Chapter 13

Methods to Study PTEN in Mitochondria and Endoplasmic Reticulum

Sonia Missiroli*, Claudia Morganti*, Carlotta Giorgi, and Paolo Pinton

Abstract

Although PTEN has been widely described as a nuclear and cytosolic protein, in the last 2 years, alternative organelles, such as the endoplasmic reticulum (ER), pure mitochondria, and mitochondria-associated membranes (MAMs), have been recognized as pivotal targets of PTEN activity.

Here, we describe different methods that have been used to highlight PTEN subcellular localization.

First, a protocol to extract nuclear and cytosolic fractions has been described to assess the "canonical" PTEN localization. Moreover, we describe a protocol for mitochondria isolation with proteinase K (PK) to further discriminate whether PTEN associates with the outer mitochondrial membrane (OMM) or resides within the mitochondria. Finally, we focus our attention on a subcellular fractionation protocol of cells that permits the isolation of MAMs containing unique regions of ER membranes attached to the outer mitochondrial membrane (OMM) and mitochondria without contamination from other organelles. In addition to biochemical fractionations, immunostaining can be used to determine the subcellular localization of proteins; thus, a detailed protocol to obtain good immunofluorescence (IF) is described. The employment of these methodological approaches could facilitate the identification of different PTEN localizations in several physiopathological contexts.

Key words PTEN, Subcellular fractionation, Nuclear extraction, Immunofluorescence, Mitochondria, Endoplasmic reticulum, Mitochondria-associated membranes (MAMs)

1 Introduction

A phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [1] is among the most commonly lost or mutated tumor suppressors in human cancers [2], and it is a key regulator of a wide range of biological functions in addition to tumor suppression. Recently, PTEN has been demonstrated to localize or associate with organelles and specialized subcellular compartments, such as the nucleus, nucleolus, mitochondria, ER, and MAMs [3–7].

*Both are contributed equally to this work

Leonardo Salmena and Vuk Stambolic (eds.), *PTEN: Methods and Protocols*, Methods in Molecular Biology, vol. 1388, DOI 10.1007/978-1-4939-3299-3_13, © Springer Science+Business Media New York 2016

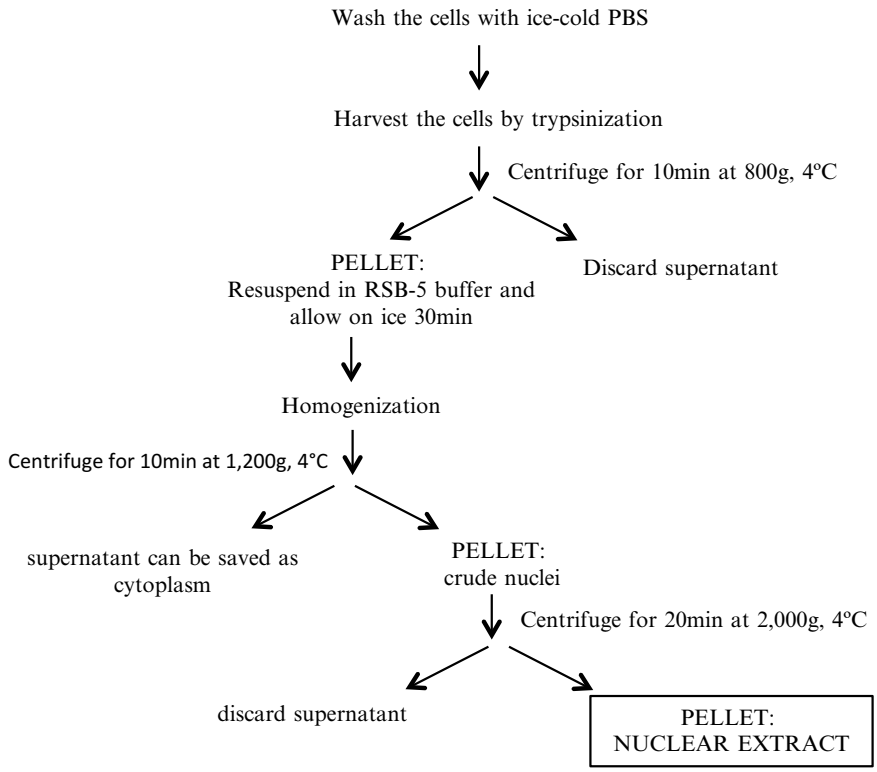

Fig. 1 Schematic steps of the nuclear extraction from cell culture

Here, we provide detailed protocols to investigate PTEN localization in the cell. A nuclear extraction protocol from cell culture can be used to determine the known localization of PTEN at the nucleus (*see* Fig. 1). Nevertheless, mitochondria isolation followed by PK digestion provides a detailed localization of a protein in the mitochondria (*see* Fig. 2). Moreover, the employment of PK enables discrimination between cytosolic proteins loosely bound to the OMM or intramitochondrial proteins integrated to outer or inner membranes. Using these different methods, it has been demonstrated that PTEN is not able to enter the mitochondria [8] and is predominately loosely bound to the OMM [3].

Using different approaches, including subcellular fractionation protocol [9] and the IF technique, Bononi et al. demonstrated that a fraction of PTEN localizes also to the ER and MAMs [3] (*see* Fig. 3, 4, 5, 6, and 7). These intracellular domains are involved in calcium (Ca^{2+}) transfer from the ER to mitochondria and apoptosis induction. Indeed, MAMs represent a specific molecular platform to receive Ca^{2+} signals from ER and transmit them to mitochondria, for the regulation of several processes including cell death [10–14].

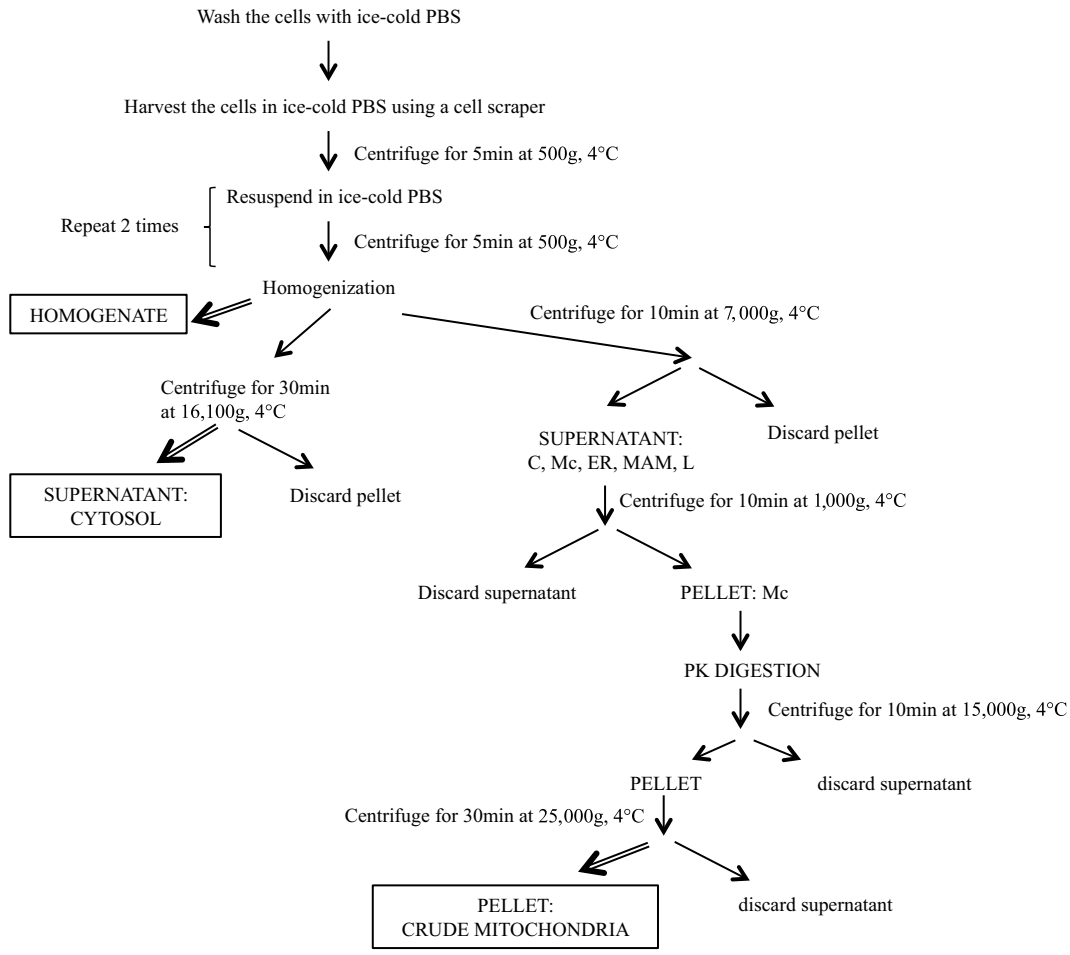

Fig. 2 Schematic representation of the mitochondrial isolation steps for the PK assay protocol. *C* cytosol; *H* homogenate; *ER* endoplasmic reticulum; *L* lysosomes; *MAMs* mitochondria-associated membranes; *Mc* crude mitochondria; *Mp* pure mitochondria; *PK* proteinase K

At the ER and MAMs, PTEN interacts with the inositol 1,4,5-trisphosphate receptors (IP3Rs) and regulates Ca^{2+} release from the ER in a protein phosphatase-dependent manner that counteracts Akt activation; thus, it can inhibit Akt-mediated phosphorylation of IP3R3 [15], which protects from Ca^{2+}-mediated apoptosis [3].

Intracellular fractionation techniques should be supported by other methods to further validate the localization of PTEN [16]. IF can be used to compare the localization of a protein of interest against known markers of intracellular structures. Here, we have provided a standard protocol of IF that can be used to simultaneously compare the localization of PTEN against known markers of the nucleus, mitochondria and ER (*see* Fig. 7). This protocol

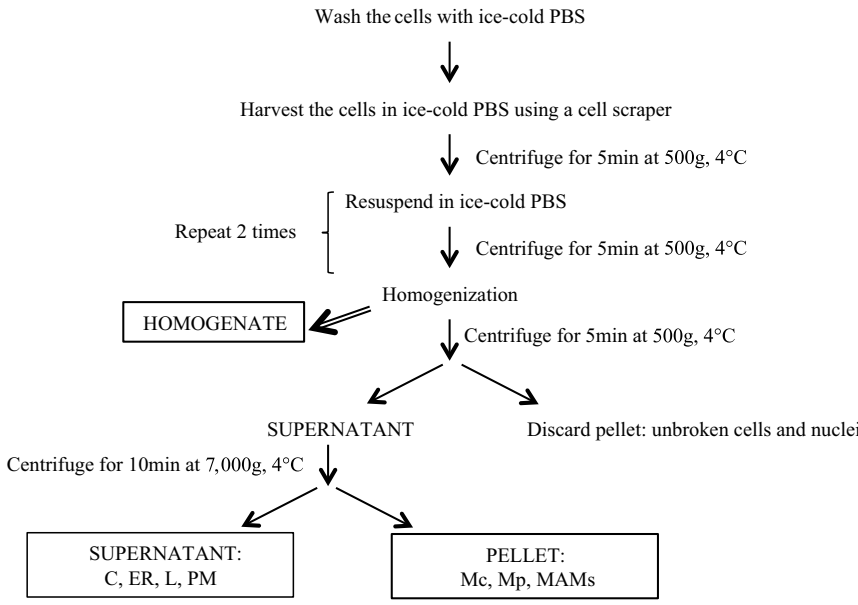

Fig. 3 Schematic steps of the subcellular fractionation protocol from cells. *C* cytosol; *ER* endoplasmic reticulum; *L* lysosomes; *MAMs* mitochondria-associated membranes; *Mc* crude mitochondria; *Mp* pure mitochondria; *PM* plasma membrane

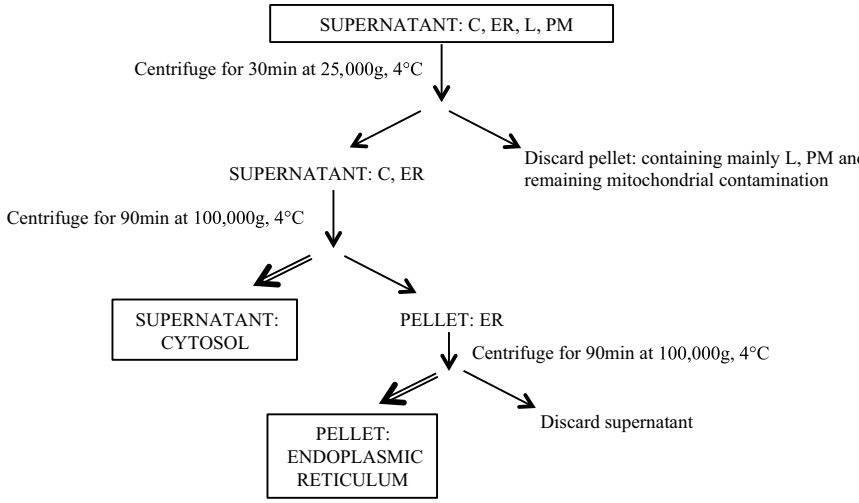

Fig. 4 Schematic steps of the subcellular fractionation protocol from cells for ER and C isolation. The final steps of the presented procedure result in the isolation of pure microsomes (endoplasmic reticulum (ER)) and cytosolic fraction. *C* cytosol; *ER* endoplasmic reticulum; *L* lysosomes; *PM* plasma membrane

can be used as a model to analyze unknown protein localization compared with markers of subcellular compartments, including MAMs. Tables 1, 2, and 3 indicate suitable alternative markers that can be used in IF localization analysis to ensure that fluorescence emission overlap is avoided.

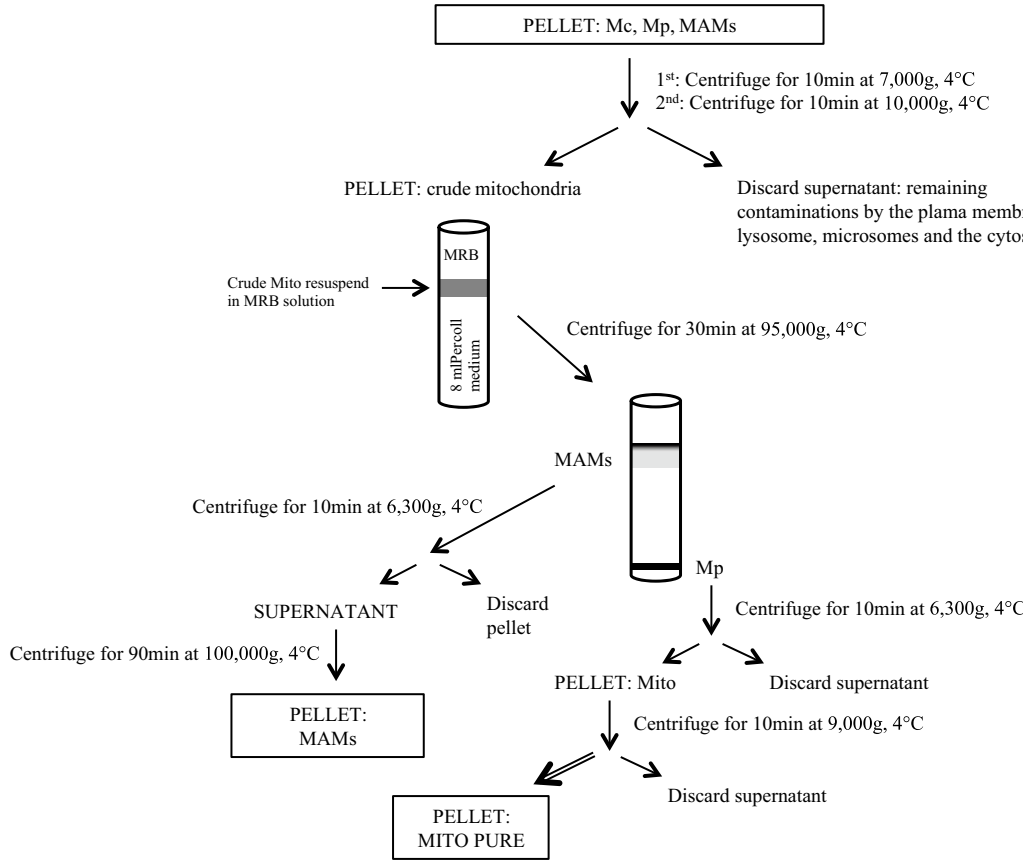

Fig. 5 Schematic steps of the subcellular fractionation protocol from cells for MAMs and Mp isolation. The final steps of the presented procedure result in the isolation of crude mitochondria, pure mitochondria, and mitochondria-associated membrane (MAM) fractions. MAMs, mitochondria-associated membranes; *Mc* crude mitochondria; *Mp* pure mitochondria

2 Materials

2.1 Nuclear Extraction from Cell Culture

2.1.1 Equipment

1. Cell culture dishes, 10 cmϕ.
2. Cell scrapers.
3. Pasteur pipette.
4. 1.5 ml Eppendorf microfuge test tubes.
5. Sigma rotor angular 6 × 30 ml.
6. Refrigerated Sigma low-speed centrifuge (Sigma (Braun), Model 2 K15, tabletop).
7. 7 ml Dounce tissue homogenizer.
8. Microcentrifuge.

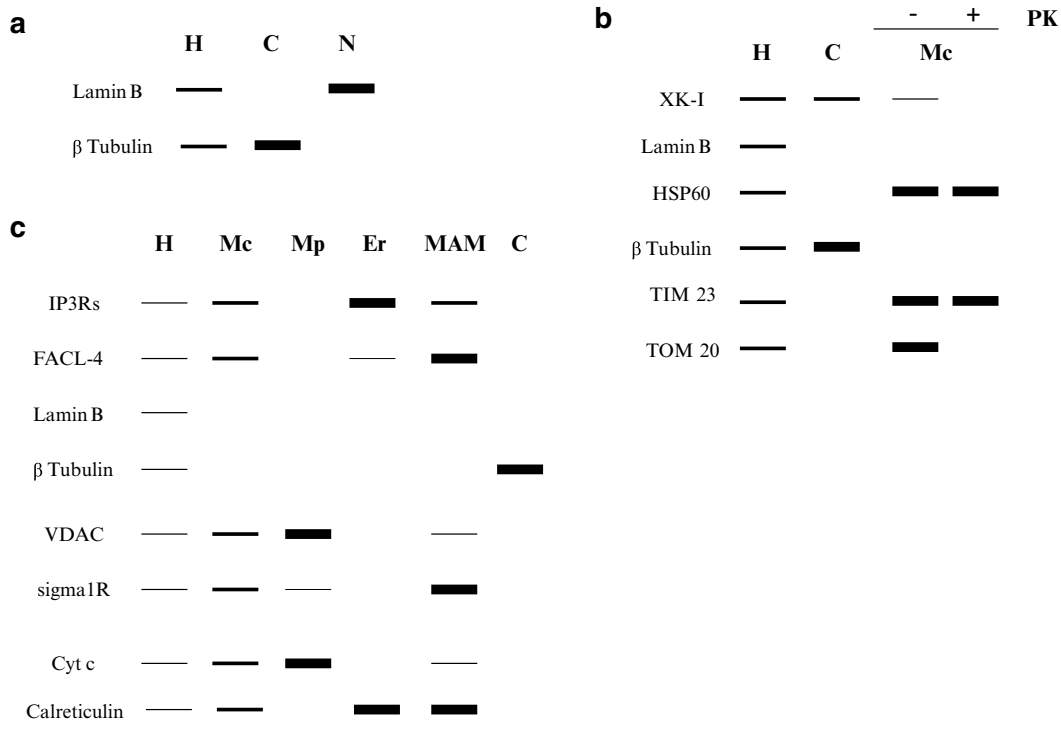

Fig. 6 Schematic representation of how markers for fractions should be distributed *H* homogenate; *Mc* crude mitochondria; *Mp* pure mitochondria fraction; *ER* endoplasmic reticulum; *MAMs* mitochondria-associated membranes; *C* cytosol; *N* nucleus. (**a**) Schematic model of markers for nuclear extraction. (**b**) schematic representation for mitochondrial isolation for PK. (**c**) Schematic representation for subcellular localization

2.1.2 Reagents

1. Dulbecco's phosphate-buffered saline (D-PBS), liquid, without Ca^{2+} and Mg^{2+}.
2. IGEPAL CA-630.
3. Sodium chloride (NaCl).
4. Mg acetate tetrahydrate (Mg acetate).
5. Magnesium chloride ($MgCl_2$).
6. Phenylmethanesulfonyl fluoride (PMSF).
7. Protease inhibitor cocktail (100×).
8. Sodium dodecyl sulfate (SDS).
9. Sodium fluoride (NaF).
10. Sodium orthovanadate (Na_3V0_4).
11. Sucrose.
12. Trizma-Base.
13. Trypsin, 0.25 % (1×) with EDTA $4Na^+$, liquid.

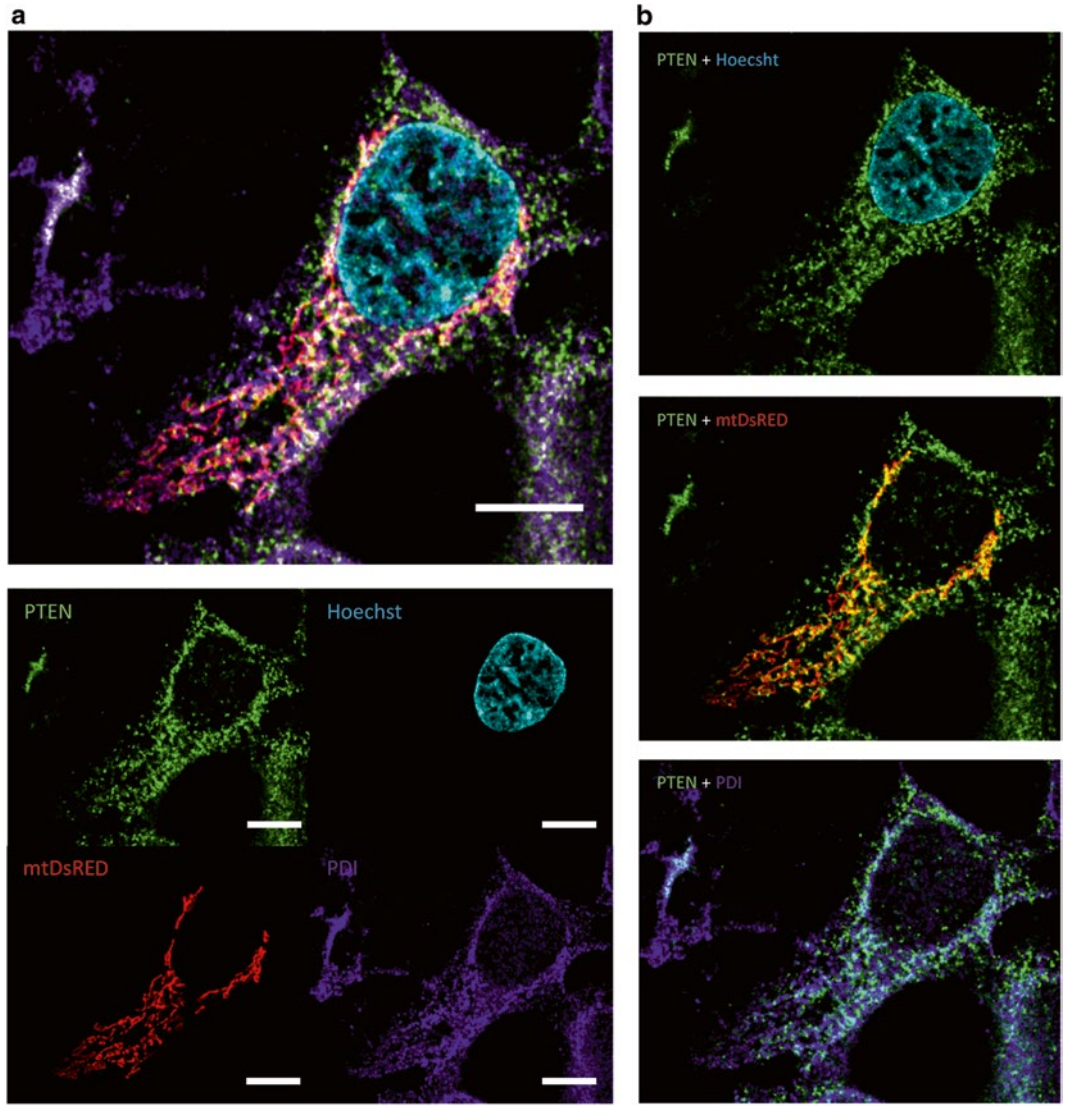

Fig. 7 Localization of PTEN by IF. (**a**) HEK-293 cells transfected with mtDsRED (mitochondria, *red*) were immunostained for PTEN (*green*), PDI (ER, *blue*), and loaded with Hoechst (nucleus, *cyan*). Representative merge images of the four channels and single images of each one are shown. (**b**) Merge images below show a succession of overlapping signals between PTEN and Hoechst, PTEN and mtDsRED, and PTEN and PDI. Scale bar, 10 mm. Figure modified from [3]

2.1.3 Reagent Preparation

1. *1 M Tris–HCl* (pH 7.4): Dissolve 121.14 g of Trizma-Base in 500 ml of bi-distilled water, adjust the pH to 7.4 with HCl, bring the solution to 1 l with bidistilled water, and store at 4 °C. The solution remains stable for a long time period.
2. *RSB-5 buffer*: 10 mM Tris–HCl, 10 mM NaCl, and 5 mM $MgCl_2$ (pH 7.4). Dissolve 0.0584 g of NaCl and 0.1017 g of $MgCl_2$ in 50 ml of bi-distilled water and add 1 ml of Tris–HCl.

Table 1
DNA constructs

	Acronym	Reference
Mitochondria	mtDsRED	
	mtGFP	[18]
Nucleus	H2BRFP	
ER	er-RFP	
	GFP-Sec61	[19]
PTEN	GFP-PTEN	
	FLAG-PTEN	[20]
	PTEN-YFP	[21]
	HA-tagget	
	PTEN	[22]
	cherry-PTEN	[23]

Table 2
Staining

Compartment	Staining
Mitochondria	Mito Tracker® Green, Red, Deep Red, Orange
Nucleus	Hoechst 33342
	Dapi
	DRAQ5™
ER	CellLight® ER-RFP

If necessary, adjust the pH to 7.4 and bring the solution to a final volume of 100 ml with bi-distilled water; store at 4 °C.

3. *0.88 M sucrose and 5 mM Mg acetate*: Dissolve 15.06 g of sucrose and 0.0536 g Mg acetate in 50 ml of bi-distilled water. Vortex carefully. If the powder is not completely dissolved in the solution, increase the temperature to put it at 37 °C. Store at −20 °C to avoid contamination.
4. *RIPA buffer*: 50 mM Tris–HCl (pH 8), 150 mM NaCl, 1 % IGEPAL CA-630, 0.5 % sodium deoxycholate (DOC), and 0.1 % SDS. Add 2.5 ml Tris–HCl 1 M (pH 8), 1.5 ml NaCl 5 M, 500 µl IGEPAL, 2.5 ml DOC stock 10 %, and 500 µl SDS 10 % and bring the solution to 50 ml with bi-distilled water.

Table 3
Antibodies

	Antibodies	Reference
Mitochondria	HSPD1 (1:100, Abcam)	[24]
ER	PDI (1 : 100, Abcam) Sec61 (1:100, Abcam) IP3R (1:50, BD Bioscience)	
MAMs	SIGMAR1 (1:100, Sigma)	
PTEN	PTEN (6H2.1) (1:200, Cascade Bioscience)	[25]
	PTEN (1:100, Cell Signaling Technology)	[7]

2.2 Mitochondria Isolation for PK Assay

2.2.1 Equipment

1. Cell culture dishes, 10 cmϕ.
2. Cell scrapers.
3. Pasteur pipettes.
4. 7 ml glass/Dounce tissue grinder.
5. 1 ml Dounce tissue grinder.
6. 1.5 ml Eppendorf microfuge test tubes.
7. Sigma rotor angular 6 × 30 ml.
8. Refrigerated Sigma low-speed centrifuge.
9. Olympus microscope LKX31.
10. Microcentrifuge.

2.2.2 Reagents

1. D-Mannitol.
2. D-PBS, liquid, without Ca^{2+} and Mg^{2+}.
3. Ethylene-bis(oxyethylenenitrilo) tetraacetic acid (EGTA).
4. HEPES.
5. Percoll.
6. PMSF.
7. Protease inhibitor cocktail (100×).
8. Sodium fluoride.
9. Sodium orthovanadate.
10. Sucrose.
11. Trizma-Base.

2.2.3 Reagent Preparation

Prepare all solutions using bi-distilled water. Extreme care should be taken to avoid contamination with ice and tap water in all preparations. Wash all glassware with bi-distilled water to avoid Ca^{2+} contamination, which can cause swelling of the mitochondria and rupture of the OMM.

1. *0.1 M EGTA/Tris* (pH 7.4): Dissolve 3.8 g of EGTA in 90 ml of bi-distilled water, adjust pH to 7.4 with Tris powder, bring the solution to 100 ml with bi-distilled water and store at 4 °C. The solution remains stable for a long time period.
2. *0.1 M Tris-MOPS* (pH 7.4): Dissolve 0.6057 g of Trizma-Base in 40 ml of bi-distilled water, adjust pH to 7.4 using MOPS powder, bring the solution to 50 ml with bi-distilled water and store at 4 °C.
3. *1 M sucrose*: In 1 l of bi-distilled water, dissolve 342.3 g of sucrose, mix well and prepare 25 ml aliquots. Store at −20 °C.
4. *Mitochondria buffer (MT)*: 200 mM sucrose, 10 mM Tris/MOPS and 0.5 mM EGTA-Tris (pH 7.4). To prepare 100 ml of MT solution, add 10 ml of 0.1 M Tris-MOPS and 100 µl of 0.1 M EGTA/Tris to 25 ml of 1 M sucrose. Bring the solution to 100 ml with bi-distilled water, adjust pH to 7.4 using Tris or MOPS powder and store at 4 °C (*see* **Note 1**).
5. *10× Proteinase K (PK)*: In 10 ml of MT buffer dissolve 10 mg of proteinase K. Store on ice (*see* **Note 2**).

2.3 Subcellular Fractionation Protocol from Cells

2.3.1 Equipment

1. Cell culture dishes, 10 or 15 cmϕ.
2. Cell scrapers.
3. Pasteur pipettes.
4. 40 ml glass/Dounce tissue grinder.
5. 1 ml Dounce tissue grinder. Loose pestle.
6. Ultra-Clear 14-ml polybrene tubes (Beckman, cat. no 344060) for SW40 rotor.
7. Polycarbonate tubes with cap assembly (Beckman, cat. no. 355618) for 70-Ti rotor.
8. 1.5 ml Eppendorf microfuge test tubes.
9. 70-Ti rotor (Fixed angle, 8 × 39 ml, 70,000 rpm, 504,000 × *g*) (Beckman, cat. no. 337922).
10. SW 40 rotor (Swinging bucket, 6 × 14 ml, 40,000 rpm, 285,000 × *g*) (Beckman, cat. no. 331302).
11. Sigma rotor angular 6 × 30 ml (Sigma, cat. no. 12139).
12. Beckman Coulter Optima L-100 XP Ultracentrifuge.
13. Refrigerated Sigma low-speed centrifuge (Sigma (Braun), Model 2 K15, tabletop).
14. Olympus microscope LKX31.
15. Microcentrifuge.

2.3.2 Reagents

1. D-Mannitol.
2. D-PBS, liquid, without Ca^{2+} and Mg^{2+}.
3. Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA).

4. HEPES.
5. Percoll.
6. PMSF.
7. Protease inhibitor cocktail (100×).
8. Sodium fluoride.
9. Sodium orthovanadate.
10. Sucrose.
11. Trizma-Base.

2.3.3 Reagent Preparation

Prepare all solutions using bi-distilled water. Extreme care should be taken to avoid contamination with ice and tap water in all preparations. Wash all glassware with bi-distilled water to avoid Ca^{2+} contamination, which can cause swelling of the mitochondria and rupture of the OMM.

1. *0.5 M EGTA* (pH 7.4): Dissolve 19 g of EGTA in 70 ml of bi-distilled water, adjust the pH to 7.4 with KOH, bring the solution to 100 ml with bi-distilled water and store at 4 °C. The solution remains stable for a long time period.
2. *0.5 M HEPES* (pH 7.4): Dissolve 59.57 g of HEPES in 400 ml of bi-distilled water, adjust the pH to 7.4 with KOH, bring the solution to 500 ml with bi-distilled water and store at 4 °C. The solution remains stable for a long time period.
3. *1 M Tris–HCl* (pH 7.4): Dissolve 121.14 g of Trizma-Base in 500 ml of bi-distilled water, adjust the pH to 7.4 with HCl, bring the solution to 1 l with bi-distilled water and store at 4 °C. The solution remains stable for a long time period.
4. *Homogenization buffer*: 225 mM D-mannitol, 75 mM sucrose, and 30 mM Tris–HCl (pH 7.4). Dissolve 12.30 g of D-MANNITOL and 7.7 g of sucrose in 250 ml of bi-distilled water and add 9 ml Tris–HCl (1 M pH 7.4). Leave the buffer for approximately 30 min at 4 °C to cool down. If necessary, adjust the pH to 7.4 with KOH (if too low) or HCl (if too high); bring the solution to a final volume of 300 ml with bi-distilled water and store at 4 °C (*see* **Note 1**).
   Immediately prior to use, add 60 µl of 0.5 M EGTA pH 7.4 and 3 ml of PMSF. EGTA is recommended to remove traces of Ca^{2+}.
5. *Mitochondria resuspending buffer (MRB)*: 250 mM D-mannitol, 5 mM HEPES (pH 7.4), and 0.5 mM EGTA. To prepare 50 ml of MRB solution, dissolve 2.28 g D-mannitol in 45 ml of bi-distilled water and add 500 µl of 0.5 M HEPES pH 7.4. Leave the buffer for approximately 30 min at 4 °C to cool down. If necessary, adjust the pH to 7.4 with KOH (if too low) or HCl (if too high); bring the solution to a final volume

of 50 ml with bi-distilled water and store at 4 °C (*see* **Note 1**). Immediately prior to use, add 50 μl of 0.5 M EGTA (pH 7.4).

6. *Percoll medium*: 225 mM D-mannitol, 25 mM HEPES (pH 7.4), 1 mM EGTA and 30 % Percoll (vol/vol). Dissolve 2.052 g D-mannitol in 25 ml of bi-distilled water and add 2.5 ml of 0.5 M HEPES (pH 7.4). Leave the buffer for approximately 30 min at 4 °C to cool down. If necessary, adjust the pH to 7.4 with KOH (if too low) or HCl (if too high); bring the basal solution to a final volume of 35 ml with bi-distilled water and store at 4 °C (*see* **Note 1**).

   Immediately prior to use, add 100 μl of 0.5 M EGTA (pH 7.4) and 15 ml of Percoll.

2.4 Cell Imaging

2.4.1 Equipment

1. Aluminum foil.
2. Cell culture dishes, 10 or 15 cmϕ.
3. Filter paper.
4. Forceps.
5. 6 wells, multi-well plate.
6. Nail polish.
7. Needle.
8. Parafilm.
9. Round glass coverslip (*see* **Note 3**).

2.4.2 Reagents

1. Bovine serum albumin (BSA) (Sigma-Aldrich, cat. no.A6003).
2. D-PBS, liquid, without Ca^{2+} and Mg^{2+}.
3. Hoechst 33342 (Life Technologies, cat. no. H3570).
4. Paraformaldehyde (Merk, cat. no. 8.18715.1000).
5. Poly-L-lysine (Sigma-Aldrich, cat. no. P36934).
6. Prolong Gold antifade reagent (Life Technologies, cat. no. P36934).
7. Triton X-100.
8. Vector encoding mtDsRED.
9. Primary antibodies: (a) mouse monoclonal to PTEN (A2B1) (Santa Cruz, cat. no. sc-7974), 1:50, and (b) rabbit polyclonal to PDI-ER marker (Abcam, cat. no. ab3672), 1:100.
10. Secondary antibodies: (a) Alexa Fluor 488 goat anti-mouse (Life Technologies, cat. no. A-11001), 1:1000, and (b) Alexa Fluor 633 goat anti-rabbit (Life Technologies, cat. no. A-21070), 1:1000.

2.4.3 Reagent Preparation

1. *Fixing solution*: 4 % paraformaldehyde. Dissolve 4 g of paraformaldehyde in 100 ml of PBS. The solution should be freshly prepared. Store at –20 °C.

2. *Permeabilization solution*: 0.1 % Triton X-100. Add 100 μl of Triton X-100 in 100 ml of PBS. Undiluted Triton X-100 is a clear viscous liquid; thus, use a wide orifice pipette tip or cut off the tip of a regular pipette tip to enlarge the opening.
3. *Blocking solution*: 1 % BSA. Dissolve 100 mg of BSA in 10 ml of PBS.

3 Methods

3.1 Nuclear Extraction from Cell Culture

Starting material: 4 human embryonic kidney (HEK) 293 cell confluent plates (10 cmϕ) or 8 MEF confluent plates (10 cmϕ).

1. Remove the medium and wash the cells with ice-cold PBS (Ca^{2+} and Mg^{2+} free).
2. Harvest the cells by trypsinization.
3. Centrifuge at 800 × *g* for 10 min at 4 °C.
4. Resuspend in 10 ml of RSB-5 buffer per gram of cell pellet.
5. Allow cells to swell on ice for 30 min (*see* **Note 4**).
6. Add NP40 to a final concentration of 0.3 %.
7. Transfer the cell suspension to a pre-cooled 7 ml Dounce tissue homogenizer. Homogenize 20 strokes using a tight pestle while maintaining the homogenizer on ice (*see* **Note 5**). An aliquot is saved as homogenate.
8. Centrifuge the homogenate at 1,200 × *g* for 10 min to sediment crude nuclei (the supernatant fraction can be saved as cytoplasm).
9. Resuspend nuclei in 20 volumes (of the pellet) of 0.88 M sucrose and 5 mM Mg acetate.
10. Centrifuge at 2,000 × *g* for 20 min.
11. Discard the supernatant and resuspend the pellet (nuclear extract) in RIPA buffer (please note: the nuclear pellet is hard to resuspend).
12. Maintain on ice for 20 min and vortex every 5 min.
13. Centrifuge at 16,000 × *g* for 10 min. Save the supernatant as nuclear homogenate.

3.2 Mitochondria Isolation for PK Assay

Starting material: 10 HEK confluent plates (15 cmϕ).

1. Remove the medium and wash the cells with ice-cold PBS (Ca^{2+} and Mg^{2+} free, supplemented with 2 mM Na_3V0_4 and 2 mM NaF).
2. Harvest the cells in ice-cold PBS using a cell scraper.
3. Centrifuge at 500 × *g* for 5 min at 4 °C.

4. Resuspend in ice-cold PBS.
5. Centrifuge at 500×*g*, 5 min at 4 °C (repeat two times and combine the cells in one tube).
6. Resuspend the cell pellet in MT buffer using a ratio of 5 ml of buffer per 1 ml of pellet (*see* **Note 6**).
7. Transfer the cell suspension in a 7 ml glass/Dounce tissue grinder.
8. Homogenize the cells with tight pestle. Every five strokes control the cell integrity under a microscope. Finish homogenization when 80–90 % of the cells are broken (*see* **Note 7**).
   Take ~50 μl of homogenate and immediately add protease inhibitor cocktail.
9. To obtain the cytosolic fraction, obtain a 200 μl aliquot of homogenate and centrifuge at 16,100×*g* for 30 min at 4 °C in a microcentrifuge. The supernatant is the cytosolic fraction.
10. From **step 8**, centrifuge at 600×*g* for 5 min at 4 °C.
11. Collect the supernatant and discard the pellet (which contains unbroken cells and nuclei); repeat 2–3 times (until pellet is no longer present).
12. Collect supernatant and centrifuge at 7,000×*g* for 10 min at 4 °C.

The supernatant is a cytosolic fraction that contains lysosomes and microsomes, whereas the pellet contains mitochondria.

13. Wash the pellet carefully with MT buffer and detach (*see* **Note 8**).
14. Centrifuge at 10,000×*g* for 10 min at 4 °C.
15. Discard the supernatant. Wash the pellet with MT buffer, detach 1 ml of buffer, and then carefully transfer in a 1 ml Dounce tissue grinder. Resuspend using the loose pestle (*see* **Note 9**).
16. Quantify the protein content.
17. Prepare equal aliquots of organelles in Eppendorf tubes: 100 μg in a final volume of 900 μl. Add 100 μl of MT buffer to one aliquot (*see* **Note 10**); add 100 μl of 10× Proteinase K to another aliquot (*see* Fig. 2).
18. Incubate on ice for 5 min.
19. Centrifuge at 15,000×*g* for 10 min at 4 °C in a microcentrifuge.
20. Discard the supernatants and wash the pellets with MT buffer. Add 1 mM PMSF and detach in 1 ml.
21. Centrifuge at 15,000×*g* for 10 min at 4 °C in a microcentrifuge.
22. Discard the supernatants and resuspend the pellets (mito crude fraction) in 50 μl of sample buffer.

3.3 Subcellular Fractionation Protocol for Cells

Starting material (10^9 cells): 40 HEK confluent plates (15 cmϕ) or 150 MEF confluent plates (10 cmϕ).

1. Remove the medium and wash the cells with ice-cold PBS (Ca^{2+} and Mg^{2+} free).
2. Harvest the cells in ice-cold PBS using a cell scraper.
3. Centrifuge at 500 × *g* for 5 min at 4 °C.
4. Resuspend in ice-cold PBS.
5. Centrifuge at 500 × *g* for 5 min at 4 °C (repeat 2 times and combine the cells in one tube).
6. Resuspend the cell pellet in Homogenization buffer using a ratio of 3 ml of buffer per 1 ml of pellet (*see* **Note 6**).
7. Transfer the cell suspension in a 40 ml glass/Dounce tissue grinder.
8. Homogenize the cells with tight pestle. Every 25 strokes, control the cell integrity under a microscope. Finish homogenization when 80–90 % of the cells are broken (*see* **Note 7**).
   Take ~100 μl of homogenate and immediately add 2 mM Na_3V0_4, 2 mM NaF and protease inhibitor cocktail.
9. Transfer the homogenate to a 30 ml polypropylene centrifugation tube and centrifuge at 600 × *g* for 5 min at 4 °C.
10. Collect the supernatant and discard the pellet (which contains unbroken cells and nuclei); repeat 2–3 times (until pellet is no longer present).
11. Collect the supernatant and centrifuge at 7,000 × *g* for 10 min at 4 °C.
    The supernatant is a cytosolic fraction that contains lysosomes and microsomes, whereas the pellet contains mitochondria (*see* Fig. 3).
    PAUSE POINT. Store the supernatant at 4 °C (on ice) if there is a plan to proceed with further separation of the cytosolic, lysosomal, and ER fractions (refers to point 25 and below).
12. Gently resuspend the pellet that contains mitochondria in 20 ml of ice-cold MRB (*see* **Note 8**); centrifuge the mitochondrial suspension at 7,000 × *g* for 10 min at 4 °C.
13. Discard the supernatant, resuspend the mitochondrial pellet as previously described in 20 ml of ice-cold MRB, and centrifuge the mitochondrial suspension at 10,000 × *g* for 10 min at 4 °C (*see* **Note 11**).
14. Discard the supernatant and gently resuspend the crude mitochondrial pellet in 1 ml of ice-cold MRB.
15. Carefully transfer in a 1 ml Dounce tissue grinder and resuspend using the loose pestle (*see* **Note 12**).

16. Add 8 ml of Percoll medium to the 14 ml thin-wall, polybrene ultracentrifuge tubes.
17. Layer the suspension of mitochondria collected on top of 8 ml Percoll medium in the ultracentrifuge tube; then, gently layer the MRB solution (approximately 3.5 ml) on top of the mitochondrial suspension to fill up the centrifuge tube (*see* **Note 13**).
18. Centrifuge at 95,000 × *g* for 30 min at 4 °C in a Beckman Coulter Optima L-100 XP Ultracentrifuge (SW40 rotor, Beckman). A dense band that contains purified mitochondria is localized at approximately the bottom of the ultracentrifuge tube (*see* Fig. 4). MAM is visible as the diffused white band located above the mitochondria.
19. Collect the MAM fraction from the Percoll gradient with a Pasteur pipette and dilute with 15 ml of homogenization buffer. Collect the pure mitochondrial band with a Pasteur pipette and dilute with 15 ml of homogenization buffer.
20. Centrifuge the MAM and mitochondrial suspension at 6,300 × *g* for 10 min at 4 °C (refrigerated Sigma low-speed centrifuge).
21. Discard the pellet obtained from the MAM and transfer the MAM supernatant to polycarbonate tubes with a cap assembly. Centrifuge at 100,000 × *g* for 90 min (70-Ti rotor, Beckman) at 4 °C.
22. Discard the mitochondrial supernatant obtained in **step 20** (which contains MAM contamination), gently resuspend the pellet in a small volume of MRB (300–500 μl), and centrifuge at 9,000 × *g* for 10 min at 4 °C in a microcentrifuge.
23. Resuspend the pellet (pure mitochondria) in 50–60 μl of homogenization buffer and immediately add 2 mM Na_3V0_4, 2 mM NaF and protease inhibitor cocktail.
24. Discard the supernatant (from **step 21**) and collect the pellet using a pipette; resuspend in a small volume of MRB (100–200 μl), and immediately add 2 mM Na_3V0_4, 2 mM NaF and protease inhibitor cocktail.
25. From **step 11**, to proceed with further separation of the cytosolic, lysosomal and ER fractions, centrifuge the supernatant at 25,000 × *g* for 30 min at 4 °C.
26. Discard the pellet that contains lysosome, plasma membrane, and remaining mitochondrial contamination. Transfer the supernatant to polycarbonate tubes with a cap assembly and centrifuge the supernatants at 100,000 × *g* for 90 min at 4 °C (*see* **Note 14**).
27. The supernatant consists of cytosolic fraction; collect 1 ml and immediately add 2 mM Na_3VO_4, 2 mM NaF and protease

inhibitor cocktail. The pellet consists of ER fraction; carefully wash with 1 ml of homogenization buffer and then detach and resuspend in 15 ml of homogenization buffer (do not pipette the pellet).

28. Centrifuge at 100,000 × *g* for 90 min at 4 °C (*see* **Note 14**).
29. Carefully discard the supernatant. Using a pipette with the cut-out end, collect the pellet (ER fraction) in a small volume of homogenization buffer (200–300 μl) (*see* Fig. 5).

    Transfer in a 1 ml Dounce tissue grinder and resuspend using the loose pestle. The result is the ER fraction. Immediately add 2 mM Na_3VO_4, 2 mM NaF and protease inhibitor cocktail.

The quality of protocol preparation can be checked by western blot analysis. Using different markers for the fractions obtained, it is possible to control the purity of each fraction and the presence of contaminations from other compartments. Figure 6 reports, as an example, the marker distribution that characterizes each fractionation from cells:

- Lamin B1 antibody is extremely useful as a nuclear loading control.
- Tubulin is used as a cytosolic marker.
- Hexokinase I (HXK-I) is used as a digestion control in mitochondrial isolation for the PK assay. It should be absent after PK treatment.
- HSP60, a constitutively expressed mitochondrial protein, localizes in the mitochondrial matrix; thus, after digestion with PK, it should be still present in the mito crude fraction.
- TIM 23, a translocase of the inner membrane, is an integral membrane protein of the mitochondrial protein translocation machinery; thus, after digestion with PK, it should be still present in the mito crude fraction.
- TOM 20, a translocase of the outer membrane, is a multisubunit protein complex that facilitates the import of nucleus-encoded precursor proteins across the OMM. After digestion with PK, it should be absent in the mito crude fraction.
- IP3Rs, an ER channel responsible for agonist-dependent ER-Ca^{2+} release, is used as an ER marker.
- Calreticulin, an ER-Ca^{2+}-buffering protein that binds misfolded proteins and prevents them from exportation from the ER to the Golgi apparatus, is used as ER and MAM markers and should be present in both fractions at comparable levels.
- Voltage-dependent anion channel (VDAC), which is localized on the OMM and is responsible for the permeability of the membrane, is used as a mitochondrial marker. It should be

present in the crude mitochondria, but it must be enriched in the Mp fraction.

- Cytochrome c (Cyt c) is a component of the electron-transfer chain localized in the mitochondrial inter-membrane space; it is used as a mitochondrial marker and should be extremely enriched in the Mp fraction.
- The sigma-1 receptor (Sig1R) as a MAM marker should be enriched in this fraction.
- Long-chain fatty-acid CoA synthase (FACL)-4 is important for the synthesis of cellular lipids and β-oxidation degradation. As a MAM marker, it should be extremely enriched in the MAM fraction.

3.4 Cell Imaging

The cell preparation, transfection methods, staining, optimal dilutions, and incubation times for the primary and secondary antibodies will need to be empirically determined. IF requires careful interpretation. There may be autofluorescence, nonspecific binding of the secondary antibody, and the primary antibody may not only bind to the expected protein. Thus, it is necessary to include controls: (1) a non-processed sample to control for autofluorescence (*see* **Note 15**); (2) a sample incubated with secondary antibody only to determine the nonspecific staining of the secondary antibody; and (3) a non-immune serum or isotype Ig (which matches the primary antibody) to determine the non-specific staining of the primary antibody.

1. Prepare the coverslip (*see* **Note 16**): place glass coverslips (24 mm in diameter) in a 6-well plate and coat them with 1 % poly-L-Lysine in PBS for 1 h at RT; wash 3 times with sterile ultrapure H_2O and allow the coverslips to dry completely (*see* **Note 17**).
2. Seed the cells on the dry coverslips (*see* **Note 18**) and allow them to grow.
3. When the cells have reached 50 % confluence, transfect them with DNA (Table 1), e.g., 3 μg of vector that encodes mtD-sRED per well (*see* **Note 19**).
4. After 36–48 h (*see* **Note 20**), remove the medium and wash 3 times with 2 ml PBS per well.
5. Stain (Table 2) the nuclei with 0.25 μl/ml Hoechst 33342 (Life Technologies) in PBS for 10 min at 37 °C in the dark (*see* **Note 21**).
6. Remove the staining solution and wash 3 times with 2 ml PBS per well.
7. To fix the cells, add 2 ml per well of fixing solution and incubate for 10 min at 37 °C (*see* **Note 22**).

8. Wash 3 times with PBS for 5 min with gentle shaking to remove the residual paraformaldehyde.
9. Add 2 ml per well of permeabilization solution (*see* **Note 23**) for 15 min at RT with gentle shaking; then wash 2 times with 2 ml PBS per well.
10. Incubate with 2 ml of blocking solution per well for 20 min at RT (*see* **Note 24**).
11. Prepare the primary antibodies (Table 3) by diluting them in blocking buffer: e.g., (a) mouse monoclonal to PTEN (A2B1) 1:50 (Santa Cruz); (b) rabbit polyclonal to PDI-ER marker 1:100 (Abcam).
12. Remove the blocking buffer by holding each coverslip by draining it onto a sheet of fiber-free paper (*see* **Note 25**); turn it cell-side down on a 50 μl drop (*see* **Note 26**) of the primary antibody solution placed on a layer of parafilm. Incubate overnight at 4 °C in the humidified chamber (*see* **Note 27**).
13. Carefully turn the coverslip cell-side up and wash 3 times for 10 min each with 2 ml of 1 % Triton X-100 with gently shaking.
14. Prepare the secondary antibodies (*see* **Note 28**) conjugated with two different fluorochromes by diluting them in blocking buffer, e.g., (a) Alexa Fluor 488 goat anti-mouse (Life Technologies) 1:1000 and (b) Alexa Fluor 633 goat anti-rabbit (Life Technologies) 1:1000.
15. Add at least 200 μl (*see* **Note 29**) of the secondary antibody mix on the cells and incubate for 1 h at RT in the dark.
16. Remove the secondary antibodies and wash 3 times for 10 min each with 2 ml of 1 % Triton X-100 with gently shaking; at the end, leave the coverslip in PBS.
17. Mounting (*see* **Note 30**): invert each coverslip on a slide that contains a drop of mounting medium (Prolong Gold antifade reagent) and allow it to dry.
18. The next day, seal the edges of each coverslip with transparent nail polish and store in the dark at 4 °C until image acquisition.
19. Acquire each field over 26 *z*-planes spaced 0.4 μm on an Axiovert 220 M microscope equipped with an ×100 oil immersion Plan-Neofluar objective (NA 1.3, from Carl Zeiss, Jena, Germany) and a CoolSnap HQ CCD camera. Image sampling was below the resolution limit and calculated according to the Nyquist calculator (*see* **Note 31**).
20. After acquisition, deconvolute the *z*-stacks with the "Parallele Iterative Deconvolution" plugin of the open source Fiji software (*see* **Note 32**).

21. Merger of the fluorescence images is the most prevalent visual method used to evaluate the colocalization of two probes (*see* **Note 33**).

A representative image of the result of this protocol is shown in Fig. 7.

3.4.1 Quantification of Co-localization

1. Open the images of the separated channel using Fiji software.
2. Background correction (*see* **Note 34**).
3. Colocalization should be measured for individual cells; thus, hand-draw a "region of interest" (ROI) over the image.
4. Estimate a global threshold to restrict the analysis to the pixel with intensity greater than the threshold value (*see* **Note 35**).
5. Measure the colocalization using Just another Colocalization Plugin (JACoP), which is a free Fiji plug-in. Select the images and the analysis to perform; there are two coefficients that can be used for the colocalization analysis: Pearson's coefficient and Manders' coefficient.
6. Include the threshold, the correct information regarding the microscope used to acquire the images and the correct wavelengths of the images; press "analyze."
7. Pearson's correlation coefficient (PCC) (*see* **Note 36**) values range from 1 for two images whose fluorescence intensities are perfectly and linearly related to −1 for two images whose fluorescence intensities are perfectly, but inversely, related to one another. Values near zero reflect the distributions of probes that are uncorrelated with one another. Manders' colocalization coefficients (MCC) measure the fraction of one protein that colocalizes with a second protein (*see* **Note 37**). The MCC values range from 0 and 1 and correspond to completely mutually exclusive and perfect colocalization, respectively. To select the most appropriate methods to evaluate the colocalization and an appreciation of the factors that must be considered for meaningful interpretation of colocalization studies, refer to a review of Dunn and colleagues [17].
8. Acquire and analyze sufficient images to perform a statistical analysis.

An alternative widespread approach that has been used to study PTEN localization is the generation of fusion proteins tagged with GFP or its derivatives. Targeting is a powerful and commonly used approach to investigate the functions and localization of proteins in eukaryotic cells. We used this strategy to target PTEN to different organelles and cell compartments, which thus generates novel tools for the investigation of the spatial complexity of PTEN functions. The available compartment-specific PTEN chimeras are reported in Table 4. In all cases, correct intracellular localization of

Table 4
Compartment-specific PTEN chimeras

Acronym	Intracellular localization
GFP-PTEN-NLS^{SV40}	Nucleus
GFP-PTEN-NES^{PKI}	Cytoplasm
snap25-PTEN	Plasma membrane (inner surface)
AKAP-PTEN	Outer mitochondrial membrane (cytoplasmatic surface)
ER-PTEN	Endoplasmic reticulum

the PTEN chimeras has been verified through IF, using antibodies against PTEN and specific markers for the various intracellular compartments.

4 Notes

1. This buffer must be prepared fresh for the experiment and must be free of protease and phosphatase inhibitor cocktails to avoid sample alterations.
2. This buffer must be prepared fresh immediately prior to use.
3. Use 1.5 mm thick coverslips because most microscope objectives are designed to work optimally with this thickness. Coverslips must be sterilized and subsequently placed in a multi-well plate: 18 mm coverslips in a 12-well plate, 12–13 mm coverslips in a 24-well plate, or 24 mm coverslips in a 6-well plate.
4. The cells should be swollen, but should not burst. It is imperative to maintain the cell suspension on ice during this step.
5. The number of required strokes depends on the cell type used. It is therefore necessary to check the homogenized cells under a microscope after every 10 strokes. Stop when >90 % of the cells have burst, thereby leaving intact nuclei, with various amounts of cytoplasmic material attached.
6. From this point, perform all procedures in a 4 °C room to minimize the activation of proteases and phospholipases.
7. Precool the glassware and homogenizer with pestle in an ice bath for 5 min prior to the initiation of the homogenization step. Pestle homogenization at a higher force can affect mitochondrial integrity; thus, the movement of the pestle should be slow and performed with "fluidity."
8. Do not pipette the pellet because it will result in mitochondria breakage.

9. It is very important to maintain the sample on ice.
10. The aliquot without PK will be considered a mito crude control sample.
11. If crude mitochondria are contaminated by other organelles, add one additional 10,000 × *g* centrifugation step for 10 min at 4 °C.
12. For the resuspension process, two or three hand-made strokes are sufficient. Store a small amount (60 μl) of crude mitochondrial fraction in 1.5 ml Eppendorf microfuge for future investigations (western blot). Add 2 mM Na_3VO_4, 2 mM NaF and protease inhibitor cocktail. Freeze at –80 °C if the sample will not be used immediately.
13. The suspension should remain 4–5 mm below the top of the tubes. Filling up the tubes is important to protect them against damage during centrifugation.
14. Ultracentrifugation was performed using a Beckman L8-70 M ultracentrifuge, 70-ti rotor (tube: Beckman, cat. no. 355618).
15. Autofluorescence can be quenched using a 100 mM glycine solution for 30 min prior to the blocking step.
16. This procedure can be used for HEK-293 and other non-adherent cell lines.
17. This passage requires at least 1 h.
18. HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS).
19. The amount of DNA depends on the transfection method: 3 μg is suggested for a standard calcium phosphate procedure.
20. This time is required to enable the protein expression of the constructs.
21. From this point, minimize the light exposure: turn off the light in the room and cover the sample with aluminum foil.
22. Cell fixing can damage the endoplasmic reticulum; if this occurs, you could reduce the time of fixing.
23. Permeabilization helps get the antibodies into now-fixed cells. In addition to Triton X-100, a range of different detergents can be used, including NP-40, Tween-20 and Saponin. Fixing and permeabilization steps affect the cell morphology and the availability of the antigen of interest. You may obtain different results with different reagents, times and concentrations; thus, there is a need for protocol optimization. The distortion of cell morphology is something to consider during image interpretation.

24. This step is essential to block unspecific binding of the antibodies. BSA or nonfat dry milk can be used as a blocking buffer. The optimization of the percentage and time of blocking depends on the antibodies used.
25. Holding the coverslip on its edge with forceps is facilitated with a needle. It is recommended to hold the coverslip on the edge to minimize damage to the cell layer.
26. Antibody incubation requires a small volume; flipping the coverslip on the drop allows tight contact between the cell and antibody.
27. The humidified chamber can be easily constructed from a 15 cmϕ cell culture dish upholstered by aluminum foil in which a layer of parafilm is placed on PBS soaked filter paper. The humidified chamber is fundamental to avoid sample drying.
28. Centrifuge the secondary antibody solutions (e.g., 2 min at 10,000 × *g, at 4 °C*) and add only the supernatant to the blocking solution. This step eliminates protein aggregates that may have formed during storage, which thereby reduces the nonspecific background staining.

    When two secondary antibodies are used, ensure that they are produced in a third different species (e.g., for mouse-anti PTEN and rabbit-anti ER, use secondary antibody anti-mouse and anti-rabbit produced in goat to avoid cross reaction between the antibodies).
29. Use the smallest amount of solution but cover all samples.
30. Mounting medium helps preserve your sample and raises the refractive index to provide good performance with oil objectives. Mountants often have scavengers, which soak up free radicals and reduce photobleaching (these sometimes can reduce the initial brightness of the samples).
31. Available at http://www.svi.nl/NyquistCalculator.
32. Freely available at http://fiji.sc/.
33. For example, colocalization of Alexa Fluor 488 and mtDsRED can be apparent in structures that appear yellow because of the combined contributions of green and red fluorescence, respectively.
34. Run process/subtract background (BG) in which a rolling ball radius of 50 pixels is suggested or select a BG ROI and Run Plugins/ROI/BG subtract from the ROI.
35. It is fundamental to select the same threshold value for the analysis of all images. The Costes method for threshold estimation is a robust and reproducible method that can be easily automated. The method has been implemented in Fiji/ImageJ plug-ins (JACoP).

36. The formula for PCC is provided for a typical image that consists of red and green channels:

$$\mathrm{PCC} = \frac{\sum_i (R_i - R) x (G_i - G)}{\sqrt{\sum_i (R_i - R)^2 x \sum_i (G_i - G)^2}}$$ where R_i and G_i refer to the intensity values of the red and green channels, respectively, of pixel i, and R and G refer to the mean intensities of the red and green channels, respectively, across the entire image.

37. For two probes, denoted R and G, two different MCC values are derived. M_1 is the fraction of R in compartments that contain G, and M_2 is the fraction of G in compartments that contain R. These coefficients are calculated as follows: $M_1 = \frac{\sum_i R_i,\mathrm{colocal}}{\sum_i R_i}$, where $R_i,\mathrm{colocal} = R_i$ if $G_i > 0$ and $R_i,\mathrm{colocal} = 0$ if $G_i = 0$ and $M_2 = \frac{\sum_i G_i,\mathrm{colocal}}{\sum_i G_i}$, where $G_i,\mathrm{colocal} = G_i$ if $R_i > 0$ and $G_i,\mathrm{colocal} = 0$ if $R_i = 0$.

Acknowledgements

The financial support of Telethon GGP11139B to P.P., the Italian Association for Cancer Research (IG-14442 to P.P. and MFAG-13521 to C.G.), the Italian Cystic Fibrosis Research Foundation (grant #19/2014), and the Italian Ministry of Education, University and Research (COFIN, FIRB, and Futuro in Ricerca) to P.P. are gratefully acknowledged.

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