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Corresponding Author:	Scott Soleimanpour, M.D. University of Michigan Ann Arbor, MI UNITED STATES
Corresponding Author's Institution:	University of Michigan
Corresponding Author E-Mail:	ssol@med.umich.edu
Order of Authors:	Gemma Pearson
	Scott Soleimanpour, M.D.
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Alisha DSouza, Ph.D.
Senior Review Editor
JoVE

Dear Dr. DSouza:

We appreciate the review of our manuscript JoVE59398 entitled, "Visualization of endogenous mitophagy complexes in situ in human pancreatic beta cells utilizing proximity ligation assay". We wish to thank you and the reviewers for their cogent and thoughtful comments on our manuscript. In this revision, we have responded to the reviewers' comments in detail, including modification of the manuscript text for clarity, expansion of detail to the protocol, and finally by addition of new unpublished data to demonstrate the feasibility of this method to survey the mitophagy pathway in cell types other than beta cells where Nrdp1 and Usp8 levels are abundant.

Thank you very much for your reconsideration of our manuscript, and we hope that you will find this improved manuscript now appropriate for publication in *JoVE*.

Sincerely,

Scott Soleimanpour, M.D.

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TITLE:

Visualization of Endogenous Mitophagy Complexes In Situ in Human Pancreatic Beta Cells Utilizing Proximity Ligation Assay

AUTHORS AND AFFILIATIONS:

Gemma Pearson[1], Scott A. Soleimanpour[1,2]

[1]Department of Internal Medicine, Division of Metabolism, Endocrinology and Diabetes, University of Michigan, Ann Arbor, MI USA

[2]VA Ann Arbor Healthcare System, Ann Arbor, MI, USA

Corresponding Author:

Scott A. Soleimanpour (ssol@med.umich.edu)

Email Addresses of Co-authors:

Gemma Pearson (gemmap@med.umich.edu)

KEYWORDS:

Pancreatic beta cells, mitophagy, proximity ligation assay, human islets, protein-interaction, mitochondria.

SUMMARY:

This protocol outlines a method for quantitative analysis of mitophagy protein complex formation specifically in beta cells from primary human islet samples. This technique thus allows analysis of mitophagy from limited biological material, which are crucial in precious human pancreatic beta cell samples.

ABSTRACT:

Mitophagy is an essential mitochondrial quality control pathway, which is crucial for pancreatic islet beta cell bioenergetics to fuel glucose-stimulated insulin release. Assessment of mitophagy is challenging and often requires genetic reporters or multiple complementary techniques not easily utilized in tissue samples, such as primary human pancreatic islets. Here we demonstrate a robust approach to visualize and quantify formation of key endogenous mitophagy complexes in primary human pancreatic islets. Utilizing the sensitive proximity ligation assay technique to detect interaction of the mitophagy regulators NRDP1 and USP8, we are able to specifically quantify formation of essential mitophagy complexes in situ. By coupling this approach to counterstaining for the transcription factor PDX1, we can quantify mitophagy complexes, and the factors that can impair mitophagy, specifically within beta cells. The methodology we describe overcomes the need for large quantities of cellular extracts required for other protein-protein interaction studies, such as immunoprecipitation (IP) or mass spectrometry, and is ideal for precious human islets samples generally not available in sufficient quantities for these approaches. Further, this methodology obviates the need for flow sorting techniques to purify beta cells from a heterogeneous islet population for downstream protein applications. Thus, we describe a valuable protocol for visualization of mitophagy highly compatible for use in

heterogeneous and limited cell populations.

INTRODUCTION:

Pancreatic beta cells produce the insulin required to maintain normal glucose homeostasis, and their failure results in the development of all forms of diabetes. Beta cells retain a robust mitochondrial capacity to generate the energy required to couple glucose metabolism with insulin release. Recently, it has become apparent that the maintenance of functional mitochondrial mass is of pivotal importance for optimal beta cell function[1-3]. In order to sustain functional mitochondrial mass, beta cells rely on quality control mechanisms to remove dysfunctional, damaged, or aging mitochondria[4]. We and others have previously demonstrated that beta cells rely on a specialized form of mitochondrial turnover, called mitochondrial autophagy (or mitophagy), to maintain mitochondrial quality control in both rodent and human islets[1,2,5]. Unfortunately, however, there was no simple method to detect mitophagy, or endogenously expressed mitophagy components, in human pancreatic beta cells.

We have recently shown that upstream regulation of mitophagy in beta cells relies on formation of a protein complex comprising the E3 ligases CLEC16A and NRDP1 and the deubiquitinase USP8[1]. NRDP1 and USP8 have been shown independently to affect mitophagy through action on the key mitophagy initiator PARKIN[6,7]. NRDP1 targets PARKIN for ubiquitination and degradation to switch off mitophagy[6], and Usp8 specifically deubiquitinates K6-linked PARKIN to promote its translocation to mitochondria[7]. Proximity ligation assay (PLA) technology has been a recent advance in the field of protein interaction biology[8], allowing visualization of endogenous protein interactions in situ in single cells, and is not limited by scarce sample material. This methodology is particularly enticing for human islet/beta cell biology, due to the sparsity of sample availability, coupled to the need for understanding physiologically relevant protein complexes within heterogeneous cell types.

Utilizing the PLA approach, we are able to observe key endogenous mitophagy complexes in primary human pancreatic beta cells, neuronal cell lines, and demonstrate the effects of a diabetogenic environment on the mitophagy pathway[1]. In summary, the overarching goal of this protocol is to analyze specific mitophagy protein complexes in tissues lacking abundant material, or where conventional protein-interaction studies are not possible.

PROTOCOL:

Use of de-identified donor human pancreatic islets is via an Institutional Review Board (IRB) exemption and in compliance with University of Michigan IRB policy. Human pancreatic islets were provided by the NIH/NIDDK-sponsored Integrated Islet Distribution Program (IIDP).

1. Human islet sample preparation

1.1. Single cell dissociation

1.1.1. Culture human islet samples (4000–6000 islet equivalents/10 mL media) for at least 1 day at 37 °C in pancreatic islet media (PIM(S)) media supplemented with 1 mM glutamine (PIM(G)),

100 units/mL antimycotic-antibiotic, 1 mM sodium pyruvate and 10 % fetal bovine serum (FBS) or human AB serum.

1.1.2. Use a dissection light microscope at 3x magnification to count individual human islets from culture. Pick 40 islets per treatment/condition of interest (such as glucolipotoxicity) into 1.5 mL tubes in islet media.

1.1.3. Centrifuge islets at 400 x *g* for 1 min at 10 °C to sediment.

1.1.4. Wash samples, by brief inversion of tubes at room temperature, twice with 1 mL phosphate buffered saline (PBS) containing 50 μM PR619 (a deubiquitinase inhibitor; to preserve ubiquitin dependent protein interactions), with centrifugation between each wash at 400 x *g* for 1 min at 10 °C.

1.1.5. Dissociate islets into single cells with 125 μL of 0.25% trypsin containing 50 μM PR619 by incubating prewarmed trypsin (37 °C) for 3 min with islet pellets. Gently disperse islets during this time with periodic gentle pipetting using a 200 μL pipette.

1.1.6. Quench the trypsin with 1 mL warmed (37 °C) PIM(S) media containing 50 μM PR619, then sediment cells by centrifuging at 400 x *g* for 1 min.

1.1.7. Wash cells by centrifugation at 400 x *g* for 1 min, twice, with PBS + 50 μM PR619.

1.1.8. Finally, resuspend cells in 150 μL PBS containing 50 μM PR619.

1.2. Single cell adherence and fixation

1.2.1. After resuspension, spin the cell solution onto frosted, charged, microscope slides using a cytocentrifuge at 28 x g for 10 min.

1.2.2. Following cytocentrifugation, outline the cellular area with a hydrophobic pen (see the **Table of Materials**) to minimize antibody/PLA solution volumes needed. Fix cells with 4% paraformaldehyde in PBS for 15 min at room temperature.

CAUTION: PFA is hazardous and must be handled with care.

1.2.3. For best results, carry out staining/PLA immediately, or within 24 h. If necessary, store samples in PBS at 4 °C until staining for no longer than 48 h.

2. Immunohistochemistry

2.1. Blocking

2.1.1. Wash cells twice with 1x PBS for 5 min at room temperature.

2.1.2. Block cell solution, to eliminate background staining, with 10% donkey serum in PBS containing 0.3% detergent (see the **Table of Materials**) for 1 h at room temperature.

2.2. Staining

2.2.1. Incubate cells with primary mouse or rabbit antibodies against USP8 (1:250) and NRDP1 (1:250) respectively (see the **Table of Materials**) diluted in phosphate buffered saline with detergent (PBT, see **Table of Materials**), at 4 °C overnight, to detect mitophagy complex signaling via PLA.

2.2.2. Co-incubate cells with a marker specific for beta cell identification that was not raised in mouse or rabbit so as to not interfere with PLA signal. In this case incubate cells with anti-goat PDX1 (1:500) in PBT. Incubate all primary antibodies overnight at 4 °C, using plastic film on top of the solution to prevent evaporation.

3. Proximity ligation assay

3.1. PLA probe and counterstain

3.1.1. Prepare the PLA probe solution according to manufacturer's instructions, i.e., make 20 µL of probe solution per sample by preparing a final concentration of 1:5 solution of both anti-mouse and anti-rabbit probe solution in PBT, and incubating for 20 min at room temperature.

3.1.2. After overnight incubation, wash cells twice with 1x PBS for 5 min each, on a rocker.

3.1.3. Just before incubation with probe solution, add anti-goat Cy5 secondary at a final concentration of 1:600 to the probe solution (for detection of endogenous PDX1). Add 20 µL probe solution to each cell condition, cover gently with plastic film and incubate at 37 °C for 1 h.

3.2. Ligation

3.2.1. Wash cells twice, at room temperature, with Buffer A (see the **Table of Materials** for the recipe) for 5 min each, on a rocker.

3.2.2. Prepare ligation solution (part of the detection reagents, see **Table of Materials**) according to manufacturer's instructions. For this, dilute ligation stock (5x) 1:5 in diethyl pyrocarbonate (DEPC)-treated water. Immediately before incubation add 0.025 U/µL ligase. Add 20 µL ligation solution to cells. Cover with plastic film and incubate at 37 °C for 30 min.

3.3. Amplification

3.3.1. Wash cells twice at room temperature with Buffer A for 2 min each.

3.3.2. Make amplification solution (part of the detection reagents, see **Table of Materials)** according to manufacturer's instructions. Dilute amplification buffer (5x) 1:5 in DEPC-treated water and keep in the dark until use. Add a 1:80 dilution of polymerase (part of the detection reagents, see **Table of Materials)** to the solution immediately prior to adding the solution to the cells.

3.3.3. Add 20 µL of amplification solution to the cells. Cover with plastic film and place in the dark at 37 °C for between 1 h 40 min to 2 h for maximum signal.

3.4. Preparation for imaging

3.4.1. Wash cells twice with Buffer B (see the **Table of Materials** for recipe) for 10 min at room temperature, on a rocker.

3.4.2. Finally, wash cells once in 0.01x Buffer B, for 2 min at room temperature on a rocker.

3.4.3. Mount samples by adding a drop of mounting media containing 4',6-diamidino-2-phenylindole (DAPI) and carefully placing a coverslip over the samples using a scalpel blade to press out any bubbles formed. Seal coverslips using clear nail polish around the edges.

3.4.4. Image samples on a microscope capable of capturing multiple focal planes, such as a laser scanning confocal microscope, or an inverted fluorescence microscope with deconvolution capabilities, taking at least 9 different focal plane images.

3.4.4.1. Capture images at 100x magnification, with approximately 0.45 µm z-stack height. Capture PLA at 550 nm excitation, 570 nm emission; counter-stain at 650 nm excitation, 670 nm emission; and DAPI at 405 nm excitation, 450 nm emission.

3.4.5. To ensure fluorescence image background signals and stray light are minimized for downstream analysis of PLA events (especially on widefield microscopes), process images utilizing a two-dimensional deconvolution (nearest neighbor) algorithm through a standard image processing software of choice.

NOTE: For Olympus use CellSens, Nikon use NIS-Elements, Leica use LAS X, Zeiss – ZEN, Metamorph, MATLAB, Huygens Software, as well as several Plugins available through Image-J. For analysis, the total number of interactions over all the z-stacks should be analyzed using Image-J software, ensuring only Pdx-1 positive cells are analyzed (section 3.5).

3.5. Quantification of PLA interactions

3.5.1. Open the free Image-J software application, and open the PLA image. Start from the first sharply in-focus PLA image.

3.5.2. Click the **image** tab, and select **adjust**, then **threshold** (**Figure 1A**). Adjust the threshold to remove all non-specific PLA signal, make a note of the threshold adjustment and try to keep it consistent throughout analysis.

3.5.3. Click the **process** tab, and select **binary**, then **make binary** (**Figure 1B**). Use the binary image to measure particles, by clicking on the "**analyze**" tab and pressing **analyze particles** (**Figure 1C**).

3.5.4. For analysis make sure the settings are as follows: **Size** = 0–Infinity, **Circularity** = 0–1.0, **Show** = Outlines, check boxes for **Display results**, and **Clear results** (**Figure 1D**). Click **OK**. Take note of the number of particles analyzed in a spreadsheet denoting sample, and z-stack position.

3.5.5. Repeat steps 3.5.2–3.5.4 until all in-focus z-stacks for the sample have been analyzed. Sum the total number of particles for the sample in the spreadsheet to quantify total number of interactions.

REPRESENTATIVE RESULTS:

We conducted initial experiments in the MIN6 pancreatic beta cell line, and the neuroblastoma cell line SH-SY5Y, to optimize and confirm both the specificity of the antibodies and the visualized protein interactions. MIN6 cells were plated onto coverslips at 30,000 cells/mL and left to adhere for 48 h, SH-SY5Y cells were plated onto coverslips at 15,000 cells/mL and left to adhere for 24 h. The PLA protocol was then followed as above, beginning at Step 1.2.3. To ensure the specificity of PLA signals, we first performed this approach in the presence of (i) no primary antibody (**Figure 2A**, **Figure 3A**), (ii) NRDP1 antibody alone (**Figure 2B**, **Figure 3B**), (iii) USP8 antibody alone (**Figure 2C**, **Figure 3C**), and (iv) both NRDP1 and USP8 antibodies (**Figure 2D**, **Figure 3D**). For ease of sample set-up, **Table 1** denotes how to properly layout experimental controls. Notably, we observe no punctate PLA signal in single primary antibody or no primary antibody control conditions, thus confirming that in situ interactions are specifically observed between NRDP1 and USP8 in the presence of both primary antibodies, in both MIN6 and SH-SY5Y cells.

We next adapted this approach for use with human islets to analyze mitophagy complex interactions specifically within primary human beta cells. Human islets are composed of a heterogenous population of functionally distinct cells, including alpha, beta, delta, and PP-cells[9]. In contrast to mouse islets where beta cells account for ~80% of islet mass, beta cells comprise a proportionally smaller range within human islets (between 28–75%)[10-12]. Thus, we sought to use PLA technology to allow us to observe the presence of NRDP1-USP8 mitophagy complexes specifically within beta cells and ensure we did not observe confounding observations from other islet cell types (which could occur from co-immunoprecipitation studies of islet lysates). Thus, it is important to ensure adequate and uniform islet dispersion to such a level that single cells can be easily discriminated and further analyzed. As seen in **Figure 4**, the dispersion protocol (section 1) is highly efficient at ensuring single cells in the microscope field of view for downstream analysis. To identify beta cells, we performed co-staining with PDX1 specific anti-sera during the PLA process. PDX1 is a vital beta cell specific transcription factor, which is found in mature insulin-producing beta cells primarily within the nucleus[13]. PDX1 staining was retained following

NRDP1:USP8 PLA in primary human islets (**Figure 4**). Importantly, we used goat PDX1 anti-sera to avoid cross-reactivity with primary antibodies used for PLA (rabbit anti-NRDP1 and mouse anti-USP8, respectively). Thus, the addition of PDX1 counterstaining allows for the specific assessment of endogenous mitophagy complexes within primary human beta cells.

Utilizing these approaches, we can compile a snapshot of the retention of the NRDP1:USP8 mitophagy complex to infer competence of the mitophagy pathway within beta cells. To expand this approach for use within environmental conditions emulating those of type 2 diabetes[14], we treated beta cell lines and primary human islets with palmitate and high glucose to induce glucolipotoxicity. Indeed, we found that the interaction of NRDP1 and USP8 was decreased following a 48 h exposure to palmitate and high glucose in both beta cell lines as well as primary human beta cells by PLA (**Figure 5** and reference[1]). This result highlights the feasibility of this assay to assess key endogenous mitophagy factors following diabetogenic stimuli.

FIGURE AND TABLE LEGENDS:

FIGURE 1: Workflow of Image J analysis for quantification of PLA interactions. The important steps of analysis are highlighted here. (**A**) Adjustment of the image threshold to ensure specific PLA signal analysis. (**B**) Conversion of image to binary data to allow easy quantification. (**C–D**) How to finalize analysis by analyzing the particles recognized by ImageJ software.

FIGURE 2: NRDP1 and USP8 specifically interact in pancreatic beta cells. High magnification (100x) images of the mouse pancreatic cell line, MIN6, are shown. (**A–D**) PLA signal for NRDP1:USP8 interaction is shown in red, and nuclei are delineated by DAPI in blue. (**A–C**) No specific punctate signal for NRDP1:USP8 interaction is seen if both (**A**) or either one (**B, C**) of the primary protein antibodies is omitted during the PLA process. However, interaction of both proteins is visible by PLA in pancreatic beta cells (**D**) when both antibodies are included.

FIGURE 3: NRDP1 and USP8 specifically interact in neuroblastoma cells. High magnification (100x) images of the human neuroblastoma cell line, SH-SY5Y, are shown. (**A–D**) PLA signal for NRDP1:USP8 interaction is shown in red, and nuclei are delineated by DAPI in blue. (**A–C**) No specific punctate signal for NRDP1:USP8 interaction is seen if both (**A**) or either one (**B, C**) of the primary protein antibodies is omitted during the PLA process. However, interaction of both proteins is visible by PLA (**D**) when both antibodies are included.

FIGURE 4: Beta cell mitophagy complexes can be identified in primary human pancreatic islets in situ by PLA. High magnification (100x) images of dispersed human islets are shown. Single cells are delineated by nuclear staining with DAPI (blue), beta cells are observed with PDX1 staining (magenta), and mitophagy complexes can be seen in both beta cells and non-beta cells (red).

FIGURE 5: The NRDP1:USP8 mitophagy complex is destabilized in beta cells by glucolipotoxicity. High magnification (100x) images of MIN6 cells treated for 48 h with either control (25 mM glucose + 0.82% BSA) or high glucose + palmitate (25 mM glucose + 0.4 mM palmitate/0.82% BSA) are shown. (**A–B**) PLA (NRDP1:USP8) signal is seen in both treatment groups. PLA signal

decreases in MIN6 beta cells after glucolipotoxicity (**B**) when compared to controls (**A**).

Table 1: Sample experimental design setup.

DISCUSSION:

Here we describe a simple and efficient approach to use NRDP1:USP8 PLA in tissues/cells of interest to quantify formation of upstream mitophagy complexes. We previously confirmed the formation of the CLEC16A-NRDP1-USP8 mitophagy complex in pancreatic beta cells by several methodologies, including co-immunoprecipitation experiments, cell-free interaction studies, and in vitro as well as cell-based ubiquitination assays, and demonstrated how this complex drives regulated mitophagic flux[1,15]. The PLA studies we report and describe here allow for a rapid, quantitative, and cell-specific view of the mitophagy pathway that is highly adaptable to primary human beta cells. Additionally, we have shown that NRDP1:USP8 PLA can be adapted for use outside of beta cell biology in SY5Y neuroblastoma cells, highlighting the potential feasibility of this technique to monitor mitophagy complexes in neuronal systems as well.

PLA is a straightforward approach; however, certain steps within the protocol are of utmost importance for ensuring clear and specific results. These include: (1) ensuring the dispersal procedure for human islets is mild enough to prevent cell lysis/damage to optimize images, (2) addition of the deubiquitinase inhibitor, PR619, immediately prior to fixation and during washes to preserve the ubiquitination state (and thus binding) of the NRDP1-USP8 complex for maximal signal observation by PLA, and (3) the objective quantification of PLA events by ImageJ to ensure an unbiased assessment of mitophagy complexes and also allow for determination of conditions whereby the changes on mitophagy may not be complete but still statistically significant/biologically relevant.

A well-known challenge within human islet studies is the concern for the heterogeneous population of both beta cells and non-beta cells. Our use of the PDX1 counterstain allows for assessment of mitophagy complex formation within beta cells (as well as PDX1 negative non-beta cells). One potential concern utilizing PDX1 as a counterstain is its expression in pancreatic delta cells[16-18], albeit to a far lower degree than in beta cells, as such other markers (i.e., NKX6.1) could be employed to target beta cells more specifically. By dispersing intact islets into single cells immediately prior to cytocentrifugation and fixation, we are also able to perform single cell mitophagy studies with only minimal disruption of the islet microenvironment during the course of drug treatments and/or glucolipotoxic exposure. However, we cannot exclude the possibility that islet dispersion could interfere with mitophagic complexes within cells independently of relevant treatments.

While more traditional protein-protein interaction studies can be employed in human pancreatic islets, such as co-immunoprecipitation and/or proteomic approaches, these procedures generally require a large amount of islet lysate, which can prove challenging given the scarcity of human islet material available. Additionally, the concern of cell type-specificity during these traditional techniques becomes more apparent or requires additional optimization of flow cytometry methods to sort pure beta cell populations[19-22]. Thus, our PLA approach provides an

attractive and practical alternative for protein-protein interaction studies of the mitophagy pathway in human beta cells. Notably, the ability to use as few as 40 total islets/condition per experiment to quantify mitophagy complex formation in thousands of individual beta-cells allows islet researchers the ability to conserve their islet preparations for other assessments from the same donor.

As NRDP1 and USP8 are ubiquitously expressed, we speculate this technique may have value for assessment of mitophagy in cell types beyond beta cells. The possibilities include other islet cell types (with relevant counterstains for alpha cells or delta cells) or cell types which heavily rely on mitophagy, such as neurons. To this end, our observation of the transferability of this technique to SY5Y neuroblastoma cells (**Figure 2**) or PDX1-negative islet cells (**Figure 4**) could suggest the utility of our technique for the mitophagy pathway in other cell types.

Despite the ease and simplicity of the NRDP1:USP8 PLA approach, there are challenges that could confound the results of this assay. While PLA is able to ascertain protein-protein interactions in very close proximity (40 nm), it does not rule out the possibility that certain experimental conditions could maintain NRDP1-USP8 proximity while disrupting interaction, which would be missed by the PLA approach. Further, while we have demonstrated that NRDP1:USP8 complex formation is a valid readout for the canonical CLEC16A/PARKIN-mitophagy pathway in pancreatic beta cells, recent studies have observed roles for PARKIN-independent mitophagy in mitochondrial quality control[23,24]. We do not yet know how NRDP1:USP8 complex formation is modified by the disruption of PARKIN-independent mitophagy. Thus, use of a complementary approach beyond those described here to assay mitophagy in beta cells would be advisable. Finally, the stability of this mitophagy complex is dependent on ubiquitination of the component proteins, such that the addition of a broad spectrum deubiquitinase inhibitor PR619 is critical to maintain ubiquitination during sample preparation. Again, manipulations of beta cells that may indirectly disrupt ubiquitination of components of the NRDP1-USP8 complex need to be considered when analyzing NRDP1:USP8 as a readout for mitophagy complex formation.

As the role of mitochondrial quality control is increasingly appreciated in not only beta cell function but also other highly metabolic cell types, the rigorous assessment of mitophagy can prove challenging and time consuming to groups wishing for a more rapid assessment of crucial mitophagy components. The PLA approach we describe is a relatively low-cost, molecular-level visualization technique that can provide quantitative analysis of mitophagy complex formation with single cell resolution in small quantity human islet samples and could be broadly applied to a variety of cell types, treatments, or disease conditions.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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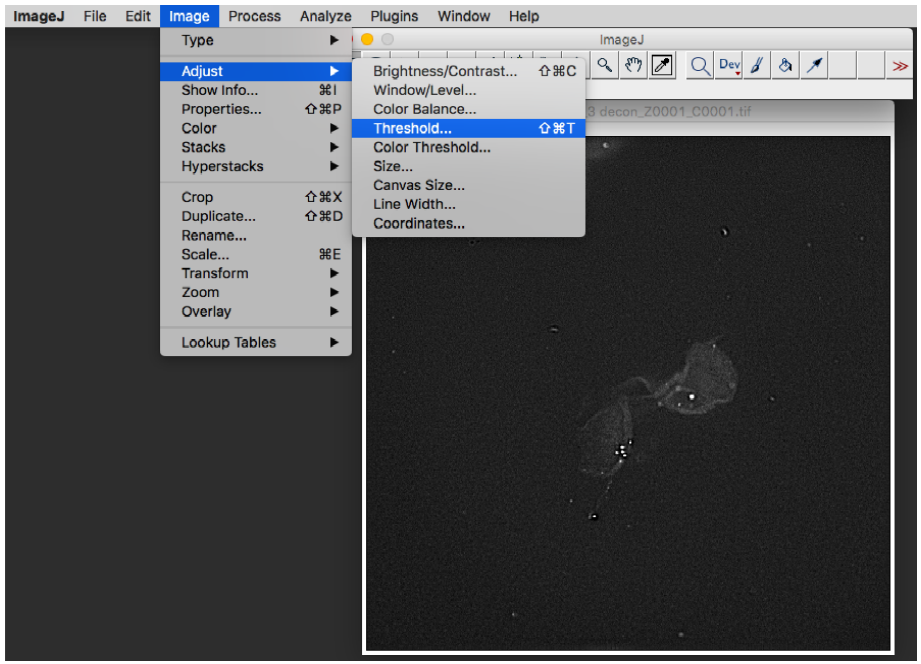
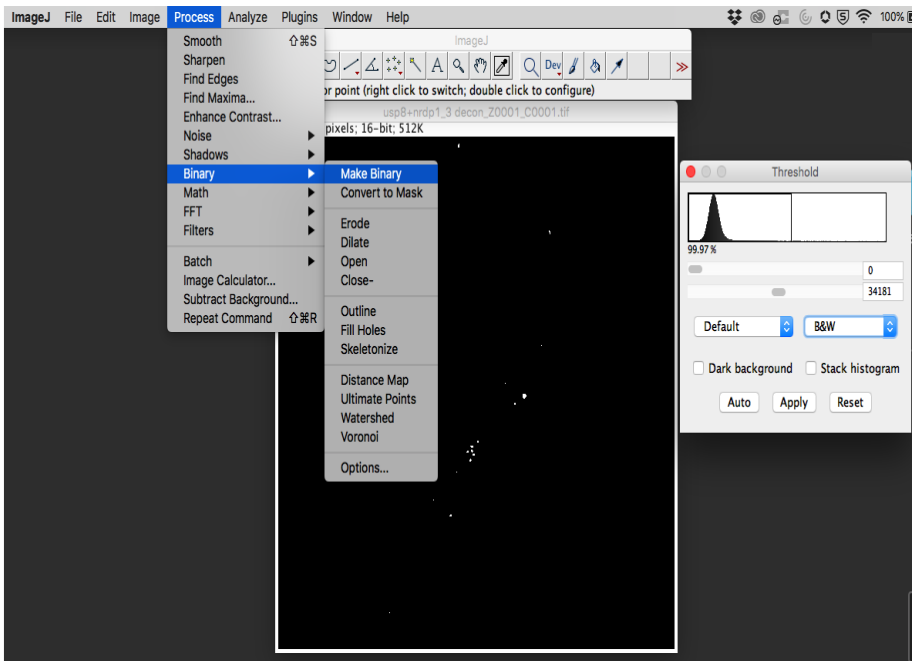
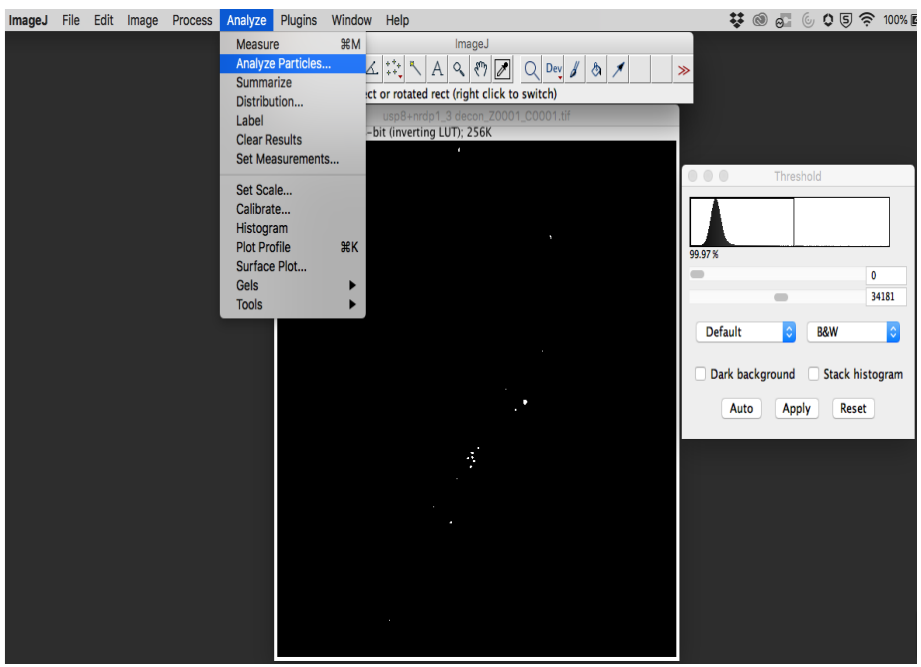
A.



B.



C.



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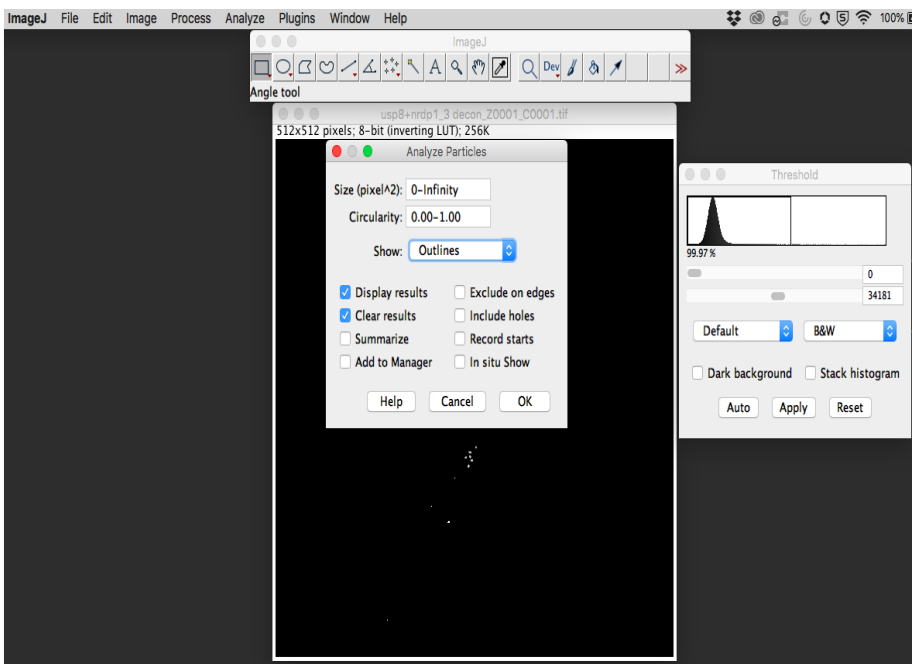

Figure 2

Click here to access/download;Figure;Fig2_new.ai

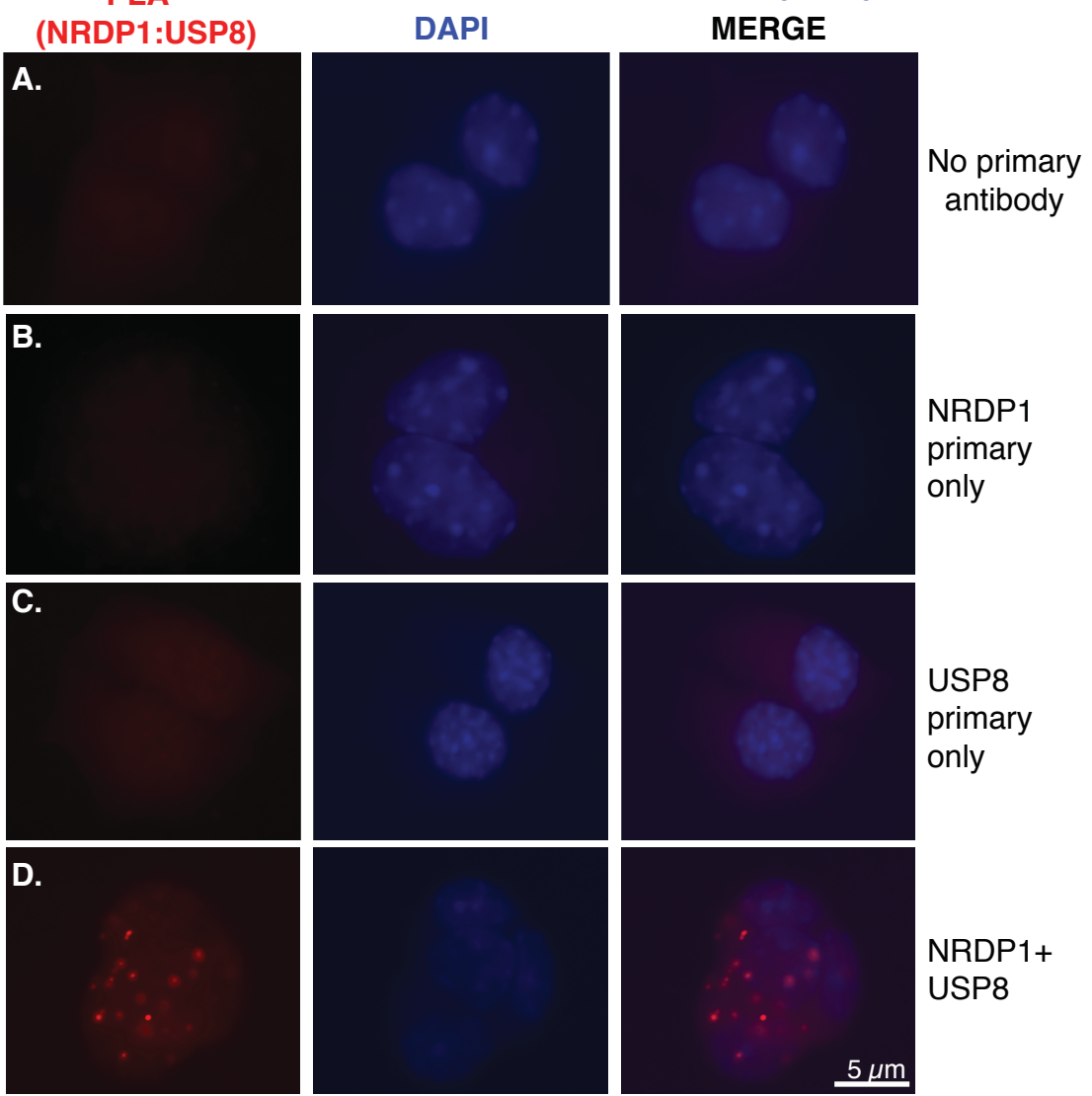

Figure 3

Click here to access/download;Figure;Fig3_new.

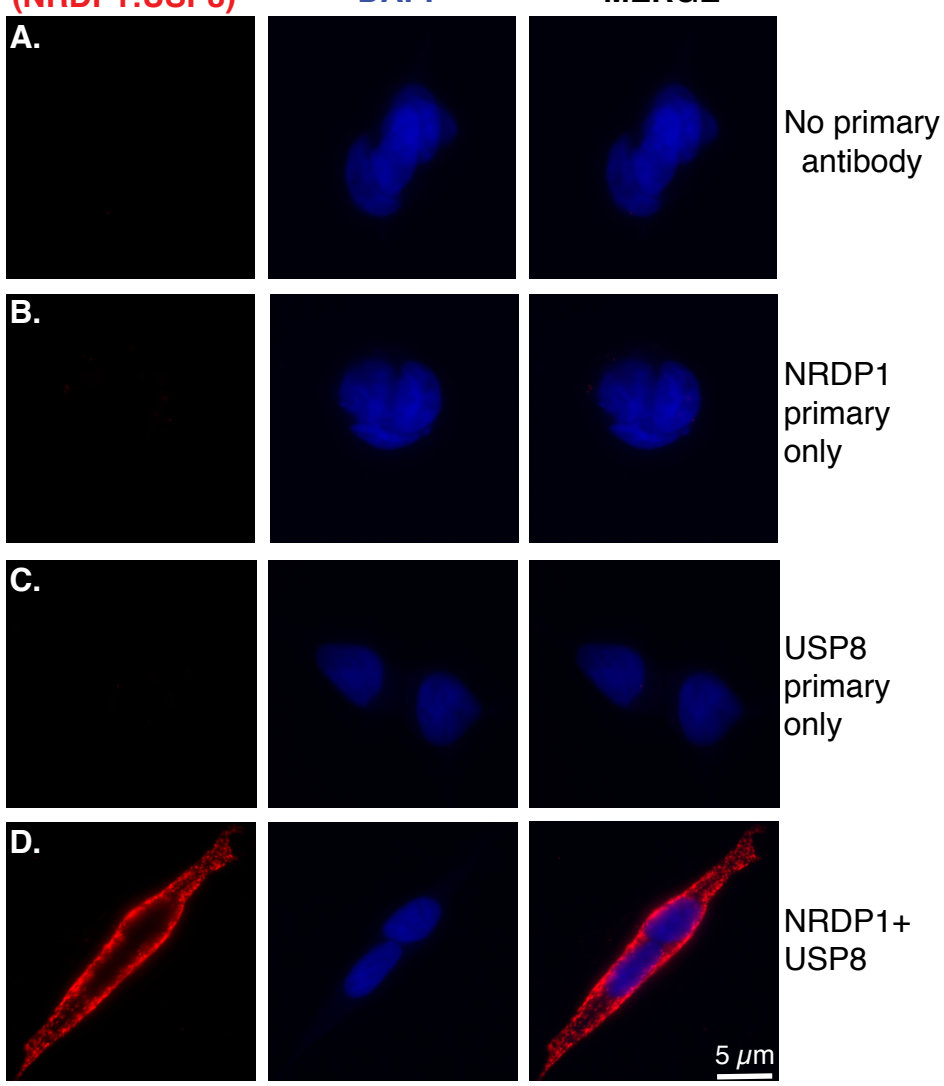

Figure 4

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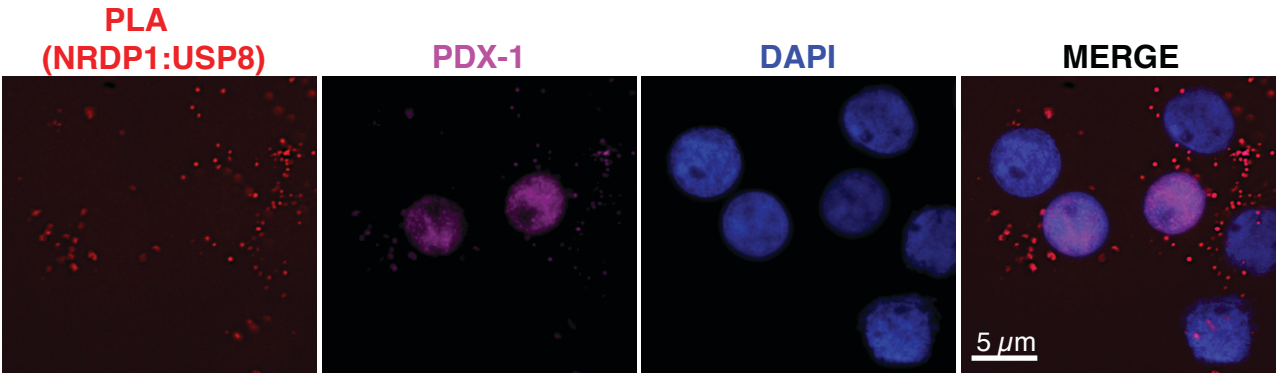
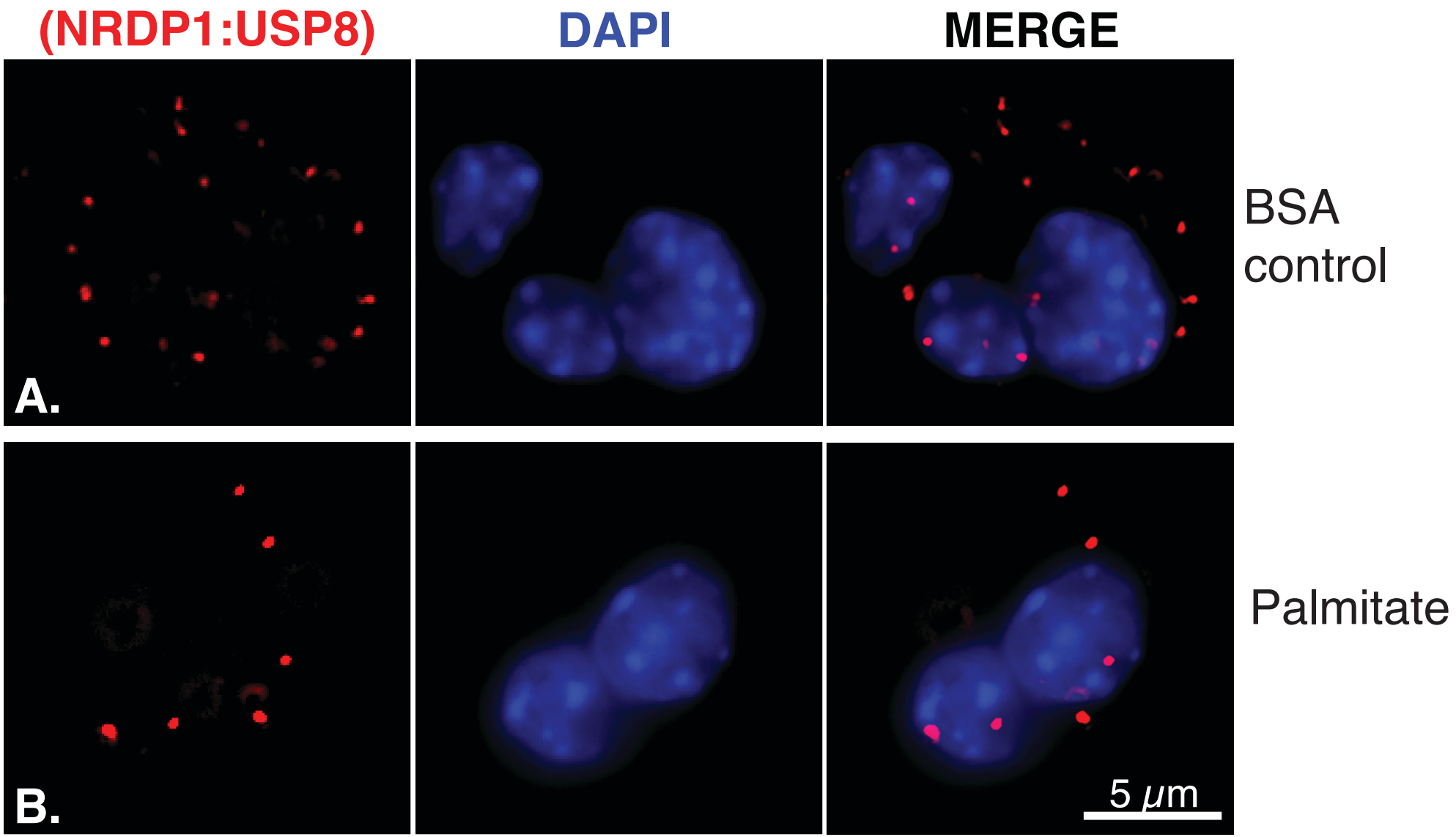


Figure 5

Click here to access/download;Figure;Fig5_new.a



Table

Click here to access/download;Table;Table 1.xlsx

SAMPLE	**ANTIBODY 1 (mouse anti-NRDP1)**	**ANTIBODY 2 (rabbit anti-USP8)**
CONTROL 1: No primary antibody (40 islets)	–	–
CONTROL 2: NRDP1 alone (40 islets)	+	–
CONTROL 3: USP8 alone (40 islets)	–	+
EXPERIMENTAL 1-x: all experimental conditions (40 islets each)	+	+

ANTIBODY 3 (goat anti-PDX1) (Optional counterstain)
+
+
+
+

Click here to access/download;Table of Materials;JoVE_Materials_Pearson et al.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.25% trypsin-EDTA 1X	Life Technologies	25200-056	
Antibiotic-Antimycotic	Life Technologies	15240-062	
Block solution	Homemade		Use 1X PBS, add 10 % donkey serum, and 0.3% Triton X-100 deterge
Buffer A	Homemade		To make 1L: Mix 8.8g NaCl, 1.2g Tris base, 500ul Tween-20, with 750
Buffer B	Homemade		To make 1L: Mix 5.84g NaCl, 4.24g Tris base, 26g Tris-HCl with 500m
Cy5-conjugated AffiniPure donkey anti-goat	Jackson Labs	705-175-147	
Detection Reagents Red	Sigma-Aldrich	DU092008-100RXN	Kit containing: ligation solution stock (5X), ligase, amplification solut
DuoLink PLA probe anti-mouse MINUS	Sigma-Aldrich	DU092004-100RXN	
DuoLink PLA probe anti-rabbit PLUS	Sigma-Aldrich	DU092002-100RXN	
Fetal bovine serum			
Goat polyclonal anti-PDX1 (clone A17)	Santa Cruz	SC-14664	RRID: AB_2162373
HEPES (1M)	Life Technologies	15630-080	
MIN6 pancreatic cell line	Gift from D. Stoffers		Mouse insulinoma cell line, utilized for cell-based assays.
Mouse monoclonal anti-USP8 antibody (clone US872)	Sigma-Aldrich	SAB200527	
Pap-pen	Research Products International	195505	

Parafilm			Use to seal antibody and probe solutions on your cells to prevent ev
PBT (phosphate buffered saline with triton)	Homemade		To make 50mL: 43.5mLddH2O, 5mL 10X PBS, 0.5mL 10X BSA(100mg,
Penicillin-Streptomycin (100X)	Life Technologies	15140-122	
Phosphate buffered saline, 10X	Fisher Scientific	BP399-20	
PIM(ABS) Human AB serum	Prodo Labs	PIM-ABS001GMP	
PIM(G) (glutamine)	Prodo Labs	PIM-G001GMP	
PIM(S) media	Prodo Labs	PIM-S001GMP	
PR619	Apex Bio	A812	
Prolong Gold antifade reagent with DAPI	Life Technologies (Molecular Probes)	P36935	
Rabbit polyclonal anti-FLRF/RNF41 (Nrdp1)	Bethyl Laboratories	A300-049A	RRID: AB_2181251
SH-SY5Y cells	Gift from L. Satin		Human neuroblastoma cell line, utilized for cell-based assays.
Sodium Pyruvate (100X)	Life Technologies	11360-070	
Triton X-100	Fisher Scientific	BP151-100	
Tween-20	Fisher Scientific	BP337-100	
Water for RNA work (DEPC water)	Fisher Scientific	BP361-1L	

nt.

)mL ddH20. pH to 7.5 with HCl, and fill to 1L. Filter solution and store at 4C. Bring to RT before experimental use

L ddH20. pH to 7.5, and fill to 1L. Filter solution and store a 4C. Bring to RT before experimental use

ion stock (5X) and polymerase.

aporation when using small solution volumes.

/mL solution), 1mL 10% triton X-100 solution in ddH20)

ALA

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Author(s): Gemma Pearson, Scott A. Soleimanpour

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CORRESPONDING AUTHOR:

Name:	Scott Soleimanpour
Department:	Internal Medicine/Metabolism, Endocrinology, and Diabetes
Institution:	University of Michigan
Article Title:	Visualization of endogenous mitophagy complexes in situ in human pancreatic beta cells utilizing proximity ligation assay.
Signature:	Digitally signed by Scott Soleimanpour DN: cn=Scott Soleimanpour, o, ou=University of Michigan, email=ssol@med.umich.edu, c=US Date: 2018.11.15 15:33:54 -05'00'
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Rebuttal Letter
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Detailed response to reviewers: JoVE5939

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We apologize for any errors in the initial submission and have corrected spelling/grammatical errors on the revised manuscript.

2. Keywords: Please provide at least 6 keywords or phrases.

These are included in the revised manuscript.

3. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

We have revised the introduction to reflect the overall goal of this method, "In summary, the overarching goal of this protocol is to analyze specific mitophagy protein complexes in tissues lacking abundant material, or where conventional protein-interaction studies are not possible." Lines 71-73.

4. Please use °C for the temperature unit. Please use the micro symbol µ instead of u and abbreviate liters to L to avoid confusion. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

5. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

6. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

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We apologize for any errors in the initial submission and have corrected all of these errors on the revised manuscript.

8. Because human islet samples are used in the protocol, please check whether an ethics statement is required.

Our use of de-identified donor human pancreatic islets is via an Institutional Review Board (IRB) exemption and in compliance with University of Michigan IRB policy. We have included this information in the revised manuscript, section 1.1.1., lines 80-82.

9. 1.1.2: Please specify the conditions.

10. 2.1.2: Please specify the conditions for blocking (time and temperature).
11. 2.2.1: Please specify the incubation temperature.
12. 3.2.1, 3.4.1: Please provide the composition of wash buffer A/B or cite the Table of Materials.
We have specified the aforementioned conditions, temperature, and buffer composition in the revised manuscript.

13. Please include single-line spaces between all paragraphs, headings, steps, etc.
Single-line spaces are included in the revised manuscript.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
We have highlighted the essential steps of the protocol for the video in the revised manuscript (in yellow).

15. Discussion: Please also discuss critical steps within the protocol.
A discussion of the critical steps within the protocol has been added to the revised manuscript. "PLA is a straightforward approach; however, certain steps within the protocol are of utmost importance for ensuring clear and specific results. These include: (1) ensuring the dispersal procedure for human islets is mild enough to prevent cell lysis/damage to optimize images, (2) addition of the deubiquitinase inhibitor, PR619, immediately prior to fixation and during washes to preserve the ubiquitination state (and thus binding) of the Nrdp1-Usp8 complex for maximal signal observation by PLA, and (3) the objective quantification of PLA events by Image J to ensure an unbiased assessment of mitophagy complexes and also allow for determination of conditions whereby the changes on mitophagy may not be complete but still statistically significant/biologically relevant." Lines 286-294.

16. Figures: Please include a space between the number and its unit (5 µm).
Spaces are now included between numbers and units in the revised manuscript.

17. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.
All figures have been uploaded individually in Editorial Manager in correctly formatted file extensions.

18. Table of Materials: Please provide lot numbers and RRIDs of antibodies, if available. Please sort the items in alphabetical order according to the name of material/equipment.
Items are now uploaded in alphabetical order and lot numbers/RRIDs (where available) have been added for all antibodies in the revised table of materials.

19. References: Please do not abbreviate journal titles.
References have been formatted appropriately in the revised manuscript.

Reviewers' comments:

Reviewer #1:

1. Pdx1 is also expressed in the mouse delta cell (at least at the mRNA level from Mark Huising's lab) and there is some expression of Pdx1 in the human delta cell (at least at the mRNA level, Stitzel lab single cell RNA-seq paper and I think Kubichek lab single cell RNA-seq paper). Therefore, figure 2 might be looking at either beta or delta cells. Perhaps, this reviewer is unaware of data to suggest that Pdx1 protein behaves differently and if so, this should be mentioned in the discussion. Even if that's not true, the technique demonstrated here would seem to be easily applied to another more beta cell specific marker (eg Nkx6.1). I don't think the authors need to necessarily do this experiment as they have shown that co-staining works. However, this potential weakness should be mentioned in the discussion.
We thank the reviewer for this important point. PDX1 is indeed expressed at low levels in delta cells while at higher levels in mature beta-cells, and thus we cannot exclude the possibility of quantification of mitophagy complexes within pancreatic delta cells. We have updated the discussion to reflect this concern and highlight that alternative beta cell counterstains (such as NKX6.1) can be successfully used for specific identification of beta cells. Lines 299-301, "One potential concern utilizing PDX1 as a counterstain is its expression in pancreatic delta cells[16-18], albeit to a far lower degree than in beta cells, such that other markers (i.e. NKX6.1) could be employed to target beta cells more specifically."

The authors show some very nice staining images on complex detection in figure 3 with palmitate and high glucose treatment. They should describe how to quantitate this data as quantitation is specifically mentioned as an advantage of this protocol.
The reviewer makes an excellent point. We have updated the methods section to include a subsection (3.5) detailing quantification of these complexes using ImageJ. We have also included a figure (new Figure 1) to highlight the important steps of analysis by Image J.

Reviewer #2:

The significance of Nrdp1-USP8-Clec16a tripartite complex is shown in their previous report (Diabetes 67, 265-277,2018), and the relationship between the complex formation evaluated by the other methods and PLA assay has been suggested. In addition, the method explained here is already described in the previous paper, and no new additional data is demonstrated in this submitted manuscript, which implies that this document is suitable only for a journal related to methodology itself.
To address this concern, we now provide new unpublished data expanding the use of this technique within SH-SY5Y neuroblastoma cells (new Figure 3) to display the general utility of this technique to quantify mitophagy complexes beyond our previous publication focused on beta cells in *Diabetes*. We believe this additional study further displays the potential for efficient assessment of upstream mitophagy complexes in multiple cell types, as well as providing the detailed methodology of the study, acquisition, and analysis of Clec16a-Nrdp1-Usp8 mitophagy complexes for general use by the scientific community.

Reviewer #3:

1. In the introduction, please provide a brief description of the role of USP8 and Nrdp1 in mitophagy so that the reader/viewer clearly understands the significance of the localization of these two proteins and how to interpret changes in proximity of these proteins in terms of the activity level of mitophagy in the cells.
A more detailed outline of Nrdp1, and Usp8 function has been added to the introduction, lines 61-64: "Nrdp1 and Usp8 have been shown independently to affect mitophagy through action on the key mitophagy initiator Parkin[6,7]. Nrdp1 targets Parkin for ubiquitination and degradation to switch off mitophagy[6], and Usp8 specifically deubiquinates K6-linked Parkin to promote its translocation to mitochondria[7].".

2. Protocol section 1.1.2: is "islet media" the PIM(S) media described in step 1.1.1? If so, it may help to clarify this and refer to the media in 1.1.1 as "islet media".
We have added islet media in parentheses in step 1.1.1, line 82.

3. Protocol section 1.2.1: Are the frosted microscope slides charged or uncharged?
They are charged, and this has been incorporated into section 1.2.1, line 102.

4. Protocol section 1.1.4: are these quick washes, or should there be incubation for a period of time or agitation for the wash, and should the washes be at room temperature?
The washes are carried out by brief inversion of the tubes at room temperature, and the manuscript has been modified accordingly, line 88.

5. Protocol section 1.1.5, what concentration of trypsin?
The concentration of trypsin (0.25%) has been included, line 92.

6. Protocol section 2.2: A table of the number of slides required and antibody combinations to be utilized would be helpful to ensure all the appropriate controls are appropriately planned for in the setup.
Table 1 has been included in the revised manuscript to assist with sample preparation and controls, and is referenced in line 209 and displayed beginning on line 272.

7. Protocol section 3.2.1: Does the wash take place at room temp?
Yes, room temperature washes are used, protocol has been updated, line 146.

8. Protocol section 3.3.1: Is the wash done in the dark? At what temp?
This step does not actually require darkness, and step 3.2.3 has been updated to reflect this change. Line 156 has been updated to mention washes are performed at room temperature.

9. Protocol section 3.4.5: Details on the microscope would be helpful to determine if other microscopes with similar features can be used. Also, presumably "9 Z-stack images" means that images are taken from 9 focal planes? This should just be stated more clearly here.
Section 3.4.5 has been updated to include details on the type of microscope and clarify the images necessary, lines 173-175.

10. Throughout the protocol, please provide centrifugation speeds in rcf instead of rpm..
Rcf has replaced rpm throughout in the revised manuscript.

11. Throughout, what concentration of PR619 should be used?
The concentration of PR619 is 50 μM and has been added to the protocol.

Reviewer #4:

Manuscript Summary:
As currently written, it is unclear whether this approach using these specific targets could be more broadly and generally applied in additional cell types and tissues by researchers in distinct fields. As this would certainly make the article more impactful, I strongly encourage the authors to consider this possibility and explore its feasibility based on other published, targeted studies or on large gene expression databases such as the Genotype-tissue expression (GTEx) consortium or other complementary databases and resources.
We thank the reviewer for making this excellent point. While we were initially interested in developing a technique for use by the beta cell and diabetes community, the expression of Clec16a, Nrdp1, and Usp8 in other cell types suggests the potential for broader use of our approach. As such, we have incorporated new data (new Figure 3) demonstrating that USP8:NRDP1 interaction can be visualized in SH-SY5Y neuroblastoma cells, which could suggest a value for our technique in the study of mitophagy in the neurobiology field. We have also amended the discussion to mention the potential for application of our approach to other cell types due the broad expression of these proteins.

Major Concerns:
1) This method describes what could be a broadly applicable targeted approach to assess dysfunctions in mitochondrial biogenesis and turnover. The authors should expand on this concept and suggest additional cell and tissue types (neurons seem an obvious one) where this approach could assess aspects of disease pathophysiology. Which other tissues express the NRDP1 and USP8 genes? Are there other potential mitophagy effector genes/proteins used in other cell types and tissues to which this general method could be applied to glean useful insights in the mitochondrial life cycle and mitophagic flux? Databases such as the genotype-tissue expression (GTEx) consortium portal could provide some of these speculative but potentially important insights and should be added where possible. Descriptions of the potential broader applications of this technique to other cell types, mitophagy pathways, and diseases could naturally fit in the last paragraph of the discussion or as a new, final paragraph.
GTEx consortium data demonstrate that both NRDP1 and USP8 are ubiquitously expressed with significant expression in neurons. The ubiquitous expression of NRDP1 and USP8 (as well as our previously published results displaying a similar broad expression of CLEC16A) is consistent with a role for key mitophagy proteins in many tissues. We do believe these findings could support the speculation that the CLEC16A-NRDP1-USP8 mitophagy pathway has roles in myriad cell types and have added this information to the revised discussion. At this time, we are unsure if our application of the PLA technique is pertinent for other mitophagy proteins/pathways, and this will

be a focus for future study. As mentioned above, we have expanded the use of this technique to show its applicability in SY5Y neuroblastoma cells (Figure 2), underscoring the potential for this technique to be utilized to survey mitophagy in cell types beyond beta cells. To this end, we have updated the revised manuscript reflecting these points as follows, "As NRDP1 and USP8 are ubiquitously expressed, we speculate this technique may have value for assessment of mitophagy in cell types beyond beta cells. The possibilities include other islet cell types (with relevant counterstains for alpha cells or delta cells) or cell types which heavily rely on mitophagy, such as neurons. To this end, our observation of the transferability of this technique to SY5Y neuroblastoma cells (Figure 2) or Pdx1-negative islet cells (Figure 4) could suggest the utility of our technique for the mitophagy pathway in other cell types." Lines 320-325.

2) Figure 3: The changes in mitophagy are not striking (i.e., all-or-nothing) in the Figure but rather seem quantitative. How do the authors assess changes in mitophagy? Is it through counting PLA speckles? Intensity of PLA speckles? Other metrics? Were multiple timepoints assessed/longitudinal analysis completed to identify trends in mitophagy changes? It seems important, and indeed could be an additional and useful component to the protocol/method, for them to describe the metrics that they use and any alternatives that can be used to assess mitophagy changes using this assay and approach.

We agree with the reviewer. Indeed, a method of quantification is vital for quantitative changes in protein interactions, as is the case with glucolipotoxicity, and this is a true advantage of the PLA approach. To this end, we measure the number of PLA speckles/puncta primarily at a single timepoint following 48 hours of glucolipotoxic exposure. We do agree that longitudinal analysis could prove important for interrogation of mitophagy in future studies. Accordingly, a subsection has been added to the revised protocol detailing the quantification of PLA speckles using ImageJ (Section 3.5, and new Figure 1).

3) Discussion, lines 211-214: does dissociation of the islets induce changes in mitophagy? Is it possible to complete these analyses in tissue slices/sections or even more simply in intact, non-dissociated islets?

While we dissociate islets immediately prior to centrifugation and fixation to minimize negative effects, it is possible that dissociation of islets could affect our outcomes. In general, numerous groups utilize gentle enzymatic dispersal of islets into single cells for many downstream studies without untoward effects (transcriptomic sequencing, flow cytometry, functional assays, imaging). We have not performed quantitative side-by-side comparisons of dissociated and non-dissociated islets and thus are unsure if this affects the final results. We are also unsure if our NRDP1 and USP8 antibodies are compatible with PLA on paraffin-embedded or cryosections of islets. In general, other groups have performed PLA on pancreatic sections, so we believe it is possible. We are also unsure if the PLA technique would be successful with intact islets, but we speculate that the inability of antibodies to penetrate cells within the core of an intact islet may prove to be a limitation of the approach. To this end, we have added the following to the revised manuscript, "However, we cannot exclude the possibility that islet dispersion could interfere with mitophagic complexes within cells independently of relevant treatments." Lines 304-306.

Do reagents such as antibodies exist that make it possible to extend these analyses to assess and quantify the differences in specific non-beta cell types such as alpha or delta? Adding context around some of these questions throughout the methods paper is recommended.

We agree with the reviewer about this interesting and important question. As noted above, we did observe PLA speckles in PDX1 negative islet cells (Figure 4), which could indeed be alpha cells, suggesting that our technique could be applicable for other islet cell types. We do believe that the PLA technique would be compatible with alpha cell-specific or delta cell-specific counterstains and thus, our technique could be used to ascertain the function of the mitophagy pathway in these cells as well as beta cells.

Minor Concerns:

1) Unclear that beta-cell should be hyphenated. Recommend to remove hyphens between "beta" and "cell" throughout the manuscript

Hyphens have been removed in beta cell throughout.

2) Summary (line 24): it doesn't seem that small "sample" numbers are limiting, as one can obtain islets from hundreds of individuals/donors. It might be more appropriate to change "small sample numbers" to "limited biological material" or "small cell numbers" or "limited clinical samples"

Line 25 has been amended to “limited biological material”.

3) Abstract, line 34: Conventional nomenclature for human proteins is all caps, no italics. Therefore, should Nrdp1 be all caps as USP8 is? If not, why?

Proteins have been amended to adhere to guidelines governing human, or mouse, nomenclature as suggested by the reviewer.

4) Abstract, line 31: to make this methods article more broadly appealing, it is suggested the authors change "utilized in primary human pancreatic islets" to "utilized in tissue samples such as pancreatic islets"

Lines 30-32 have been updated to be more inclusive, “Assessment of mitophagy is challenging and often requires genetic reporters or multiple complementary techniques not easily utilized in tissue samples, such as primary human pancreatic islets”.

5) Abstract, line 36: suggest changing "inputs" to "factors" or "conditions"

Line 38 has been updated to factors.

6) Abstract, line 38: suggest deleting ,"which are" to make the sentence "need for large quantities of cellular extracts required for other…"

Line 39 has been updated, “which are” deleted.

7) Introduction, line 59: remove commas flanking "Clec16a and Nrdp1"

Line 60, commas removed.

8) Introduction, line 60: change "has been a recent advancement" to "has been a recent advance"

Line 64, updated to “has been a recent advance”.

9) Protocol: Add degrees symbol between number and "C" for all entries; change "spin" to "centrifuge"

Degree symbols have been added to all temperatures, and spin replaced by centrifuge throughout.

10) Protocol, 1.1.1: Many protocols culturing human islets in Prodo media use the human AB serum instead of 10% FBS. Is FBS really recommended over the Prodo AB serum media additive component? Does it matter or not? Might be useful to add "10% FBS or human AB serum". Also, denote this media composition as "islet media" so it's clear in 1.1.2 what the "islet media" composition is.
Step 1.1.1 has been amended to include the use of either 10% FBS or human AB serum, as recommended by Prodo Laboratories. We have found no detrimental consequences of use of FBS for these studies. Media composition is added to the revised manuscript.

11) Protocol, 1.1.4, what is the concentratin of PR619?
The concentration of PR619 is 50 μM. This has been added to the protocol.

12) Protocol, 1.1.5, change "with PR619" to "containing PR619" and delete the comma following PR619. Also indicate the concentration of PR619 in this trypsin solution
Section 1.1.5 has been amended accordingly and includes the concentration of PR619 (50 μM).

13) 1.1.6, delete "quenched" after trypsin; change "including PR619" to "containing PR619"
Section 1.1.6 has been amended accordingly.

14) 1.1.7, indicate concentration of PR619 in PBS
The concentration of PR619 has been added (line 97).

15) 1.1.8, is this PBS with or without PR619?
This PBS does contain PR619 and the protocol has been amended to reflect this (line 98).

16) 1.2.1. Are the microscope slides treated with poly lysine or other solution to help the cells adhere to the glass surface? Add a space between 500 and rpm.
The slides are uncoated, but they are charged. This information has been added to line 102.

17) 1.2.2: remove "was", indicate vendor for pap-pen, change "antibody/PLA solution use" to "antibody/PLA solution volumes needed"
Section 1.2.2. has been amended, and the hydrophobic pen has been added to the table of materials.

18) 1.2.3: 4% PFA in PBS? Other diluent?
The PFA is made in PBS, and the protocol has been amended to reflect this, line 106.

19) 1.2.4: indicate maximum time the samples could be stored in PBS at 4 degree C (24 hours as indicated in first sentence or longer?)
The maximum time should be no longer than 48 hours in PBS, and this has been added to section 1.2.4 (line 108).

20) Indicate vendors for antibodies

Vendors are listed within the table of materials, and this has been referenced in the revised protocol (line 121).

21) 2.2.1: is the Triton X-100 concentration 0.3% as above or 0.1%, or other?
A reference to the table of materials has been added, which denotes the composition of the PBT buffer (lines 122).

22) 3.1.1: please indicate if the 1:5 solution is the final concentration/dilution, or if it's some other mixture of 1:5 dilutions of each of the mouse/rabbit probe solutions
This has been clarified to ensure readers understand to make a final concentration of 1:5 of the probes as follows, "by preparing a final concentration of 1:5 solution of both anti-mouse and anti-rabbit probe solution in PBT" (lines 135-137).

23) 3.1.3: is the 1:600 dilution of the Cy5 secondary the final concentration/dilution of the antibody in the probe solution? Or is it some other final concentration?
1:600 is the final concentration of Cy-5 antibody, and section 3.1.3. has been updated to clarify this, "add anti-goat Cy5 secondary at a final concentration of 1:600 to the probe solution" (lines 139-140).

24) 3.2.1: refer to wash buffer A as "Buffer A" instead to be consistent with the table listing materials, solutions, and reagents
Buffer names have been updated to correlate to the materials table, lines 146, 156, 166, and 168.

25) 3.2.2: what is the stock concentration of ligase? It might be easier to just indicate the final concentration of ligase that should be in the solution. Change "DEPC water and just before incubation" to "DEPC water. Immediately before incubation, add ligase…"
Line 149 has been updated accordingly. The final concentration of ligase (0.025 U/μL) has been included.

26) 3.3.1: same comment as 3.2.1 above with Wash Buffer A
Amended as above (#24).

27) 3.3.2: Delete "That is"; Change "Just before addition to cells…" to "Add a 1:80 dilution of polymerase to the solution immediately prior to adding the solution to the cells
Section 3.3.2. has been amended accordingly, lines 157-160.

28) 3.3.3: suggest changing text to "37C for between 1 hour, 40 minutes to 2 hours…"
Section 3.3.3. has been changed to reflect this.

29) 3.4.1: same comment as 3.2.1 and 3.3.1 above about renaming "Wash Buffer B" to "Buffer B" for clarity and consistency
Buffers changed for consistency throughout.

30) 3.4.2: Is 0.01X correct? Or was 1X or 0.1X perhaps meant instead?
0.01X is correct for this final wash step.

31) 3.4.5: What other scopes would be compatible with this imaging? Confocal, widefield, spinning disk confocal, other? Recommend changing "on an Olympus IX81" to "on an Olympus IX81 or equivalent [confocal, widefield with deconvolution software/capabilities, etc.] microscope"
Section 3.4.5. has been updated to highlight the microscopes compatible with this technique, lines 173-175.

32) 3.4.6: remove "was utilized"; change "background was minimized" to "background is minimized" and "z-stacks were then analyzed" to "z-stacks should be analyzed"; change "positive cells were analyzed" to "positive cells are analyzed"
3.4.6. grammatical errors have been amended as recommended.

33) It appears the convention for the beta cell line is "MIN6" instead of "Min6" (see PMID 2163307 and other publications)
MIN6 has been adopted throughout as requested.

34) Representative results: line 147 change "on to" to "onto"
This has been updated in line 204.

35) Line 148: delete "onwards"
36) Line 149: delete "at all"
37) Line 150: change "(Fig1C), and 4) both…" to "(Fig1C), or 4) both…"
38) Line 151: change "single or no primary antibody conditions,…" to "single primary antibody or no primary antibody control conditions,…"
39) Line 154: change "within human islets and to analyze" to "with human islets to analyze"
40) Line 157: change "~80% of the islet mass" to "~80% of islet mass"
41) Line 158: change "smaller fraction" to "smaller range", add Brissova et al., 2005 (J Histochem Cytochem) to the references cited, and provide the range shown in Figure 7 or written in the text.
42) Line 161: change "which would occur from co-immunoprecipitation" to "which could occur in co-immunoprecipitation"
43) Figure 1 legend (line 187): change "proteins in present" to "proteins is present"
For items #35-43, all changes have been made in the revised manuscript as described.

44) Figure 3 legend: please indicate the amount of time MIN6 cells were exposed to the control or glucolipotoxic conditions. Also indicate the concentration of palmitate used
MIN6 cells were exposed to the control or glucolipotoxic conditions for 48 hours. 0.4 mM palmitate was used for cell treatments. We have added this information in the revised manuscript.

45) Discussion: line 204, italicize "in vitro"
This has been updated in line 279.

46) Line 205: change "However, the PLA studies we reported" to "…PLA studies we report…"
This has been updated in line 280.

47) Line 220: suggest adding Arda et al., 2016 (Cell Metabolism) to the list of references for flow cytometry approaches to enrich specific islet cell types in addition to studies currently cited

We agree with the reviewer and have added this reference to the revised manuscript.

48) Line 224: delete either "provides" or "allows" from the sentence

49) Line 234: change "in the presence of disruption" to "by the disruption"

50) Line 237: delete "DUB", which isn't defined and hasn't been used previously and replace with the full text of the acronym's meaning, presumably "deubiquitnase"?

51) Line 246: change "quantitative analysis of mitophagy..." to "quantitative analysis of mitophagy complex formation with single cell resolution in small quantity...."

52) Table: change "Sigma-Aldritch" to "Sigma-Aldrich" or to their new name "Millipore Sigma"

For items #48-52, all changes have been made in the revised manuscript as described.