Published in final edited form as:

Methods Mol Biol. 2017 ; 1656: 131–142. doi:10.1007/978-1-4939-7237-1_7.

Methods to visualize MAVS subcellular localization

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Abstract

The mitochondrial antiviral signaling (MAVS) protein is a central adaptor protein required for antiviral innate immune signaling. To facilitate its roles in innate immunity, MAVS localizes to multiple intracellular membranous compartments, including the mitochondria, the mitochondrial-associated ER membrane (MAM), and peroxisomes. Studies of MAVS function therefore often require an analysis of MAVS localization. To detect MAVS protein on intracellular membranes, biochemical fractionation to isolate MAMs, mitochondria, or peroxisomes can be used. Further, immunofluorescence with antibodies against specific membrane markers can be used to visualize MAVS distribution throughout the cell. Here, we describe the biochemical fractionation and immunofluorescence protocols used to detect MAVS subcellular localization.

Keywords

MAVS; mitochondria; MAM; peroxisomes; endoplasmic reticulum; fractionation; immunofluorescence; interferon

Introduction

Mitochondrial antiviral signaling protein (MAVS, has been referred to as VISA, Cardif, or IPS-I) is an innate immune adaptor protein important for activation of intracellular antiviral innate immunity and subsequent viral clearance. During RNA virus infection, MAVS is activated when a cytosolic pattern recognition receptor, such as RIG-I, binds to MAVS via interactions between the caspase activation and recruitment domains (CARD) for each protein. This initiates a downstream signaling cascade culminating in the transcriptional induction of type I and type III interferon (IFN), as well as IFN-stimulated genes (ISGs) (Reviewed in ((1)). MAVS is a classical C-terminal tail-anchored membrane protein, and its domains are comprised of this C-terminal transmembrane domain, an N-terminal CARD, and a proline rich region (2). The transmembrane domain of MAVS tethers it to multiple intracellular membranes, namely the mitochondria, peroxisomes, and a specialized

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19. In order to see peroxisomal-MAVS localization, use at least a 63X objective for imaging with multiple averaging and a long pixel dwell.

subdomain of the ER called the mitochondrial--associated ER membrane (MAM) (2--5). This membranous localization is critical for MAVS function. Indeed, cleavage of MAVS near its transmembrane domain by the NS3--NS4A protease of hepatitis C virus releases the protein into the cytosol and prevents downstream signaling to type I IFN (6--9).

Multiple techniques have been established that allow for the detection and visualization of MAVS subcellular localization. These include biochemical techniques such as subcellular fractionation to isolate MAM, mitochondria, or peroxisomes, as well as immunofluorescence followed by confocal microscopy. These assays have been crucial in determining where MAVS localizes and understanding the role of MAVS localization in orchestrating its antiviral signaling function. Further, the MAM/mitochondrial fractionation technique outlined below allows for pure mitochondrial isolation, whereas standard/crude mitochondrial fractionation have contaminating ER, highlighting the importance of the MAM/mitochondrial isolation described herein (4,10). Here, we outline the protocols for several assays used to investigate MAVS localization.

2. Materials

2.1 MAM Fractionation

All reagents and equipment should be pre--cooled to 4°C and kept on ice unless otherwise noted.

1. Cells--eight 15 cm² dishes of Huh7 cells plated at 8×10^6 cells/dish the day before (90% confluency when harvested) (*See Note 1*)
2. 15 cm² tissue culture dishes
3. Phosphate--buffered saline (PBS)
4. Polyethylene cell lifter with 3 cm blade length
5. Overhead stirrer
6. Potter--Elvehjem homogenizer or a 7 ml dounce homogenizer
7. Tabletop centrifuge (Eppendorf 5430 R) with fixed angle rotor, accommodates tubes up to 2 ml
8. Ultracentrifuge with SW41 rotor
9. 14 × 89--mm SW41 ultraclear centrifuge tubes (*See Note 2*)
10. Sucrose homogenization buffer: *To 100 ml ultrapure H₂O, add:* 17.1 g sucrose (0.25 M final), 20 ml 100 mM HEPES (10 mM final), adjust to pH 7.4 with 1 N NaOH, add ultrapure H₂O to 200 ml. Sterilize by autoclaving for 20 min using a liquid cycle. Can store 2--3 months at 4°C.

Notes

1. The number of dishes used needs to be optimized according to the cell type used for fractionation, but eight dishes should be enough for most cell lines.

11. Mannitol buffer A: *To 100 ml ultrapure H₂O*, add: 9.13 g mannitol (0.25 M final), 38 mg EGTA (0.5 mM final), 10 ml 100 mM HEPES (5 mM final), adjust to pH 7.4 with 1 N NaOH, add ultrapure H₂O to 200 ml. Sterilize by autoclaving for 20 min using a liquid cycle. Can store 2—3 months at 4°C.
12. Mannitol buffer B: *To 100 ml ultrapure H₂O*, add: 8.22 g mannitol (0.225 M final), 76 mg EGTA (1 mM final), 50 ml 100 mM HEPES (25 mM final), adjust to pH 7.4 with 1 N NaOH, add ultrapure H₂O to 200 ml. Sterilize by autoclaving for 20 min using a liquid cycle. Can store 2—3 months at 4°C.
13. Stock isotonic Percoll, 90% (v/v): 9 vol Percoll to 1 vol 2.5 M sucrose (0.25 M final). Sterilize by autoclaving for 20 min using a liquid cycle. Can store 2—3 months at 4°C.
14. 30% (v/v) Percoll suspension in Mannitol buffer B: 1 vol autoclaved 90% (v/v) stock isotonic Percoll to 2 vol autoclaved Mannitol buffer B. Can store 2—3 months at 4°C.
15. 20--gauge needle
16. 1 and 3 ml syringes
17. Amicon Ultra—4 centrifugal Filter Unit with Ultracel—10 membrane
18. Protease Inhibitor
19. Halt phosphatase inhibitor
20. Reagents and equipment for SDS--PAGE analysis

2.2 Peroxisomal Fractionation

All reagents and equipment should be pre--cooled to 4°C and kept on ice unless otherwise noted.

1. 15 cm² culture dishes
2. Polyethylene cell lifter with 3 cm blade length
3. Tabletop centrifuge (Eppendorf 5430 R) with fixed angle rotor, accommodates tubes up to 2 ml
4. Protease Inhibitor
5. Halt phosphatase inhibitor
6. Two chamber gradient maker
7. MOPS buffer: 4.18 g 3--(*N*-morpholino)propanesulfonic acid (MOPS), add ultrapure water to final volume of 200 ml. Can store at room temperature.
8. Homogenization medium (HM): 0.25 M sucrose, 1 mM disodium EDTA, 5 mM MOPS, 0.1% (v/v) ethanol, adjust to pH 7.2 with 1 M NaOH, add ultrapure water to desired final volume. Store up to 2 days at 4°C.

9. High-density diluent (HD): 1 mM EDTA, 5 mM MOPS, 0.1% (v/v) ethanol, adjust to pH 7.4 with 1 M NaOH, add ultrapure water to desired final volume. Store up to 2 days at 4°C.
10. 10% (w/v) Nycodenz in HM
11. 40% (w/v) Nycodenz in HD
12. 50% (w/v) Nycodenz in HD
13. 10⁸ Huh7 cells (*See Note 3*)
14. Ball-bearing homogenizer
15. 5—10 ml dounce homogenizer, Wheaton type B
16. Tabletop centrifuge with swinging bucket rotor, such as the Thermo Scientific Legend XTR to accommodate 15—50 ml tubes
17. Ultracentrifuge with vertical rotor
18. Ultraclear sealable tubes compatible with an ultracentrifuge with a fixed angle rotor and can accommodate up to 15 ml
19. 20-gauge needle
20. 5 ml syringe with metal cannula
21. Reagents and equipment for SDS-PAGE analysis

2.3 Immunofluorescence Assay for MAVS

Images can be obtained using any confocal microscope. Please refer to the specific guidelines for your confocal microscope and its respective computer software.

1. Millicell EZ clear chamber slides for maximum volume of 1.7 ml (*See Note 4*)
2. Huh7 cells--Cells plated onto chamber slides such that the cells are approximately 60% confluent the following day (*See Note 5*)
3. PBS
4. 4% paraformaldehyde (PFA, methanol-free diluted to 4% in PBS). Keep in light-sensitive container.
5. 0.1% Triton X-100 in PBS. Store at 4°C.
6. Blocking buffer: 10% fetal bovine serum (FBS) or other blocking reagent diluted into PBS. Store at 4°C.
7. Antibodies: MAVS (mouse, 1:100), PMP70 (rabbit, 1:100), TOM20 (rabbit, 1:100), Hoechst (1:500), Alexa Fluor rabbit-conjugated secondary (1:500),

2. An alternative to collecting fractions by pipetting is using a fraction collector. If using a fraction collector, such as the Biocomp gradient station fraction collector, for the biochemical fractionations described here, use 14 × 89-mm open-top polyclear tubes.
3. Any mammalian cell line can be used for this protocol, but the number of cells can be adjusted to increase yield.
4. Alternatively, cover glasses can be used for plating and staining cells. 12-mm round cover glasses can fit into a 24 well tissue culture plate.

Alexa Fluor mouse-- conjugated secondary (1:500). Primary and secondary antibodies should be diluted in blocking buffer. Store antibodies according to manufacturer's guidelines.

8. Orbital shaker
9. Confocal microscope
10. Ultrapure water
11. Rectangular cover glass
12. Mounting medium

3. Methods

3.1 MAM Fractionation

This protocol was adapted from Bozidis et al. Basic Protocol 2 (*II*). Antibody companies and dilutions can be found from Horner et al, *PNAS* 2011 (*4*). All procedures and reagents should be kept at 4°C throughout the protocol.

1. Seed cells 16--24 h before fractionation. For Huh7 cells, seed eight 15 cm² dishes at 8×10^6 cells/dish.
2. When cells are 90% confluent, wash cell monolayers with 10 ml PBS per dish. Aspirate PBS.
3. Add 10 ml ice--cold PBS per dish and scrape using a sterile cell lifter.
4. Transfer cells to two 50 ml conical tubes, combining all the dishes (40 ml per conical). Save 1 ml for whole cell lysate and harvest using standard cell lysis buffer.
5. Pellet cells by spinning for 10 min at 1000 x g in a tabletop centrifuge at 4°C.
6. Aspirate supernatant and resuspend the pellets in a total of 7 ml ice--cold sucrose homogenization buffer containing protease and phosphatase inhibitors (1:100 each).
7. Attach the pre--cooled pestle for the Potter-Elvehjem homogenizer to an overhead stirrer and gently homogenize by 10 up--and--down strokes at 500 rpm. Transfer homogenate to a 15 ml conical tube (*See Note 6*).
8. Pellet the nuclei, cell debris, and intact cells by centrifugation at 600 x g for 5 min in a tabletop centrifuge. Discard the pellet and keep the supernatant.
9. Transfer supernatant to 1.5ml microfuge tubes (~5 per sample) and centrifuge for 10 min at 10,300 x g at 4°C using a tabletop centrifuge with a fixed angle rotor. This pellet contains the mitochondria and MAM--save for step 14. Continue to next step with the supernatant.

5. Mammalian cell lines can be used for immunofluorescence. Determine the cell number to plate that will reach ~60% confluency the next day.

10. Centrifuge the supernatant at least two additional times under the same conditions until a pellet is no longer visible. This supernatant contains the microsomes and cytosol fractions (*See Note 7*). Discard the pellet after each spin in this step.
11. Pellet the microsomes by pipetting the supernatant from step 10 into an SW41 ultraclear tube filled to 1 cm from the top and balance to the same weight. Then, spin this for 60 min at 100,000 x g at 4°C using a Beckman SW41 swinging bucket rotor. Keep the resulting microsome pellet for step 13 and use the supernatant in step 12 (*See Note 8*).
12. Collect supernatant by pipetting into a 15 ml conical. Concentrate the supernatant using an Amicon protein concentrator according to manufacturer's guidelines to a final volume of ~500µl. This is the final cytosol fraction (*See Note 9*).
13. Resuspend the microsome pellet from step 11 in 300 µl of Mannitol buffer B. This is the final microsome fraction.
14. Resuspend and combine the pellets from step 9 in 300 µl of ice--cold Mannitol buffer A.
15. Homogenize using three strokes in a 2 ml Potter--Elvehjem homogenizer at 500 rpm (*See Note 10*).
16. Carefully layer homogenate on top of 10 ml of 30% Percoll suspension in a 14 × 89--mm SW41 ultraclear tube.
17. Centrifuge for 65 min at 95,000 x g at 4°C in an SW41 rotor (*See Note 11*).
18. Collect banded fractions using a 20--gauge needle and a 1 or 3 ml syringe. Collect fractions 1 (mixed membranes) and 2 (MAM) (as seen in Figure 1A) using a 1 ml syringe by puncturing the wall of the tube above the fraction and slowly suction the fractions with a fan-like movement, avoiding other bands. Collect fraction 3 (mitochondria) by puncturing below the bands and slowly suction using a 3 ml syringe with a fan--like movement, avoiding other bands.
19. Carefully remove the 20--gauge needle from the tube by gently pulling the needle away from the tube. Dispose of the needle in an appropriate biohazard disposal container designated for "Sharps".
20. Transfer each sample (fraction 2 and 3) to a 14 × 89--mm ultraclear SW41 tube. Dilute fractions with 5 vol of ice--cold Mannitol buffer B (~10 ml total) to dilute the Percoll.

6. Keep cells on ice during homogenization. Check efficiency of homogenization under the microscope by pipetting 10 µl of lysate onto a microscope slide and visualizing homogenization under a microscope. A shiny ring around the nuclei indicates cells are intact. Cells should be ~90% homogenized, if not repeat homogenization at 1000 rpm. If using a dounce homogenizer, do 20--40 strokes.

7. Additional centrifugation is required to separate microsomes from residual mitochondria and MAM.

8. Pellets can be stored on ice and centrifuged at the same time as the MAM fraction (step 24).

9. Amicon concentrators are routinely used for concentrating fractions to increase the protein concentration. Other concentration techniques, such as acetone concentration or methanol/chloroform concentration, can be used as an alternative approach.

10. If using a dounce homogenizer, use 10 strokes.

21. Pellet the MAM and mitochondria (fraction 2 and 3) by centrifuging for 10 min at 6,300 x g using an SW41 rotor.
22. Aspirate supernatant and wash an additional time in at least 5 ml ice--cold Mannitol buffer B. Spin for 10 min at 6,300 x g using an SW41 rotor (*See Note 12*).
23. Aspirate the supernatant from step 22 and resuspend the final mitochondrial pellet in 50µl of ice--cold Mannitol buffer B.
24. Centrifuge the MAM fraction (fraction 2 from step 18) for 60 min at 100,000 x g at 4°C in an SW41 rotor to pellet the MAM.
25. Resuspend the final MAM pellet in 300 µl in ice--cold Mannitol buffer B.
26. Quantify protein concentrations of the fractions using a standard protein quantification assay, such as a bicinchoninic assay (BCA), according to the manufacturer's guidelines.
27. Following protein quantification, fractions can be frozen at -80°C or used for SDS--PAGE analysis. For SDS--PAGE analysis, load 2 µg of each fraction and 10--20 µg of the whole cell lysate.
28. Immunoblot for Microsomes/ER: calnexin; Mitochondria: OxPhos; Outer membrane of mitochondria; VDAC; MAM: FACL4; cytosol: Tubulin (see Figure 1B).

3.2 Peroxisomal Fractionation

This protocol is slightly modified from this reference (*12*). Keep all reagents and equipment at 4°C, unless otherwise noted.

1. Collect cells by aspirating off media, washing with cold PBS, and adding 2 ml of cold PBS to the 15 cm² culture dishes.
2. Using a cell lifter, scrape the cells and transfer 1.8 ml of the cold PBS + cell suspension to a 15 ml conical tube for fractionation. Also, transfer 200 µl of the cold PBS + cell suspension to a 1.5 ml microfuge tube for whole cell lysate. Spin the cell suspension for fractionation in the 15 ml conical tube for 5 min at 1000 x g in a tabletop centrifuge. In the meantime, also spin the suspension for the whole cell lysate in the 1.5 ml microfuge tube for 5 min at 1000 x g in a tabletop microfuge.
3. Aspirate off the PBS from both tubes in step 2. The pellet in the 1.5 ml microfuge tube will be used for whole cell lysate analysis in SDS--PAGE and can be directly lysed using standard cellular lysis conditions. Proceed with the cell pellet in the 15 ml conical tube.

11. To ensure generation of the Percoll gradients, use an acceleration program (e.g., 500 rpm for 3 min) at the start of the spin and a similar deceleration program at the end.

4. Resuspend this cell pellet in the 15 ml conical tube, which should contain approximately 10^8 cells, with 5 ml of HM containing protease and phosphatase inhibitors (1:100 each) and transfer to an ultraclear centrifuge tube compatible with a vertical rotor ultracentrifuge. Homogenize the cells using five strokes from each handle of the ball--bearing homogenizer.
5. Centrifuge homogenate 5 min at 500 x g in a tabletop centrifuge that can accommodate 15 ml tubes.
6. Remove and transfer the supernatant to a new tube. Keep the collected supernatant on ice. Keep the cell pellet for step 7.
7. Resuspend the cell pellet in 5 ml of HM using five strokes in a 7 ml dounce homogenizer.
8. Using the homogenate from step 4, repeat steps 5, 6, and 7.
9. Pool the collected supernatants, which should total 15 ml, and centrifuge for 10 min at 6000 x g in a tabletop centrifuge.
10. Collect the supernatant and centrifuge again for 15 min at 20,000 x g in an ultracentrifuge with a vertical rotor. There should be a cell pellet at the bottom of the ultracentrifuge tube following centrifugation. This pellet is the light mitochondrial pellet.
11. Aspirate off the supernatant and resuspend the light mitochondrial pellet in 4 ml of homogenization medium using four strokes of the pestle of the dounce homogenizer.
12. Prepare a 10 ml linear gradient from equal volumes of the 10% and 40% Nycodenz in a vertical rotor ultracentrifuge--compatible tube using a two chamber gradient maker.
13. Using a syringe and metal cannula, underlayer the gradient from step 9 with 0.5 ml of 50% Nycodenz.
14. Add 2 ml of the pellet suspension from step 10 on top of the gradient.
15. Centrifuge 35 min at 75,000 x g in an ultracentrifuge with a vertical rotor, using either a controlled acceleration/deceleration centrifugation program or a centrifugation with the brake turned off at 500 x g (2000 rpm).
16. Collect 750 μ l fractions. Hold the ultracentrifuge tube over a microfuge tube, and carefully poke a hole in the bottom of the ultracentrifuge tube with a 20--gauge needle. Allow approximately 750 μ l to drip into a microfuge tube, and then collect the subsequent fractions in new microfuge tubes. There should be approximately 11 collected fractions.
17. Dilute the collected fractions with 2 ml of HM and centrifuge 20 min at 30,000 x g. Keep the supernatant.
18. Quantitate total protein of the diluted fractions and adjust the volume of the samples with HM such that the total protein concentration is 1--5 mg/ml.

19. Proceed to SDS--PAGE analysis. Figure 1C shows the MAVS--containing fractions that contain the peroxisomal marker PMP70 in fraction 11 and the mitochondrial marker Cox--1 in fraction 3.

3.3 Immunofluorescence Assay for MAVS

1. Plate Huh7 cells onto chamber slides such that they are 60% confluent on the following day. If only endogenous protein is being visualized, there is no need for transfection (*See Note 13*).
2. The day after plating, harvest the cells, wash with 500 μ l of PBS, and aspirate the PBS.
3. Add 400 μ l of 4% PFA to each well. Incubate at room temperature for 30 min (*See Note 14*).
4. Following incubation, aspirate off PFA, wash twice with 500 μ l of PBS (*See Note 15*).
5. Add 200 μ l of 0.1% Triton X--100 to each well. Gently rock on an orbital rocker at room temperature for 15 min.
6. Wash three times with 500 μ l of PBS for 5 min.
7. To each well, add 200 μ l of blocking buffer. Gently rock on an orbital shaker at room temperature for 1 hour.
8. Aspirate off the blocking buffer and add 200 μ l of your desired primary antibody diluted in blocking buffer into the appropriate wells of the chamber slides. Gently rock on an orbital shaker at room temperature for two hours (*See Note 16*).
9. Aspirate off the blocking buffer and wash three times with 400 μ l of PBS for 5 min.
10. After the final wash, add 200 μ l of Alexa Fluor rabbit-conjugated or mouse-conjugated secondary antibody diluted in blocking buffer to the respective wells. Gently rock on an orbital shaker at room temperature for one hour. (*See Note 17*).
11. Wash three times with 500 μ l of PBS for 5 min.
12. After the final wash, dismantle the chamber slide such that the plastic wells are removed and only the slide remains. Allow the slide to dry, which should take about 10 min.

12. Centrifuging the MAM fraction will remove any residual mitochondria.

13. If over--expressed protein is being visualized, the next day following plating, transfect using appropriate transfection reagent and plasmids. Then perform the immunostaining protocol the next day after transfection, as described above.

14. To reduce the risk of cell loss, 10% PFA can be added directly to the culture media, without aspirating and washing with PBS, to a final PFA percentage of 4%.

15. Chamber slides can be stored at 4°C following fixation in PBS.

16. Alternatively, primary antibodies can be left on overnight, gently shaking on a rocker at 4°C.

13. Mount a glass cover slip onto the chamber slide using a drop of mounting medium onto each corner of the cover slip.
14. Allow the mounting medium to dry overnight in a dark container, such as a drawer. Image using a confocal microscope (*See Notes 18 and 19*). Figure 2 shows representative images of MAVS localized to either mitochondria (Tom20) or peroxisomes (PMP70).

Acknowledgement

We thank the Duke University Light Microscopy Core Facility for assistance with imaging and analysis. Research in the Horner laboratory is supported by funds from the National Institutes of Health (NIH) (R01AI125416 and R21AI124100) and a Duke School of Medicine Whitehead Scholarship. Additional funding sources include the Ford Foundation (CV) and NIH T32CA009111 (DCB).

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17. Alexa Fluor antibodies can be light--sensitive. Keep chamber slides covered in aluminum foil or turn off the lights in the area the chamber slides are rocking.

18. If you are imaging your cells on the same day as staining them, allow slide to dry for at least four hours so that the cover slip will not move during focusing adjustments during the confocal imaging.

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Figure 1. MAM/mitochondrial and peroxisomal fractionation.

(A) Percoll gradient showing the fractions obtained in step 18 following the spin of the MAM/mitochondria through the Percoll gradient. (B) Immunoblot of a MAM/mitochondrial fractionation of Huh7 cells, showing MAVS, Cox--1 (mitochondria), tubulin (cytosol), FACL4 (MAM), and calnexin (ER/microsomes). (C) Representative immunoblot of fractions 3 and 11 (out of 11 total fractions) from a peroxisomal fractionation of Huh7 cells showing Cox--1 (mitochondria), Pex19 (peroxisomes), and MAVS. Mito, mitochondria; Pex, peroxisomes.

Figure 2. Confocal microscopy analysis at 63X magnification of Huh7 cells immunostained for (A) endogenous MAVS and TOM20 (outer mitochondrial membrane) or (B) endogenous MAVS and PMP70 (peroxisomes). White arrows indicate co-localization of MAVS with either mitochondria or peroxisomes. Zoom panels below each set are from the area indicated by the white box. Scale bar—10 μm.