Chapter 3

Isolation of Synapse Sub-Domains by Subcellular Fractionation Using Sucrose Density Gradient Centrifugation: Purification of the Synaptosome, Synaptic Plasma Membrane, Postsynaptic Density, Synaptic Membrane Raft, and Postsynaptic Density Lattice

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Abstract

A protocol presents a purification of postsynaptic density (PSD), from rat brain by subcellular fractionation using solubilization of membrane with Triton X-100 and sucrose density centrifugation. The protocol also includes purification of other synapse sub-domains such as synaptosome, synaptic plasma membrane (SPM), synaptic membrane raft, PSD lattice, P_1 (nuclei and cell debris), P_2 (crude mitochondria fraction), S_3 (soluble fraction), and P_3 (microsomal fraction). The PSD purification method presented in this text is the one established by Siekevitz group. The PSDs obtained by this method are mainly excitatory type I PSDs. These methods are useful for biochemical analyses such as identification of proteins associated with these sub-domains by proteomics methods and western blotting, and morphological analyses at the electron microscopic level. The purification protocol for the synaptic membrane raft using sucrose gradient ultracentrifugation is a useful means by which to analyze the relationship between the PSD and synaptic membrane raft by isolating both preparations simultaneously.

Key words Synaptosome, Synaptic plasma membrane, Postsynaptic density, Postsynaptic membrane raft, PSD lattice, Subcellular fractionation, Detergent-insoluble cytoskeleton, Detergent-insoluble membrane

1 Introduction

Isolation of subcellular compartment is a useful approach to analyze the subcellular complexes at the molecular level. We describe methods to isolate synapse sub-domains including P_1 (nuclei and cell debris), P_2 (crude mitochondria fraction), synaptosome, synaptic plasma membrane (SPM), postsynaptic density (PSD), synaptic membrane raft, and PSD lattice by subcellular fractionation using density gradient centrifugation. History of development of method for isolation of synaptic complex and PSD is concisely summarized

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previously [1]. The method for PSD purification established by Siekevitz's laboratory [2–4] has been widely used. The methods introduced in this text are basically the same as those used in his laboratory. Both short and long procedures are stated. The method is applicable to brains from at least dog, rat, mouse, and human [2, 5, 6], different brain regions [4, 7–10] and brains in various developmental stages [11]. Protein yield of synaptosome, SPM, and PSD (short and long procedures, respectively) are approximately 14.7, 6.2, and 0.26 and 0.1 mg per 1 g original forebrain of adult rat, respectively, but may fluctuate by unknown factor(s). Percentages of PSD protein per amount of total protein in the forebrain, synaptosome, and SPM are summarized in Table 1. Protein yields of these fractions change depending on the age of the animals used [11]. The short procedure, in which PSD is purified from Triton X-100 (TX-100)-treated synaptosomes, has been widely used and is now a standard method. Protein profiles of the PSD isolated by short and long procedures in one-dimensional gel are similar but not identical (Fig. 1) [2, 12]. Contents of neurofilament proteins, at least partly contaminants [12], are higher in the PSD prepared by short procedure [12]. Protein yield is also different [12]. Major constituent proteins are the same between the two preparations, while the mass spectrometric (MS) analysis revealed that only 60–75% proteins in these two PSD fractions are common (T. Suzuki, unpublished data). Purified PSD fraction also contains mRNAs encoding various kinds of proteins [13].

In the early methods to prepare synaptic junctional complex and PSD, *p*-iodonitrotetrazorium violet (INT) was used to separate mitochondria by producing heavy formazan in mitochondria [14–16]. However, it was found that INT causes undesirable oxidation of proteins and artificially cross-links synaptic proteins [16–19]. Structures of the isolated PSD are tightened by disulfide bonds formed during the PSD isolation using INT. It is suggested that the artificial disulfide bonding of PSD proteins during isolation may occur even in the absence of INT [1, 20, 21]. Artificial cross-linking of postsynaptic proteins during isolation gives resistance of the isolated PSD to various treatments including detergent solubilization [1, 20, 21]. Blocking of disulfide formation is required for preparing PSD for analyses of its structural and physiological properties.

It is desirable to prepare synaptic sub-compartments from freshly dissected brains. PSD fraction can also be prepared from frozen brains [3], which is convenient, in particular, when purifying it from human specimens. However, special attention should be paid when collecting brain tissue, because some proteins, in particular Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), accumulate to PSD in a short duration after decapitation [22]. Accumulation of CaMKII is accelerated at room temperature or 37 °C. Tubulin also accumulates to PSD fraction in a relatively

Table 1
Summary of PSD protein content

Subfractions	Protein recovery mg/g FB (mean ± S.D.)	(%)	(%)	(%)
Total protein	92.6 ± 21.1 (n = 3)	100		
Synaptosome	14.7 ± 4.2 (n = 5)	15.9	100	
SPM	6.24 ± 1.77 (n = 4)	6.74	42.4	100
PSD	0.263 ± 0.109 (n = 11)	0.284	1.79	4.21

FB forebrain
PSD was purified from frozen forebrains of rat (6 weeks old, male) by short protocol
Values were calculated from the protein yields for total homogenate, synaptosome, SPM, and PSD prepared from adult Wistar rat forebrain

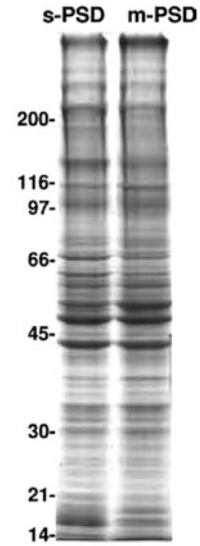


Fig. 1 Protein profiles of PSD fractions purified by short and long procedures. PSD fractions was purified from rat forebrains (Wistar male, 6 weeks old) and separated by 7–17% gradient polyacrylamide gel. s-PSD and m-PSD refer to PSD fractions prepared by short and long procedures via TX-100 treatment of synaptosome and SPM, respectively. Molecular weights are shown in kDa on the left

long time period at 4 °C after decapitation [23]. Attention should also be paid to "cold-induced exodus of postsynaptic proteins" [24]. Exposure of neuron to coldness causes rapid disassembly of unstable microtubules that are present in the spine and associated with PSD. Various proteins also exit from PSD, and spine morphology, at least some, may change by this microtubule disassembly.

The method stated in this text is useful to prepare the fraction enriched in the PSDs of asymmetric type I excitatory synapses, but not of the inhibitory neurons, such as those in the cerebellum. Protein yield of cerebellar PSD fluctuates; sometimes very low due to unknown reasons. Preparation of type II inhibitory PSD has been reported [25]. Method for the purification of PSD using sonication but not detergent has also been reported [26], but up to now the method has been reported only once to the best of author's knowledge.

"One-Triton" PSD and "Two-Triton" PSD are prepared as a pellet after centrifugation of TX-100-treated synaptosomes [27–29]. "One-Triton" PSD contains detergent-resistant membrane (DRM) with light buoyant density, which is also TX-100 insoluble at 4 °C and floats on the 1.0 M sucrose layer [25, 30]. Recently, it is demonstrated that "One Triton PSD" also contains type II GABAergic inhibitory PSD [25].

Nonionic detergent TX-100 is usually used to purify PSD fraction. High-quality TX-100 should be used. Other detergents, such as deoxycholate (DOC) [31, 32], *n*-octyl β-D-glucoside (OG) [1, 33], and *N*-lauroyl sarcosinate (NLS) [16, 27, 32], have also been used. NLS, a strong ionic detergent, nearly completely solubilizes PSD components when oxidation is prevented with 1 mM *N*-ethylmaleimide (NEM) during isolation of PSD [1]. DOC-insoluble PSD shows clearly a lattice-like core PSD structure [32, 34], which is broken after NLS treatment [32]. OG is effective to solubilize rapidly the whole membrane [35, 36] and generally does not affect protein–protein interactions.

Presynaptic structure is unstable in alkaline solution, while postsynaptic structures are resistant [37]. Therefore, the synaptic junctional structures composed of both pre- and postsynaptic cytoskeletal structures can be prepared when synaptosome is solubilized with TX-100 at slightly acidic conditions [37].

Membrane rafts are distributed in both pre- and postsynaptic sites in all neuronal components, including axons, dendrites, and somas, in both immature and mature neurons. We term detergent-insoluble materials with light buoyant density purified from SPM at a low temperature the synaptic membrane rafts. The postsynaptic membrane raft (PSR) and PSD are two major postsynaptic signaling domains that interact physiologically, and it is thought that PSRs may be indispensable to PSD function [38]. PSRs may be essential components of the postsynaptic signaling machinery connected to PSDs, providing membrane anchor sites for PSD cytoskeletons/scaffolds, as well as signaling platforms and sites for membrane fusion and vesicular trafficking. Thus, it is likely that postsynaptic activities require both PSDs and PSRs. PSRs may play a role in synaptogenesis, growth, and maturation of developing PSDs, and support and regulate functions and plasticity of mature

PSDs. The notion of lipid/membrane rafts [39] was first proposed in 1997 [40]. PSRs and PSDs can be separated by density in vitro, though they are both detergent-insoluble at low temperatures and interact with one another [41–43].

In a recent concept, PSDs can be divided into two areas: the "PSD core" and the "PSD pallium," typically located at depths of 30–50 nm from the postsynaptic membrane and further 50–60 nm towards the cytoplasmic side, respectively [44] (Fig. 2). Purified PSD contains not only a PSD core but also a PSD pallium.

A network structure called the "junctional lattice" or the "PSD lattice" was identified long time ago by the extraction of the SPM, synaptic junction, or TX-100-insoluble PSD using the relatively strong detergent DOC (Fig. 3), and was proposed to be an under-

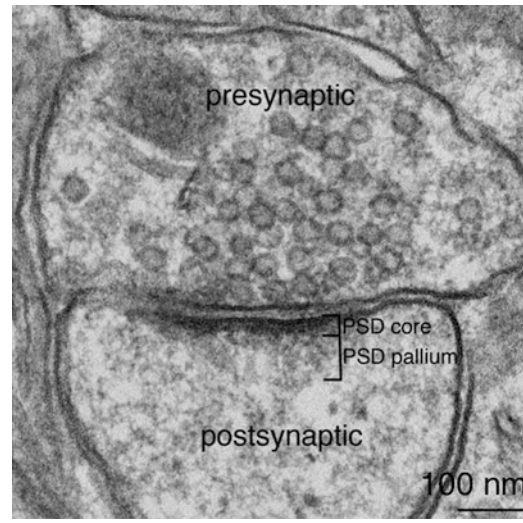


Fig. 2 Morphology of a typical type I excitatory synapse. The "PSD core" and "PSD pallium" regions are indicated. The image is from Dosemeci et al. [44]

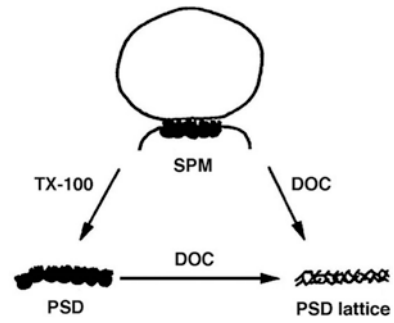


Fig. 3 The PSD lattice is visualized after treatment of the synaptic subfraction with DOC. The "PSD lattice" was identified in the 1970s by extraction of the SPM, synaptic junction, or TX-100-insoluble PSD using the relatively strong detergent DOC. The image is from Matus and Taff-Jones [32] with permission

lying structure of the PSD [31, 32, 34, 45]. However, until recently, the key components and molecular organization of the PSD lattice have not been determined [31, 32, 46].

2 Materials

Use distilled, double-distilled, distilled-and-deionized, or equivalent grade water. Using ultrapure water sometimes results in low protein yield of PSD (*see* **Note 1**). All chemicals should be of reagent grade. All stocks and working solutions are kept at −20 to −30 °C between uses to prevent bacterial and fungal growth. Make sure to mix up the solutions homogeneously after defreezing them, especially those containing dense sucrose solutions. All solutions should be kept at 4 °C or on ice during the subfractionation. TX-100 is susceptible to autoxidation upon exposure to air (*see* **Note 2**). Store the unused solution sealed and also avoid storage in direct light.

PSD material is extremely sticky to glass and cellulose nitrate and tend to aggregate very easily [2]. Therefore, usage of plastic (polyallomer) tubes and pipettes, in particular, after TX-100 treatment, is necessary to avoid undesirable absorption of PSD proteins to glasses.

Add protease inhibitors, phosphatase inhibitors, oxidization inhibitors, or RNase inhibitors as required. Addition of protease inhibitors results in increased yield of PSD proteins. It is desirable to purify PSD in the presence of iodoacetamide (IAA) or *N*-ethylmaleimide (NEM), which prevents harmful oxidation during the purification [1, 20, 21]. PSDs prepared in the presence of IAA (2 mM in solution A and solution B) are different from those prepared in the absence of IAA in detergent solubility, aggregation state of PSD, and possibly dynamic properties of PSD. Addition of dithiothreitol interferes with endogenous disulfide bondages necessary for the formation of normal PSD configuration [20, 21].

2.1 Preparation of P_1, P_2, Synaptosome, and PSD Fraction (Short Procedure)

1. 1 M $MgCl_2$ stock. Dissolve 20.33 g of $MgCl_2 \cdot 6H_2O$ (MW, 203.30) in 100 mL H_2O.
2. 1 M $CaCl_2$ stock. Dissolve 14.70 g of $CaCl_2 \cdot 2H_2O$ (MW, 147.02) in 100 mL H_2O.
3. 100 mM $NaHCO_3$ stock. Dissolve 1.68 g of $NaHCO_3$ in H_2O and make up to 200 mL with H_2O.
4. 1 M Tris–HCl (pH 8.1) stock. Dissolve 24.2 g of Tris(hydroxymethyl)aminomethane in H_2O (~150 mL) and adjust pH to 8.1 by HCl, and make up to 200 mL with H_2O.

5. 0.5 M HEPES/KOH (pH 7.4) stock.
   Dissolve 11.8 g of HEPES, adjust pH to 7.4 with KOH solution and make up to100 mL with H_2O.
6. Solution A (0.32 M sucrose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM $NaHCO_3$). Dissolve 109.6 g of sucrose in H_2O. Add 10 mL of 100 mM $NaHCO_3$, 1 mL of 1 M $MgCl_2$ and 0.5 mL of 1 M $CaCl_2$. Make up to 1000 mL with H_2O.
7. Solution B (0.32 M sucrose, 1 mM $NaHCO_3$). Dissolve 109.6 g of sucrose in H_2O. Add 10 mL of 100 mM $NaHCO_3$. Make up to 1000 mL with H_2O.
8. 1% TX-100, 0.32 M sucrose, 12 mM Tris–HCl (pH 8.1). Dissolve 109.6 g of sucrose in H_2O. Add 10 g of TX-100 (Sigma) and 12 mL of 1 M Tris–HCl (pH 8.1). Make up to 1000 mL with H_2O.
9. 1% TX-100, 150 mM KCl. Dissolve 2 g of TX-100 and 2.26 g of KCl in H_2O. Make up to 200 mL with H_2O.
10. 10 mM HEPES/KOH (pH 7.4)-40% glycerol. Dilute 4 mL of 0.5 M HEPES/KOH (pH 7.4) in H_2O (~80 mL). Add 80 g of glycerol and make up to 200 mL with H_2O.
11. Sucrose solution (1.0, 1.4, 1.5 and 2.1 M). Dissolve sucrose (68.5 g, 95.8 g, 102.7 g and 143.8 g for 1.0, 1.4, 1.5, 2.1 M sucrose solutions, respectively) in H_2O. Add 2 mL of 0.1 M $NaHCO_3$ to each solution and make up to 200 mL with H_2O.
12. 1 mM $NaHCO_3$. Dilute 100 mM $NaHCO_3$ into H_2O. 400 mL/20 g of starting brain is required.
13. Plastic disposable pipettes. e.g., Liquipette, polyethylene transfer pipettes of 4 mL capacity, thin stem, 7 mL capacity, with scale, and 6 mL capacity 9″ long (Elkay, Shrewsbury, MA), or other plastic Pasteur pipette such as 3 mL (with scale).

2.2 Preparation of SPM and PSD Fraction (Long Procedure)

The long procedure requires solutions used in Subheading 2.1 and additional solutions listed below.

1. Sucrose solutions (0.85, 1.0 and 1. 2 M). Dissolve sucrose (58.2 g, 68.5 g, and 82.2 g for 0.85, 1.0, and 1. 2 M sucrose solutions, respectively) in H_2O, add 2 mL of 0.1 M $NaHCO_3$ and make up to 200 mL with H_2O.
2. 0.5 mM HEPES/KOH (pH 7.4). Dilute 0.5 M stock in H_2O. About 250–500 mL/20~25 g brain is required for SPM preparation.
3. 1 mM $NaHCO_3$. Dilute 100 mM $NaHCO_3$ into H_2O. About 150 mL/20–25 g of starting brain is required.

2.3 Preparation of S_3 and P_3 Fraction

No additional reagent or solution is necessary.

2.4 Preparation of Synaptic Membrane Raft from SPM

1. TNE buffer: 20 mM Tris–HCl (pH 7.4) containing 150 mM NaCl and 1 mM EDTA. Store at 4 °C.
2. 2× detergent stock solution in TNE buffer. Weigh and dissolve detergent in TNE buffer. Detergent concentration is weight/volume. Store at 4 °C. Examples of detergents include TX-100, OG and 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate (CHAPSO).
3. Sucrose solutions (80%, 30%, and 5%) in TNE buffer. Dissolve sucrose in TNE buffer (160 g, 60 g, and 10 g for 80%, 30%, and 5% sucrose solutions, respectively in 200 mL). Degas the sucrose solution by vacuum pump until no air bubbles form. Deaeration is required to prevent disturbance of the sucrose gradient caused by bubbles during sucrose density gradient (SDG) ultracentrifugation.
4. 1 M IAA. Weigh ~20 mg of IAA and add an appropriate volume of H_2O to make a 1 M solution. Mix at room temperature until no crystals remain. Let stand at room temperature before use. Prepare on the day of use.
5. Protease inhibitor cocktail (P8340, Sigma-Aldrich, St Louis, MO). Store the stock reagent at −30 °C. Thaw at room temperature on the day of use.

2.5 Preparation of PSD Lattice from SPM

No additional reagent or solution is necessary (*see* Subheading 2.4).

3 Methods

3.1 Preparation of P_1, P_2, Synaptosome, and PSD Fraction (Short Procedure) from Rat Forebrain

The method is based on those developed by Siekevitz's group [2–4]. Protocol for PSD purification (short procedure) using 20–25 g forebrain as starting material is described below. The maximum amount of forebrains is about 25 g due to the limitation of capacity of ultracentrifuge. All the processes are carried out at 4 °C. The procedure is outlined in Fig. 4.

1. Collect rat forebrains by decapitation and quick dissection (*see* **Note 3**). Place forebrains immediately after dissection in a beaker placed on ice. Weigh the pooled brains (weight of the container is better be measured before pooling tissues). Proceed for **step 2** or freeze and keep the forebrains at −80 °C until use.
2. Chop forebrains into small pieces (about less than 2 × 2 × 2 mm) with scissors. When using frozen brains, dip frozen brains into small amount of cooled solution A (~ a few mL) in a beaker, chop or scrape them by scissors. Add solution A to make

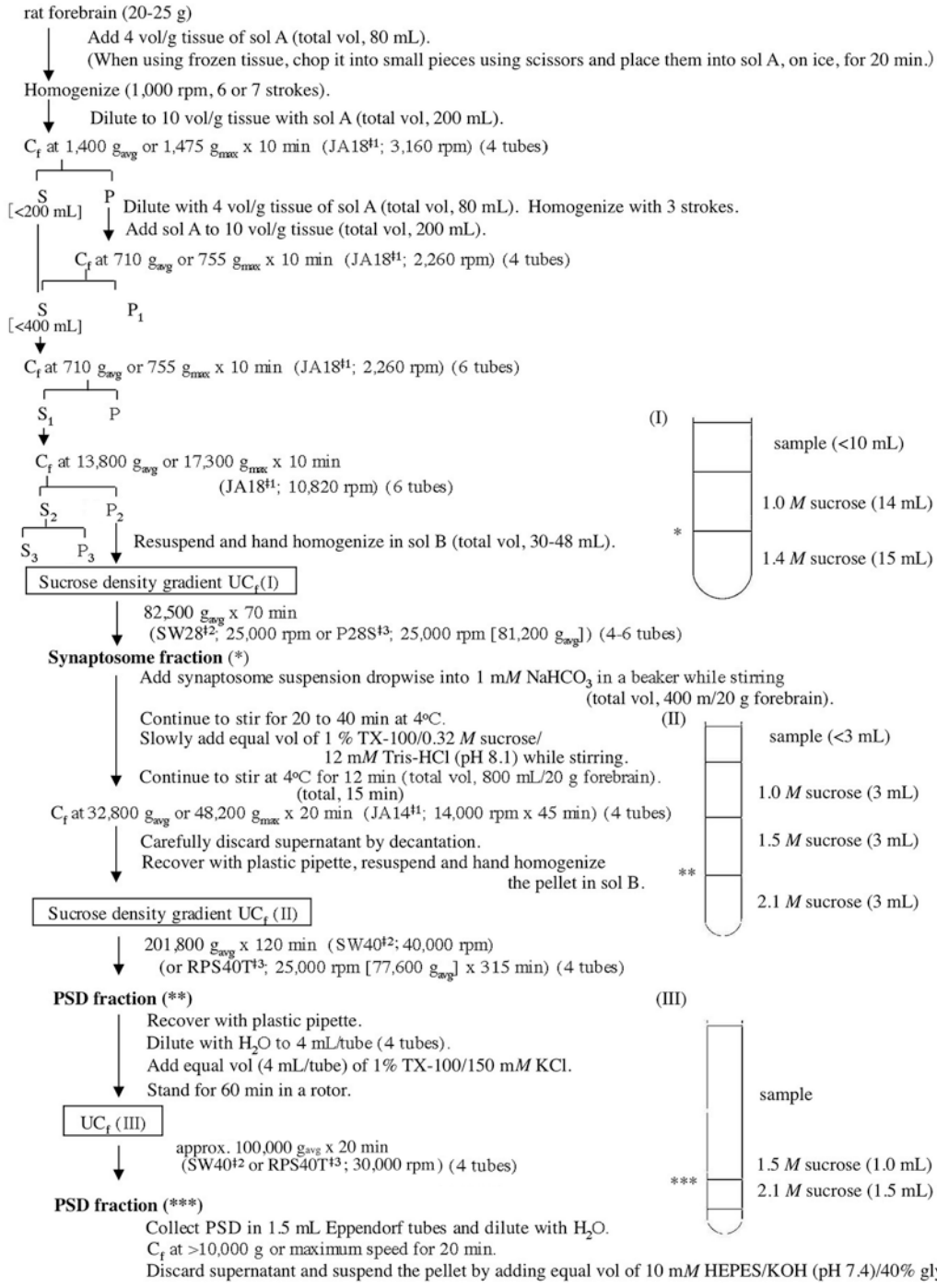


Fig. 4 Purification of synaptosome and PSD by subcellular fractionation using sucrose density gradient centrifugation (PSD purification by short procedure). Examples of centrifugation conditions (rotors and speed) are indicated. Rotors marked with ‡1, ‡2 and ‡3 are those for centrifuges of Avanti J-25 (Beckman), L5–50 (Beckman) and Himac CP60E (Hitachi), respectively. Steps using ultracentrifuge are numbered with roman characters and surrounded with square. Volumes and number of centrifuge tubes used are those for purifying PSD from 20–25 g forebrains of rats. ∗, ∗∗ and ∗∗∗ Positions where synaptosome and PSD before and after TX-100/KCl treatment, respectively, are collected. *C*f centrifugation, *UC*f ultracentrifugation, *av*, average, *sol* solution, *vol* volume

80 mL suspension. Keep the suspension on ice for at least 20 min when using frozen brains (*see* **Note 4**).

3. Homogenize the suspension at 1000 rpm with 6 or 7 up-and-down motions with a motor-operated Teflon/glass homogenizer using a loose-fitting pestle (*see* **Note 5**) while cooling the container in ice water. Recover suspension into a new beaker and dilute to 200 mL with solution A. (Start preparing sucrose layers necessary at **step 9** during centrifugations at **steps 3** (or **4**)–**8**.)
4. Centrifuge at 1400 × g_{av} or 1475 × g_{max} for 10 min (JA18; 3160 rpm, 4 tubes). Save supernatant in a beaker placed on ice or at 4 °C.
5. Dilute the pellet with solution A and make 80 mL suspension. Homogenize with 3 strokes as in **step 3**. Dilute with solution A to 200 mL.
6. Centrifuge at 710 × g_{av} or 755 × g_{max} for 10 min (JA18; 2260 rpm, 4 tubes). Collect supernatant. Pellet is P_1.
7. Combine supernatants obtained in **steps 4** and **6** and centrifuge at 710 × g_{av} or 755 × g_{max} for 10 min (JA18; 2260 rpm, 6 tubes) (*see* **Note 6**).
8. Collect supernatant (S_1) and centrifuge at 13,800 × g_{av} or 17,300 × g_{max} for 10 min (JA18; 10,820 rpm, 6 tubes). Supernatant and pellet obtained in this step are S_2 and P_2, respectively.
9. Resuspend the P_2 and gently hand homogenize with a Dounce homogenizer or Teflon-glass homogenizer in solution B (~48 mL). Layer the suspension on gradients composed of 1.0 and 1.4 M sucrose, and centrifuge at 82,500 × g_{av} for 70 min (SW 28, 25,000 rpm, 4–6 tubes) (*see* **Note 7**).
10. Collect the bands in the interface between 1.0 and 1.4 M sucrose layer (synaptosome fraction) (*see* **Note 8**) into a small beaker with a plastic pipette of 4 mL capacity with thin stem (*see* **Note 9**). Measure the volume of the synaptosome suspension, if necessary. Protein concentration of synaptosome just after recovery from the interface band is approx. 5 mg protein/mL. Save aliquot of synaptosome suspension after dilution to make about 2.5 mg/mL (just an example), if necessary.
11. Pour 1 mM $NaHCO_3$ into a large beaker to make the final volume after mixing of the synaptosome suspension 400 mL/20 g starting forebrains (*see* **Note 10**). Place a stirrer bar into the beaker. Add synaptosome suspension dropwise into 1 mM $NaHCO_3$ in a beaker while stirring. Continue to stir for about 20–40 min at 4 °C (*see* **Note 11**).

12. Add slowly 400 mL/20 g of starting forebrains of 1% TX-100/0.32 M sucrose/12 mM Tris–HCl (pH 8.1) (final 0.5% TX-100, 0.16 M sucrose, 6 mM Tris–HCl) with constant stirring. Take 1 min to add the TX-100 solution. Continue to stir at 4 °C. Total time of treatment with TX-100 (from starting addition of TX-100 to starting next centrifugation) should be 15 min. Therefore, transfer the solution to the transparent centrifuge tubes (*see* **Note 12**) at about 12 min after starting addition of TX-100 (*see* **Note 13**).
13. Centrifuge at 32,800 × g_{av} or 48,200 × g_{max} for 20 min (JA14; 14,000 rpm × 45 min, four 250 mL tubes). (Prepare sucrose layers required at **step 14** by using a plastic Pasteur pipette with scale.) Discard upper large portion of supernatant by slow decantation. Discard supernatant using a plastic pipette of 6 mL with 9″-long so that about 2 mL supernatant remains in the tube. Be very careful not to disturb the pellet. Recover pellet with plastic pipette by peeling and aspirating the pellet as a mass. Collect the pellet as small a volume as possible. Resuspend the pellet in solution B. Gently hand homogenize the pellet with a Dounce homogenizer or loose Teflon-glass homogenizer.
14. Layer the solution on gradients composed of 1.0, 1.5, and 2.1 M sucrose, and centrifuge at 201,800 × g_{av} for 120 min (SW40; 40,000 rpm or RPS40T; 25,000 rpm 315 min, 4 tubes) (*see* **Notes 14** and **15**). (In the latter case, next step begins next morning.)
15. Recover PSD fraction (**) with a plastic pipette (4 mL with thin stem) into 15 mL plastic tube. Dilute with cold H_2O to 4 mL/1 tube and mix homogeneously. Add equal volume [4 mL/tube] of 1% TX-100/150 mM KCl (final 0.5% TX-100, 75 mM KCl) and mix homogeneously. Stand for 60 min (*see* **Note 16**).
16. Layer the solution on gradients composed of 1.5 and 2.1 M sucrose, and centrifuge at approx. 100,000 × g_{av} for 20 min (SW40 or RPS40T; 30,000 rpm, 2 tubes) (*see* **Note 14**).
17. Retrieve PSD fraction (***) with a plastic pipette (4 mL with thin stem) into 1.5 mL Eppendorf microfuge tubes. Dilute with more than an equal volume of cold H_2O (*see* **Note 17**). Centrifuge at >10,000 × g for 20 min. (Swing rotor is favorable).
18. Discard supernatant and weigh the PSD material. Add equal amount of 10 mM HEPES/KOH (pH 7.4)/40% glycerol and mix homogeneously (*see* **Note 18**). Divide into small aliquots and keep them in plastic tubes at −80 °C until use.

3.2 Preparation of SPM and PSD Fraction (Long Procedure)

Protocol (long procedure) for PSD purification using 20–25 g forebrain as starting material is described below. All the processes are carried out at 4 °C. The procedure is outlined in Fig. 5. **Steps 6–12** are the same as **steps 12–18** of short procedure except for volumes of the samples and the number of centrifuge tubes used.

1. Prepare synaptosome fraction following the protocol described in Subheading 3.1.
2. Pour 0.5 mM HEPES/KOH (pH 7.4) into a large beaker to make the final volume after mixing the synaptosome suspension 400 mL. Place a stirrer bar into the beaker. Add synaptosome suspension (~50 mL) dropwise into the HEPES/KOH buffer in a beaker while stirring. Continue to stir for about 45 min at 4 °C.
3. Centrifuge at 32,800 × g_{av} or 48,200 × g_{max} for 20 min (JA14; 14,000 rpm × 45 min). Collect pellet and resuspend in solution B as stated in Subheading 3.1, **step 13**.

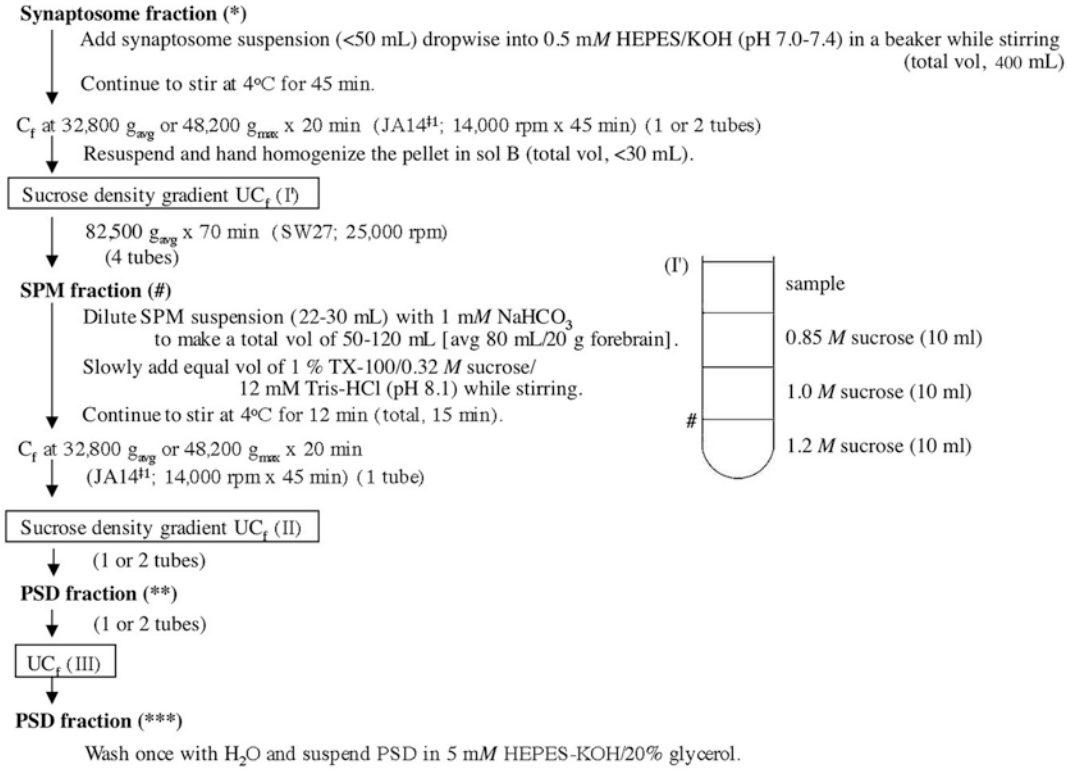


Fig. 5 Purification of SPM and PSD by subcellular fractionation using sucrose density gradient centrifugation (PSD purification by long procedure). Protocol to prepare synaptosome and steps after sucrose gradient centrifugation (II) are essentially the same as those shown in Fig. 4. Comments and abbreviations are the same as in Fig. 4

4. Layer the suspension on gradients composed of 0.85, 1.0 and 1.2 M sucrose, and centrifuge at 82,500 × g_{av} for 70 min (SW 28, 25,000 rpm). Use 4 tubes.
5. Collect SPM in the 1.0–1.2 M sucrose interface (#). Volume of this suspension is usually 20–30 mL (∗approx. 3–4 mg protein/mL).
6. For subsequent purification of PSD, dilute SPM suspension with 1 mM $NaHCO_3$ (final vol, 50–120 mL [average 80 mL/20 g forebrain]).
7. Treat the SPM suspension by adding equal volume of TX-100 as stated in Subheading 3.1, **step 12**.
8. Centrifuge at 32,800 × g_{av} or 48,200 × g_{max} for 20 min (JA14; 14,000 rpm × 45 min, 1 tube). Collect pellet and resuspend in solution B as stated in Subheading 3.1, **step 13**.
9. Layer the solution on the top of the sucrose gradient and centrifuge at 201,800 × g_{av} × 120 min (SW40, 40,000 rpm or RPS40T; 25,000 rpm 315 min, 1 tube).
10. Recover PSD fraction (∗∗) as stated in Subheading 3.1, **step 15**.
11. Centrifuge at approx. 100,000 × g_{av} for 20 min (SW40 or RPS40T; 30,000 rpm, 1 tube) as stated in Subheading 3.1, **step 16**.
12. Retrieve PSD fraction (∗∗∗), process and save as stated in Subheading 3.1, **steps 17** and **18**.

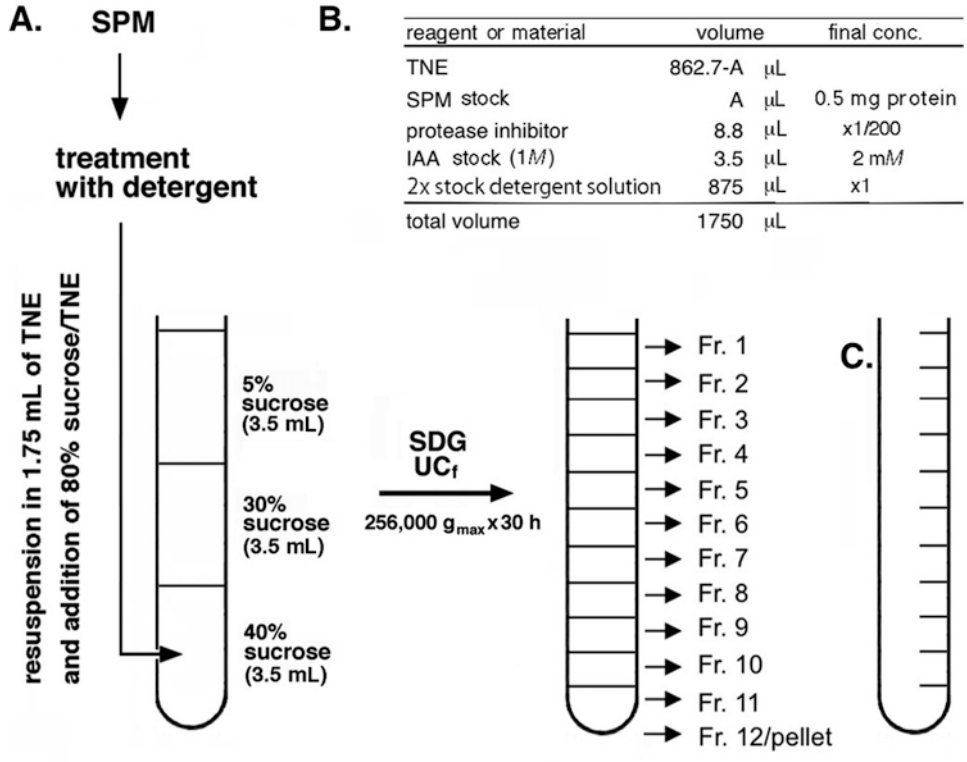
3.3 Preparation of S_3 and P_3 Fraction

Centrifuge S_2 material at 100,000 × g for 1 h. Supernatant and pellet obtained are S_3 and P_3 fractions, respectively.

3.4 Preparation of Synaptic Membrane Raft from SPM

The protocol provided in this chapter is useful for the purification of the synaptic membrane raft, simultaneous purification of the PSD, and investigation of relationship between the synaptic membrane raft and the PSD. It is desirable to use SPM prepared in the presence of 2 mM IAA. Both freshly prepared SPM and SPM stored at −30 °C in the presence of 50% glycerol can be used. All processes are carried out at 4 °C. The standard protocol, outlined in Fig. 6, uses 500 μg of SPM protein. Typically, 20 and 1.5 μL of each fraction are used for the SDS-PAGE and GM1 immuno-dot blot, respectively. Therefore, more than 40 repetitions of SDS-PAGE (stained with silver or SYPRO Ruby) and western blotting can be carried out.

Researchers can modify the protocol by using different types of detergents and various concentrations of detergent (typically ranging from 0.05–5%) (*see* **Note 19**). The protein profile of SDG and the protein components in the membrane raft and PSD fractions differ depending on the detergent used and the ratio of detergent



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reagent or material	volume		final conc.
TNE	862.7-A	µL	
SPM stock	A	µL	0.5 mg protein
protease inhibitor	8.8	µL	x1/200
IAA stock (1*M*)	3.5	µL	2 m*M*
2x stock detergent solution	875	µL	x1
total volume	1750	µL	

Fig. 6 Purification protocol of synaptic membrane rafts by sucrose density gradient centrifugation. (**a**) Protocol using the SPM as the starting material. (**b**) Example of detergent treatment. The total SPM protein amount during detergent treatment is 0.5 mg. Change the types and concentration of detergent as required. Detergent treatment is carried out at 4 °C for 30 min with gentle rotational mixing. (**c**) Centrifuge tube marked with the volumes of each fraction (955 µL for each fraction). Mark after every addition of 955 µL H_2O

to protein. Typical examples of SDG protein distribution are shown in Fig. 7. The potency of the holding raft-PSD complex differs with detergent type and their concentrations. For example, low concentration TX-100 (e.g., 0.15%) maintains the membrane raft–PSD complex [38, 41]. OG dissociates the synaptic membrane raft and the PSD [42]. OG and CHAPSO tend to maintain the membrane raft integrity better than TX-100 [43]. Fraction 12/pellet contains the PSD [41]. Fractions 8–11 are mixtures of soluble and cytoskeletal proteins. The membrane rafts are typically distributed in fractions 4–6 after treatment with 0.15% TX-100 (Fig. 7) [38, 41, 43]. Identify the membrane raft-containing fractions by GM1 immuno–dot blot assay.

1. Mix SPM (0.5 mg protein) (*see* **Note 20**), TNE buffer, protease inhibitor cocktail, and IAA in a 15 mL screw-cap plastic tube as indicated in Fig. 6b. Mix gently but well.

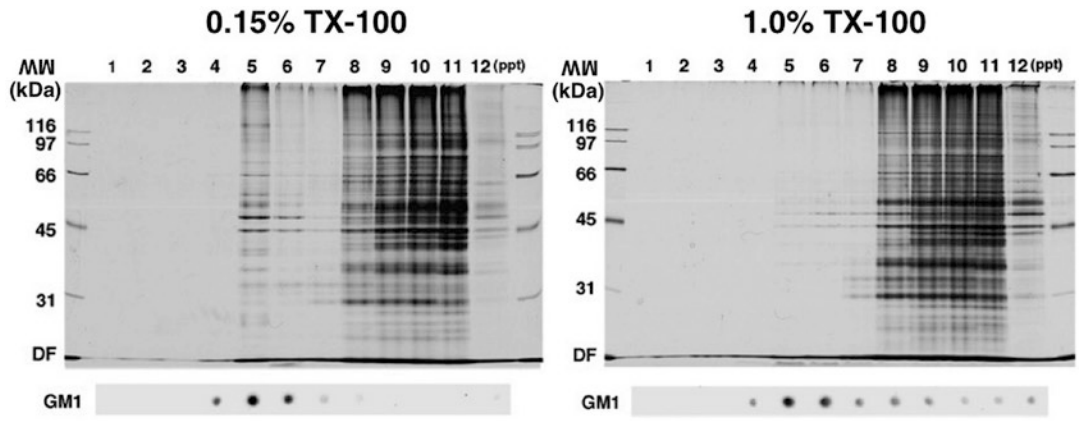

Fig. 7 Electrophoretic profile of detergent-treated SPM after SDG ultracentrifugation. Representative examples are shown. Proteins were stained with silver. The lower images are the GM1 distribution on the sucrose gradient revealed by dot blot analysis using horseradish peroxidase-conjugated cholera toxin B subunit, which specifically binds to GM1 ganglioside, a membrane raft marker. Membrane rafts are distributed on fractions 4–6 in the 0.15% TX-100 treatment. Fraction 12/pellet contains the PSD. It should be noted that the raft fractions in the 0.15% TX-100 treatment contain the synaptic membrane raft-PSD complexes [38, 41]. Fractions 8–11 are a mixture of soluble and cytoskeletal proteins. The protein distribution profiles on the SDG depend on the detergent-to-protein ratio. In this protocol, the detergent-to-protein ratios (w/w) are 5.25 and 35, respectively, for the 0.15% and 1.0% TX-100 treatments. Fraction numbers are shown at the tops of the gels, and the molecular weights (MW, in kDa) are on the left. DF and ppt refer to the dye front and pellet, respectively. The images were extracted from a previous publication [41]

2. Add 875 μL of 2× stock detergent solution to the tube. Close the screw cap of the tube, mix well, and continue gentle mixing using a rotator for 30 min.
3. Prepare 1.75 mL 80% sucrose/TNE (2.3 g) in an ultracentrifuge tube while detergent treatment is underway.
4. Transfer the detergent-treated sample to the ultracentrifuge tube containing 1.75 mL 80% sucrose/TNE and mix well.
5. Gently overlay the TNE buffers containing 30% sucrose and 5% sucrose sequentially without disturbing the interfaces of the sucrose solutions.
6. Centrifuge at 256,000 × g_{max} for 30 h.
7. Collect the 11 fractions (955 μL for each fraction) from the top using a 1 mL pipette. Follow the solution levels using the pre-marked lines on a second ultracentrifuge tube (Fig. 6c) (*see* **Note 21**). Collect fraction 12/pellet by repeated flushing with the recovery solution by pipette (*see* **Note 22**). Mix each fraction, except for fraction 12, gently by brief vortex to make the solutions homogeneous (*see* **Note 23**).

3.5 Preparation of PSD Lattice from SPM

Purification and characterization of PSD lattice, presumably a backbone structure for excitatory PSD in the mammalian central nervous system, was reported in 2018 [47]. However, the preparation, and therefore, the protocol had disadvantage, in particular, for the identification of protein composition, because the preparation contained not a few SDS-insoluble proteins. After the initial paper on the PSD lattice [47], the initial protocol was improved and the insolubility problem was solved. The new PSD lattice preparation is considered to be more physiological than the previous one and enabled identification of the component proteins by SDS-PAGE and western blotting (Suzuki et al., in preparation). This chapter describes the improved protocol. A protocol for purification of the PSD lattice in the forebrain SPM shown in Fig. 8 uses 3 mg of forebrain SPM protein as a starting material. Use SPM prepared in the presence of 2 mM IAA. First half of the protocol is the same as that for synaptic membrane raft purification. All processes are carried out at 4 °C. Detergents other than OG were not tested.

1. Incubate the SPM protein (3 mg) (see **Note 20**) in TNE buffer containing protease inhibitor cocktail, 2 mM IAA, and 1%OG in a 50 mL screw-cap plastic tube to make a total volume of 10.5 mL (1.75 mL ×6) (*see* **Note 24**). Close the screw cap of the tube, mix well, and continue gentle mixing using a rotator for 30 min.
2. Mix to the detergent-treated solution with equal volume (10.5 mL) of TNE buffers containing 80% sucrose and devide the mixture into 6 ultracentrifugation tubes. Overlay with TNE buffers containing 30% sucrose and then 5% sucrose (Each tube contains 3 layers of 3.5 mL sucrose solution), and centrifuge at 256,000 × g_{max} for 30 h at 4 °C.
3. Slowly aspirate the solution localizing in the fractions 1–10 from the top, using a plastic pipette by following the premarked volume lines on a second tube (Fig. 6c) (*see* **Note 25**).
4. Collect upper and lower portions of fraction 11 (1%OG-11U and1%OG-11B, respectively) (typically 825 μLD and 130 μL, respectively) separately (*see* **Notes 26** and **27**). Resuspend the pellet (1%OG-12) in 955 μL of 5 mM HEPES/KOH (pH 7.4) containing 50%glycerol.
5. Dilute 1%OG-11U and 1%OG-11B by 4 fold with 5 mM HEPES/KOH (pH 7.4), centrifuge at 100,000 × g_{max} for 30 min at 4 °C. Resuspend the pellets in 1 mL of 5 mM HEPES/KOH (pH 7.4), ultracentrifuge again, and resuspend the final pellets (1%OG-11U-IS and 1%OG-11B-IS, respectively) in 100 μL of 5 mM HEPES/KOH (pH 7.4) containing 50%glycerol (*see* **Notes 28** and **29**).
6. The preparations were stored unfrozen at −30 °C.

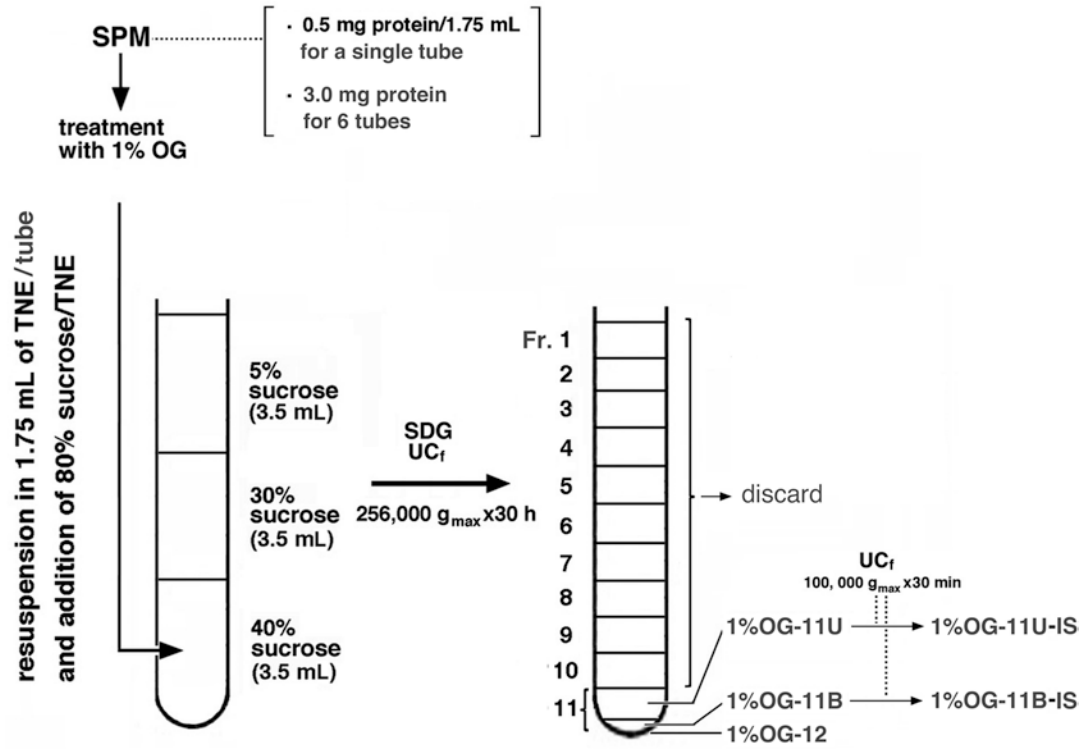

Fig. 8 The purification protocol of the PSD lattice by sucrose density gradient centrifugation. The protocol shown is an improved version of the previous one [47]. (Suzuki et al., in preparation). First half of the protocol is the same as that for synaptic membrane raft purification until the process of SDG ultracentrifugation shown in Fig. 6. We refer 1%OG-11U-IS as PSD lattice preparation. Protein components in these two preparations are nearly the same as far as SDS-PAGE profiles are compared (Suzuki et al., in preparation). U, B, IS, and UC_{ff} refer to upper, bottom, insoluble, and ultracentrifugation, respectively

4 Notes

1. Subtle changes in ionic strength and metal concentration may affect sedimentation of subcellular organelles and protein complexes. It is not necessary to use ultrapure water, such as nanopure or miliQ water, for this subfractionation, and the usage of ultrapure water may sometimes result in low yield of synaptosome and PSDs. Some unidentified factor(s) affects the sedimentation and/or are necessary for stabilization of PSD protein complex.
2. Commercial TX-100 has been found to contain impurity with oxidizing activity [48].
3. If brains are homogenized or rapidly frozen in liquid nitrogen within 30 s to 1 min after decapitation, content of CaMKII, both α and β, are very low in the PSD fraction [22]. Neurofilament content is increased in such PSD preparation.

4. Defrozen and chopped brains should be kept in cooled solution A for at least 20 min to depolymerize actin cytoskeleton. Inadequate depolymerization causes unfavorable sedimentation.
5. Literature [1, 2] recommends loose homogenizer (e.g., Teflon-glass homogenizer with a clearance of 0.25 mm or Dounce homogenizer with a loose-fitting pestle) to preserve morphological integrity of PSD. However, 0.25 mm clearance homogenizer does not appear to be a must.
6. It is very difficult to separate clearly the supernatant and pellet from total brain homogenate by centrifugation at $755 \times g_{max}$. Therefore, the first centrifugation was carried out at $1475 \times g_{max}$. Supernatant obtained in the first centrifugation and the second centrifugation at $755 \times g_{max}$ are combined, centrifuged again at $755 \times g_{max}$, and thus S_1 fraction was obtained. Removing $755 \times g_{max}$ pellet is important to minimize contamination of nuclear materials to synaptic fractions [49]. Methods omitting this step (e.g., one-step purification of synaptosome) cannot avoid large amounts of contamination of nuclear proteins.
7. The first sucrose gradient was originally composed of 0.85, 1.0, and 1.2 *M* sucrose [2, 4], but was replaced by those composed of 1.0 and 1.4 M sucrose with equivalent result [3].
8. Use fresh unfrozen brain as starting tissues for functional analysis of synaptosome. It is required to incubate synaptosome suspension in normotonic buffer to bring the terminals to a physiological steady state [50]. Synaptosomes recovered from the sucrose gradient and are not incubated in normotonic buffer are shrunken due to high osmotic pressure.
9. Using disposable plastic pipettes to collect synaptosome, SPM, and PSD enriched bands after sucrose gradient centrifugation is convenient. See also Subheading 2.1, **item 13**. Be careful not to warm the plastic pipette (it means protein sample) by holding it with warm hand or fingers with wide contact areas for long time.
10. Fixed volume (400 mL for 20 g starting tissue) of the synaptosome suspension just before the TX-100 treatment is based on the protein concentration [2] estimated by the A_{260} and A_{280} using nomogram (distributed by California Corporation for Biochemical Research, LA) based on the equation by Warburg and Christian [51]. Dilute synaptosome solution by 40 times for measurement of A_{260} and A_{280}. (This also applies to SPM solution). The protein concentration estimated by Warburg-Christian method is about fourfolds of the value obtained by Lowry method using BSA as standard. (The values were

4.3 ± 1.2 [n = 7] and 3.3 ± 1.2 [n = 12] folds for synaptosome and SPM fractions, respectively.) Therefore, protein concentration of the synaptosome suspension in 400 mL/20 g original forebrain is approximately 1 mg protein/mL (not 4 mg protein/mL as written in the original paper). Volume should be changed when starting from other parts of the brain, such as cerebellum.

11. This process is required before treatment with TX-100 and important to obtain good yield of PSD proteins, although the reason is unknown. Omitting this process may bring low yields of PSD.
12. Use transparent centrifuge tube to see the pellet clearly with the naked eye. The pellet obtained is very soft and easy to disturb. It is required to collect the pellet in a small volume to load on the top layer of the next sucrose density gradient.
13. The duration of TX-100 treatment affects the recovery of PSD.
14. PSD is extremely sticky to glass and cellulose nitrate tubes [2]. Use polyallomer centrifuge tubes [2] to prevent adhere PSD to the tubes.
15. Keep temperature to be around 4 °C during ultracentrifugation. Raise of temperature loses some enzyme activity.
16. Inadequate treatment at this step leaves membrane materials to the final PSD preparation.
17. Repeat wash once or twice if complete removal of TX-100 is required.
18. Glycerol should be added to prevent artificial aggregation of the PSD proteins during storage at −80 °C. Again, PSD material is extremely sticky to glass and cellulose nitrate, and tend to aggregate very easily, in particular, after freezing and defreezing.
19. Consider the critical micelle concentration (CMC) of the detergents. In particular, the CMC of OG is high (20–25 mM/0.585–0.7%).
20. Use SPM prepared in the presence of 2 mM IAA, because the subsequent protocol is carried out in the presence of IAA.
21. Collection using a 1 mL pipette is unreliable because the sucrose density differs from fraction to fraction (the gradient ranges from 5% to 40% from the top of the tube to the bottom), resulting in the recovery of different volumes by pipette. Take advantage of the pre-marked levels on a second ultracentrifuge centrifuge tube for this step.

22. Vigorous vortexing of the PSD-containing solution may cause denaturation of the PSD proteins and increase insolubility of the PSD protein complex. The recovery solution should contain 40–50% glycerol to prevent irreversible aggregation of PSDs (*see* also **Note 18**).
23. The solution recovered by pipette from the sucrose gradient is not homogeneous even in small fractions. Therefore, mixing by brief vortex is required. Avoid vortexing fraction 12/pellet, which contains the PSD.
24. This amount is sixfold of the standard-scale purification protocol for synaptic membrane raft shown in Fig. 6.
25. Positions of 11 fractions (955 μL each) were marked on a second centrifuge tube and numbered from the top, as shown in Fig. 6.
26. Pre-mark the surface level of 130 μL on a second centrifuge tube after the addition of 130 μL H_2O. Be very careful not to contaminate the PSDs in the 1%OG-IS-11B/PSD lattice fraction by touching the tip of pipette with the PSD-containing pellet.
27. The volume of bottom portion (therefore, that of upper portion, too) critically affects on the protein recovery and sparseness/density of the PSD lattice structure in the upper portion. It is better not to collect bottom portion more than 200 μL. Otherwise, protein recovery may be too low for subsequent biochemical analyses. More densely packed PSD lattice is recovered in the more bottom portion.
28. The pellet materials were not visible with the naked eye during the last purification process.
29. Collect 50 μL of suspension twice and make final volume 100 μL. The final solutions were not hand-homogenized nor vortexed to avoid any loss and protein denaturation.

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