverslip (see step 11i).
16. Immunostaining for insulin.
 - a. Wash slides with 1 × PBS for 5 min × 2.
 - b. Wash slides with 1 × PBS-T for 5 min, 1 time.
 - c. Block slides for 1 h at 20°C in hydrophobic chamber with 10% goat serum diluted in 1 × PBS-T. Make sure to cover tray to avoid tissue from drying out.
 - d. Incubate with primary antibody (Insulin antibody made in Guinea Pig, Dako, cat# A056401-2, diluted 1:2000) for 16 h at 4°C. Primary antibody is diluted in blocking solution.
 - e. Wash slides with 1 × PBS for 5 min × 2.

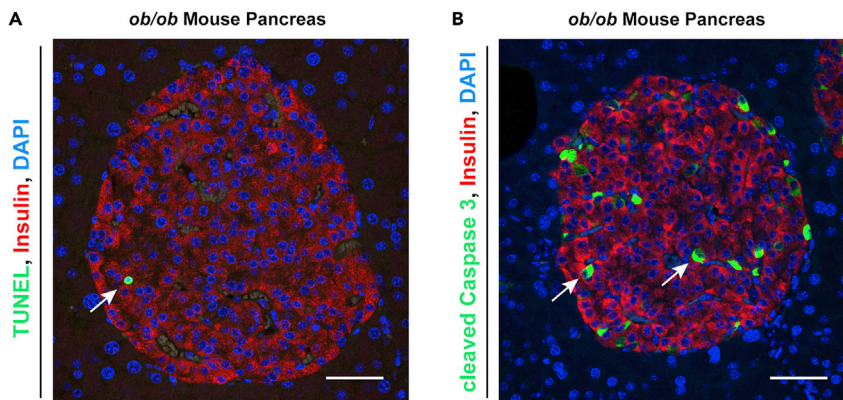


Figure 5. Detecting apoptosis in pancreatic tissue

(A and B) TUNEL (panel A) and cleaved caspase-3 (panel B) staining in mouse pancreatic tissue. Representative islets are shown from 23 week old *ob/ob* mice. β -cells are demarcated by an insulin costain. White arrow denotes TUNEL+ or cleaved caspase 3+ cell. DAPI counterstains nuclei. Scale bar is 50 μ m. For representative quantification of these experiments, please see Figure 5G and 5H of (McKimpson et al., 2021).

- f. Wash slides with 1 \times PBS-T for 5 min \times 1.
 - g. Incubate each slide with secondary antibody (1:1000, diluted in blocking serum, recommended: Goat anti-Guinea Pig 568, Invitrogen, cat# A-11075) for 1 h at 20°C.
 - h. Wash slides with 1 \times PBS for 5 min \times 2.
 - i. Wash slides with 1 \times PBS-T for 5 min \times 1.
 - j. Coverslip with mounting media with DAPI (VECTASHIELD Vibrance Antifade Mounting Medium with DAPI, cat# H-1800-10).
17. Image samples. An example of TUNEL staining in pancreatic tissue is shown below (Figure 5A). For sample quantification, please see Figure 5H of (McKimpson et al., 2021). [Troubleshooting 2](#) and [5](#).

Measuring cell death with cleaved caspase-3 immunostaining

⌚ Timing: 12 days, 22 h

This step describes how to detect activation of β -cell death in paraffin pancreatic tissue sections by coimmunostaining for active caspase-3 and insulin (to demarcate β -cells). Caspase-3 is activated by cleavage during apoptosis. Accordingly, this measurement is meant to compliment TUNEL staining as a second indicator that a death program has been activated in a cell. This protocol can be adapted to frozen tissue sections by omitting the deparaffinization and antigen retrieval steps.

18. Prepare pancreatic tissue from mice as described in step #12.
19. Prepare tissue sections for staining as described in step #13.
20. Immunostaining for cleaved caspase-3.
 - a. Block slides for 1 h at 20°C in hydrophobic chamber with 10% goat serum diluted in 1 \times PBS-T. Make sure to cover tray to avoid tissue from drying out.
 - b. Incubate with primary antibody (cleaved caspase-3 antibody made in rabbit, Cell Signaling, cat# 9661, diluted 1:100) for 16 h at 4°C. Primary antibody is diluted in blocking solution.
 - c. Wash slides with 1 \times PBS for 5 min \times 2 and 1 \times PBS-T for 5 min \times 1.
 - d. Incubate each slide with secondary antibody (1:1000, diluted in blocking serum, recommended: Goat anti-Rabbit 488, Invitrogen, cat# A-11034) for 1 h at 20°C.
 - e. Wash slides with 1 \times PBS for 5 min \times 2 and 1 \times PBS-T for 5 min \times 1.

- f. Incubate slides with insulin primary antibody (Insulin antibody made in Guinea Pig, Dako, cat# A056401-2, dilute 1:2000) for 2 h at 20°C. Primary antibody is diluted in blocking solution.
 - g. Wash slides with 1 × PBS for 5 min × 2 and 1 × PBS-T for 5 min × 1.
 - h. Incubate each slide with secondary antibody (1:1000, diluted in blocking serum, recommended: Goat anti-Guinea Pig 568, Invitrogen, cat# A-11075) for 1 h at 20°C.
 - i. Wash slides with 1 × PBS for 5 min × 2 and 1 × PBS-T for 5 min × 1.
 - j. Coverslip with mounting media with DAPI (VECTASHIELD Vibrance Antifade Mounting Medium with DAPI, cat# H-1800-10).
21. Image samples. An example of cleaved caspase-3 staining is shown above in [Figure 5B](#). For sample quantification, please see [Figure 5G](#) of ([McKimpson et al., 2021](#)). [Troubleshooting 2](#) and [6](#).

EXPECTED OUTCOMES

Examples of expected absolute values are referenced from ([McKimpson et al., 2021](#)).

In cell culture, rates of cell death obtained will vary depending on cell type and the concentration and duration of treatment. Chemical death inducers often trigger more cell death than physiological stimuli. For the recommended cell death stimuli thapsigargin and tunicamycin at the conditions indicated in MIN6 cells, one can expect ~20%–30% cell death when measured using propidium iodide (see bar 4 in [Figures 2D and 2E](#) in ([McKimpson et al., 2021](#))). Under similar conditions, rates of TUNEL+ cells in cultured cells should be similar to the number of propidium iodide+ cells (bar 4 in [Figure 2F](#) in ([McKimpson et al., 2021](#))). On other hand, inducing death with the physiologic stimuli palmitate will trigger ~10%–15% TUNEL+/total cells (see bar 4 in [Figure 2H](#) in ([McKimpson et al., 2021](#))). In all of the above instances, the basal rates of cell death in a cell line should be minimal (i.e., less than 5%). Absolute numbers of cell death may vary experiment to experiment, so it is important to perform statistics between untreated and treated control for each experiment to first confirm that death is triggered as expected.

It is common for there to be more variation in the amount of cell death measured in isolated islets compared to cultured cells. In addition, diabetic islets will likely be more susceptible to cell death after stimulus compared to wild type islets. For example, expected rates of TUNEL+ cells in islets treated with 1.2 mM palmitate for 3 days are ~10%–15% for wild type mice (bar 2 of [Figure 5D](#) in ([McKimpson et al., 2021](#))) and ~20%–25% for leptin deficient *ob/ob* (bar 2 in [Figures 5E and 7E](#) in ([McKimpson et al., 2021](#))) mice. Pancreatic islets isolated from diabetic mice will also have a higher baseline of cell death (see bar 1 of [Figures 5E and 7E](#) (*ob/ob* islets); compared to bar 1 of [Figure 5D](#) (wild type islets) in ([McKimpson et al., 2021](#))).

The rate of apoptotic cell death varies significantly by tissue. For example, some tissues, including the pancreas, have low basal levels of cell death, although these numbers do increase with stress stimuli, as is the case with diabetes. For example, when β -cell death is measured using TUNEL in the pancreas of *ob/ob* mice, one can expect ~0.5%–2.5% of β -cells undergoing apoptosis (see bar 1 of [Figure 5H](#) in ([McKimpson et al., 2021](#))). In addition, caspase-3 activation is a more sensitive detector of cell death compared to TUNEL. Therefore, one should expect a higher proportion of cells immunoreactive for cleaved caspase-3 compared to TUNEL. For example, ~10% of β -cells of *ob/ob* mice are positive for cleaved caspase-3 (see bar 1 of [Figure 5g](#) in ([McKimpson et al., 2021](#))).

LIMITATIONS

TUNEL only measures cells actively undergoing cell death, which is low in pancreatic β -cells. Therefore, it is necessary to quantify a large number of cells (or pancreatic islets) in order to obtain an accurate representation of cell death in the experimental conditions being tested. In addition, because numbers can be easily skewed, it is important to randomly quantify experiments.

If pooling results from multiple experiments is required, it may be necessary to normalize absolute values. As such, as basal levels of cell death are often low, it is important to normalize the control sample after death stimuli (and not the control sample with no death inducer). For example, see Figure S3, panel A in (McKimpson et al., 2021). Treatments are normalized to Vector cells treated with thapsigargin (bar 3).

TROUBLESHOOTING

Problem 1

It is difficult to detect cells positive for cell death markers (propidium iodide and TUNEL) in cell culture experiments or isolated islets (steps 2c, 7, and 11k).

Potential solution 1

It is possible that the death inducer may not be strong enough to induce a significant amount of cell death. To test this, increase the length and/or concentration of treatment. Note that it is important to reach statistical significance of death treatments compared to control in experiments.

Problem 2

It is difficult to detect cells positive for cell death markers in pancreatic tissue (steps 17 and 21).

Potential solution 2

It is possible that the tissue that has low basal levels of cell death. To test this, include a positive control, such as the intestine, in the experiment. A suggested technical control for TUNEL staining is pre-treatment of the tissue with DNase I prior to TUNEL staining.

Problem 3

In live cell experiments, all cells are positive for both propidium iodide and Hoechst (step 2c).

Potential solution 3

It is possible that the time spent imaging cells was too long. Both Hoechst and PI can be toxic to live cells after a prolonged period of time. If this problem is occurring regularly, stagger the timing of propidium iodide staining such that fewer cells need to be imaged at any given time. It is also possible that the death stimuli is too severe. To test this, decrease the length and/or concentration of treatment.

Problem 4

There is an abnormally high amount of TUNEL+ cells in isolated islets (step 11k).

Potential solution 4

There are a few possibilities for this. First, it is possible that islet culture conditions were not ideal. If this is the case, one would expect to see elevated levels of TUNEL+ cells in both control and treatment samples. Many unhealthy islets will have a larger number of cells positive for TUNEL in the core of the islet, which should not be included in quantification for the experiment. If elevated levels of TUNEL+ cells are only in the treatment condition, it is possible that the death stimuli is too severe. To test this, decrease the length and/or concentration of treatment.

Problem 5

There are an abnormally high amount of TUNEL+ cells in fixed pancreatic tissue samples (step 17).

Potential solution 5

Extended proteinase K treatment may lead to false positives. In addition, if images are overexposed, nuclei may seem positive for TUNEL, when in fact they are not. A suggested control to test for these is including pancreatic tissue in which key components of the cell death signaling pathway are deleted and/or dysfunctional, and consequently, should have very low levels of TUNEL+ cells. We

recommend a caspase 3 mutant (Rongvaux et al., 2014). Additionally, a technical control to help determine what is background staining is staining one slide with only the reaction mix which contains the TUNEL label solution (and no TdT).

Problem 6

There is an abnormally high amount of cleaved-caspase 3+ cells in fixed pancreatic tissue samples (step 21).

Potential solution 6

As noted above in potential solution 5, a suggested genetic negative control to test for real cleaved-caspase 3 signal is including pancreatic tissue isolated from mice harboring a caspase 3 mutant defective in cell death signaling (Rongvaux et al., 2014). Note, it is expected to have higher rates of cleaved-caspase 3+ cells compared to TUNEL+ cells.

Problem 7

There is significant experiment to experiment variation in the absolute numbers of cell death markers induced (steps 7 and 11k).

Potential solution 7

With palmitate treatment, it is possible that the palmitate did not dissolve completely into solution. Prior to filtering the palmitate media after 16 h dissolving step, check the media for solid material. If solid palmitate is still visible to the eye, allow the palmitate to dissolve longer and increase the shaking speed. It is also important to make fresh palmitate each time. With chemical inducers, aliquot stock solutions into small single-use volumes so that a fresh aliquot can be used for each experiment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Wendy M^cKimpson at wm2347@cumc.columbia.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets/code.

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AUTHOR CONTRIBUTIONS

R.N.K. and W.M.M. designed the experiments and analyzed the data. W.M.M. performed the experiments. R.N.K. and W.M.M. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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