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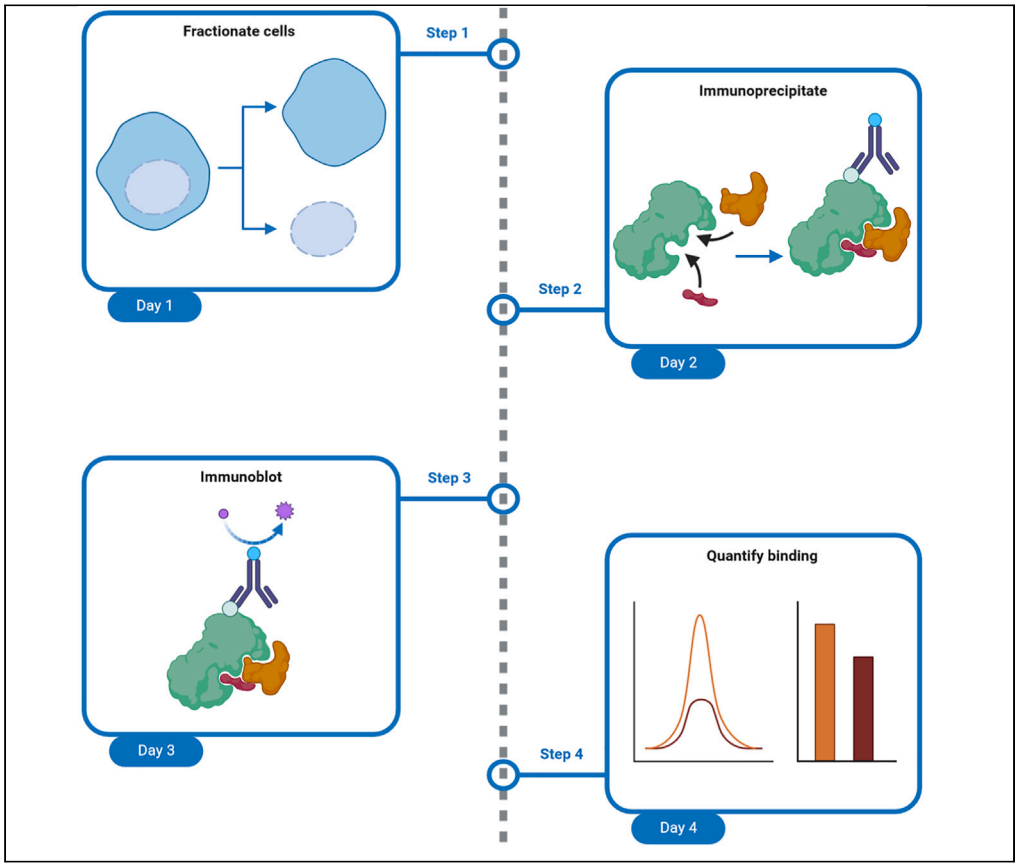
Protocol to determine the subcellular localization of protein interactions in murine keratinocytes



Protein activities and interactions are determined by their subcellular localization. Elucidating the network of protein-protein interactions at a spatial resolution is essential for understanding the complexity of protein functions, their regulation, and cellular processes. Here, we present a protocol to determine the subcellular localization of protein interactions in non-transformed murine keratinocytes. We describe steps for nucleus/cytoplasm fractionation, immunoprecipitation from these fractions, and immunoblotting. We then detail binding quantification.

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

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Highlights

Quick assay to identify subcellular localization of protein-protein interactions

Step-by-step guide for high-quality and reproducible sample preparation

Robust protocol to fractionate, purify, immunoblot, and quantify proteins

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Protocol to determine the subcellular localization of protein interactions in murine keratinocytes

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SUMMARY

Protein activities and interactions are determined by their subcellular localization. Elucidating the network of protein-protein interactions at a spatial resolution is essential for understanding the complexity of protein functions, their regulation, and cellular processes. Here, we present a protocol to determine the subcellular localization of protein interactions in non-transformed murine keratinocytes. We describe steps for nucleus/cytoplasm fractionation, immunoprecipitation from these fractions, and immunoblotting. We then detail binding quantification.

For complete details on the use and execution of this protocol, please refer to Müller et al. (2023).[1]

BEFORE YOU BEGIN

Timing: 2–3 h

The protocol below describes the specific steps using non-transformed murine keratinocytes. Although the author did not use alternate cells, nucleus/cytoplasm fractionation with similar experimental conditions was performed in HEK293T and COLO320 cells.[2] Thus, the protocol can also be used to reveal the subcellular protein-protein interactions in other cell lines. However, the optimal incubation conditions and buffer amounts may vary from cell line to cell line.

1. Before the start of the experiment, grow murine keratinocytes stably expressing the green fluorescent protein (GFP)-fusion protein of interest to 80%–90% confluence.

   Note: Murine keratinocytes were grown on Collagen I coated cell culture dishes in low calcium medium at 32°C in 5% CO_2 and 90% humidity. The medium contained no antibiotics. See table in materials and equipment section for a detailed recipe.

   Note: If possible, use GFP fusion proteins. GFP trap immunoprecipitation can then be performed, allowing the rapid and efficient one-step immunopurification. The GFP fusion proteins can be stably expressed or transiently overexpressed. Alternatively, overexpression of other epitope-tagged proteins (e.g. HA, FLAG, etc.) can be used.[3,4] High quality antibodies that are already coupled to agarose beads are available for these tags, making immunoprecipitation very efficient. However, keep in mind that epitope tags can interfere with protein function and possibly disrupt protein interactions. Therefore, the expected intracellular localization of fusion proteins should be compared to the localization of the endogenous protein if

antibodies to the protein of interest are available.[1] In addition, enrichment of endogenous proteins can also be performed by immunoprecipitation, if a suitable antibody is available.[5] The presented protocol was optimized for purifying GFP-tagged fusion proteins and their interaction partners by a GFP trap immunoprecipitation approach.

2. Prepare hypotonic buffer. Make sure that sufficient quantities are available before starting cell lysis.
   a. See table in materials and equipment section for a detailed recipe.
   b. This buffer can be stored at +4°C for six months.

   Note: A cell placed in a hypotonic solution swells and expands until it finally bursts by a process known as cytolysis. A hypotonic buffer is therefore used to lyse the cells and release the cytoplasmic fraction. Treatment with a hypotonic buffer is combined with homogenization to efficiently break up the cells.

   Note: Hypotonic buffer contains KCl. Use KCl or the alternate $MgCl_2$ in hypotonic buffer.[6] K^+ or Mg^{2+} protects nuclei from breakdown of the nuclei envelope. Furthermore, KCl maintains the conformation of cytosolic proteins.

   Note: No sterilization by autoclaving or filtering of the buffer is needed.

3. Prepare hypertonic buffer. Make sure that sufficient quantities are available before starting cell lysis.
   a. See table in materials and equipment section for a detailed recipe.
   b. This buffer can be stored at +4°C for six months.

   Note: Use hypertonic buffer to isolate the nuclear fraction. When a cell is placed in a hypertonic solution, water flows out of the cell and the cell loses volume.

   Note: The hypertonic buffer contains NaCl. High salt content contributes to the dissolution of nuclear membranes and the hypercondensation of chromatin. Since chromatin is destabilized, DNA is released from nucleosome particles in free, uncomplexed form.[7]

   Note: No sterilization by autoclaving or filtering of the buffer is needed.

4. Prepare immunoprecipitation buffer. Make sure that sufficient quantities are available before starting immunoprecipitation.
   a. See table in materials and equipment section for a detailed recipe.
   b. This buffer can be stored at +4°C for six months.

   Note: Depending on the stability of the interaction to be analyzed, the immunoprecipitation buffer may require the use of different detergents. While mild non-ionic detergents preserve many protein-protein interactions, the ionic detergent sodium dodecyl sulfate (SDS) disrupts most protein-protein interactions (and therefore can be problematic for immunoprecipitations).

   Note: No sterilization by autoclaving or filtering of the buffer is needed.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GFP rabbit polyclonal antibody	Rockland	Cat#600-401-215; RRID: AB_828167
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (1:1,000)	Sigma-Aldrich	Cat#G8795; RRID: AB_1078991
Histone H3 rabbit monoclonal antibody (1:1,000)	Cell Signaling Technology	Cat#4499; RRID: AB_10544537
Nono/p54nrb mouse monoclonal antibody (1:500)	BD Transduction Laboratories	Cat#611279; RRID: AB_398807
Phospho-retinoblastoma protein (serine 807/811) rabbit monoclonal antibody (1:1,000)	Cell Signaling Technology	Cat#8516S; RRID: AB_11178658
Plakophilin 3 guinea pig polyclonal customized peptide specific antibody (1:20,000)	Peptide Specialty Laboratories	N/A
Peroxidase AffiniPure Donkey Anti-Guinea Pig IgG (1:15,000)	Jackson ImmunoResearch	Cat#706-035-148; RRID: AB_2340447
Peroxidase AffiniPure Donkey Anti-Mouse IgG (1:20,000)	Jackson ImmunoResearch	Cat#715-035-150; RRID: AB_2340770
Peroxidase AffiniPure Donkey Anti-Rabbit IgG (1:40,000)	Jackson ImmunoResearch	Cat#711-035-152; RRID: AB_10015282
α-Tubulin mouse monoclonal antibody (1:1,000)	Sigma-Aldrich	Cat# T6199; RRID: AB_477583
Chemicals, peptides, and recombinant proteins		
Adenine	Merck Chemicals GmbH	Cat#A8626
Bovine serum albumin (BSA)	Carl Roth	Cat#8076.3
Bromphenol blue	Merck Chemicals GmbH	Cat#1081220005
Chelex 100 chelating resin	Bio-Rad	Cat#142-2842
Cholera toxin from *Vibrio cholerae*	Sigma-Aldrich	Cat#C8052
Collagen I, rat tail	Corning	Cat#354236
Coumaric acid	Carl Roth	Cat#9908.1
D-(+)-glucose solution	Sigma-Aldrich	Cat#G8769
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D2650
Dithiothreitol (DTT)	Promega	Cat#P1171
DMEM/Ham's F12 (3.5:1.1) medium containing 50 μM $CaCl_2$; customized formulation, w/o: Glucose, w/o: L-Glutamine, w/o: Phenol red, w/o: Sodium pyruvate, w: 3.096 g/L $NaHCO_3$	Pan Biotech	N/A
EDTA	Carl Roth	Cat#8040.2
EGTA	Carl Roth	Cat#3054.3
Epidermal growth factor (EGF)	Sigma-Aldrich	Cat#E4127
Fetal bovine serum (FBS) Superior	Sigma-Aldrich	Cat#S0615
GFP-Trap agarose	Chromotek	Cat#gta-20
Glutamate solution	Sigma-Aldrich	Cat#G8541
Glycerol	Carl Roth	Cat#3783.1
Glycine	Carl Roth	Cat#HN07.1
Halt™ protease and phosphatase inhibitor cocktail	Thermo Fisher Scientific	Cat#78444
HEPES	Carl Roth	Cat#9105.3
Hydrocortisone	Sigma-Aldrich	Cat#H4001
IGEPAL CA-630	Sigma-Aldrich	Cat#I8896
Insulin solution	Sigma-Aldrich	Cat#I9278
KCl	Carl Roth	Cat#HN02.3
Luminol	Carl Roth	Cat#7708.2
NaCl	Carl Roth	Cat#HN00.1
PageRuler™ Plus Prestained protein ladder	Thermo Fisher Scientific	Cat#26619
Pierce™ protein A plus agarose	Thermo Fisher Scientific	Cat#22811
Ponceau S	Carl Roth	Cat#5938.2
SDS	Carl Roth	Cat#0183.1
Skimmed milk powder	Carl Roth	Cat#T145.2
Sodium pyruvate solution	Sigma-Aldrich	Cat#S8636
Sulfosalicylic acid	Carl Roth	Cat#4119.1
Trichloroacetid acid	Carl Roth	Cat#3744.2
Tris-HCl	Invitrogen	Cat#15567027

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tween-20	Carl Roth	Cat#9127.2
Western blot ultra-sensitive HRP substrate	Takara Bio	Cat#T7104A
β-mercaptoethanol	Carl Roth	Cat#4227.3
Experimental models: Cell lines		
WT + PKP3 keratinocytes (murine keratinocytes *PKP3*$^{+/+}$ expressing PKP3-WT-EGFP)	Müller et al.[1]	N/A
WT + GFP keratinocytes (murine keratinocytes *PKP3*$^{+/+}$ expressing EGFP)	Müller et al.[1]	N/A
Recombinant DNA		
EGFP-pLVX-IRES-puro	Müller et al.[1]	N/A
PKP3-WT-EGFP-pLVX-IRES-puro	Müller et al.[1]	N/A
Software and algorithms		
BioRender	BioRender	https://biorender.com/
Fiji (ImageJ)	Schindelin et al.[8]	https://imagej.net/software/fiji/downloads
Other		
Cell scraper	TPP	Cat#99003
Centrifuge Mikro 220R	Hettich GmbH & Co. KG	Cat#2205
Centrifuge tubes 1.5 mL	Sarstedt	Cat#72.690.001
Fusion-SL-3500.WL imaging system	Peqlab Biotechnologie GmbH	N/A
Heating thermo shaker MHR 23	Hettich Benelux B.V.	Cat#HETTBE_15005
Hypodermic needle (G 26 × 1" / ø 0,45 × 25 mm)	Braun Melsungen	Cat#4657683
Ice box (Styrofoam)	N/A	N/A
Inverted microscope Primovert	Carl Zeiss Microscopy GmbH	Cat#491206-0005-000
Mini Trans-Blot cell for wet blot approach	Bio-Rad Laboratories	Cat#1703930
Nitrocellulose blotting membrane (pore size 0.2 µm)	Thomas Geyer GmbH & Co. KG	Cat#10600001
Objective LD Plan-Achromat 40×/0.5 Ph2 for Primo	Carl Zeiss Microscopy GmbH	Cat#415500-1616-000
Overhead rotator (Stuart rotator SB3)	Cole-Parmer	Cat#11496548
Pipettes, variable volume, 1–10 µL, 20–200 µL, 100–1000 µL	Starlab	Cat#S1110-3000, Cat#S1111-1006, Cat#S1111-6001
Rocking shaker (VWR 12620 platform rocker)	VWR International	Cat#444-0756
Scanner (Epson Perfection V600 Photo)	Epson	Cat#B11B198032
Syringe, 1 mL	Becton Dickinson	Cat#303172
Syringe filter, Filtropur S plus, pre-filter, pore size 0.2 µm	Sarstedt	Cat# 83.1826.002
Syringe filter, Filtropur S, pore size 0.2 µm	Sarstedt	Cat# 83.1826.001
Vortex mixer (Vortex-Genie 2)	Scientific Industries	Cat#SI-0236

MATERIALS AND EQUIPMENT

Low calcium medium for murine keratinocytes

Reagent	Final concentration	Add to 500 mL
DMEM/Ham's F12 (3.5:1.1) medium containing 50 µM $CaCl_2$	1×	460 mL
Ca^{2+}-free FBS	10%	50 mL
Sodium pyruvate	1 mM	5 mL
Glutamate	1 mM	5 mL
Adenine	0.18 mM	1,000 µL
Hydrocortisone	0.5 mg/mL	250 µL
Insulin	5 mg/mL	250 µL
Epidermal growth factor (EGF)	10 ng/mL	500 µL
Cholera toxin	100 nM	5 µL
D-(+)-glucose	1 mg/mL	1,200 µL

The complete media can be stored at +4°C for three months.

Hypotonic buffer			
Reagent	Stock concentration	Final concentration	Add to 250 mL
HEPES pH 7.9	1 M	10 mM	2.5 mL
KCl	1 M	10 mM	2.5 mL
EDTA	0.5 M	0.1 mM	50 µL
EGTA	0.5 M	0.1 mM	50 µL
ddH_2O		N/A	244 mL

This buffer can be stored at +4°C for six months. Right before use, add protease and phosphatase inhibitor cocktail to a final 1× concentration and DTT to a final concentration of 0.5 mM (see key resources table and other solutions section, respectively).

Hypertonic buffer			
Reagent	Stock concentration	Final concentration	Add to 250 mL
HEPES pH 7.9	1 M	20 mM	5 mL
NaCl	3 M	0.42 M	35 mL
EDTA	0.5 M	1 mM	500 µL
EGTA	0.5 M	1 mM	500 µL
ddH_2O		N/A	209 mL

This buffer can be stored at +4°C for six months. Right before use, add protease and phosphatase inhibitor cocktail to a final 1× concentration and DTT to a final concentration of 0.5 mM (see key resources table and other solutions section, respectively)

Immunoprecipitation buffer			
Reagent	Stock concentration	Final concentration	Add to 500 mL
Tris-HCl pH 7.5	1 M	20 mM	10 mL
NaCl	3 M	137 mM	22.83 mL
EDTA	0.5 M	2 mM	2 mL
Glycerol		10%	50 mL
IGEPAL CA-630		1%	5 mL
ddH_2O		N/A	410.17 mL

This buffer can be stored at +4°C for six months. Right before use, add protease and phosphatase inhibitor cocktail to a final 1× concentration (see key resources table and other solutions section).

4× Sample buffer			
Reagent	Stock concentration	Final concentration	Add to 50 mL
Tris/HCl pH 6.8	1 M	250 mM	12.5 mL
SDS		8%	4 g
β-mercaptoethanol		10%	5 mL
Glycerol		30%	15 mL
Bromphenol blue		0.25%	0.1 g
ddH_2O		N/A	17.5 mL

This buffer can be stored at −20°C for six months.

SDS electrophoresis buffer			
Reagent	Stock concentration	Final concentration	Add to 1 L
Tris/HCl pH 8.5		25 mM	30 g
Glycine		0.192 M	144 g
SDS		0.1%	10 g
ddH_2O		N/A	1 L

This buffer can be stored at 20°C–25°C for six months.

10× tris-buffered saline (TBS)			
Reagent	Stock concentration	Final concentration	Add to 5 L
Tris/HCl pH 7.6		100 mM	62 g
NaCl		1 mM	292,2 g
ddH_2O		N/A	5 L
This buffer can be stored at 20°C–25°C for six months.			

10× TBS with Tween-20 (TBST)			
Reagent	Stock concentration	Final concentration	Add to 5 L
Tris/HCl pH 7.6		100 mM	62 g
NaCl		1 mM	292,2 g
Tween-20		1%	50 mL
ddH_2O		N/A	4,950 L
This buffer can be stored at 20°C–25°C for six months.			

10× Ponceau S staining solution			
Reagent	Stock concentration	Final concentration	Add to 100 mL
Ponceau S		2%	2 g
Trichloroacetid acid		30%	30 g
Sulfosalicylic acid		30%	30 g
ddH_2O		N/A	100 mL
This buffer can be stored at 20°C–25°C for six months.			

Chemiluminescence solution 1			
Reagent	Stock concentration	Final concentration	Add to 50 mL
Tris/HCl pH 8.5	1 M	100 mM	5 mL
Luminol	2 M	20 mM	500 µL
Coumaric acid	88.8 mM	0.4 mM	225 µL
ddH_2O		N/A	45 mL
This buffer can be stored at +4°C for one month.			

Chemiluminescence solution 2			
Reagent	Stock concentration	Final concentration	Add to 50 mL
Tris/HCl pH 8.5	1 M	100 mM	5 mL
H_2O_2		0.02%	30 µL
ddH_2O		N/A	45 mL
This buffer can be stored at +4°C for one month.			

Other solutions	
Name	Reagents
Adenine	90 mM adenine: add 0.304 g adenine in 75 mL 150 mM HCl. Sterile filtrate with a syringe filter (Filtropur S, pore size 0.2 µm). Store at −20°C for one year.
Ca^{2+}-free FBS	Ca^{2+}-free FBS: to remove calcium ions, FBS was pretreated with 8 g/50 mL Chelex-100 at +4°C for 16 h–24 h on an overhead rotator. Procedure was 1× repeated followed by sterile filtration with a syringe filter (Filtropur S plus, pore size 0.2 µm).

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Name	Reagents
Cholera toxin	1 μM choleratoxin: add 1 mg choleratoxin in 1.18 mL sterile ddH_2O. Sterile filtrate with a syringe filter (Filtropur S, pore size 0.2 μm). Store at +4°C for one year.
Coumaric acid	88.8 M coumaric acid in DMSO: add 0.3 g coumaric acid in 20 mL DMSO. Store at −20°C for one year.
DTT	100 mM stock solution of DTT in water from company: store in small aliquots at −20°C for three months.
EGF	10 μg/mL EGF: add 0.1 mg EGF in 10 mL serum-free low calcium medium. Sterile filtrate with a syringe filter (Filtropur S, pore size 0.2 μm). Store at −20°C for one year.
Halt™ protease and phosphatase inhibitor cocktail	100× stock solution from company: store at +4°C for one year. The protease inhibitors target aminopeptidases, cysteine, and serine proteases. The phosphatase inhibitors target serine/threonine and protein tyrosine phosphatases. Add the cocktail at 10 μL/mL directly to the buffer to produce a 1× final concentration.
Hydrocortisone	1 mg/mL hydrocortisone: add 500 mg hydrocortisone in 50 mL ethanol. Sterile filtrate with a syringe filter (Filtropur S, pore size 0.2 μm). Store at −20°C for one year.
Luminol	2 M luminol in DMSO: add 0.8 g luminol in 20 mL DMSO. Store at −20°C for one year.
1× Ponceau S staining solution	1× Ponceau S staining solution: add 10 mL 10× Ponceau S staining solution in 90 mL ddH_2O. Store at 20°C–25°C for two months.
1× Sample buffer	1× Sample buffer: add 250 μL 4× Sample buffer in 750 μL ddH_2O. Store at 20°C–25°C for two months.
1× TBS	1× TBS: add 100 mL 10× TBS in 900 mL ddH_2O. Store at 20°C–25°C for two months.
1× TBST	1× TBST: add 100 mL 10× TBST in 900 mL ddH_2O. Store at 20°C–25°C for two months.
3% BSA/1× TBST	3% BSA solution: add 3 g BSA in 100 mL 1× TBST. Store at +4°C for one week.
3% skimmed milk/1× TBST	3% skimmed milk solution: add 3 g skimmed milk in 100 mL ddH_2O. Store at +4°C for one week.

STEP-BY-STEP METHOD DETAILS

Nucleus/cytoplasm fractionation

Timing: 2 h

In this step the author describes in detail the nucleus/cytoplasm fractionation of proteins (Figure 1).

⚠ CRITICAL: All preparation steps must be performed on ice for the following reasons: (1) most proteins are very sensitive to temperature increase and higher temperatures can cause proteins to precipitate (prevention of denaturation is necessary), (2) elevated temperatures increase the activity rate of proteases (prevention of proteolysis is necessary), and (3) elevated temperatures increase the activity rate of phosphatases (prevention of dephosphorylation is necessary). To avoid denaturation, proteolysis, or dephosphorylation, keep all solutions and samples on ice, e.g.in styrofoam ice boxes. The author performed all preparation steps at a 20°C–25°C bench. Perform incubation on an overhead rotator at +4°C in a cold room.

1. Aspirate the growth medium from the tissue culture dish.
2. Wash the cells three times with ice-cold 1× PBS.
3. Aspirate the last of PBS.
4. Add to each dish 800 μL of the hypotonic buffer to lyse the cells.
   a. Right before use, add protease and phosphatase inhibitor cocktail to a 1× final concentration to the hypotonic buffer.

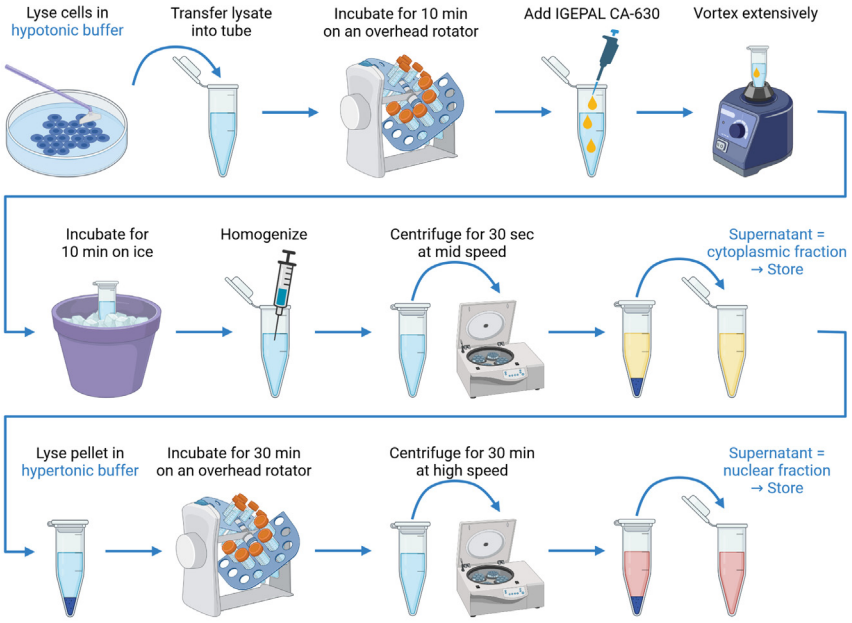


Figure 1. Workflow of nucleus/cytoplasm fractionation of proteins
Created with biorender.com.

b. Right before use, add DTT to a final concentration of 0.5 mM to the hypotonic buffer.

Note: The author used a protease and phosphatase inhibitor cocktail from a company (see key resources table), which is stored at +4°C. This avoids repeated freeze/thaw cycles. Add 8 µL of the 100× protease and phosphatase inhibitor cocktail to 800 µL hypotonic buffer to produce a 1× final concentration.

Note: The author used a 100 mM stock solution of DTT in water from a company (see key resources table), which is stored in small aliquots at −20°C. The author recommends using DTT stocks within three months to prevent loss of potency. Add 4 µL of the 100 mM DTT stock solution to 800 µL hypotonic buffer to a final concentration of 0.5 mM.

Note: DTT is a reducing agent, which maintains the thiol groups of cytosolic proteins and prevents them from oxidation to disulfide bridges.

Note: The volume of 800 µL hypotonic buffer is sufficient to perform cytoplasmic fractionation of 6 × 10^5 cells. Although this protocol was optimized for the use of non-transformed murine keratinocytes, it should be applicable to other cell lines as well. However, optimal incubation conditions and amounts of buffers may vary between cell lines.

5. Detach the cells from the tissue culture dish with a cell scraper.

Note: No sterilization of the cell scraper by autoclaving is needed. The author recommends cleaning with 90% ethanol and subsequent air drying right before use.

6. Place the cell suspension in a 1.5 mL centrifuge tube kept on ice.
7. Incubate the lysate for 10 min at +4°C on an overhead rotator.
8. Add IGEPAL CA-630 to a final concentration of 3%, e.g., add 24 µL IGEPAL CA-630 when performing the fragmentation with 800 µL hypotonic buffer/lysate.

Alternatives: IGEPAL CA-630 is a mild detergent, which can be used to dissolve proteins without denaturing them. The use of detergents allows the extraction of proteins based on their relative solubility. Other nonionic detergents, e.g. Nonidet P-40 or Triton X-100, can also be applied prior to homogenization.[9] In addition, you can change the detergent concentration to match the solubility of your protein of interest. Keep in mind that detergents which are milder than IGEPAL CA-630 or Nonidet P-40 can result in lower efficiency of fractionation.[10] Higher concentrations may result in a loss of protein-protein interactions.

⚠ CRITICAL: IGEPAL CA-630 is a highly viscous liquid. The author recommends dropwise pipetting of the total volume into the lysate. Vortex the lysate-IGEPAL CA-630 mix very well until IGEPAL CA-630 is completely dissolved.

9. Incubate the lysate for 10 min on ice.
   a. Vortex the lysate-IGEPAL CA-630 mix for 10 s three times during the incubation step.
10. Homogenize the cells.

Note: The author recommends homogenization of cells using hypodermic needles by about ten iterations of up and down of the syringe. A syringe with a volume of 1 mL is sufficient for the indicated buffer volume. In this method, cells are lysed by forcing them through the narrow lumen of the needle. Upon exit, the cells are subjected to shear forces, which leads to their lysis. The author used disposable hypodermic needles with a thickness of 26 gauges (0.45 × 25 mm) for keratinocytes cultured under conditions to prevent cell-cell contact formation. The author recommends needles with a thickness of 21 gauges (0.8 × 40 mm) for homogenization of bigger cells with increased numbers of cell-cell contacts, e.g. squamous epithelial cells of the oral mucosa, ovarian follicle cells, or keratinocytes maintained in medium with calcium which induces cell-cell contact formation. Although this protocol was optimized for the use of non-transformed murine keratinocytes, it should be applicable for other cell lines as well. Optimal homogenization methods may vary depending on the cell line used.

Note: At this point you may want to control cytoplasmic lysis by microscopy. Apply a small volume of the lysate (1–2 μL) on a slide and check for lysed cells but intact nuclei using a phase-contrast microscope. The author used the objective LD Plan-Achromat 40×/0.5 Ph2 for Primo (WD = 2.7 mm at D = 1 mm) to visualize cell lysis (see key resources table).

11. Clear the lysates by centrifugation for 30 s at 14,000 × *g* and +4°C.

Note: Mid speed centrifugation results in a huge pellet that contains cell debris, unbroken cells, some large subcellular components (cytoskeleton, mitochondria) and intact nuclei. The supernatant will contain the cytosol and the organelles in free suspension.[11]

12. Collect the supernatant as the cytoplasmic fraction in a new 1.5 mL centrifuge tube kept on ice.
   a. To 40 μL of the cytoplasmic fraction add 13.3 μL of 4× SDS loading buffer.
      i. Boil lysate at 95°C for 5 min on a heating thermo shaker.
      ii. Store the lysates at −20°C until loading the SDS-PAGE gel for immunoblotting.

Note: This sample is the cytoplasmic fraction input of the immunoprecipitation reaction.

   b. Store the remaining volume (760 μL) of the cytoplasmic fraction at −20°C for later use or keep on ice for immediate performance of the immunoprecipitation. This sample will be used for the immunoprecipitation reaction.
13. Wash the pellet three times in hypotonic buffer by centrifugation for 1 min at 14,000 × *g* and +4°C.
   a. Right before use, add protease and phosphatase inhibitor cocktail to a 1× final concentration to the hypotonic buffer.

b. Right before use, add DTT to a final concentration of 0.5 mM to the hypotonic buffer.

Note: The author recommends washing the pellet by adding approximately 500 µL of hypotonic buffer to the cell pellet and vortexing at low speed until the pellet detaches from the bottom of the centrifuge tube. Be careful not to resuspend the pellet.

Note: The author used a protease and phosphatase inhibitor cocktail from a company (see key resources table). Add 5 µL of the 100× protease and phosphatase inhibitor cocktail to 500 µL hypotonic buffer to produce a 1× final concentration.

Note: The author used a 100 mM stock solution of DTT in water. Add 2.5 µL of the 100 mM DTT stock solution to 500 µL hypotonic buffer to a final concentration of 0.5 mM.

⚠ CRITICAL: Carefully remove the wash hypotonic buffer through a hypodermic needle or pipette tip. Aspiration of the supernatant with the help of a pump may cause the loss of the pellet. Be careful not to lose the pellet.

14. Remove the last of hypotonic buffer.
15. Add to each pellet 400 µL of the hypertonic buffer to lyse the nuclei.
    a. Right before use, add protease and phosphatase inhibitor cocktail to a final 1× concentration to the hypertonic buffer.
    b. Right before use, add DTT to a final concentration of 0.5 mM to the hypertonic buffer.

Note: The volume of 400 µL hypertonic buffer is sufficient to perform nuclear fractionation of 6 × 10^5 cells. This protocol was optimized for the use of non-transformed murine keratinocytes. However, it should be applicable for other cell lines as well. Optimal incubation conditions and amounts of buffers may vary between cell lines.

Note: The author used a protease and phosphatase inhibitor cocktail from a company (see key resources table). Add 4 µL of the 100× protease and phosphatase inhibitor cocktail to 400 µL hypertonic buffer to produce a 1× final concentration.

Note: The author used a 100 mM stock solution of DTT in water. Add 2 µL of the 100 mM DTT stock solution to 400 µL hypertonic buffer to a final concentration of 0.5 mM.

16. Incubate the pellet in hypertonic buffer for 30 min at +4°C on an overhead rotator.

Note: At this point you may want to control nuclear lysis by microscopy. Apply a small volume of the lysate (1–2 µL) on a slide and check for intact nuclei using a phase-contrast microscope.

17. Clear the lysate by centrifugation for 30 min at 18,000 × *g* and +4°C.

Note: High speed centrifugation results in a small pellet that contains nuclei debris, unbroken nuclei and insoluble intermediate filaments. The supernatant will contain the nucleoplasm and the DNA in free solution.

18. Collect the supernatant as the nuclear fraction in a new 1.5 mL centrifuge tube kept on ice.
    a. To 40 µL of the nuclear fraction add 13.3 µL of 4× SDS loading buffer.
        i. Boil lysate at 95°C for 5 min on a heating thermo shaker.
        ii. Store the lysates at −20°C until loading on a SDS-PAGE gel for immunoblotting.

Note: This sample is the nuclear fraction input of the immunoprecipitation reaction.

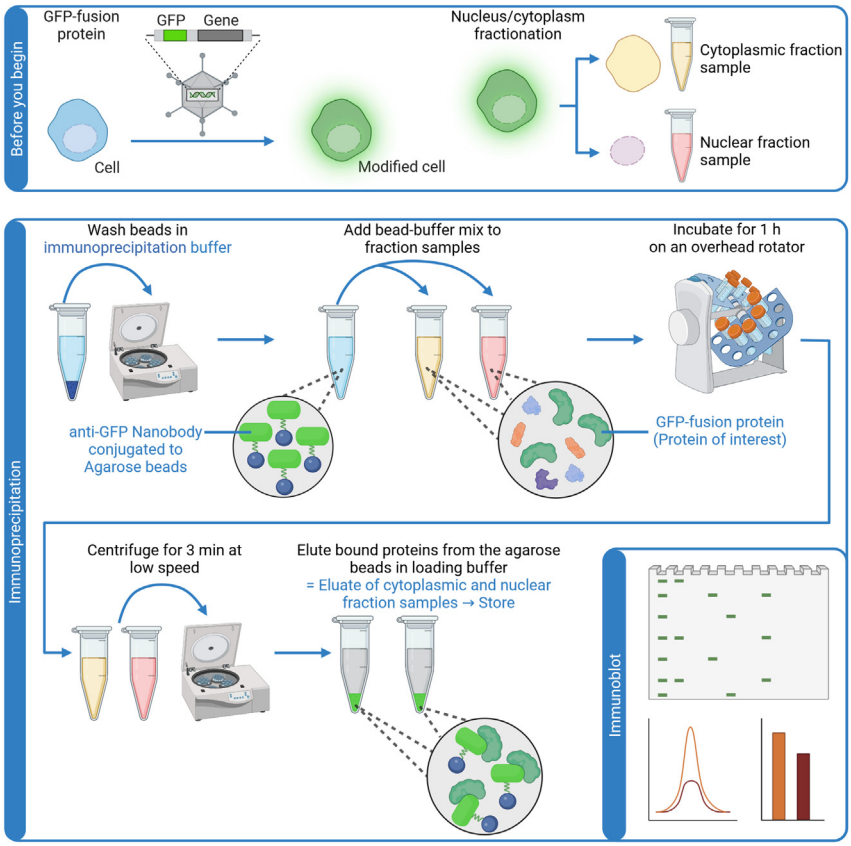


Figure 2. Workflow of GFP-Trap immunoprecipitation
Created with biorender.com.

b. Store the remaining volume (360 µL) of the nuclear fraction at −20°C for later use or keep on ice when immediately performing the immunoprecipitation. This sample will be used for the immunoprecipitation reaction.

Alternatives: When performing nucleus/cytoplasm fractionation without an additional immunoprecipitation, add the required quantity of 4× SDS loading buffer to both fraction samples (800 µL cytoplasmic fraction, 400 µL nuclear fraction). Boil lysate at 95°C for 5 min and store at −20°C until use for SDS-PAGE and immunoblotting.

Pause point: When not immediately performing the immunoprecipitation, store both fraction samples at −20°C for later use (for up to one week).

Immunoprecipitation

Timing: 90 min

In this step the author describes in detail the immunoprecipitation from cytoplasmic and nuclear fractions using GFP-Trap agarose beads (Figure 2). GFP-Trap agarose is an anti-GFP Nanobody conjugated to beads, which can be used for immunoprecipitation of GFP-fusion proteins and their interacting factors. Hence, the incubation step required for coupling antibodies to protein A/G agarose beads is omitted.

Alternatives: Immunoprecipitation of GFP-fusion proteins can also be performed using an anti-GFP rabbit polyclonal antibody and protein A agarose beads (see key resources table, respectively). Use 50 μL of settled protein A agarose (100 μL resin slurry). This amount of resin is sufficient to bind 25–250 μg of antibody (the amount may vary depending on the source of the protein A agarose). First, incubate the cytoplasmic and nuclear fraction sample with the appropriate amount of anti-GFP antibody at +4°C on an overhead rotator. The incubation time may vary between 2 h and 16 h–24 h. Next, wash the protein A agarose beads three times in immunoprecipitation buffer. Add 100 μL of the protein A resin slurry to the cytoplasmic and nuclear fractions containing the preformed antibody-complexes. Incubate for 4 h at +4°C on an overhead rotator. Collect and wash the beads by centrifugation. Discard the supernatant and elute the antibody-antigen complex from the resin by adding 70 μL of 1× SDS loading buffer to each sample. Boil the beads at 95°C for 5 min on a heating thermo shaker and store at −20°C until performing SDS-PAGE and immunoblotting.

Alternatives: Immunoprecipitation can also be performed in the absence of GFP-fusion proteins. Instead of the pull-down assay using GFP-Trap agarose beads or protein agarose beads in combination with anti-GFP antibody, perform immunoprecipitation of endogenously expressed proteins. Affinity purify antigens using a specific antibody. Use appropriate protein A, protein G or protein A/G agarose beads, which depends on the optimal binding affinity of the antibody used for immunoprecipitation.[3,4] Incubate the antibody with the cytoplasmic or nuclear fraction sample followed by incubation with the beads to capture your protein of interest and its interacting factors.

⚠ CRITICAL: To avoid denaturation, proteolysis, or dephosphorylation of proteins, keep all solutions and samples on ice e.g.in styrofoam ice boxes. The author performed all preparation steps at a 20°C–25°C bench. Perform incubation on an overhead rotator at 4°C in a cold room.

19. Thaw the samples of the cytoplasmic and nuclear fraction on ice.

Alternatives: Store the samples on ice (step 12b and step 18b) to avoid freeze/thaw cycles. The activity of some enzymes is sensitive to repeated freeze/thaw cycles.

20. Place appropriate amounts of GFP-Trap Agarose beads in new 1.5 mL centrifuge tubes.

⚠ CRITICAL: Beads will tend to stick to the sides of the pipette tip. Cut 5 mm from the tip end to minimize the loss of beads in the tip and to facilitate pipetting.

Note: The author routinely uses 15 μL of the GFP-Trap Agarose bead slurry for every immunoprecipitation reaction. The author used GFP-Trap Agarose beads, which have a binding capacity of 15–18 μg of recombinant GFP per 15 μL bead slurry. When performing more immunoprecipitation reactions, the author recommends generating a master mix. Multiply 15 μL of the GFP-Trap Agarose bead slurry with the number of the samples (and add two more samples as a reserve). For example, for six immunoprecipitation reactions, use 120 μL GFP-Trap Agarose bead slurry.

21. Wash the beads three times in immunoprecipitation buffer by centrifugation for 1 min at 4,000 × *g* and +4°C.
    a. Right before use, add protease and phosphatase inhibitor cocktail to a final 1× concentration to the immunoprecipitation buffer.

Note: The author routinely uses 150 μL of immunoprecipitation buffer for each washing step of 15 μL GFP-Trap Agarose bead slurry.

Note: The author used a protease and phosphatase inhibitor cocktail from a company (see key resources table). Add 1.5 μL of the 100× protease and phosphatase inhibitor cocktail to 150 μL immunoprecipitation buffer to produce a 1× final concentration.

Note: Washing of the GFP-Trap Agarose beads minimizes nonspecific binding. The author recommends washing the beads by adding the immunoprecipitation buffer and vortexing at low speed until the bead pellet detaches from the bottom of the centrifuge tube. Be sure to fully resuspend the beads in immunoprecipitation buffer. Centrifuge at low speed to avoid crashing the beads.

⚠ CRITICAL: Remove the immunoprecipitation buffer. Be careful not to touch and/or lose the pellet. This is easily avoided using a hypodermic needle. Slowly lower the opening of the side of the tube opposite the pellet and aspirate the buffer. Aspiration of the supernatant with the help of a pump may cause the loss of beads.

22. Remove the last of immunoprecipitation buffer.
23. Add immunoprecipitation buffer to the GFP-Trap Agarose beads.

Note: The author recommends adding immunoprecipitation buffer to a final volume of 100 μL for each 15 μL GFP-Trap Agarose bead slurry. When generating a master mix in step 20, multiply 100 μL of immunoprecipitation buffer with the appropriate number. For example, for six immunoprecipitation reactions, 120 μL GFP-Trap Agarose bead slurry were washed. Add immunoprecipitation buffer to a final volume of 800 μL (orient yourself by the marking on the centrifuge tube).

24. Add 100 μL GFP-Trap Agarose bead-immunoprecipitation buffer mix to the cytoplasmic and nuclear fraction samples.
25. Incubate for 1 h at +4°C on an overhead rotator.
26. Collect the agarose beads by centrifugation for 3 min at 4,000 × *g* and +4°C.
27. Wash the beads three times in immunoprecipitation buffer by centrifugation for 1 min at 4,000 × *g* and +4°C.

Note: The author recommends washing the beads by adding approximately 500 μL of immunoprecipitation buffer and vortexing at low speed until the bead pellet detaches from the bottom of the centrifuge tube. Be sure to fully resuspend the beads in immunoprecipitation buffer.

⚠ CRITICAL: Remove the immunoprecipitation buffer through a hypodermic needle. Be careful not to lose the pellet.

28. Remove the last of immunoprecipitation buffer using a hypodermic needle.
29. Add 70 μL of 1× SDS loading buffer to each sample.
    a. Boil the beads at 95°C for 5 min on a heating thermo shaker.
    b. Store at −20°C until performing SDS-PAGE and immunoblotting.

Note: The entire 70 μL sample is the eluate of the immunoprecipitation reaction. For SDS-PAGE, 15–20 μL of each sample will be loaded onto the stacking gel. Accordingly, 70 μL of the eluate sample is sufficient for 3–4 SDS-PAGE gels.

SDS-PAGE and immunoblotting

Timing: 2 days

In this step the author describes in short SDS-PAGE and immunoblotting procedures, which are used to detect and characterize precipitated proteins from cytoplasmic and nuclear fractions. The protocol was adapted from Mahmood and Yang (2021).[12] If not mentioned otherwise, all steps can be performed at 20°C–25°C.

30. Perform SDS-PAGE to separate proteins under denaturing conditions.
    a. Fill the buffer tank with SDS electrophoresis buffer.
    b. Load 15–20 μL of each sample in pockets of the stacking gel.
    c. Load 4 μL of a protein standard in one pocket.
    d. Run the gel with 80 V for 30 min and 100 V for approximately 2–3 h (depending on the size of the gel and acrylamide concentration).
31. Transfer proteins to a nitrocellulose blotting membrane using Mini Trans-Blot cells for a wet blot approach at 110 V for 70 min.

Note: The required transfer time may vary depending on the size of your protein.

32. Stain the membrane in Ponceau S solution for 5 min.
33. Destain the membrane in ddH_2O for 5 min.
34. Document the stained membrane using a scanner.

Note: Ponceau S staining is not mandatory. The author recommends performing Ponceau S staining to quickly detect protein bands on the immunoblot membrane. This is helpful to control the efficiency of the transfer and for total protein normalization.

35. Wash the membrane in 1× TBST until completely destained (approximately 3 min).

Note: Perform all washing and antibody incubation steps on a rocking shaker to ensure even agitation.

36. Block the membrane in 3% skimmed milk/1× TBST or 3% BSA/1× TBST on a rocking shaker for 30 min.

Note: Using milk or BSA depends on the primary antibody. Different antibodies have different binding strengths. The author routinely uses the dilution and blocking solution recommended by the company from which the antibody was purchased.

37. Probe the membrane with the appropriate primary antibody on an overhead rotator at +4°C for 16 h–24 h in a cold room.

Primary antibodies for immunoblotting

Antibody	Dilution	Solution
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody	1:1,000	3% skimmed milk/1× TBST
Histone H3 rabbit monoclonal antibody	1:1,1000	3% BSA/1× TBST
Nono/p54nrb mouse monoclonal antibody	1:500	3% BSA/1× TBST
Phospho-retinoblastoma protein (serine 807/811) rabbit monoclonal antibody	1:1,000	3% BSA/1× TBST
Plakophilin 3 guinea pig polyclonal customized peptide specific antibody	1:20,000	3% skimmed milk/1× TBST
α-tubulin mouse monoclonal antibody	1:1,000	3% skimmed milk/1× TBST

38. Next day, wash membranes in 1× TBST three times for 10 min.
39. Incubate membranes with the appropriate horseradish peroxidase-conjugated secondary antibody (diluted in 3% skimmed milk/1× TBST) for 1 h.

Secondary antibodies for immunoblotting		
Antibody	Dilution	Solution
Peroxidase AffiniPure Donkey Anti-Guinea Pig IgG	1:15,000	3% skimmed milk/1× TBST
Peroxidase AffiniPure Donkey Anti-Mouse IgG	1:20,000	3% skimmed milk/1× TBST
Peroxidase AffiniPure Donkey Anti-Rabbit IgG	1:40,000	3% skimmed milk/1× TBST

40. Wash membranes twice in 1× TBST for 10 min.
41. Wash membranes once in 1× TBS (without detergent) for 10 min.
42. Treat membranes with enhanced chemiluminescence western blotting substrate (equal parts chemiluminescence solution 1 and 2) or ultra-sensitive substrate from a company (see key resources table).
43. Detect signals using an imaging system.

EXPECTED OUTCOMES

This protocol describes a biochemical approach to identify the subcellular localization of protein-protein interactions using the nucleus/cytoplasm fractionation combined with GFP-Trap immunoprecipitation. Prepare the cytoplasmic and nuclear fractions from your cells. To make sure that cell fractionation was successful, analyze both fractions by immunoblotting using marker proteins that localize either exclusively in the nucleus or the cytoplasm, respectively. Then use these fractions to affinity-purify the GFP-tagged protein of interest. You can then probe for interactions by immunoblotting with several antibodies directed to the putative interaction partners. Measuring the integrated density of the immunoblot bands allows quantification of the subcellular distribution of proteins and their putative interaction partners. A successful experimental approach should show cytoplasmic and nuclear fractions without cross contamination between both fractions. For example, the nuclear marker should not be detected in the cytoplasmic fraction. Conversely, the cytoplasmic marker should not be detected in nuclear fraction.

An example of association of the retinoblastoma protein (phosphorylated at serine 807/811) with GFP-tagged plakophilin 3 as the protein of interest is shown in Figure 3, adopted from Müller et al.[1] α-tubulin is used as a cytoplasmic marker, Nono/p54nrb is used as a nuclear marker. A GFP-tag overexpressing cell line (not fused to plakophilin 3) is shown as a control. Plakophilin 3 was isolated from both nuclear and cytoplasmic fractions, but occurred predominantly in the cytoplasmic fraction. The phosphorylated form of retinoblastoma protein was co-purified with plakophilin 3, with a preference for an association in the cytoplasm.

QUANTIFICATION AND STATISTICAL ANALYSIS

An example of quantification is shown in Figure 4. For quantification of immunoblots, the author uses the ImageJ tool "Gel Analysis". To make sure that cell fractionation was successful, normalize the cytoplasmic and nuclear fraction markers of the input samples against Ponceau S. Define a single region of interest by selecting the "rectangle" tool from ImageJ and drawing a frame around the entire first lane of the Ponceau S staining of the input samples (Figure 4A, left panel). Use the same frame for all of the lanes across the membrane. Use a new frame for a blank region of the membrane (Figure 4A, right panel. Record the measurements. The "Mean" values represent the mean grey values. Export these data into a spreadsheet (e.g., Microsoft Excel). Quantify the band intensities of the cytoplasmic marker (e.g., α-tubulin) and nuclear marker (e.g., Nono/p54nrb) of the input samples (Figure 4B). Drawing a frame around all lanes of the protein creates a histogram with a series of

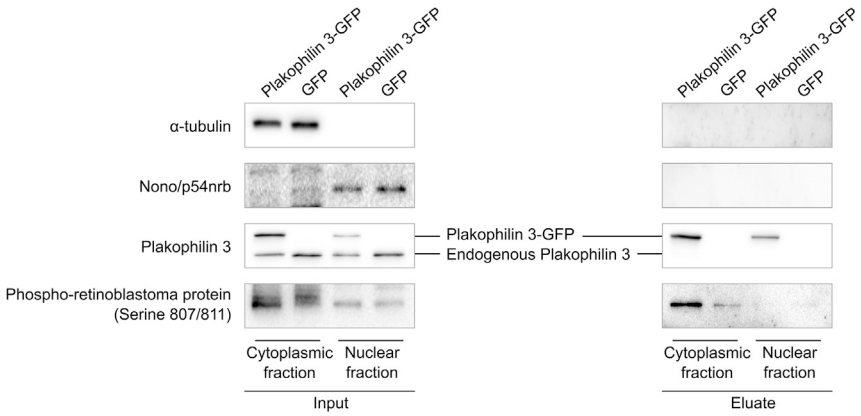


Figure 3. Expected outcome of the subcellular localization of protein-protein interactions using the nucleus/cytoplasm fractionation, followed by GFP-Trap immunoprecipitation from these fractions

α-tubulin is used as a cytoplasmic marker, Nono/p54nrb is used as a nuclear marker. Adapted from Müller et al.[1]

line graphs relating the density of the color in each band. Measure the area of each histogram and export these data.

Perform the calculations (Figure 4C). Invert the pixel density for all data of the Ponceau S staining in new columns. The inverted value is expressed as "Blank minus Mean", where "Mean" is the value recorded by ImageJ. For example, if the pixel density recorded by ImageJ is 200.646 for the entire lane and 231.495 for blank then the inverted value should return 30.849. Normalize these values to the cytoplasmic fraction of plakophilin 3-GFP cells.

Normalize the area readings from the cytoplasmic marker to the cytoplasmic fraction of plakophilin 3-GFP cells. Finally, take a ratio of the normalized intensities of the cytoplasmic marker over the normalized intensities of the Ponceau S staining. A bar plot (Figure 4C upper right panel) shows the detection of the cytoplasmic marker mainly in the cytoplasmic fractions of both cell lines. Repeat the measurement for the nuclear marker by normalization to the nuclear fraction of plakophilin 3-GFP cells (Figure 4C lower panels). A bar plot shows the detection of the nuclear marker mainly in the nuclear fractions of both cell lines. A successful subcellular fractionation should show cytoplasmic and nuclear fractions without cross contamination between both fractions.

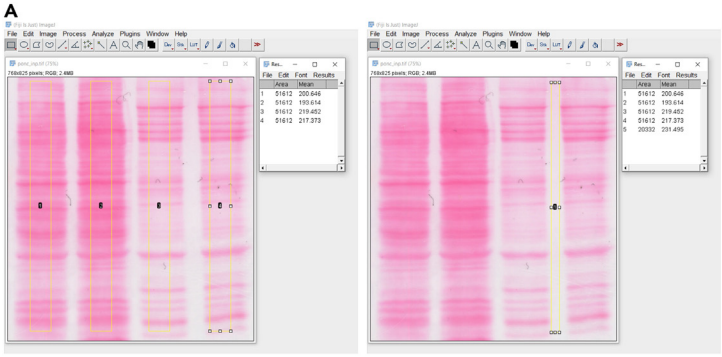
For analysis of the subcellular localization of interaction partners, quantify the band intensities of the protein of interest (e.g., phospho-retinoblastoma protein) of the eluate samples (Figure 4D). Take a ratio of the area reading from plakophilin 3-GFP cells over the area reading from GFP-tag overexpressing cells (not fused with plakophilin 3). This is the relative level of proteins co-precipitated with plakophilin 3 compared to unspecific binding of proteins with GFP-tag and shows the subcellular protein-protein interactions. The author determines statistical significance of protein-protein interactions between cytoplasmic and nuclear fractions using Student's unpaired two-tailed t test. The author recommends performing at least three independent experiments.

LIMITATIONS

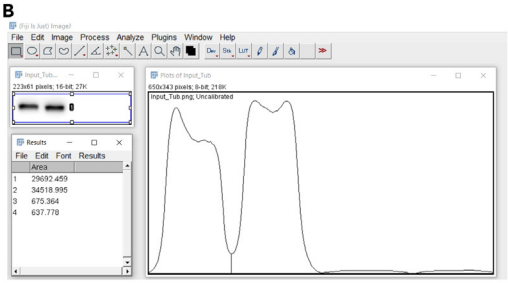
The shown experimental approach has been applied to identify the putative interacting partners of proteins in distinct subcellular locations. A major advantage of this protocol is a fast and inexpensive performance. Nonetheless, transient or weak interaction partners may not co-precipitate. Thus, the outcome does not project the full range of biological complexity.

A major limitation is that the outcome depends on the efficiency of the nuclear/cytoplasmic fractionation. Cytoskeletal organization varies between different cell lines. Since cytoskeletal proteins can

A

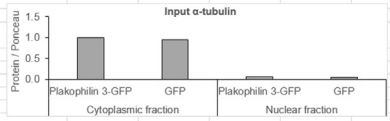


B

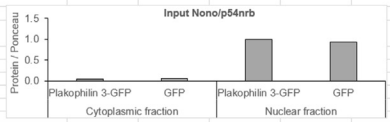


C

			Input Ponceau S staining			Input α-tubulin		
		Lane	Mean	Blank minus Mean	Relative to cytoplasmic fraction of Plakophilin 3-GFP	Area	Relative to cytoplasmic fraction of Plakophilin 3-GFP	Relative to Ponceau S staining
Cytoplasmic fraction	Plakophilin 3-GFP	1	200.646	30.849	1.00	29692.459	1.00	1.00
	GFP	2	193.614	37.881	1.23	34518.995	1.16	0.95
Nuclear fraction	Plakophilin 3-GFP	3	219.452	12.043	0.39	675.364	0.02	0.06
	GFP	4	217.373	14.122	0.46	637.778	0.02	0.05
		Blank	231.495					



			Input Ponceau S staining			Input Nono/p54nrb		
		Lane	Mean	Blank minus Mean	Relative to nuclear fraction of Plakophilin 3-GFP	Area	Relative to nuclear fraction of Plakophilin 3-GFP	Relative to Ponceau S staining
Cytoplasmic fraction	Plakophilin 3-GFP	1	200.646	30.849	2.56	2430.823	0.12	0.05
	GFP	2	193.614	37.881	3.15	3572.723	0.18	0.06
Nuclear fraction	Plakophilin 3-GFP	3	219.452	12.043	1.00	19743.966	1.00	1.00
	GFP	4	217.373	14.122	1.17	21547.966	1.09	0.93
		Blank	231.495					



D

			Eluate Phospho-retinoblastoma protein	
		Lane	Area	Plakophilin 3-GFP relative to GFP
Cytoplasmic fraction	Plakophilin 3-GFP	1	36464.329	4.07
	GFP	2	8960.915	1.00
Nuclear fraction	Plakophilin 3-GFP	3	611.163	0.76
	GFP	4	801.941	1.00

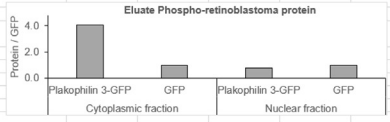


Figure 4. Example of immunoblot quantification

(A) Measurement of the mean gray value of all lanes and a blank membrane region of the Ponceau S staining of the input samples.

(B) Measurement of the histogram area of the bands of the cytoplasmic and nuclear markers.

(C) Calculation of normalized intensities of the subcellular markers over the normalized intensities of the Ponceau S staining.

(D) Normalization of the band intensities of the protein of interest of the eluate samples.

attach to the nuclear envelope[13] and thereby contaminate the nuclear fraction, homogenization methods may require optimization for each cell line. Thus, the control of the quality of the fractions is very important and can be ensured by phase-contrast microscopy and detection of cytoplasmic and nuclear markers.

One disadvantages of using GFP-tagged proteins to determine the subcellular protein-protein-interactions is that the proteins observed are both overexpressed and tagged with a relatively large, albeit inert, protein. The GFP-tag can be added to the carboxy or amino terminus of the protein, which can induce different phenotypes depending where it appears on the protein.[14] GFP -tagged driven mislocalization of a protein might affect its functions, e.g., protein-protein interactions. Ideally, you may want to control the correct localization of the tagged protein by co-staining with a specific antibody and immunofluorescence analysis of co-localization. Additionally, you can perform further experiments using alternate epitope tags (e.g., FLAG, HA, Myc), which are smaller and have little or no effect on the structure of the resulting fusion protein. However, the author recommends performing further experiments, such as immunofluorescence studies, to confirm the protein localization derived from the fractionation results.

TROUBLESHOOTING

Problem 1

Cell lysis may be incomplete. The cells may not be homogenized (related to step 10). This will lead to a contamination of the nuclear fraction with whole cells and thus cytoplasmic proteins.

Potential solution

- Using detergents such as IGEPAL CA-630 before homogenization increases the efficiency of cell lysis. Vortex the lysate-IGEPAL CA-630 mix very well (step 9). This may require several minutes. Only when the IGEPAL CA-630 is completely dissolved, the homogenization will be highly efficient.
- Try to homogenize the cells with different methods, because optimal homogenization methods may differ between cell lines. Homogenization with hypodermic needles by about ten iterations up and down of the syringe lysed murine keratinocytes. This syringe-based homogenization method is easy to handle, is suitable for small volumes, gives reproducible result, and does not require specialized equipment. A syringe with a volume of 1 mL is sufficient for the indicated buffer volume. The author used disposable hypodermic needles with a thickness of 26 gauges (0.45 × 25 mm, brown) for small cells with rare cell-cell contacts. The author recommends using needles with a thickness of 21 gauges (0.8 × 40 mm, green) when homogenization of cells with strong cell-cell contacts. Try a variety of needle gauge sizes to increase the efficiency of homogenization. If syringe-based homogenization does not work in general, perform homogenization procedures using higher mechanical forces, e.g., dounce homogenization.[15] For more difficult material, use ultrasonic based homogenization or a French pressure cell press.[16] However, keep in mind that some homogenization methods are not well suited for small sample sizes.
- Be careful not to disintegrate nuclei from the very beginning. You may control this by applying a small volume of the lysate (1–2 μL) on a slide and checking for intact nuclei using a phase-contrast microscope.

Problem 2

The cytoplasmic and nuclear fractions show cross contamination between both fractions (related to step 12 and step 18).

Potential solution

- Cross contaminations between the two fractions can be detected by staining for cytoplasmic and nuclear markers in the immunoblot. A cytoplasmic marker should not be detected in the nuclear fraction. Conversely, a nuclear marker should be absent from the cytoplasmic fraction. Try to use several markers, because appropriate cytoplasmic and nuclear markers may differ between cell lines. Useful cytoplasmic markers are α-tubulin, β-actin, pyruvate kinase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (see Figure 5A), whereas the proteins Nono/p54nrb, lamin A/C/B1, nucleoporin, histones and histones-associated proteins can be used as nuclear markers.[1,10,17]
- Optimize buffer concentrations, incubation times, and centrifugation parameters to improve the quality of the fractions. For recovery of the cytoplasmic fraction, centrifuge at mid speed (maximum 14,000 × *g*, step 11), because high speed centrifugation may promote nuclear lysis and thus nuclear contamination of the cytoplasmic fraction. For purification of the nuclear fraction, additional washes of the pelleted nuclei in the hypotonic buffer (step 13) can be performed.

Problem 3

The cytoplasmic and nuclear fractions show unequal densities (related to step 43).

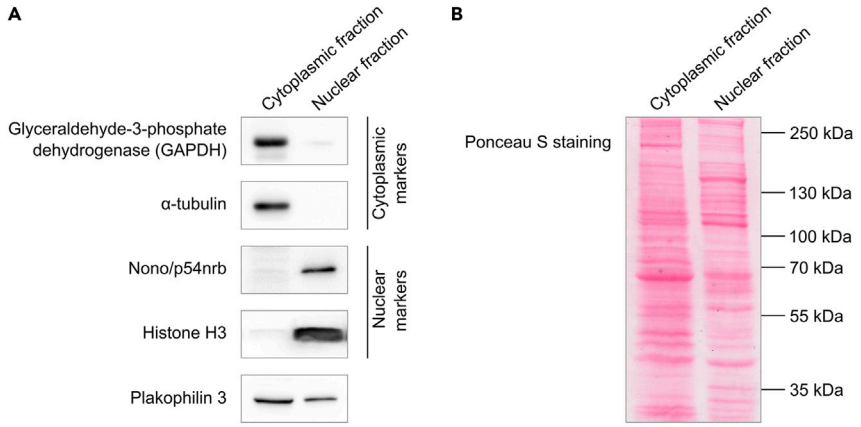


Figure 5. Troubleshooting of nucleus/cytoplasm fractionation

(A) Use the appropriate cytoplasmic and nuclear markers to avoid detection of cross contamination between both fractions.

(B) Ponceau S staining can be used as loading control of cytoplasmic versus nuclear fraction.

Potential solution

- Very unequal densities of the two fractions make comparison of the immunoblot band intensities difficult. As a consequence, the quantification of the subcellular distribution of proteins and their putative interaction partners will not be accurate. Before performing immunoblotting, determine the protein concentration in each sample in order to ensure appropriate loading of the SDS gel in the subsequent steps. For example, the Bradford assay provides fast and simple protein quantification.[18]
- Perform Ponceau S staining to detect protein bands on the immunoblot membrane (step 32). Ponceau S staining is a rapid and reversible staining method that can be used as loading control (see Figure 5B).
- If the density of the nuclear fraction sample is low, optimize nuclear lysis (see problem 1 and its potential solutions). If nuclei are efficiently solubilized, the pellet after high speed centrifugation (step 17) should be considerably smaller than that observed after mid speed centrifugation (step 11).
- The nucleoplasm to cytoplasm ratio depends on cell type and growth conditions. For example, it may vary between ~1% for mature adipocytes[19] and ~ 50% for very small cells including highly proliferating tumor cells.[20] Keep in mind that the nucleoplasm to cytoplasm ratio may vary depending on cell lines.
- Optimize the amount of hypotonic and hypertonic buffer to match your cell type and achieve similar protein concentration in both extracts.

Problem 4

There are no or very weak bands upon immunoblot detection (related to step 43).

Potential solution

- Try to use GFP-tagged fusion proteins to perform GFP-Trap immunoprecipitation. When performing a GFP-Trap immunoprecipitation, no additional antibody is required. Thus, no heavy and light antibody chains contaminate the fractions. This results in a low background and highly effective immunoprecipitation.
- Optimize the handling of the samples. Avoid elevated temperatures, which can cause protein degradation during the experiment.

- Increase the amount of cells for the nucleus/cytoplasm fractionation, but keep the amount of buffers constant.
- Optimize the IGEPAL CA-630 amount (step 8). Non-ionic detergents such as IGEPAL CA-630, Nonidet P-40, or Triton X-100 can be applied prior to homogenization.[9] High detergent concentrations might interfere with weak protein interactions and might result in less specific detection in immunoblots, but decreased background signals. However, some cell types may require higher concentrations of detergent for efficiently homogenization.[10]
- Compare the quality of the protein transfer from the SDS gel to the blotting membrane to colored molecular weight standards (step 30c).
- Increase the amount of antibody to detect the target protein.
- Use the appropriate immunoblot substrate, which enables the detection of picogram amounts of antigen.

Problem 5

Immunoblot detection of the protein of interest reveals a high unspecific background signal (related to step 43). Such a high noise badly affects the detection of the protein of interest.

Potential solution

- Increase the incubation time of blocking the membrane (step 36). Blocking of the membrane is an essential step of immunoblotting, as it prevents antibodies from binding to the membrane non-specifically.
- Wash the nitrocellulose blotting membrane thoroughly (step 38, step 40, step 41).
- The primary antibody may have low affinity for your protein of interest. Optimize the concentration of the primary antibody (step 37). Use as little antibody as possible since higher concentrations favor unspecific binding. Incubation at +4°C reduces background signals. The incubation time of the antibody should not exceed 24 h.
- The secondary antibody may bind non-specifically. Use the secondary antibody alone (without a preincubation with the primary antibody). If bands develop, reduce the concentration of the secondary antibody or choose an alternate secondary antibody (step 39).
- Use the appropriate immunoblot substrate, which enables the detection of picogram amounts of antigen (step 42).
- Avoid drying out of the membrane during processing. Perform all washing and antibody incubation steps on a rocking shaker to ensure even agitation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lisa Müller (lisa.mueller@uk-halle.de).

Materials availability

No materials were generated in this study.

Data and code availability

No data or code was generated in this study.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

L.M. performed the experiments, developed and optimized the protocol, and wrote the manuscript.

DECLARATION OF INTERESTS

The author declares no competing interests.

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