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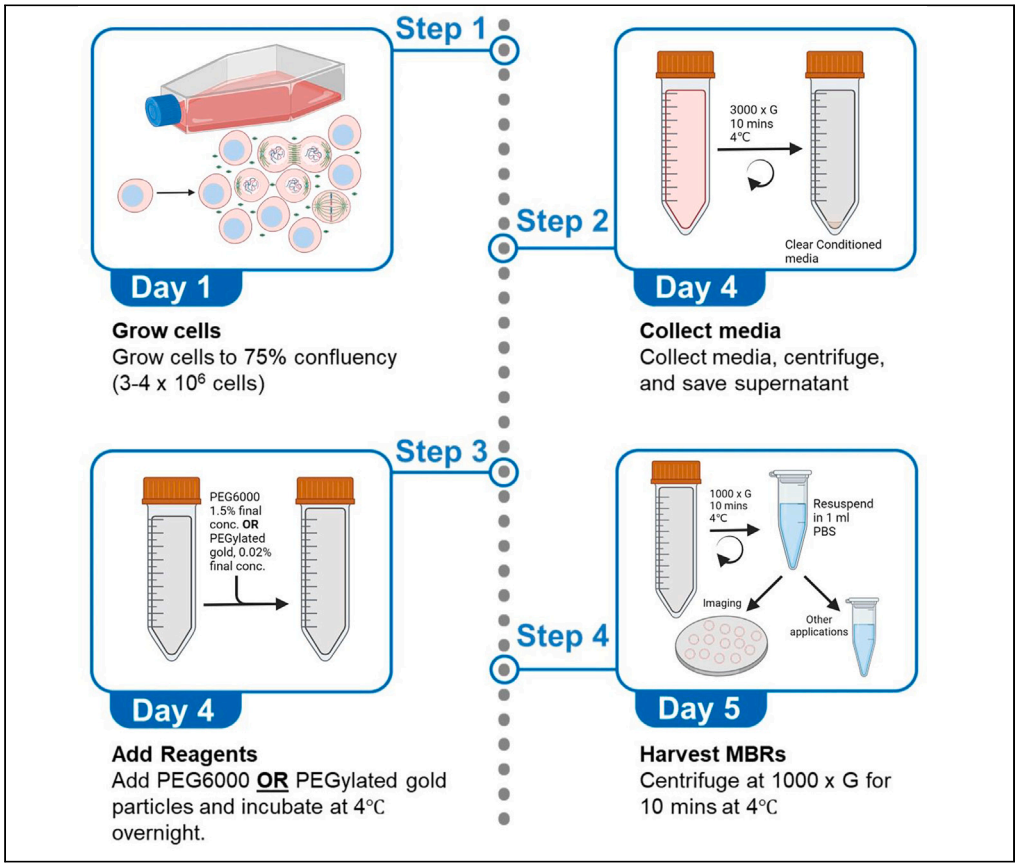
A protocol for isolating and imaging large extracellular vesicles or midbody remnants from mammalian cell culture



Traditionally, midbody remnants (MBRs) are isolated from cell culture medium using ultracentrifugation, which is expensive and time consuming. Here, we present a protocol for isolating MBRs or large extracellular vesicles (EVs) from mammalian cell culture using either 1.5% polyethylene glycol 6000 (PEG6000) or PEG5000-coated gold nanoparticles. We describe steps for growing cells, collecting media, and precipitating MBRs and EVs from cell culture medium. We then detail characterization of MBRs through immunofluorescent antibody staining and immunofluorescent imaging.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Two inexpensive and simple protocols for isolating large EVs/MBRs

Isolate large EVs/MBRs from cell culture medium

Prepare samples for characterization using different microscopy techniques

Use of MKLP1 protein as a marker for isolated large EVs or MBRs

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A protocol for isolating and imaging large extracellular vesicles or midbody remnants from mammalian cell culture

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SUMMARY

Traditionally, midbody remnants (MBRs) are isolated from cell culture medium using ultracentrifugation, which is expensive and time consuming. Here, we present a protocol for isolating MBRs or large extracellular vesicles (EVs) from mammalian cell culture using either 1.5% polyethylene glycol 6000 (PEG6000) or PEG5000-coated gold nanoparticles. We describe steps for growing cells, collecting media, and precipitating MBRs and EVs from cell culture medium. We then detail characterization of MBRs through immunofluorescent antibody staining and immunofluorescent imaging.

BEFORE YOU BEGIN

The protocol below describes the specific steps for isolating midbody remnants (MBRs), or large extracellular vesicles (EVs), from the culture medium of human cells using polyethylene glycol 6000 (PEG6000), or PEG5000 coated gold nanoparticles, precipitation. This protocol is optimized for HeLa-CCL-2 cells, HeLa Kyoto-mitotic kinesin-like protein 1 (MKLP1)-GFP cells[1] and Dulbecco's Modified Eagle Medium (DMEM)/F12 culture medium. It has also been successfully used with DMEM culture medium. Other cell lines and culture media may be suitable alternatives; however, additional protocol modifications may be necessary. All cell procedures are performed in a Class II biosafety cabinet using sterile technique. The HeLa cells are thawed at least 7 days before MBR isolation and cultured in a 37°C humidified incubator with 5% CO_2, with regular passaging. The cells are regularly screened for mycoplasma.

Before beginning the isolation, prepare medium, solutions, and poly-L-lysine coated coverslips.

Preparation of poly-L-lysine coating of cover glasses (coverslips)

Timing: 1 day

MBRs, or large EVs, are small particles that do not adhere well to glass. Thorough cleaning of the glass coverslips with sterile water and sonication removes any surface contamination. Treating the coverslip with poly-L-lysine promotes adhesion of MBRs by electrostatic interactions between the cell membrane and the poly-L-lysine. Attachment to the coverslip is essential for the later stages of the protocol which involve immunostaining of MBRs (see problem 1).

1. Wash 18-mm circular coverslips in sterile distilled water.
2. Sonicate the coverslip in 1 M KOH solution for 30 min.
3. Wash five times with sterile distilled water.
4. Wash once with 100% ethanol and dry.
5. Place each coverslip into a well of a 12-well plate.
6. Cover the surface of each coverslip with 50 μL poly-L-lysine and incubate at either 2°C–8°C for 12–18 h or 37°C for 1 h.
7. Wash the coverslips three times with sterile distilled water.
8. Store the coated coverslips at −20°C for up to 6 months.

Note: Perform steps 3–8 in a Biosafety cabinet using sterile technique and forceps to handle the coverslips. Wash the coverslips by dipping into the relevant solution.

Preparation of Reynolds lead citrate solution for electron microscopy

Timing: 1 h

Reynolds lead citrate is used as an enhancer for heavy metal staining in electron microscopy. One of the techniques used to identify isolated MBRs is imaging with a transition electron microscope.

9. Add 1.33 g of lead nitrate (80 mM) and 1.76 g of sodium citrate dihydrate (120 mM) to 30 mL of sterile distilled water.
10. Shake vigorously and incubate for 30 min at 20°C–25°C with intermittent mixing.
11. Add 8 mL of 1 N sodium hydroxide and bring up to a final volume of 50 mL with sterile distilled water.
12. Store the solution in a glass bottle at 20°C–25°C for up to 6 months.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-MKLP1	Novus Biologicals	Cat#NBP2-56923
Mouse anti-CD9	Santa Cruz Biotechnology	Cat#SC-13118
Mouse anti-α-tubulin Alexa Fluor 555	Millipore Sigma	Cat#05-829X-555
Alexa Fluor 488 goat anti-mouse IgG	Jackson ImmunoResearch	Cat#115-545-003
Alexa Fluor 647 goat anti-rabbit IgG	Jackson ImmunoResearch	Cat#111-605-003
Chemicals, peptides, and recombinant proteins		
DMEM/F12 medium	Gibco	Cat#11330-057
DMEM	Gibco	Cat#11965092
Fetal bovine serum (FBS)	Fisher Scientific	Cat#26140079
Penicillin/Streptomycin	Life Technologies	Cat#15140-122
Geneticin	Gibco	Cat#10131-035
Phosphate-buffered saline (PBS) no calcium no magnesium	Gibco	Cat#14190250
0.25% Trypsin-EDTA (1X)	Gibco	Cat#25200-056
Poly(ethylene glycol), BioUltra, 6000 (PEG6000)	Millipore Sigma	Cat#81253
Gold nanoparticles, 30 nm diameter, methyl terminated, PEG 5000 coated	Sigma-Aldrich	Cat#765732
Poly-L-lysine solution, mol. wt. 150,000–300,000, 0.01%, sterile-filtered, BioReagent, suitable for cell culture	Millipore Sigma	Cat#P4832
KOH	Millipore Sigma	Cat#221473

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ethanol	Fisher Scientific	Cat#BP2818500
PIPES disodium salt	Millipore Sigma	Cat#P3768
HEPES	Promega	Cat#H5302
EGTA	Millipore Sigma	Cat#E4378
$MgSO_4$	Millipore Sigma	Cat#M5921
Paraformaldehyde (PFA) 37%	Electron Microscopy Sciences	Cat#15714-5
Triton X-100	Millipore Sigma	Cat#T9284
Bovine serum albumin (BSA)	Millipore Sigma	Cat#A2153
Tween 20	Millipore Sigma	Cat#P2287
Fluoro-Gel mounting medium with TES buffer	Electron Microscopy Sciences	Cat#17985-30
VECTASHIELD Vibrance Antifade Mounting Media with DAPI	Vector Laboratories	Cat#H-1800
Glutaraldehyde 10% EM grade	Electron Microscopy Sciences	Cat#16120
Paraformaldehyde 16% solution	Electron Microscopy Sciences	Cat#15710
Sodium phosphate dibasic	Electron Microscopy Sciences	Cat#21182
Sodium phosphate monobasic	Ted Pella Inc	Cat#19542
Osmium tetroxide	Electron Microscopy Sciences	Cat#19100
Potassium ferrocyanide	Millipore Sigma	Cat#P9387
Ethanol (for TEM)	Decon Laboratories Inc	Cat#2701
Acetone 99.8%, Extra Dry AcroSeal (AC)	Acros Organics	Cat#326800010
EMbed 812/DER 736 Kit	Electron Microscopy Sciences	Cat#14130
EMbed 812 resin	Electron Microscopy Sciences	Cat#14900
Uranyl acetate	Electron Microscopy Sciences	Cat#22400
Reynolds lead citrate-lead nitrate	Acros Organics	Cat#423855000
Reynolds lead citrate-sodium citrate dihydrate	Chem-Impex	Cat#01265
Reynolds lead citrate-sodium hydroxide 1N	Acros Organics	Cat#12460010
Experimental models: Cell lines		
HeLa	ATCC	Cat#CCL-2
HeLa MKLP1-GFP (Douglas et al. 2010)	Gift from Mishima laboratory	N/A
Other		
T75 flask	Fisher Scientific	Cat#FB012937
T175 flask	Fisher Scientific	Cat#12-556-011
150 mm dish	Fisher Scientific	Cat#FB012925
50 mL tubes	Falcon	Cat#352098
Steriflip tube top filter unit	Sigma Millipore	Cat#SE1M179M6
Circular cover glass, #1.5, diameter = 18 mm, thickness = 0.16–0.19 mm	Electron Microscopy Sciences	Cat#101413-518
Glass slide	Fisher Scientific	Cat#12-550-413
12-well tissue culture plate	FabLab	Cat#FL7111
1.5 mL tube	Fisher	Cat#05-408-129
Centrifuge	Eppendorf	Cat#5810R
Swinging bucket	Eppendorf	Cat#022638866
Revolve microscope	Echo	RVL2-X
Confocal microscope	Nikon	A1R-Si+
Structured illumination microscope (SIM)	Nikon	ECLIPSE-Ti-E
Ultramicrotome	Reichert Technologies	Ultracut E
Formvar-coated 2 × 1 mm slot Cu grids	Electron Microscopy Sciences	Cat#FF2010-Cu
Transmission electron microscope (TEM)	Philips	CM120
TEM digital camera	AMT	Biosprint 12

MATERIALS AND EQUIPMENT

Medium and solutions are filter-sterilized through a membrane of 0.2 μm or smaller pore size.

HeLa-CCL-2 and HeLa-MKLP1-GFP culture medium		
Reagent	Final concentration	Volume/Mass
DMEM/F12 medium	N/A	445 mL
FBS	10%	50 mL
Penicillin/Streptomycin	1%	5 mL
Geneticin - for MKLP1-GFP culture	750 μg/mL	15 μL/mL
Store at 2–8°C for up to 4 weeks.		

PHEM buffer		
Reagent	Final concentration	Volume/Mass
Sterile distilled water	N/A	up to 500 mL
PIPES	60 mM	9.07 g
HEPES	25 mM	3.25 g
EGTA	10 mM	1.9 g
MgSO4	4 mM	495 mg
2 M KOH (adjust pH 7.0)	N/A	N/A
Store at 2–8°C for up to 1 year.		

PHEM-paraformaldehyde (PFA)		
Reagent	Final concentration	Volume/Mass
PHEM buffer	N/A	44.85 mL
PFA (37%)	3.7% (v/v)	5 mL
Triton-X-100	0.3% (v/v)	150 μL
Store at −20°C for up to 4 weeks.		

Sodium phosphate buffer (PB)		
Reagent	Final concentration	Volume/Mass
Sodium Phosphate monobasic	0.019 M	227.96 mg
Sodium Phosphate dibasic	0.081 M	2.17 g
Sterile distilled water	N/A	Up to 100 mL
Store at 2–8°C for up to 1 year.		

Immersion-fix sodium phosphate solution		
Reagent	Final concentration	Volume/Mass
Sodium phosphate buffer (PB)	0.1 M	6.25 mL
PFA (16%)	2% (v/v)	1.25 mL
Glutaraldehyde (10%)	2.5% (v/v)	2.5 mL
Store at 2–8°C for up to 1 year.		

Post-fix sodium phosphate solution		
Reagent	Final concentration	Volume/Mass
Sodium phosphate buffer (PB)	0.1 M	Up to 100 mL
Osmium tetroxide	1%	1 g
Potassium ferrocyanide	1%	1 g
Store at 2–8°C for up to 1 year.		

- For TEM microscopy, an ultramicrotome (Reichert-Jung, Ultracut E), and Formvar coated 2 × 1 mm slot Cu grids (Electron Microscopy Sciences, Cat. no. FF2010-Cu) are required.
- PHEM blocking buffer: add 1.5 g BSA (3% w/v) to the PHEM buffer in a final volume of 50 mL.

Store at 2–8°C for up to 1 year.

- 30% PEG (w/v): add 15 g PEG6000 to sterile phosphate-buffered saline (PBS) to a final volume of 50 mL.

Store at 2–8°C for up to 6 months.

- PBS-T: add 1 mL of Tween 20 (0.2% [v/v]) to 499 mL of PBS.

Store at 16–25°C for up to 1 year.

STEP-BY-STEP METHOD DETAILS

HeLa-CCL-2 and HeLa-MKLP1-GFP cell passaging and maintenance

Timing: up to 7 days

This protocol is optimized for isolating large EVs or MBRs from cell culture medium. The cells should be in the active growth phase before plating into T175 flasks for medium collection and MBR isolation (steps 8–10 followed by either steps 11–13 or steps 14–16). HeLa-CCL-2 and HeLa-MKLP1-GFP cells are cultured similarly but may proliferate at differing rates.

1. Passage the cells when they are approximately 70% confluent (3–4 × 10^6 cells).
2. Remove medium, add 0.25% Trypsin-EDTA, and incubate at 37°C for 5–10 min until cells detach.
3. Pipette the cells up and down to obtain a single-cell suspension.
   a. Mix resuspended cells with an equal volume of DMEM/F12 medium.
   b. Centrifuge at 300 × *g* for 5 min.
   c. Remove supernatant, and resuspend in DMEM/F12 medium.
4. Split cells 1:5 (6–8 x 10^5 cells) into a T75 flask in DMEM/F12 medium.

Note: We suggest cells are split when they reach 60%–70% confluency to ensure they remain in the active growth phase with high levels of mitosis. Geneticin should be added to the MKLP1-GFP medium for one passage every few weeks to maintain selection. MKLP1-GFP HeLa cells have a geneticin resistant cassette linked to MKLP1-GFP. The addition of geneticin antibiotic will eliminate cells without the MKLP1-GFP gene and maintain a culture of MKLP1-GFP positive cells.

HeLa-CCL-2 and HeLa-MKLP1-GFP cell culture on coverslips

Timing: 1–3 days

To visualize cells and MBRs together, HeLa-CCL-2 and HeLa-MKLP1-GFP cells can be cultured on coverslips and characterized by immunofluorescence or an assay of choice. MKLP1-GFP cells should be in a non-selection medium (i.e., without geneticin).

5. Plate cells on poly-L-lysine coated coverslips in a 12-well plate in 1 mL DMEM/F12 medium (3–4 x 10^4 cells per well).
6. Incubate the cells until they reach 60%–70% confluency.
7. Characterize the cells using immunofluorescence (steps 41–49) or an assay of choice.

Note: We suggest cells are fixed when they reach a confluency of 60%–70% to ensure the cells remain in the active growth phase with high levels of mitosis, enabling the detection of MBRs.

Collection of HeLa-CCL-2 and HeLa-MKLP1-GFP medium

Timing: 3–6 days

Cells should be 60%–90% confluent before harvesting medium for MBR isolation. HeLa-CCL-2 and HeLa-MKLP1-GFP cells are cultured similarly but may proliferate at differing rates. MKLP1-GFP cells should be in a non-selection medium (i.e., without geneticin) for collection.

8. Plate HeLa cells (6–8 × 10^5 cells per flask) in T175 flasks in 20 mL DMEM/F12 medium.
9. Once cells are approximately 75% confluent (6–8 × 10^6 cells), vigorously shake the flask from side to side 10 times to detach as many midbodies from the cells as possible.
   a. Harvest the medium into a 50 mL conical tube.
   b. Centrifuge the medium at 1000 × *g* for 10 min to remove dead cell debris.
10. Carefully remove the supernatant (cleared conditioned medium) and transfer to a new 50 mL conical tube.

Note: If the medium turns yellow, indicating a buildup of waste products from the cells, replace it with fresh medium and incubate for at least 24 h before harvesting. If cells are cultured in 150 mm dishes rather than T175 flasks (step 8), shaking the dish cannot be performed easily without medium loss. Therefore, vigorous pipetting of the medium over the surface of the dish multiple times should be performed to dislodge the MBRs.

⚠ CRITICAL: Cells should be cultured in T175 flasks for at least 2 days and no longer than 5 days before collecting medium. This ensures that the cells have had enough time to undergo at least 2 cycles of mitosis to produce MBRs, while also ensuring that the cells are not either overconfluent, which could introduce dead cell debris to the medium, or senescent and not proliferating, therefore producing no MBRs (see problem 1).

Option 1: Skop Lab 1.5% PEG6000

Timing: 1 day

Cleared conditioned medium from step 10 can be used to isolate MBRs/large EVs with 1.5% PEG6000 which precipitates the MBRs/large EVs from the medium, allowing them to be centrifuged and concentrated in a smaller volume.

11. To the cleared conditioned medium from step 10 add 30% PEG6000 solution to yield a final PEG6000 concentration of 1.5% (v/v), and incubate for 12–18 h at 2–8°C.
12. Centrifuge at 1000 × *g* for 10 min at 4°C to pellet the PEG/MBR.
13. Resuspend the PEG/MBR pellet in 1 mL sterile PBS.

Note: There is a chance that small extracellular vesicles in cell culture medium from FBS might also precipitate, but the proposed isolation protocol uses very low concentrations of PEG6000. PEG6000 was tested on fresh medium containing FBS, no isolated EVs were observed.

Pause point: The PEG/MBR PBS solution can be used immediately for further analysis, stored at 4°C for up to 3 weeks or at −80°C for up to 2 months.

Option 2: Skop Lab 30 nm gold nanoparticle

Timing: 1 day

Cleared conditioned medium from step 10 can be used to isolate MBRs/large EVs using 30 nm gold nanoparticles which precipitates the MBRs/large EVs from the medium, allowing them to be centrifuged and concentrated in a smaller volume.

14. To the cleared conditioned medium from step 10 add PEG5000 coated 30 nm gold particles solution to yield a final concentration of 0.02% (v/v), and incubate for 12–18 h at 2–8°C
15. Centrifuge at 1000 × *g* for 10 min at 4°C to pellet the gold/MBR.
16. Resuspend the gold/MBR pellet in 1 mL sterile PBS.

Note: There is a chance that small extracellular vesicles in cell culture medium from FBS might also precipitate, but the proposed isolation protocol uses very low concentrations of gold nanoparticles to ensure that small exosomes and other impurities are not precipitated. When the gold nanoparticles were tested on fresh medium containing FBS, no isolated EVs were observed.

Pause point: The gold/MBR PBS solution from step 16 can be used immediately for further analysis, stored at 4°C for up to 3 weeks or at −80°C for up to 2 months.

MBR fixation for transmission electron microscopic (TEM) imaging

Timing: 2–3 days

MBR size and structure can be studied using electron microscopy.

17. Centrifuge the PEG/MBR PBS solution from step 13 or gold/MBR PBS solution from step 16 at 1000 × *g* for 10 min at 4°C to pellet the MBRs.
    a. All subsequent steps for TEM are performed on the PEG/MBR or gold/MBR pellet.
    b. If the pellet can no longer be visualized, centrifuge at 1000 × *g* for 10 min.
18. Immersion-fix the MBR pellet in immersion fix sodium phosphate solution for 2 h at 20–25°C.
19. Rinse the MBR pellet five times for 5 min in PB.
20. Post-fix the MBR pellet in post fix sodium phosphate solution for 1 h at 20–25°C
21. Rinse five times for 5 min in PB.
22. Dehydrate the MBR pellet in a graded ethanol series (35%, 50%, 70%, 80% and 90%) diluted in dH_2O for 5 min each at 20–25°C.
23. Dehydrate the MBR pellet in 95% ethanol diluted in dH_2O for 10 min at 20–25°C.
24. Dehydrate the MBR pellet in 100% ethanol twice for 10 min at 20–25°C.
25. Dehydrate the MBR pellet in dry acetone (AC) twice for 7 min at 20–25°C.
26. Incubate the dehydrated MBR pellet with 10% EMbed 812 in AC for 60 min at 20–25°C.
27. Incubate the dehydrated MBR pellet with 25% EMbed 812 in AC for 60 min at 20–25°C.
28. Incubate the dehydrated MBR pellet with 50% EMbed 812 in AC for 12–18 h at 20–25°C.
29. Incubate the dehydrated MBR pellet with 75% EMbed 812 in AC for 60 min at 20–25°C.
30. Incubate the dehydrated MBR pellet with 100% EMbed 812 for 45 min at 60°C.
31. Incubate the MBR pellet in fresh EMbed 812 for 48 h at 60°C for embedding and polymerization.
32. Section the MBR pellet on an ultramicrotome into 100-nm sections.
33. Collect the sections on 2 × 1 mm slot copper grids.
34. Post-stain with 8% uranyl acetate in 50% ethanol for 15 min at 60°C.
35. Post-stain with Reynolds lead citrate for 10 min at 60°C.
36. Image sections at 80 kV, 40,000× magnification with a transmission electron microscope.

Table 1. Antibodies for immunofluorescence

Antibody	Dilution	Supplier
Primary antibody		
Anti-MKLP1	1:2000	Novus Biologicals
Anti-CD9	1:500	Santa Cruz Biotechnology
Secondary antibody		
Alexa Fluor 488 goat anti-mouse	1:500	Jackson ImmunoResearch
Alexa Fluor 647 goat anti-rabbit	1:500	Jackson ImmunoResearch
Alexa Fluor 555 anti-α-tubulin	1:500	Millipore Sigma

MBR attachment to poly-L-lysine coated coverslip

Timing: 30 min

MBRs from step 13 or 16 can be attached to poly-L-lysine-coated coverslips using the enhanced electrostatic interaction between the cell membrane and the poly-L-lysine for characterization by immunofluorescence or an assay of choice. Without the poly-L-lysine coating the MBRs do not attach well to glass and can be lost from the coverslip (see problem 1).

37. Using forceps place one poly-L-lysine coated coverslip into each well of a 12-well plate.
38. Add 500–1000 μL of PEG/medium solution to each coverslip.
39. Centrifuge the plate at 1000 × *g* for 10 min in a tabletop centrifuge using a swinging bucket.
40. Carefully aspirate the supernatant without disturbing the coverslip.

Note: The coverslips can be used for analysis pre-fixation and immunofluorescent staining after fixation.

Characterizing MBRs: Immunofluorescent antibody staining

Timing: ≤ 2 days

Midbody remnants (MBRs) are released when a cell undergoes mitosis. MBRs or large extracellular vesicles (EV) can be distinguished from other EVs by the presence of MKLP1, a midbody marker protein (Patel et al., submitted). MBRs, either isolated or attached to cells, can be characterized by immunofluorescent staining. Here, the MBRs were stained for MKLP1,[1–4] a widely used marker for midbodies, and CD9, a tetraspanin protein found on the surface of all classes of extracellular vesicles.[5–7]

41. Fix the MBR coverslips from step 40 or cell and MBR coverslips from step 7 with PHEM-PFA for 15 min at 20–25°C.
42. Wash the fixed coverslips three times for 5 min with PBS.
43. Block the coverslips with PHEM blocking buffer for 45–60 min at 37°C or 2 h at 20–25°C.
44. Incubate coverslips with primary antibody (Table 1) diluted in PHEM blocking buffer for 12–18 h at 4°C.
45. Wash coverslips three times for 5 min with PBS-T.
46. Incubate the coverslips with secondary antibody (Table 1) diluted in PHEM blocking buffer for 30 min at 37°C, 1 h at 20–25°C, or 12–18 h at 4°C.
    a. If required, fluorophore-conjugated antibodies can be added along with the secondary antibodies.
47. Wash the coverslips four times for 5 min with PBS-T.
48. Mount the coverslips onto slides with a drop of Fluoro-Gel mounting medium for the isolated MBRs or Vectashield mounting medium for cells with MBRs.

49. Image the MBRs with a 20× objective, or higher, as required.

Note: We used a Nikon Structured illuminated microscope (N-SIM) with 100× objective and an Echo Revolve microscope with a 20× objective to obtain images of MBRs.

Characterizing MBRs: Immunofluorescent imaging

Timing: ≤ 2 days

MBRs obtained from step 13 or 16 from HeLa-MKLP1-GFP cells can be directly characterized by immunofluorescence imaging because the MBRs are GFP-positive.

50. Fix the MBR-containing coverslips with PHEM-PFA for 15 min at 20–25°C.
51. Wash the fixed coverslips three times for 5 min with PBS.
52. Mount the coverslips onto slides with a drop of Fluoro-Gel mounting medium.
53. Image the MBRs with a 20× objective, or higher, as required.

Note: If required, the MKLP1-GFP MBRs can also be stained with CD9, following steps 44–47. If there are a large number of small vesicles that are CD9 positive (exosome marker) and MKLP1- GFP negative please refer to problem 2. We used a Nikon Structured illuminated microscope (N-SIM) with a 100× objective and an Echo Revolve microscope with a 20× objective to obtain images of MBRs. Please refer to problem 1 if there are low numbers of MKLP1 positive vesicles visible.

EXPECTED OUTCOMES

The protocol presented here is adapted and optimized from previously published protocols for isolating extracellular vesicles (EVs) including exosomes.[8–11] PEG6000 has been used to precipitate exosomes and large EVs. Modifying the PEG concentration can effectively isolate large extracellular vesicles or MBRs with a uniform membrane structure (Figure 1). PEG coated gold and iron (II,III) oxide nanoparticles have been used to isolate exosomes and extracellular vesicles.[6,9] Modifying the concentration of the gold nanoparticles can effectively isolate large extracellular vesicles or MBRs from cell culture media (Figure 3).

High PEG6000 concentrations (8%, currently used in the field) distort large EVs and MBRs, giving them a swollen, deformed appearance (Figures 1B and 1C). With 1.5% PEG6000, the large EVs/MBRs are smaller and circular and have a brighter MKLP1-GFP intensity (Figure 1C), which mimics those observed in midbodies (MB) and MBRs in cells (Figure 2B). Higher PEG concentrations interfere with cell membrane fluidity by modifying lipids on the surface membrane.[11–15] Hence, higher PEG concentrations can compromise the structure and activity of the MBR surface membrane, making it appear dull and misshapen (Figure 1C). PEG6000 is a hydrophilic polymer which dissolves in water based solution, this reduces the solubility of larger particles in solution, such as large EVs/MBRs.[8] Therefore, these particles precipitate and can be pelleted when centrifuged at low speeds (1000 × *g* for 10 min). A PEG6000 concentration of 1.5% is too low to precipitate small EVs or exosomes, which remain in solution when centrifuged at low speeds. Thus, the PEG concentration correlates negatively with the size of particles precipitated and higher PEG concentrations foster smaller off-target extracellular vesicles.[8]

There are two proposed mechanisms by which PEG-coated gold nanoparticles may function in isolating MBRs. Firstly, PEG-coated gold nanoparticles may weigh down exosomes in solution, making them easier to pellet at low-speed centrifugation.[9] Secondly, the branched chains of PEG on PEG-coated Fe_3O_4 nanoparticles can increase their surface-to-volume ratio and trap small proteins and impurities, enabling pure exosomes to be pelleted during centrifugation.[6]

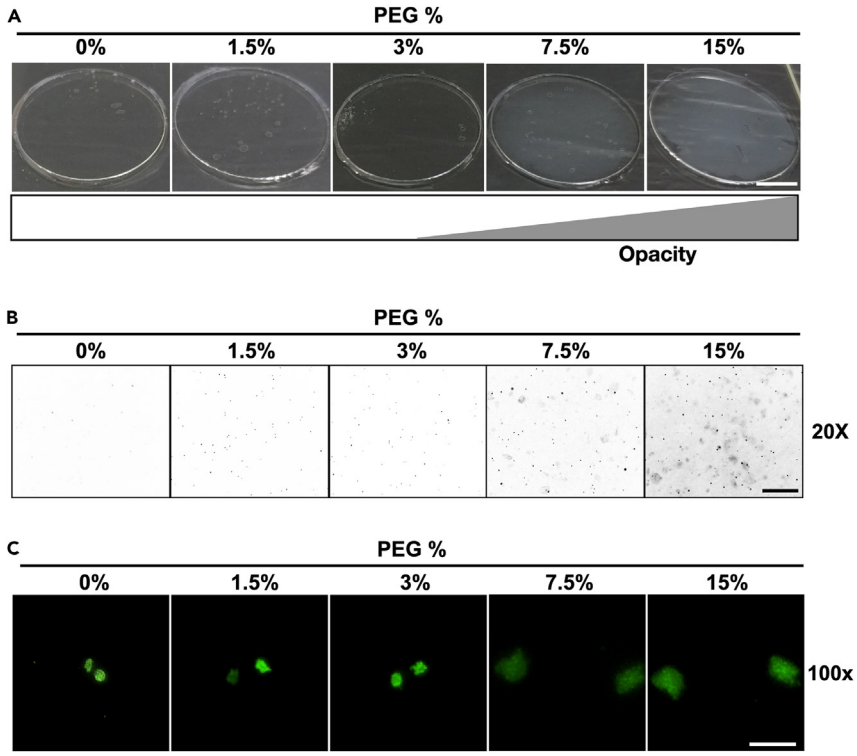

Figure 1. Increasing the concentration of PEG6000 distorts the MKLP1-GFP signal and MBR structure

(A) Cleared conditioned medium from HeLa-MKLP1-GFP cells was incubated with increasing concentrations of PEG6000 and settled onto coverslips. Increasing PEG concentration resulted in decreased transparency. The scale bar represents 5 mm.

(B) Higher PEG6000 concentrations caused an increased, blurred fluorescent background. The coverslips were imaged at 20× magnification on a Revolve microscope. The scale bar represents 100 μm.

(C) MBRs isolated from HeLa-MKLP1-GFP cell culture medium by increasing PEG6000 concentrations imaged at 100× magnification by N-SIM Z projection. The scale bar represents 10 μm.

To show that the isolated particles are large EVs or MBRs, the isolated particles can be labeled with CD9 and MKLP1 (Figure 2A). This staining shows an overlap between CD9 and MKLP1; however, the CD9 channel has a cloudy background that is missing from the MKLP1 channel. TEM confirms that the particles isolated by the current protocol are MBRs with a dark core formed by an electron-dense region that the electrons from the TEM cannot pass through (Figure 2C). The images are similar to previously published reports,[3,13,16–18] which showed the midbody and MBR as a tubulin-rich, electron-dense dark region.

We tested different concentrations of PEG-coated gold nanoparticles in clear conditioned media and compared their results (Figure 3A). A concentration of 0.02% v/v of PEG-coated gold nanoparticles is sufficient to yield a high concentration of MBRs. We identified isolated MBRs using MKLP1, a highly specific MBR marker, through HeLa CCL-2 MBR staining and the use of the MKLP1-GFP HeLa cell line (Figures 3B and 3C).

Skop Lab 1.5% PEG6000 and Skop lab 30 nm gold nanoparticles are both effective protocols of producing a highly concentrated solution of large EVs/MBRs from cell culture medium. They both isolate particles that maintain biological properties, such as translation. The advantage of Skop Lab 1.5% PEG6000 is that it is very cost effective and isolates a larger number of MBRs than the Skop Lab 30 nm gold nanoparticle protocol. However, the Skop Lab 30 nm gold nanoparticle protocol uses 0.02% (v/v) of reagent versus PEG6000 which uses 1.5% (v/v)

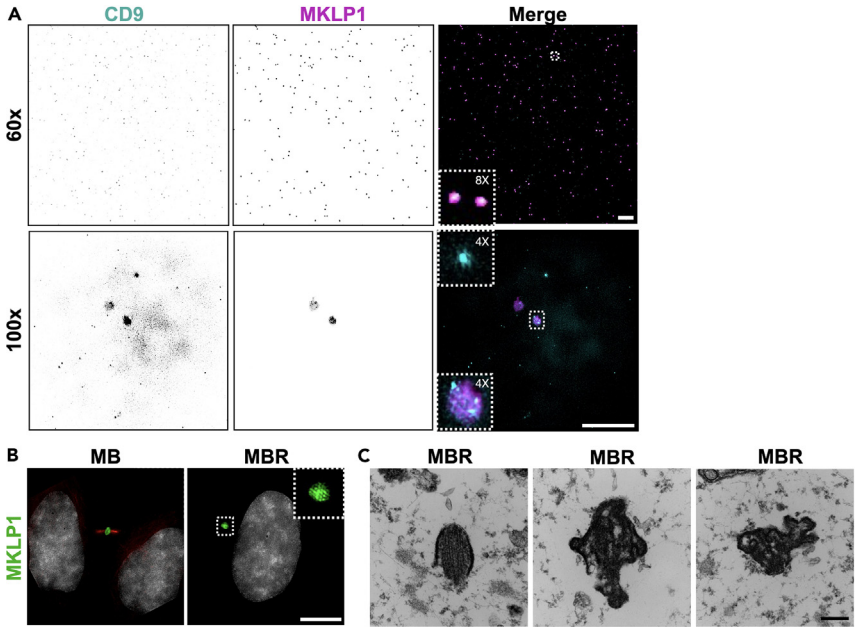

Figure 2. Isolated MBRs are midbodies (MB) formed during the late telophase stage of mitosis

(A) Representative images of MBRs isolated from HeLa-CCL-2 cells stained with anti-MKLP1 (magenta) and anti-CD9 (cyan). Images were taken at 60× magnification on a confocal microscope. The scale bar represents 24 μm, and the inset represents an 8× enlargement of the dotted square. The same slide images of isolated MBRs from HeLa-CCL-2 cell culture labeled with anti-MKLP1 (Magenta) and anti-CD9 (Cyan) were taken at 100× magnification on an N-SIM microscope. The scale bar represents 10 μm, and the inset represents a 4× enlargement of the dotted square.

(B) Representative images of mitotic HeLa-CCL-2 cells labeled with anti-MKLP1 (green), anti-α-tubulin 555 (red), and DAPI (gray). The green midbody in the first image attaches two daughter cells in the final stages of mitosis. Green MBR is taken up by a cell in interphase post-mitosis. Images were taken at 100× magnification on an N-SIM microscope. The scale bar represents 10 μm.

(C) Representative images of isolated MBRs from HeLa-CCL-2 cells using transmission electron microscopy confirm the electron-dense property of MBs at 40,000× magnification. The scale bar represents 500 nm.

of reagent. This reduces the amount of contaminating particles within the final large EV/MBR solution (Patel et al.).

The purpose of this study was to improve MBR or large EV isolation protocols by using MKLP1 to identify large EVs/MBRs (1–2 μm) from smaller EVs or exosomes (30–500 nm) (Figure 2). Moreover, we wanted to reduce the cost and time required for MBR isolation compared to protocols currently on the market that are used to isolate all sizes of EVs. These assays require access to three types of ultracentrifuges, making the isolation more expensive. The 1.5% PEG6000 and 30 nm gold nanoparticle protocols and use of MKLP1 as a marker for large EVs are more cost-effective and easier to identify large EVs, making these protocols more accessible to all types of laboratories.

QUANTIFICATION AND STATISTICAL ANALYSIS

MBR isolation by these protocols were repeated a minimum of 25 times, and MBRs (5–20 × 10^5) were successfully isolated on each occasion from a T75 flask.

LIMITATIONS

Cell confluency may be variable throughout the flask. Therefore, we use an average confluency in the protocol. Differences in seeding cell number and growth time may alter the confluency, which can affect the MBR yield during isolation.

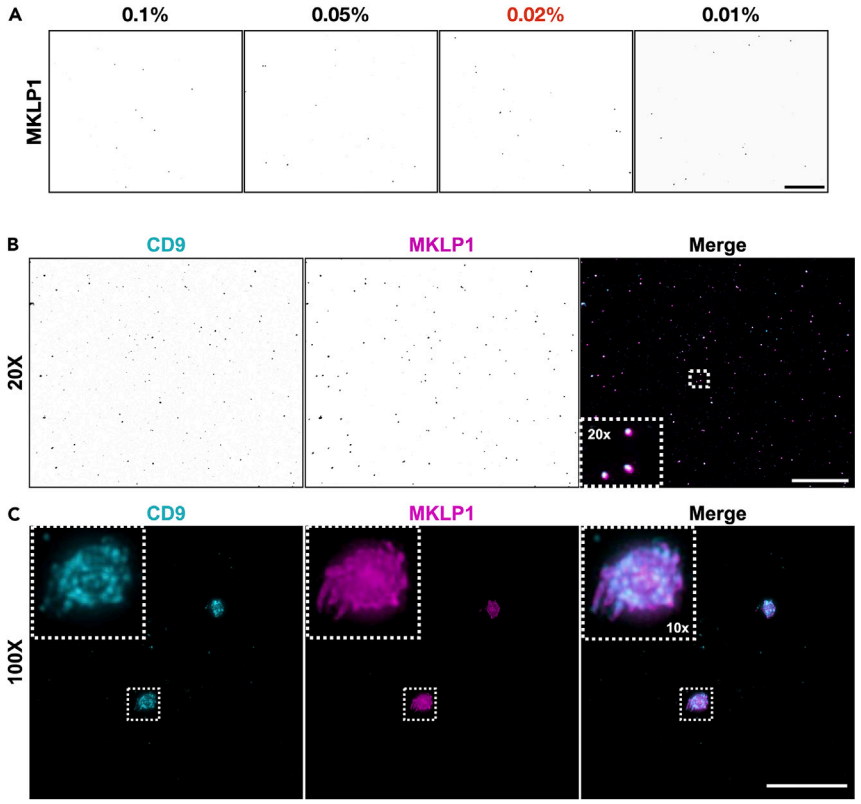

Figure 3. Comparison of GOLD PEG5000 nanoparticle concentrations in the isolation of midbody remnants (MBRs)

(A) HeLa CCL-2 clear conditioned medium was incubated with decreasing concentrations of PEG-coated gold nanoparticles overnight at 4°C. MBRs were stained with anti-MKLP1 and imaged at 20× magnification. The scale bar represents 90 μm.

(B) HeLa MKLP1-GFP clear conditioned medium was incubated with PEG5000-coated gold nanoparticles at a 1:5000 (v/v) dilution overnight at 4°C. MBRs were stained with anti-CD9 and imaged at 20× magnification. The scale bar represents 90 μm.

(C) HeLa MKLP1-GFP clear conditioned medium was incubated with PEGylated gold nanoparticles at a 1:5000 dilution (v/v) overnight at 4°C. MBRs were stained with anti-CD9 and imaged at 100× magnification. The scale bar represents 10 μm. Inset shows a 10× zoomed view of the MBR.

TROUBLESHOOTING

Problem 1

When viewed by microscopy, there is poor MBR yield with few particles labeled with anti-MKLP1/GFP (related to characterizing the MBRs and MBR attachment to poly-L-lysine coated coverslip).

Potential solution

- The density of MBRs in the medium may have been low prior to adding the PEG6000 or 30 nm gold nanoparticle solution. To obtain higher numbers of MBRs, ensure the cells are at least 75% confluent before medium collection (Step 9) and shake the flask vigorously to detach MBRs from the cells and flask surface.
- The poly-L-lysine coverslips may have been stored incorrectly (i.e., too long), or the poly-L-lysine solution used was expired. Make new coated coverslips with fresh poly-L-lysine solution and repeat attachment of MBRs to coverslips.
- When fixing, washing, and staining the coverslips, carefully add and remove the solutions down the side of the well rather than direct pipetting onto the coverslips as shear force could potentially detach the MBRs.

Problem 2

Many small CD9-positive exosomes are attached to the coverslip alongside the MBRs (related to step 36).

Potential solution

When attaching the MBRs onto the coverslips, the plate may be spun at a lower speed of 500 × *g* for 10 min, ensuring only the larger MBRs attach to the coverslip and any small exosomes remain suspended in solution.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Ahna Skop (Skop@wisc.edu).

Materials availability

This study did not generate any unique materials.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.P., S.A.P., and A.R.S.; investigation, S.P., S.A.P., A.-G.N.D., and A.M.M.; writing, S.P., S.A.P., E.E.T., and A.R.S.; supervision, A.R.S.

DECLARATION OF INTERESTS

Patents are pending (P230134US01) for the intellectual property described in this protocol.

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