Biochemical Fractionation of Brain Tissue for Studies of Receptor Distribution and Trafficking

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ABSTRACT

An important tool for studying the regulation of synapses is a rapid and reliable means of separating synaptic and intracellular proteins. This unit presents a technique for analysis of brain tissue which relies on differential centrifugation to separate proteins present at synaptic sites from those found in intracellular cytoplasmic and vesicular pools. The method is efficient in that only small amounts of tissue, such as might be obtained from a small region of a rodent brain, are required. It is reproducible and, in conjunction with immunoblot or immunoprecipitation techniques, can produce reliable quantitative data. The protocol will be of interest to those conducting a variety of different studies related to the localization and trafficking of brain receptors and signaling molecules. Curr. Protoc. Neurosci. 42:1.16.1-1.16.16. © 2008 by John Wiley & Sons, Inc.

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INTRODUCTION

Receptor trafficking refers to the movement of proteins from one subcellular compartment to another. Intracellular trafficking and synaptic clustering of receptors play a fundamental role in controlling appropriate localization of receptors in neurons, which in turn is critical to the regulation of neural signaling.

The Basic Protocol describes a method of differential centrifugation to separate proteins present at synaptic sites from those found in intracellular cytoplasmic and vesicular pools. This biochemical technique can be used to investigate the distribution of receptors within different subcellular compartments and under different physiological conditions or disease states. By combining centrifugation with immunoprecipitation, the method can also be used to assess compartment-specific post-translational modifications, particularly protein phosphorylation, which may affect receptor activation, subcellular distribution, and synaptic targeting. Similarly, differential centrifugation together with immunoprecipitation can be used to assess macromolecular assemblies of receptors with scaffolding proteins and signaling molecules involved in the regulation of receptor function. Support Protocols 1 to 4 describe procedural details for such immunoprecipitation studies.

The methods described here are reliable, quantitative, and reproducible; they have been successfully used in the striatum, cortex, and cerebellum of both rodent and primate brains, and should be relevant for studies related to the localization and trafficking of receptors and signaling molecules in various regions of the brain.
STRATEGIC PLANNING

The Basic Protocol describes a subcellular fractionation procedure. Using immunoblotting procedures (also described in detail in the Basic Protocol) the distribution of proteins in different subcellular compartments can be determined, and the localization of such proteins following different pharmacological treatments, or in different disease states, can be compared. Support Protocol 4 describes a method to study either the association of two different proteins within one subcellular compartment or the phosphorylation of a protein within a subcellular compartment. For coimmunoprecipitation studies for the investigation of interactions between two proteins, the subcellular compartment of interest should be solubilized under non-denaturing conditions (see Support Protocol 1) before proceeding to the general immunoprecipitation procedure in Support Protocol 4. For the investigation of protein phosphorylation, denaturing conditions should be used for protein solubilization (see Support Protocol 2) before proceeding to Support Protocol 4 for immunoprecipitation. For proteins with weak or transient interactions (e.g., the association of a protein with an enzyme) the sample can be chemically cross-linked using dithiobis(succinimidylpropionate) (DSP; see Support Protocol 3) prior to subcellular fractionation and subsequent immunoprecipitation studies. Figure 1.16.1 is a flow diagram showing appropriate sequences of protocols that should be followed for studies of receptor abundance, receptor composition, or protein phosphorylation.

SUBCELLULAR FRACTIONATION OF BRAIN TISSUES

The following subcellular fractionation procedure essentially describes a sequence of centrifugations that separates the starting material (e.g., dissected brain regions) into different subcellular compartments, including a synaptosomal membrane fraction, a synaptic vesicle-enriched fraction, a light-membrane-enriched fraction, and cytosolic...
fractions. See Figure 1.16.2 for a schematic showing the various steps that constitute the procedure.

Samples must always be kept at 4°C during the subcellular fractionation procedure and during subsequent handling of fractionated samples. TEVP buffers are used for the subcellular fractionation procedure. They should be prepared in advance, chilled, and adjusted to pH 7.4 at 4°C. The basic TEVP buffer contains NaF, Na₃VO₄, EDTA, and EGTA, as well as protease inhibitors. The addition of NaF and Na₃VO₄ reduces the actions of phosphatases, while EDTA, EGTA, and protease inhibitors decrease proteolysis. For the initial homogenization step, TEVP buffer containing 320 mM sucrose is used, and for the hypoosmotic lysis step, TEVP buffer containing 35.6 mM sucrose is applied. For resuspension of pellet fractions before transferring into a 1.5-ml microcentrifuge tube, TEVP buffer with no sucrose added is used.

The volumes given for resuspension of pellet samples are approximate for a starting frozen weight of 100- to 200-mg tissue, and yield protein values of 5 to 10 µg/µl for the pellet samples. When a larger starting weight of tissue is used, buffer volumes should be increased proportionally.

**NOTE:** All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for care and use of laboratory animals.

**Materials**

- Laboratory rats (either gender)
- Chilled isopentane (−35°C to −45°C)
- TEVP buffer, 4°C (see recipe)
- TEVP buffer containing 320 mM sucrose, 4°C (see recipe)
- TEVP buffer containing 35.6 mM sucrose, 4°C (see recipe)
- 4× sample buffer (see recipe)
- SDS-acrylamide gels *(CPMB UNIT 10.2)*
- Broad-range molecular weight markers
Polyvinylidene difluoride (PVDF) membrane (e.g., BioRad PVDF immunoblotting membrane)
TBST buffer (see recipe)
Non-fat milk powder
Primary antibodies against antigens of interest
0.1% sodium azide
HRP-conjugated secondary antibodies
Chemiluminescent detection reagent (e.g., Western Lightning from Perkin-Elmer)
Tissue homogenizer (e.g., Tissuemizer)
Transfer pipet
Vortex
Large and small ultracentrifuge tubes (e.g., Beckman 38.5-ml and 6.5-ml polyallomer tubes)
Ultracentrifuge
Probe sonicator
Microplate protein assay kit (e.g., BioRad DC protein assay kit, BioRad)
Microplate reader
Heating block or boiling water bath
SDS-acrylamide gel electrophoresis (SDS-PAGE) equipment
Electrophoresis transfer equipment
Chemiluminescence-detecting film (e.g., Hyperfilm ECL from Amersham)
Automatic film developer
Additional reagents and equipment for euthanasia of the mouse (APPENDIX 4H) and SDS-PAGE (CPMB UNIT 10.2)

Dissect rat brain tissues
1. Euthanize rat according to institutionally approved procedure (e.g., pentobarbital overdose or decapitation; see APPENDIX 4H), immediately remove the brain, and dissect the regions of interest over wet ice (e.g., striata, cortex, hippocampus, and cerebellum).
2. Snap freeze the dissected tissue in chilled isopentane (−35°C to −45°C), and store up to 6 months at −80°C.

Perform differential centrifugation
3. Defrost 100- to 200-mg frozen weight rat brain tissue (e.g., approximately two rat striata, two rat hippocampi, or half of a rat cortex) for 5 min in 5 ml ice-cold TEVP buffer, pH 7.4, containing 320 mM sucrose, on wet ice.
4. Homogenize tissue for 10 to 15 sec using a tissue homogenizer (e.g., Tissuemizer) on a medium load setting. Remove a 500-µl sample of the homogenate (H) and transfer into a microcentrifuge tube.
5. Centrifuge the remaining homogenate for 10 min at 800 × g, 4°C, in ultracentrifuge tubes (38.5 ml).
6. Carefully separate the supernatant (S1) from the pellet (P1) by gently removing the supernatant either by decanting or using a transfer pipet. Transfer supernatant into a clean 38.5-ml ultracentrifuge tube.
   The P1 pellet contains nuclei and large debris.
7. Add 1 ml TEVP buffer to the remaining pellet (P1) and gently vortex to resuspend. Remove a 500-µl aliquot and transfer into a microcentrifuge tube. Discard the remainder.
8. Centrifuge the S1 supernatant for 15 min at 9,200 × g, 4°C, in ultracentrifuge.
9. Remove a 300-µl sample of the supernatant (S2) and transfer into a microcentrifuge tube. Transfer the remaining supernatant to a small (6.5-ml) ultracentrifuge tube and place on wet ice.

10. Briefly rinse the P2 pellet using 1 ml TEVP buffer containing 35.6 mM sucrose, and then discard the buffer.

   This step minimizes cross-over contamination between fractions. The P2 pellet contains crude synaptosomal membranes.

11. Resuspend the P2 pellet in 2 ml TEVP buffer containing 35.6 mM sucrose. Vortex gently for 5 to 10 sec to dislodge the pellet from the side of the tube and to break it up. Place the centrifuge tube containing the sample on wet ice and leave for 30 min.

   As the 35.6 mM sucrose buffer is hypo-osmotic to the tissue sample, lysis to release synaptic vesicles and other cytoplasmic organelles occurs.

12. Vortex the P2 sample gently and remove 300 µl and transfer into a microcentrifuge tube.

13. Centrifuge the remaining P2 fraction for 20 min at 25,000 × g, 4°C, in ultracentrifuge.

14. Following centrifugation transfer the supernatant (LS1) into a small (6.5-ml) ultracentrifuge tube, and transfer a 300-µl sample into a microcentrifuge tube. Rinse the LP1 pellet briefly in 1 ml TEVP buffer and discard this wash buffer. Resuspend the LP1 pellet in 1 ml TEVP buffer by vortexing for 5 to 10 sec and transfer the sample into a microcentrifuge tube.

   The LP1 pellet contains synaptosomal membranes.

15. Centrifuge the S2 and LS1 supernatant fractions for 2 hr at 165,000 × g, 4°C, in ultracentrifuge.

16. Transfer a 500- to 1000-µl sample of the supernatants (S3 and LS2) into microcentrifuge tubes. Discard the remaining supernatant. Briefly rinse the P3 and LP2 pellets with TEVP buffer and discard wash buffer. Resuspend the pellets in 50 to 100 µl TEVP buffer and transfer into clean microcentrifuge tubes.

   The P3 pellet contains light membranes, while the LP2 pellet is enriched with synaptic vesicles.

17. Sonicate all pellet fractions for 5 to 10 sec, using a probe sonicator.

18. Perform a protein assay on each pellet and supernatant fraction before freezing and storing at −80°C (stable up to 6 months).

   It is typical to have low protein concentrations in the LS1, LS2, and S3 supernatant fractions.

**Detect proteins in isolated subcellular fractions by SDS-PAGE**

19. Defrost each fractionated sample on wet ice and then briefly vortex.

20. Using the protein concentrations determined previously, prepare samples of protein in sample buffer (~1 to 10 µg/µl concentrations are used for the detection of NMDA receptor subunits) and boil for 5 min on a heating block or in boiling water bath.

   For the supernatant fractions (LS1, S3, LS2), where protein levels are very low, add as much sample as possible. All samples should contain the same amount of protein for equal gel loading.

21. Load samples onto an SDS-polyacrylamide gel and include a lane of broad-range molecular weight markers. For the detection of higher-molecular-weight proteins,
use a lower percent polyacrylamide gel, and vice versa. Separate proteins using SDS-gel electrophoresis (*CPMB UNIT 10.2*).

22. Transfer proteins onto PVDF membrane (*UNIT 5.19*).

23. Remove membranes from transfer equipment, and wash in TBST buffer for 5 min on a shaking platform.

24. Incubate membranes in blocking solution (5% non-fat milk in TBST buffer) for 30 to 60 min at room temperature on a shaking platform.

25. Transfer membranes into a solution containing the antibodies of interest in blocking solution containing 0.1% sodium azide, and incubate on a shaking platform at 4°C overnight.

   *The addition of sodium azide, which acts as a preservative, allows the primary antibody to be reused multiple times.*

26. Wash the membranes for 5 min in TBST buffer at room temperature on a shaking platform. Repeat wash in fresh TBST two more times.

27. Incubate with HRP-biotinylated secondary antibody in blocking solution for 1 hr at room temperature on a shaking platform.

28. Perform three 5-min washes in TBST, followed by three 10-min washes.

29. Quickly blot dry membrane to remove excess buffer solution (e.g., using a Kimwipe) and then incubate in chemiluminescence detection reagent, as per manufacturer’s instructions.

30. Expose blot to chemiluminescence-detecting film for various amounts of time and develop film in an automatic film developer.

**SUPPORT PROTOCOL 1**

**NON-DENATURING PROTEIN SOLUBILIZATION FOR COIMMUNOPRECIPITATION OF PROTEIN COMPLEXES**

Following the subcellular fractionation procedure, the interaction of a protein of interest with another protein can be investigated using coimmunoprecipitation. The proteins are solubilized using non-denaturing conditions to preserve the association of the proteins.

**Additional Materials** (*also see the Basic Protocol*)

- Subcellular fractions of interest (from the Basic Protocol)
- Deoxycholate (DOC) buffer (10% sodium deoxycholate in 500 mM Tris-Cl, pH 9.0)
- Buffer T at 4°C (see recipe)
- Dialysis buffer at 4°C (see recipe)
- 36°C water bath
- Dialysis cassettes (e.g., Slide-A-Lyzer Dialysis cassette, Pierce)
- Magnetic stir bar
- Stir plate

**Prepare samples**

1. Defrost on wet ice the fractions of interest acquired from the subcellular fractionation procedure (the Basic Protocol).

2. Remove ∼100 µg of each sample and transfer to new microcentrifuge tubes.

3. To each sample add one-tenth volume of DOC buffer to achieve a final concentration of 1% sodium deoxycholate.
4. Incubate samples for 30 min at 36°C with mild shaking.
5. Transfer samples onto wet ice and add one-tenth volume of ice-cold Buffer T.
6. Transfer samples into dialysis cassettes according to manufacturer’s instructions.

**Prepare for dialysis**

7. Place dialysis cassettes containing samples into a beaker containing 1 liter of ice-cold dialysis buffer and a magnetic stir bar. Dialyze samples overnight at 4°C on a stir plate set on low.

8. Following dialysis, transfer samples from the dialysis cassettes into centrifuge tubes on ice.

9. Centrifuge in an ultracentrifuge for 40 min at 36,000 × g, at 4°C.

10. Transfer the supernatants into clean microcentrifuge tubes on wet ice.

   *The supernatant should be clear. If it is turbid then centrifuge again.*

**Determine protein concentrations**

11. Determine the protein concentration of supernatants.


   *If not using for immunoprecipitation immediately, then aliquots of the sample can be frozen and stored up to 6 months at −80°C.*

**DENATURING PROTEIN SOLUBILIZATION FOR IMMUNOPRECIPITATION STUDIES OF PROTEIN PHOSPHORYLATION**

For phosphorylation studies, protein extracts should be solubilized under denaturing conditions to break macromolecular protein complexes into individual proteins for efficient precipitation of tyrosine-phosphorylated receptors.

**Additional Materials** *(also see Basic Protocol)*

- Subcellular fraction samples of interest (from the Basic Protocol)
- 20% sodium dodecyl sulfate (SDS) solution

1. Defrost on wet ice the fractions of interest (from the Basic Protocol).

2. Remove ~100 µg of each sample and transfer to new microcentrifuge tubes.

3. Add one-twentieth sample volume of 20% SDS (final concentration of 1% SDS) to solubilize the protein, and sonicate each sample for 5 sec.

   *The solubilized samples should now be kept at room temperature to prevent precipitation of the SDS.*

4. Centrifuge samples for 5 min at 15,000 × g, 4°C.

   *This step removes insoluble material*

5. Transfer the supernatant to a clean microcentrifuge tube, and determine the protein concentration of each supernatant.

   *The supernatant can be used for precipitation studies, such as described in Support Protocol 4.*
**DSP-MEDIATED CROSS-LINKING OF PROTEINS FOR COIMMUNOPRECIPITATION STUDIES**

To chemically cross-link proteins to identify weak or transient protein interactions, dithiobis{succinimidylpropionate} (DSP)-mediated cross-linking prior to biochemical fractionation and subsequent immunoprecipitation can be carried out. DSP reacts with primary amines to form stable amide bonds at pH 7 to 9.

**Additional Materials (also see the Basic Protocol)**

- Frozen weight rat brain tissue (from the Basic Protocol)
- 100 mM sodium borate buffer, pH 8.0, at 4°C
- Dithiobis [succinimidylpropionate] (DSP)
- Dimethylsulfoxide (DMSO)
- 1 M Tris·Cl, pH 7.6 (*APPENDIX 2A*)
- 15-ml Falcon centrifuge tubes

1. Defrost 100 to 200 mg frozen weight rat brain tissue (e.g., approximately two rat striata, two rat hippocampi, or half of a rat cortex) for 5 min in ice-cold sodium borate buffer on wet ice. Use ~2 ml buffer per 100 mg frozen weight tissue.

2. Homogenize tissue for 10 to 15 sec on a tissue homogenizer (e.g., Tissuemizer) with medium load setting.

3. Centrifuge for 10 min at 800 × g at 4°C.  
   *This step clears the sample cell debris.*

4. While the sample is centrifuging, prepare a fresh solution of 10 mM dithio-bis(succinimidylpropionate) (DSP) in 1 ml DMSO.

5. Remove the supernatant and transfer to a clean 15-ml Falcon tube.

6. Add the DSP stock solution to the supernatant to a final concentration of 200 µM.

7. Shake the sample gently for 10 to 15 min at 4°C.

8. Terminate the cross-linking reaction by adding 1 M Tris·Cl (pH 7.6) to a final concentration of 100 mM.

9. Shake the sample gently for 10 to 15 min at 4°C.

   *Biochemical fractionation, as described in the Basic Protocol, can now be performed using the cross-linked sample.*

**IMMUNOPRECIPITATION OF PHOSPHORYLATED PROTEINS AND COIMMUNOPRECIPITATION OF PROTEIN COMPLEXES**

The following procedures describe a basic immunoprecipitation protocol that can be used for coimmunoprecipitation of protein complexes of interest using extracts solubilized under non-denaturing conditions, or for precipitation of phosphorylated proteins using protein extracts solubilized under denaturing conditions. The immunoprecipitating antibody is initially precoupled to either Protein A (PA) Sepharose beads (for rabbit polyclonal antibodies) or Protein G (PG) Sepharose beads (for mouse monoclonal antibodies).

Steps 5 to 16 (cross-linking antibody to Protein Sepharose beads) are not essential, however, if coelution of immunoglobulins (IgGs) from the immunoprecipitating antibody is not desired (for example, if the molecular weight of the immunoprecipitated protein...
of interest is similar to the heavy chain of IgG), then chemical cross-linking between the antibody and the PA Sepharose beads can be performed.

**Materials**

PA or PG Sepharose beads (resuspended as a 20% slurry stock in 100 mM sodium borate buffer, pH 8.0, containing 0.02% sodium azide as a preservative)

100 mM sodium borate buffer, pH 8.0, at 4°C

Immunoprecipitating antibody (for the detection of tyrosine phosphorylated proteins, the BD Biosciences monoclonal phosphotyrosine PY20 antibody is recommended)

20 mM dimethylpimelimidate dihydrochloride

200 mM sodium borate buffer, pH 9.0, at 4°C

200 mM ethanolamine, pH 8.0

Deionized water

Solubilized protein samples (e.g., from Support Protocols 1 or 2)

RIPA buffer at 4°C (see recipe)

1.5-ml microcentrifuge tubes

Microcentrifuge

**Precouple antibody to protein Sepharose beads**

1. Transfer 50 µl bead volume of PA or PG Sepharose into a 1.5-ml microcentrifuge tube, and add 800 µl of ice-cold 100 mM sodium borate buffer, pH 8.0, to wash the beads.

2. Centrifuge briefly (~15 sec) in a microcentrifuge at maximum speed, 4°C, and then use a pipet to remove the supernatant from the beads. Discard supernatant.

3. Add 20 µg of the immunoprecipitating affinity-purified antibody to the beads, and dilute with 600 µl 100 mM sodium borate buffer, pH 8.0.

4. Incubate the PA/PG Sepharose bead-antibody suspension overnight at 4°C with gentle rotation.

**Cross-link antibody to protein Sepharose beads**

5. Just before use, prepare a fresh solution of 20 mM dimethylpimelimidate dihydrochloride in 200 mM sodium borate buffer, pH 9.0, and adjust the pH to 8.5 using HCl.

6. Following overnight incubation, centrifuge the PA/PG Sepharose-antibody coupled beads in a microcentrifuge (preferably at 4°C) for 15 sec at maximum speed. Remove and discard the supernatant.

   To remove the supernatant, it is recommended that a 200-µl pipet tip placed over the end of a 1000-µl pipet tip be used. The smaller aperture of the 200-µl tip will reduce accidental aspiration of the beads.

7. Add 800 µl of ice-cold 100 mM sodium borate buffer, pH 8.0. Gently invert the sample, centrifuge as in step 6, and repeat wash step for a total of three washes.

   Keep the tubes containing the PA/PG Sepharose-antibody coupled beads on ice as much as possible between centrifugation/washing steps to prevent the dissociation of the antibody from the Sepharose beads.

8. Add 600 µl of the dimethylpimelimidate dihydrochloride solution and incubate the PA/PG Sepharose-antibody-coupled beads for 30 min at room temperature with gentle rotation.
9. Centrifuge the sample briefly (~15 sec) and remove the supernatant.

10. Prepare a solution of 200 mM ethanolamine in deionized water, pH 8.0.

11. Wash the sample twice with 600 µl 200 mM ethanolamine.

12. Add 600 µl of 200 mM ethanolamine and incubate for 2 hr at room temperature with gentle rotation.

   *This step blocks nonspecific sites on the PA/PG Sepharose-antibody-coupled beads.*

13. Centrifuge the sample briefly (~15 sec) and carefully remove the supernatant.

14. Add 800 µl of 100 mM sodium borate buffer, pH 8.0, to the beads, and centrifuge briefly again.

15. Remove the supernatant and repeat this washing step twice.

   *At this point the PA/PG Sepharose-antibody-coupled beads are ready for use for immunoprecipitation, but if desired can be stored for several months in 100 mM sodium borate buffer, pH 8.0, containing 0.02% sodium azide at 4°C without any noticeable loss in activity. If using antibody-coupled beads that have been stored frozen, they should be washed prior to subsequent immunoprecipitation in 100 mM sodium borate buffer containing no sodium azide.*

**Immunoprecipitate antigen**

To couple the antigen to the PA-antibody coupled beads, remove any sodium borate buffer from the beads and add a suitable (100 to 300 µg) amount of solubilized protein sample to the beads [as prepared under denaturing conditions (for detection of phosphorylated proteins), or non-denaturing conditions (for coimmunoprecipitation studies) as described in Support Protocols 1 and 2].

16. Dilute the protein sample 20-fold with ice-cold RIPA buffer (~600 to 1000 µl).

17. Incubate the antigen with the antibody-coupled beads for 2 to 3 hr at 4°C with gentle rotation.

18. Briefly centrifuge the sample and remove the supernatant.

19. Add 800 µl of RIPA buffer to the antigen-antibody-coupled beads, and centrifuge briefly (preferably at 4°C).

20. Repeat this wash step twice.

21. After the final washing step, remove all buffer from the beads and add 50 µl of 2× sample buffer.

22. Boil the sample for 5 min and agitate the sample every minute to mix.

23. Vortex the sample vigorously for 2 min.

24. Microcentrifuge sample 5 min at maximum speed, 4°C.

25. Remove the supernatant from the beads and transfer to a microcentrifuge tube.

   *The supernatant now contains the immunoprecipitated proteins of interest. It is recommended to use a gel-loading pipet tip for this step to prevent uptake of the PA-Sepharose beads.*

   *The supernatant can now be used for SDS-gel electrophoresis and immunoblotting. The membrane obtained from the immunoblotting procedure will be incubated with a primary antibody, which recognizes the protein of interest.*
REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Buffer T
500 mM Tris-Cl (pH 9.0 at 4°C; APPENDIX 2A)
1% (v/v) Triton X-100
1% (w/v) sodium deoxycholate
Store up to 3 months at 4°C

Dialysis buffer, 10x
500 mM Tris-Cl, pH 7.0 (APPENDIX 2A)
1% (v/v) Triton X-100
Adjust to pH 7.0 using HCl, at 4°C
Dilute with dH2O to make a 1x solution
Store up to 3 months at 4°C

RIPA buffer, 2x
150 mM NaCl
50 mM Na2SO4
2 mM EDTA
1% (w/v) sodium deoxycholate
1% (v/v) Triton X-100
Adjust to pH 7.2 using HCl, at 4°C
Dilute with an equal volume of dH2O to make a 1x solution
Store up to 3 months at 4°C

Sample buffer, 2x
125 mM Tris-Cl, pH 6.8 (APPENDIX 2A)
4% SDS
100 mM DTT
15% glycerol
0.02% (w/v) bromphenol blue
Freeze aliquots at −20°C for up to one year. Defrost to room temperature and mix well (vortex) prior to using.

Sample buffer, 4x
250 mM Tris-Cl, pH 6.8 (APPENDIX 2A)
8% SDS
200 mM DTT
30% glycerol
0.02% (w/v) bromphenol blue
Freeze aliquots at −20°C for up to one year. Defrost to room temperature and mix well (vortex) prior to using.

TBST buffer, 10x
250 mM Tris base
1.4 M NaCl
0.1% (v/v) Tween 20
Adjust to pH 7.6 using HCl, at room temperature
Dilute with dH2O to make a 1x solution
Store up to 3 months at 4°C
**TEVP buffer**

- 10 mM Tris base
- 5 mM NaF
- 1 mM Na$_3$VO$_4$
- 1 mM EDTA
- 1 mM EGTA

Adjust to pH 7.4 using HCl (chill buffer to 4°C before measuring pH)

Store up to 3 months at 4°C

**TEVP buffer + 35.6 mM sucrose**

To TEVP buffer (see recipe) add 35.6 mM sucrose

Adjust to pH 7.4 using HCl (chill buffer to 4°C before measuring pH)

Store up to 3 months at 4°C

**TEVP buffer + 320 mM sucrose**

To TEVP buffer (see recipe) add 320 mM sucrose

Adjust to pH 7.4 using HCl (chill buffer to 4°C before measuring pH)

Store up to 3 months at 4°C

**COMMENTARY**

**Background Information**

There is a significant amount of literature published on the importance of intracellular trafficking in the regulation of a variety of ionotropic and G-protein-coupled receptors (e.g., Carroll and Zukin, 2002; Malenka, 2003; Isaac et al., 2004; Luscher and Keller, 2004; Perez-Otano and Ehlers, 2005; Bernard et al., 2006). Recent studies have shown that abnormal trafficking of receptors might underlie some neurological disorders, such as Parkinson’s disease, Alzheimer’s disease, and addiction (Dunah and Standaert, 2001; Wolf et al., 2003; Snyder et al., 2005).

The protocols described in this unit provide a useful technique for the isolation of various subcellular fractions from brain tissue. These methods are reliable, convenient, and reproducible, and are useful for studies of the localization and trafficking of receptors and signaling molecules, including the detection of post-translational modifications such as phosphorylation, palmitoylation, and glycosylation of proteins. The biochemical fractionation protocol has been successfully used to quantitatively study the localization and trafficking of receptor complexes (e.g., glutamate receptors) between synaptic sites and intracellular cytoplasmic and vesicular compartments in both rodent and primate tissues (Dunah and Standaert, 2001, 2003; Hallett et al., 2005). In addition, this biochemical technique can be used to investigate modifications in the properties of receptors under varying pathophysiological conditions, such as in disease states (Dunah et al., 2000; Hallett et al., 2005).

**Critical Parameters**

The major disadvantage of the subcellular fractionation technique is that a relatively large amount of starting tissue is needed to obtain adequate protein in the final LP2 (synaptic-vesicle-enriched) fraction. In general at least 100 mg of starting material is needed to obtain sufficient protein in the LP2 fraction for several polyacrylamide gels (~50 µl of sample with a protein concentration of 2 to 5 µg/µl is typical). If small regions of tissue are used (for example, mouse striatum), then tissue can be pooled from several animals to obtain an adequate starting weight of tissue, resulting in sufficient amounts of protein in the various subcellular fractions, especially the LP2. This pooling step is particularly important in quantitative co-immunoprecipitation studies in which large amounts of proteins are required. If the LP2 fraction is not required for study, then it is possible to obtain sufficient P2 and LP1 fractions with less starting material.

**Troubleshooting**

The commonly encountered problems with the biochemical subcellular fractionation procedure, and suggestions for addressing such difficulties, are described in Table 1.16.1. Advice on frequently encountered problems with polyacrylamide gel electrophoresis (CPMB UNIT 10.2A) and immunoprecipitation (UNITS 5.24 & 5.25) techniques is widely published in the literature.
Table 1.16.1 Troubleshooting Biochemical Fractionation of Brain Tissue

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause</th>
<th>Solution</th>
</tr>
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<tbody>
<tr>
<td>Low protein yields in pellet fractions</td>
<td>Insufficient starting material</td>
<td>Use at least 100 mg tissue to obtain adequate protein in LP2 fraction</td>
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<tr>
<td></td>
<td>Pellet samples resuspended in excessive volume of buffer</td>
<td>Resuspend pellets in smaller volumes of buffer. The pellet resuspension volumes recommended in the Basic Protocol are a guideline, and can be modified to suit individual experimental requirements.</td>
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<tr>
<td></td>
<td>Not all pellet material was properly resuspended</td>
<td>It may be difficult to observe small pellets in the centrifuge tubes after centrifugation, in particular the LP2 pellet fraction. To facilitate visualization of the pellet, observe the pellet immediately following removal of the centrifuge tube from the rotor, and outline it on the outside of the tube with a pen. To facilitate resuspension of the LP2 and P3 pellets, remove the very end of a 200-µl pipet tip with a diagonal cut. The sharper end will permit easier scraping of the pellet from the side of the centrifuge tube.</td>
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<tr>
<td>Faint bands, or absence of detectable bands of expected size on immunoblot; observance of proteolytic products at lower molecular weight than the expected size</td>
<td>Proteolytic breakdown of proteins due to improper handling of samples, or lack of protease and phosphatase inhibitors</td>
<td>Include protease and phosphatase inhibitors in TEVP buffers used for biochemical fractionation procedure; perform all centrifugation steps at 4°C; keep samples on ice at all times [the only exception is when protein samples are solubilized in SDS (as described in Support Protocol 2), and subsequently the sample is stable at room temperature.</td>
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Figure 1.16.3 Example characterization of the biochemical fractionation procedure. The isolated subcellular compartments from striatal rat tissues were separated by SDS-PAGE, transferred to PVDF membranes, and the blots probed with antibodies against synaptophysin, calnexin, and β-tubulin. H, total homogenate; P1, nuclei and large debris; P2, crude synaptosomal membrane fraction; P3, light membrane fraction; LP1, synaptosomal membrane fraction; LP2, synaptic vesicle-enriched fraction. S1, S2, LS1, and LS2 are supernatants from P1, P2, LP1, and LP2, respectively. Synaptophysin is concentrated in P3 (lane 6), LP1 (lane 8), and LP2 (lane 10) fractions. Calnexin is significantly enriched in P3 (lane 6). β-tubulin was detected in both cytosolic and membrane-associated compartments.

Anticipated Results
The protocols in this unit produce reliable separation of distinct subcellular compartments. This can be demonstrated by examination of marker proteins for various compartments such as synaptophysin, calnexin, and β-tubulin (Fig. 1.16.3). Synaptophysin is a synaptic vesicle glycoprotein and is highly concentrated in the synaptosomal membrane (LP1, lane 8), synaptic vesicle-enriched (LP2, lane 10), and light membrane (P3, lane 6) fractions (Fig. 1.16.3, top row); whereas, calnexin,
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Figure 1.16.4  Subcellular localization of glutamate receptor subunits and their interacting proteins. The isolated biochemical fractions from striatal rat tissues were resolved by SDS-PAGE and resulting blots probed with antibodies against NR1, NR2A, NR2B, PSD-95, GluR2/3, and GRIP. The glutamate receptor subunits NR1, NR2A, NR2B, and GluR2/3 are enriched in the LP1 (lane 8) and are also found in P3 (lane 6) and LP2. PSD-95 and GRIP are mainly detected in the synaptosomal membrane compartment (LP1, lane 8).

a calcium-binding protein, which is an integral membrane protein of the endoplasmic reticulum (David et al., 1993), is highly enriched in the light membrane compartment (P3, lane 6) relative to the LP1 and LP2 fractions (Fig. 1.16.3, middle row). The soluble microtubule-associated protein β-tubulin, is present in the cytosolic fractions (LS1, lane 9, S3, lane 7, and, LS2, lane 11), as well as the membrane-associated subcellular compartments (LP1, lane 8, P3, lane 6, and LP2, lane 10; Fig. 1.16.3, bottom row).

The efficiency of separation is also illustrated by the ability of the method to reveal the proper compartments containing glutamate receptors and associated anchoring and signaling components. The glutamate receptors NR1, NR2A, NR2B, and GluR2/3 are integral membrane proteins and found to be mainly confined to the three distinct membrane-linked compartments (LP1, LP2, and P3; Fig. 1.16.4). A similar distribution pattern is seen for the NMDA receptor anchoring protein, PSD-95, and the AMPA receptor binding protein GRIP (Fig. 1.16.4), correlating with previous reports that revealed glutamate receptor localization to post-synaptic sites and intracellular vesicular compartments (Petraila et al., 1994). The post-synaptic density is contained in LP1 and thus constitutes proper localization sites for both glutamate receptors, including anchoring and signaling molecules (Kennedy, 1993; Ziff, 1997). The LP2 fraction contains synaptic vesicles and trafficking organelles for membrane proteins and neurotransmitter receptors (Sudhof, 1995; Takamori et al., 2000), and the glutamate receptors present might constitute those in transit for targeting at the postsynaptic density. On the other hand, the P3 fraction contains cellular organelles such as

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endoplasmic reticulum, Golgi apparatus, and mitochondria (Schapira, 1998), and might represent newly synthesized glutamate receptors destined for post-translational modifications.

Immunoprecipitation can be used to study the coprecipitation of two proteins, or for post-translational modifications of proteins, such as phosphorylation, in a particular fraction of interest. Figure 1.16.5 shows an example of such an experiment, whereby fractionated striatal protein extracts (LP1 fraction) have been immunoprecipitated with an anti-phosphotyrosine antibody and the precipitates subsequently immunoblotted with antibodies selective for NMDA receptor subunits. The “Input” (lanes 1 through 3) represents the solubilized protein sample obtained following the denaturing solubilization procedure, and contains both phosphorylated and nonphosphorylated proteins. The “Pellet” extract (lanes 4 through 6) represents the immunoprecipitated sample obtained from the final immunoprecipitated pellet, and therefore contains only tyrosine-phosphorylated proteins.

Time Considerations
The subcellular fractionation procedure described in Basic Protocol, including preparation of relevant buffers, can be performed in 1 day. Subsequent immunoblotting can be carried out over 2 days, and immunoprecipitation experiments including immunoblotting, over 3 days.

Samples obtained from the subcellular fractionation procedure can be safely stored at −80°C for several months without any obvious degradation in protein quality or phosphorylation. However, samples should not be exposed to repeated freeze-thaw cycles, and if the sample will be required for several experiments, it is recommended to aliquot it into smaller volumes suitable for the sizes of the experiments. Protein samples that have been solubilized in either sodium dodecyl sulfate (SDS), or sodium deoxycholate (DOC), can also be frozen at −80°C and subsequently reused. Samples solubilized in SDS should be allowed to warm to room temperature and briefly sonicated before reuse, as the SDS will have precipitated after freezing/cooling.

Literature Cited


Dunah, A.W. and Standaert, D.G. 2001. Dopamine D1 receptor-dependent trafficking of striatal

Figure 1.16.5 Tyrosine phosphorylation of NMDA receptor subunits. Soluble extracts from synaptosomal membrane fraction of rat striatum were precipitated with anti-phosphotyrosine antibody. Different amounts of samples from the input (lanes 1 through 3) and pellet (lanes 4 through 6) were immunoblotted with antibodies specific for tyrosine-phosphorylated NR2A and NR2B subunits.


