Chapter 8

Preparation of Gap Junctions in Membrane Microdomains for Immunoprecipitation and Mass Spectrometry Interactome Analysis

Stephanie Fowler, Mark Akins, and Steffany A.L. Bennett

Abstract

Protein interaction networks at gap junction plaques are increasingly implicated in a variety of intracellular signaling cascades. Identifying protein interactions of integral membrane proteins is a valuable tool for determining channel function. However, several technical challenges exist. Subcellular fractionation of the bait protein matrix is usually required to identify less abundant proteins in complex homogenates. Sufficient solvation of the lipid environment without perturbation of the protein interactome must also be achieved. The present chapter describes the flotation of light and heavy liver tissue membrane microdomains to facilitate the identification and analysis of endogenous gap junction proteins and includes technical notes for translation to other integral membrane proteins, tissues, or cell culture models. These procedures are valuable tools for the enrichment of gap junction membrane compartments and for the identification of gap junction signaling interactomes.

Key words Cx32, Endogenous immunoprecipitation, Gap junctions, Integral membrane protein, Interactome, Membrane fractionation, Membrane microdomains, Network analysis, Opti-Prep gradient, Proteomics

1 Introduction

Gap junctions were originally identified as intercellular membrane channels, yet are now understood to be complex signaling platforms with functional diversity extending far beyond metabolic coupling [1–3]. Gap junction plaques are composed of networks of integral scaffolding proteins with cytoskeletal contacts that recruit intracellular and nuclear localized enzymes and substrates (kinases, phosphatases, etc.) [4]. Sequestering multiprotein complexes at gap junction plaques is hypothesized to regulate the spatiotemporal proximity of signaling mediators, ensuring their proper subcellular localization for efficient signaling cascade generation [5, 6].

Most of our knowledge of gap junction protein interactions comes from studies of ubiquitously expressed Cx43 channels [6].

Mathieu Vinken and Scott R. Johnstone (eds.), *Gap Junction Protocols*, Methods in Molecular Biology, vol. 1437,
DOI 10.1007/978-1-4939-3664-9_8,

There is significant diversity in connexin interactomes including calcium sensing proteins, other junction proteins, membrane channels, enzymes, cytoskeletal proteins, transcription factors, and proteins involved in intracellular trafficking [5]. Many of the interactions are shared amongst different connexin family members, and certain connexin isoforms can interact with each other in heteromeric and/or heterotypic configurations, thereby altering network interactions [7–9].

Connexin interaction data has been gathered using a variety of techniques, including colocalization imaging, immunoprecipitation (IP) assays, affinity binding assays, and biochemical techniques [2]. Limitations common to many of these techniques include high false-positive (and false-negative) interactions, inability to detect transient/weak interactions, and masking of less abundant proteins by highly expressed proteins. Spurious interactions often occur during the mixing of cellular compartments upon cell lysis, generating nonphysiological interaction environments [10, 11]. Further, the use of ectopic epitope-tagged proteins may interfere with localization/posttranslational modification of bait proteins while the tag itself may mask sites of protein–protein interaction, again generating nonphysiological interactions [12, 13]. Despite these limitations, *in vivo* systems are usually preferred over synthetic systems for multiprotein complex detection, as proteins in synthetic systems may not assemble or traffic properly, and crucial accessory proteins may not be present to enable full complex assembly [14].

To address these technical limitations, the present work describes a procedure for fractionating both light and heavy cellular membranes from tissue using detergent-free OptiPrep™ density gradients to identify microdomains enriched for any membrane protein of interest. We identify here the membrane microdomains that are enriched for the liver gap junction protein Cx32 and describe how to analyze endogenous Cx32-associated interaction proteins following gentle membrane solvation, IP, and tandem mass spectrometry (MS/MS). Technical guidance for interaction protein validation and network analysis is included, and options for examining integral membrane protein interactions in other tissues and cell culture models are discussed.

2 Materials

2.1 Mouse Liver Tissues

1. Mice: wild-type (WT) and knockout (KO, negative control) animals for dissection (*see* **Note 1**).
2. For identification of Cx32 complexes: 1–3 g of isolated mouse liver material from 2 to 3 mice per centrifuge tube (*see* **Notes 2** and **3**).
3. For the identification of less abundant PM microdomains more starting material is required, e.g., 8–10 g liver wet weight recommended (*see* **Note 4**).

2.2 Tissue Homogenization and OptiPrep Membrane Fractionation

1. 10 mM phosphate buffered saline (PBS): 10 mM phosphate, 137 mM NaCl, pH 7.2, filter sterilized.
2. Light membrane homogenization buffer (LHB): 0.25 M sucrose, 2 mM $MgCl_2$, 20 mM HEPES-NaOH (*see* **Note 5**). Adjust pH to 7.4 and add 1 mM sodium fluoride, 50 μg/mL aprotinin, 1 mM sodium orthovanadate, 1 mg/mL PMSF immediately *prior* to use. Make 500 mL buffer just *prior* to use, filter sterilize, and keep on ice.
3. Heavy membrane homogenization buffer (HHB). 250 mM sucrose, 1 mM EDTA, 20 mM HEPES-NaOH (*see* **Note 6**). Adjust pH to 7.4 and add 1 mM sodium fluoride, 50 μg/mL aprotinin, 1 mM sodium orthovanadate, 1 mg/mL PMSF immediately *prior* to use. Make 500 mL buffer just *prior* to use, filter sterilize, and keep on ice.
4. Potter-Elvehjem glass homogenizer with loose and tight fitting teflon pestles (30 mL).
5. 15 and 50 mL tubes.
6. Glass pasteur pipettes.
7. OptiPrep (OP) density gradient solution (Sigma).
8. OP Diluent: 0.25 M sucrose, 120 mM HEPES, pH to 7.4 with NaOH. Make 50 mL and filter sterilize.
9. 50% working solution of OP in LHB or HHB: 5 parts OP with 1 part OP diluent.
10. Protein concentrating centrifuge tubes, e.g., Amicon Ultra-4 centrifugal unit.
11. 10% detergent of choice in LHB and HHB, e.g., triton X-100, NP-40, digitonin, CHAPS (*see* **Notes 7** and **27**).
12. Refrigerated high-speed centrifuge with swinging bucket rotor fitting 15 mL tubes (capable of at least 3000 × *g*).
13. Rotor for high-speed centrifuge fitting 50 mL polypropylene bottles with screw-on caps.
14. Ultracentrifuge with swinging bucket rotor for 13 mL tubes.
15. 13.2 mL (14 × 89 mm) polypropylene ultracentrifuge tubes for swinging bucket rotor.

2.3 Agarose Bead Preparation

1. Protein G and/or Protein A agarose beads (*see* **Note 8**).
2. Monoclonal or polyclonal antibody directed against connexin/membrane protein of interest. These should be validated for use in immunoprecipitation studies.
3. 10 mM PBS: 10 mM phosphate, 137 mM NaCl, pH 7.2, filter sterilized.
4. Tube rotator.
5. Refrigerated tabletop centrifuge.

2.4 Immunoprecipitation (IP) Complex Preparation for MS/MS

1. IP buffer 1: 20 mM Tris, 137 mM NaCl, 2 mM EDTA, 1% NP-40, pH 7.4. Make 50 mL just before use and store up to 1 week at 4 °C.
2. IP buffer 2 (no detergent) : 20 mM Tris, 137 mM NaCl, 2 mM EDTA, pH 7.4. Make 50 mL just before use and store up to 1 week at 4 °C.
3. Ammonium hydroxide elution buffer: 0.5 M NH_4OH, 0.5 mM EDTA.
4. Centrifugal vacuum concentrator, e.g., speedvac (Thermo Scientific).
5. 6× Sample buffer: 350 mM Tris–HCl, pH 6.8, 5% glycerol, 10% SDS, 100 mM DTT, 0.002% bromophenol blue.
6. 2× SDS sample buffer: 6× sample buffer diluted to 2× with buffer containing 350 mM Tris–HCl, 0.28% SDS, pH 8 buffer.
7. β-Mercaptoethanol (BME).
8. Precast 4–12% Bis–Tris gels gradient gels.
9. Antibodies for fraction characterization: Cx32, Flotillin, Na^+K^+ATPase, Calnexin, Golgin97, LAMP1, CoxIV.

2.5 Silver Staining and Sample Preparation for MS/MS

1. Clean, acid washed glassware rinsed with at least three changes of double distilled water (ddH_2O).
2. 5% Acetic acid/methanol solution: 25 mL acetic acid in 475 mL ddH_2O and 500 mL methanol.
3. 0.02% Sodium thiosulfate: 0.2 g sodium thiosulfate in 1 L ddH_2O.
4. 0.1% Silver nitrate solution: 0.1 g $AnNO_3$ in 100 mL ddH_2O.
5. 0.01 Formaldehyde in 2% sodium carbonate: 150 μL 37% formaldehyde and 10 g sodium carbonate in 500 mL ddH_2O.
6. 1% Acetic acid: 10 mL acetic acid in 990 mL ddH_2O.
7. #11 scalpel blades.
8. MS/MS Facility of choice.

2.6 Considerations for Reducing Contamination in Downstream MS/MS Analyses (See Note 9)

1. Nonpowdered, nitrile gloves cleaned with 70% ethanol. Gloves should be changed often.
2. A clean lab coat and low-shedding clothing, e.g., no wool.
3. Nonautoclaved, filtered pipette tips.
4. Laminar flow hood or a keratin-free room if possible.
5. Low-bind eppendorf tubes.
6. Clean, acid washed glassware rinsed with at least three changes of ddH_2O.
7. Noncontaminated cell cultures.
8. Filter sterilized solutions (*see* **Note 10**).
9. Clean and sterile workspace (*see* **Note 11**).

2.7 Validation and Analysis of Protein Interactions

1. Network analysis software, e.g., ingenuity systems pathway analysis software, version 8.8, Ingenuity Systems Inc.

3 Methods

3.1 Tissue Homogenization and OP Membrane Fractionation (See Notes 12 and 13)

1. Prepare WT and KO animals for dissection.
2. Carefully excise the gallbladder (filled with digestive enzymes), remove the livers, and immediately place into ice-cold LHB (*see* **Notes 14** and **15**). Recommended starting material is 2–3 livers (3 g total wet weight) per genotype.
3. Mince tissue with a razor blade on a teflon chopping board until homogenous pieces of around 2 mm^2 are achieved.
4. Wash liver pieces with three changes of 30 mL ice-cold LHB in a 50 mL tube. At each wash, invert the liver pieces in the fresh wash solution, allow the liver pieces to settle at the bottom of the tube, and pour off the supernatant.
5. Discard the final wash supernatant and add LHB to the liver pieces at 4× the volume per wet weight of tissue, e.g., 12 mL LHB per 3 g liver tissue.
6. Swirl the tube to suspend the liver pieces in LHB and pour the entire contents into a 30 mL glass homogenizer. Transfer any leftover liver pieces to the homogenizer using forceps.
7. Homogenize gently on ice until very smooth. This will take about 5 strokes with a loose-fitting pestle and 20–30 strokes with a tight-fitting teflon pestle (*see* **Notes 16** and **17**).
8. Transfer homogenized lysate to 15 mL falcon tubes and centrifuge at 800 × *g* for 5 min at 4 °C to remove nuclei and cellular debris.
9. Collect the supernatant and repeat centrifugation at 800 × *g* to collect the final postnuclear supernatant (PNS).
10. Reserve a 100 µL aliquot of the PNS at −80 °C for later analysis.
11. Transfer the PNS to polypropylene centrifuge bottles for high speed centrifuge, and centrifuge supernatants at 8000 × *g* for 10 min to pellet intact mitochondria. Reserve pellet on ice.
12. Remove the supernatant to a clean bottle and repeat centrifugation at 8000 × *g* for 10 min to remove the majority of intact mitochondria. Reserve pellet on ice.
13. Carefully transfer the postmitochondrial supernatants (PMS) into prechilled 15 mL tubes (*see* **Note 18**).
14. Reserve a 100 µL aliquot of the PMS at −80 °C.
15. Concentrate the PMS down to 1.4 mL using a centrifugal unit with 3 kDa molecular weight cutoff.

16. Centrifuge as recommended by the manufacturer at 4 °C for 30 min (*see* **Note 19**).
17. Prepare the HM pellet (mitochondrial and mitochondrial membranes) and dilution of OP solutions while the PMS is concentrating.
18. To prepare the heavy membranes for flotation, gently combine the mitochondrial pellets stored on ice with 2 mL HHB using a 1 mL pipette with the tip cut off.
19. Add 28 mL HHB to the resuspended pellets and centrifuge at 4 °C for 10 min at 8000 × *g*.
20. Discard the supernatant and carefully resuspend the pellet a second time in 2 mL HHB.
21. Add 28 mL HHB and pellet the membranes a final time for 4 °C for 10 min at 8000 × *g*.
22. Resuspend the heavy membrane pellet (HMP) very carefully in 250 μL HHB using a 1 mL pipette with the tip cut off. The final volume of the resuspended pellet should be 400–500 μL (*see* **Note 18**).
23. Reserve a 50 μL aliquot of the resuspended HMP at −80 °C for later analysis.
24. During preparation of the HMP for flotation and while concentrating the PMS, prepare appropriate volumes of OP dilutions from the 50% OP working solution as required. Dilute 50% OP with LHB or HHB to prepare the appropriate volumes of OP dilutions for the LM and HM flotations (LM: 25, 22.5, 20, 15, and 10%; HM: 30, 26, 24, 22, and 20%) as shown in Fig. 1. Chill all solutions on ice.
25. Following concentration of the PMS, reserve a 100 μL aliquot at −80 °C for later analysis.
26. Dilute 1.24 mL of the concentrated PMS into 1.86 mL 50% OP working solution to generate 3.1 mL of 30% PMS/OP solution. Mix well by inversion.
27. Dilute 372 μL of the HMP into 2.728 mL 50% OP working solution to generate 3.1 mL of 44% HM/OP solution. Mix well by inversion.
28. Add 3 mL of the 30% PMS/OP solution and 3 mL of the 44% HM/OP solution to separate chilled 13.2 mL polyallomer centrifuge tubes on ice, ensuring to add each solution to the bottom of the tube without touching the sides.
29. Carefully overlay the appropriate volume and concentration of OP dilution solutions on top of the 30% PMS/OP and 44% HM/OP layers by placing the tip of a 1 mL pipette at the interface of the bottom layer and slowly drawing it up the side of the tube as the lighter density solution is released.

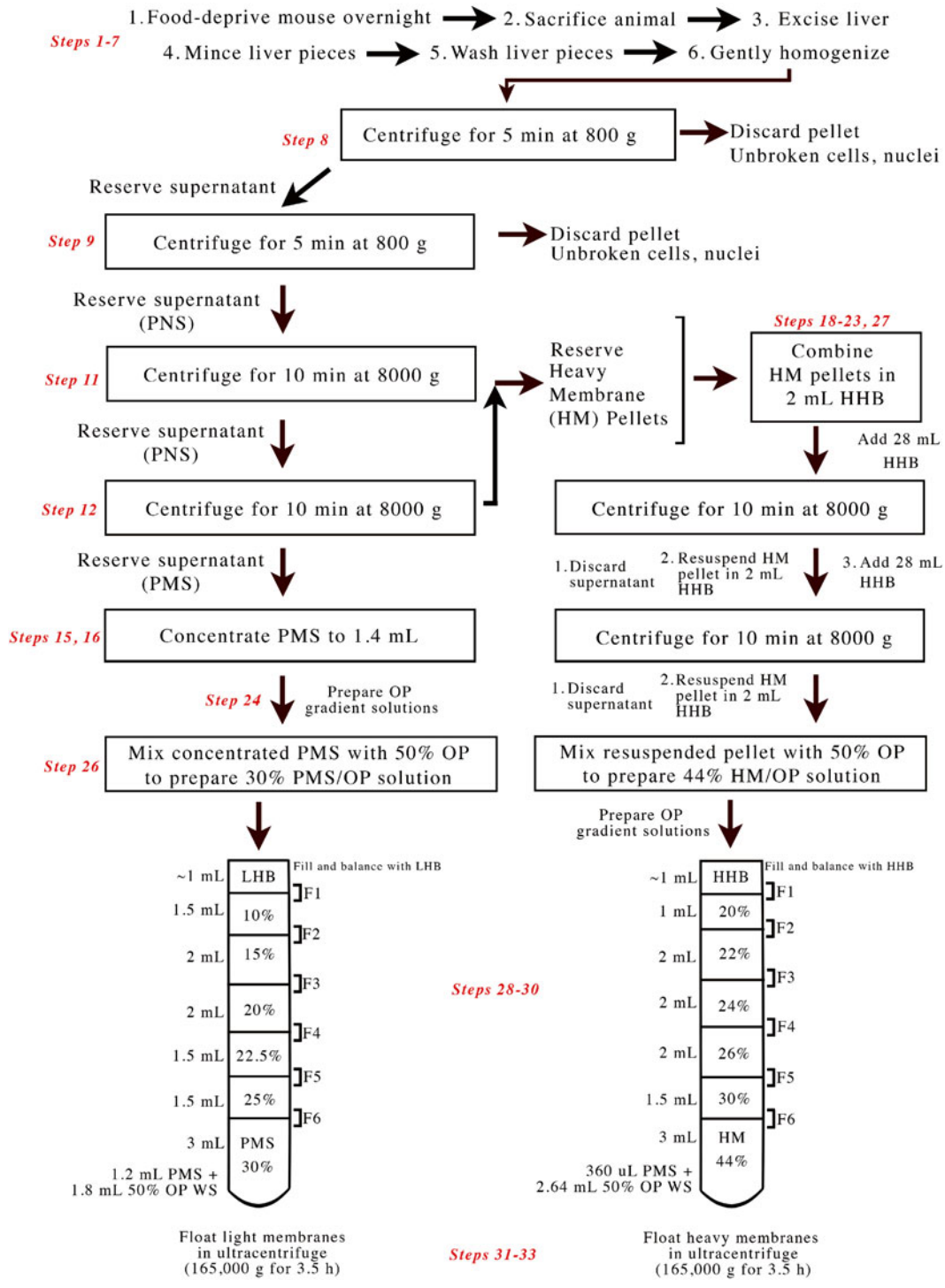

Fig. 1 *Membrane fractionation experimental flow-through*. Briefly, whole liver tissue is separated into light membrane (LM) and heavy membrane (HM) fractions by differential centrifugation. Nuclei are cleared following two centrifugations at 800 × *g*, and HM and mitochondria are cleared from the PNS following two centrifugations at 8000 × *g*. LM and HM are floated in 30%-0%, and 44%-0% OP gradients, respectively, to generate six different density microdomains per tube. The LM preparation contains the majority of plasma-membrane (PM)-associated gap junction proteins, concentrated in the 20-0% OP layers. The HM may be floated to collect the PM and lipid raft domains from the top two interfaces, and to isolate mitochondria and lysosomes for further downstream processing

30. Continue overlaying the remaining density solutions as shown in Fig. 1, making sure that each layer sits discretely on top of the previous layer (*see* **Notes 20** and **21**).
31. Place filled tubes into chilled swinging buckets using a vertical support (*see* Fig. 2a, b), weigh the assemblies in matching pairs (±10 mg) with the opposite tube and bucket using LHB or HHB.
32. Tighten the lids onto the swinging buckets and attach each bucket to the rotor.
33. Centrifuge overnight at 165,000 × *g* at 4 °C for 3.5 h (*see* **Note 22**).

3.2 Agarose Bead Preparation (See Note 23)

1. To reduce shear forces on the agarose beads, cut off the first few mm of the plastic pipette tip using a razor blade sterilized with 70% ethanol.
2. Prepare protein G or protein A agarose beads by rinsing 1 mL bead slurry (50% beads, 50% PBS) with 10 volumes of PBS (*see* **Note 8**).
3. Invert to mix and pellet beads at 1000 × *g* for 5–10 s (discard wash supernatant) and repeat wash to fully remove bead preservative.
4. After final wash, pellet beads at 1000 × *g* for 5–10 s and discard supernatant. Regenerate the original bead volume (1 mL) by adding half the bead volume of PBS.
5. Add between 20 and 50 μg of a primary antibody of choice per 1 mL of beads (*see* **Note 24**) and rotate with inversion overnight at 4 °C (*see* **Notes 25** and **26**).

3.3 Fraction Collection and Preparation for IP

1. Carefully remove centrifuge tubes from the swinging buckets and set tubes in vertical stands. Membrane domains float to the interfaces between the OP layers (Fig. 2c, d).
2. Using a 1 mL pipette, collect the interfaces in 0.5–1.5 mL volumes by pipetting from the top of the tube.
3. Transfer the interfaces to chilled 1.5 mL eppendorf tubes.
4. Collect identical fraction volumes from WT and KO fractionations.
5. At this point samples can be either stored frozen at −80 °C for immunoblotting or maintained chilled for IP.

3.4 Characterizing Samples by Immunoblotting

1. Thaw samples and add nonionic detergent of choice to a final concentration of 1% to each fraction (*see* **Note 27**).
2. Incubate fractions for 30 min at 4 °C with inversion rotation.
3. Centrifuge fractions at 16,100 × *g* in a refrigerated tabletop centrifuge for 20 min at 4 °C to clear the lysates of any unsolubilized material.
4. Pipette the supernatants into clean tubes.

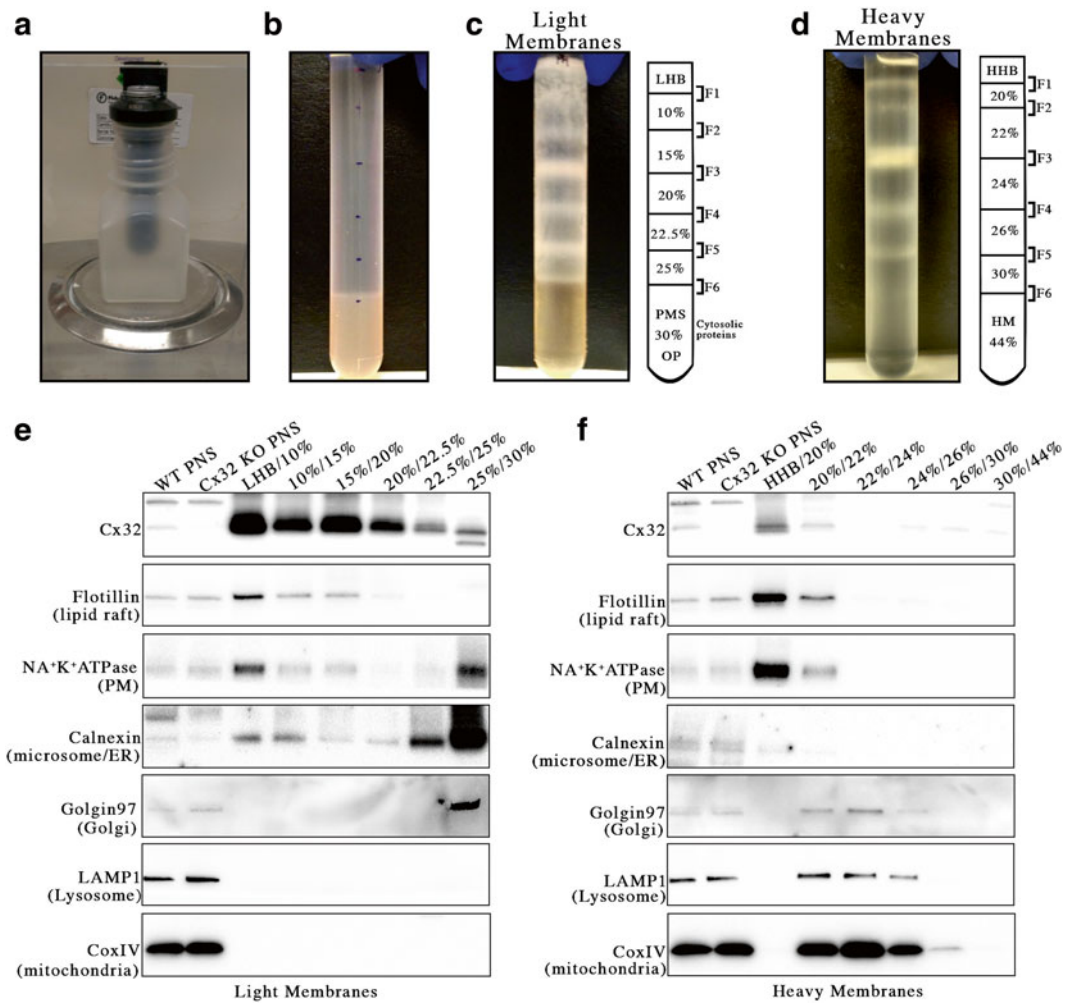

Fig. 2 *Typical set up and results from membrane fractionation.* It is critical to properly balance samples for ultracentrifugation to ±10 mg. To balance swinging buckets, place the swinging bucket, filled centrifuge tube, and lid into a plastic jar (or similar device) to balance it with an opposing bucket (**a**). An example of a fractionation tube with bottom-loaded LM sample, just prior to centrifugation with gradient interfaces marked (**b**). In LM fractionation, LM are light in color, almost pure white in the lightest fractions and cytosolic proteins are found in the 30 % OP layer at the bottom of the LM flotation (**c**). In HM fractionation, HM domains are darker in color compared to the LM domains, and the majority of the material bands at the 22/24 % interface (**d**). Western blot analysis of LM preparations containing gap junction proteins (Cx32), lipid rafts (Flotillin), plasma membranes (Na^+K^+ATPase), microsomes/ER (Calnexin), the majority of golgi membranes (Golgin97), and do not contain lysosomes (LAMP1) or mitochondria (CoxIV) (**e**). Western blot analysis of HM preparations containing gap junction proteins, lipid rafts, and plasma membranes in the lightest two fractions (24-0 % OP). The majority of the material is mitochondrial and located between the 26–20 % OP layers (**d**). Golgi membranes and lysosomes cofractionate with the mitochondria

5. Assay each fraction for protein concentration using a detergent compatible protein assay kit. Use a BSA standard made up in the same detergent buffer as the fractions.

6. Prepare an immunoblot of each solubilized fraction and include a sample of the original PNS solution (*see* **Note 28**).
7. Blot the membrane for the bait protein and several organelle markers (optional) (*see* **Note 29**).
8. From the immunoblots, identify all fractions in which the bait protein is found, or only fractions containing the bait protein and a certain organelle markers for use in IP studies (Fig. 2e, f).

3.5 Preparation of Samples for Immunoprecipitation

1. Based on previous immunoblotting identification, pool together the fractions of interest into 15 mL centrifuge tubes.
2. Add nonionic detergent of choice to a final concentration of 1 % to the pooled fractions.
3. Incubate samples with inversion rotation for 30 min at 4 °C.
4. Centrifuge fractions at 16,100 × *g* in a refrigerated centrifuge for 20 min to clear the lysates of any unsolubilized material.
5. Pipette the supernatants into clean tubes.
6. To exchange the buffer to an IP-compatible buffer, place supernatants in a 3 kDa centrifugal device and centrifuge according to the manufacturer recommendations at 4 °C to reduce the volume to a minimum of 50 µL (centrifugation time depends on total volume).
7. Discard the flow through, add 3.95 mL IP buffer 1 to the filter device and repeat the above centrifugation. Add IP buffer 1 to the concentrated sample to generate ~5 mg/mL lysate.
8. Reserve the final sample in a clean tube on ice (*see* **Notes 30** and **31**).

3.6 Immunoprecipitation Complex Preparation for MS/MS

1. To preclear the lysates, prepare 100 µL bead slurry per 1 mL of lysate.
2. Rinse the bead slurry with 10 volumes of PBS, invert to mix, and centrifuge at 1000 × *g* for 5–10 s.
3. Discard the supernatant, add 10 more volumes of PBS, and resuspend beads by inversion.
4. Divide the resuspended bead slurry into two tubes (for WT and KO lysates) and centrifuge each bead tube at 1000 × *g* for 5–10 s.
5. Remove the PBS, leaving the bead matrices only.
6. Immediately add the pooled fraction lysates to the rinsed bead pellets (*see* **Note 32**).
7. Rotate with inversion for 1 h at 4 °C (*see* **Note 33**).
8. Centrifuge the reactions at 1000 × *g* for 10 s and pipette the supernatants to clean tubes.
9. Centrifuge supernatants for 1 min at 1000 × *g* to fully clear the lysates of beads and pipette the precleared pooled fraction lysates into clean tubes.

10. Recover the previously prepared antibody coated beads and fully resuspend by gently tapping and inverting the tubes.
11. Transfer equal volumes of bead slurry to separate tubes for the WT and KO lysates.
12. Let beads settle by gravity and discard the bead supernatants.
13. Immediately add precleared pooled fraction lysates to each bead matrix and incubate overnight at 4 °C with inversion rotation.
14. Centrifuge samples at 1000 × *g* for 10 s.
15. Remove supernatant, aliquot into 1 mL volumes, and store at −80 °C for later analysis as the post-IP lysate.
16. Carefully pipette 1 mL PBS onto the beads and invert gently to mix.
17. Centrifuge the beads at 1000 × *g* for 5–10 s and discard the supernatant. Repeat the bead washing procedure 2 times for a total of 3 washes. Completely remove the final wash supernatant (*see* **Note 34**).
18. Elute protein complexes from the beads by adding 1 bead volume of ammonium hydroxide elution buffer (e.g., 200 µL for 200 µL bead slurry). Invert the beads to mix and centrifuge at 1000 × *g* for 5–10 s. Repeat this process while collecting and pooling the elution volumes until <1 mL of ammonium hydroxide buffer is collected. Perform a final centrifugation at 1000 × *g* for 1 min to ensure no beads remain in the final elution.
19. Speedvac this solution to lyophilize the protein complexes (*see* **Note 35**).
20. Reconstitute the lyophilized protein in 60 µL 2× SDS sample buffer containing 10% BME. Boil for 5 min, or vortex well and incubate at RT for 30 min *prior* to loading on a gel.
21. Load up to 50 µL of eluted proteins onto a precast 4–12% gradient gel (*see* **Note 36**).
22. Prepare and load 0.5 µL of unstained protein ladder (according to manufacturer's specifications) on each end of the gel.
23. Leave 1 lane space in between each WT and KO sample and between each ladder and the samples. Pipette an equal volume of sample buffer in empty gel lanes.
24. Do not run the dye front off the gel, as potential interacting partners could be lost.
25. Add approximately 100 mL of 5% acetic acid/methanol fixative solution to a clean glass dish with lid and transfer the gel into the fixative immediately following electrophoresis.
26. Cover the glass container and incubate with gentle shaking in fixative for 30 min.

27. Rinse twice with ddH_2O for 2 min each.
28. To minimize background, wash gel overnight in ddH_2O with shaking at 4 °C.

3.7 Silver Staining and Sample Preparation for MS/MS (Day 4)

1. Sensitize the gel for 2 min in approximately 100 mL of 0.02% sodium thiosulfate solution and rinse twice for 30 s each with ddH_2O.
2. Incubate the gel in approximately 100 mL fresh 0.1% silver nitrate solution for 30 min.
3. Wash gel twice in ddH_2O for 30 s.
4. Develop the gel with two changes of freshly prepared 0.01% formaldehyde in 2% sodium carbonate solution. The first application of sodium carbonate solution will change to a brownish-yellow color after a few minutes of shaking. Discard this solution and replace with fresh sodium carbonate. During this second wash, the gel will develop color more quickly, so watch carefully for the appearance of bands. Do not overdevelop the gel, as this impairs the ability to identify proteins within the gel bands by MS/MS.
5. When the gel is sufficiently stained, discard the developing solution and add 1% acetic acid to stop the reaction. The gel may be stored in 1% acetic acid in the fridge for several weeks. A representative silver stained gel image can be found in [7] (*see* **Note 37**).
6. Wash new plastic overhead sheets with 70% ethanol and sandwich the stained gel between the 2 sheets.
7. Image the gel and determine which bands will be analyzed.
8. Excise protein bands with a #11 scalpel blade (*see* **Note 38**).
9. Excise equivalent regions of each WT and KO gel lanes, cut each piece into smaller pieces approximately 3 mm^2 and transfer to separate 0.6 mL eppendorf tubes of mass spectrometry quality.
10. Add a region up to 25 kDa to each tube and continue this process until all regions of interest are collected from both genotypes.
11. Send samples for MS/MS analyses to a preferred MS facility. Ensure that this sample preparation will meet the facility's particular sample specifications.

3.8 Validation and Analysis of Protein Interactions

1. Repeat experiment to perform mass spectrometry on replicate IPs (minimum 2, preferably 3 replicates) (*see* **Note 39**).
2. Prioritize the validation of both previously identified protein interactions and novel interactions using a network analysis software package.

3. Following prioritization of protein interactions, perform reciprocal coimmunoprecipitations (co-IPs) to confirm that bait proteins and MS-identified proteins are present in the same complex (*see* **Note 40**). Representative reciprocal co-IP images can be found in [7].
4. To begin the co-IP, incubate agarose beads with primary antibodies overnight (*see* **Note 41**).
5. Following overnight bead coupling, homogenize liver tissue using IP buffer 2 with fresh protease inhibitors at 4× the tissue weight in a glass homogenizer (*see* **Note 42**).
6. To generate the PNS, centrifuge homogenate at 800 × *g* for 5 min at 4 °C two times to clear unbroken cells and nuclei.
7. If mitochondrial protein interactions are not of interest, generate the PMS by centrifuging the supernatant at 8000 × *g* for 10 min at 4 °C 2×, discarding the mitochondrial pellet each time.
8. Add nonionic detergent at the same concentration used to solubilize the membrane fractions in the original experiment.
9. Incubate samples with inversion rotation for 30 min at 4 °C.
10. Centrifuge at 16,000 × *g* for 20 min at 4 °C. Reserve supernatants and adjust to 5 mg/mL protein with fresh IP buffer 1, following protein assay using a detergent compatible kit.
11. Add between 250 and 1000 μg of total protein for each co-IP to antibody/bead slurry and incubate with inversion at 4 °C overnight.
12. Centrifuge beads at 1000 × *g* for 1 min.
13. Remove supernatant, aliquot into 1 mL volumes, and store at −80 °C for later analysis as the post-IP lysate.
14. Carefully pipette 1 mL PBS onto the beads and invert gently to mix.
15. Centrifuge the beads at 1000 × *g* for 5–10 s and discard the supernatant. Repeat the bead washing procedure two times for a total of three washes. Completely remove the final wash supernatant.
16. Elute the protein complexes in two bead volumes (50–100 μL) 2× SDS sample buffer + 10% BME.
17. Perform co-IP immunoblots, including the pre- and post-IP PNS samples, at least 1 KO sample and each of the experimental IP samples.
18. To determine success of the IP, blot for the bait protein using an antibody raised in a different animal, to avoid detection of the IgG signal.
19. Strip the blot and reprobe for potential interacting proteins. An antibody of a different species from the IP antibody is also preferred.

20. Revisit the network analysis when you have validated (or "unvalidated") several of the MS-identified proteins to generate new hypotheses about the protein of interest.

4 Notes

1. If heavier membranes from mitochondrial pellets are to be analyzed, food-deprive the animals overnight to deplete the liver of glycogen. Glycogen cofractionates with mitochondria and will prevent the isolation of pure mitochondrial-associated membrane fractions [15].
2. A single liver from an adult mouse of around 25–30 g has a wet weight of approximately 1–1.5 g. Thus, 2–3 adult mouse livers per centrifuge tube are required for the identification of Cx32-based interactions.
3. This method is applicable to other tissues but will require optimization of the tissue amount required.
4. For more than three livers, set up identical centrifuge flotations using 13 mL tubes, or scale up the flotation, e.g., to accommodate up to 9 g wet weight liver using 38.5 mL tubes in a larger swinging bucket rotor.
5. Including $MgCl_2$ in the LHB helps in the isolation of intact plasma membrane sheets. Always use LHB when working with any of the light membrane supernatants or fractions *prior* to detergent solubilization.
6. HHB contains EDTA to chelate cations, as mitochondria are uncoupled in the presence of $Ca2^{+}$. Always use HHB when working with any of the heavy membrane pellets or fractions *prior* to detergent solubilization.
7. Detergent choice will be based on the proteins requiring isolation in each particular fraction, e.g., Triton X-100 insoluble lipid raft proteins from the lightest interfaces in each tube (Fig. 2).
8. Antibody/Agarose bead compatibilities. Protein G and A agarose beads bind strongly to most human and mouse IgG proteins; however, Protein A is usually preferred for binding rabbit IgG proteins. Some IgG subtypes do not bind strongly to Protein A or Protein G.
9. Protein identities resulting from MS/MS analyses rely heavily on the quality of the sample being investigated. MS/MS identifies the most abundant species in a sample and if low abundance species are of interest, it is especially important to limit sample contamination. Common contamination sources in MS analyses include bacteria from infected cell culture samples,

oils, plastics, fibers, dust, skin constituents from instruments and human handlers, and detergent/chemical residues used to clean laboratory equipment. For these reasons, always use good laboratory practice.

10. Prepare all solutions and buffers fresh and maintain sterility of stock solutions throughout the procedure. Keep lids closed and only open in a laminar flow hood.
11. The workspace and pipettes should be cleaned often with 70% ethanol and low-lint wipes.
12. Pilot experiments should be performed to characterize the fractions and determine which membrane domains contain your protein of interest and/or organelle marker of interest. These samples should not be used for the IP as they will have to be frozen to allow time for fraction analysis by immunoblotting. Freeze-thawing the samples may disrupt protein complexes. For the pilot experiment, omit the agarose bead preparation.
13. The complete procedure requires 4 consecutive lab days to generate a sample for MS/MS. Some of the different sections of the protocol require to be run continuously, e.g., antibody coating of beads on day 1 for use on day 2.
14. KO tissue is required for these studies.
15. Keep all samples on ice or at 4 °C at all times.
16. It is critical to homogenize gently with fluid motions and to prevent the introduction of air bubbles. Protection of organelle integrity during homogenization is essential for isolation of pure membrane fractions.
17. Modifications for cell culture and other tissues. Cell culture: remove cells from incubator and place on ice. Wash cells three times with ice-cold PBS and aspirate fully between each wash to remove all traces of serum. Add 1 mL cold LHB with protease inhibitors and scrape cells from the plate (Use 1 mL for every 5 plates scraped). Add this lysate to subsequent plates and scrape to pool lysates. Pass cells 8–10 times through a 23–25 g needle to break open cells instead of using a glass homogenizer and continue to PNS isolation. In certain cases of cells or tissue containing low amounts of mitochondria, the entire PNS supernatant may be fractionated in the light membrane schematic. Other tissues: Harder tissues such as heart and muscle will require more strokes in the glass homogenizer to achieve adequate disruption. Rotary blade homogenization may cause cross-contamination of subcellular fractions [16].
18. The membranes contained within PMS and HM pellets can be purified from soluble cytosolic proteins by ultracentrifugation at 100,000 × *g* for 50 min at 4 °C following 10× dilution with LHB or HHB, preflotation. Discard the supernatant containing

cytosolic proteins and resuspend light and heavy membrane pellets in 3 mL of 30% or 44% OP solutions, respectively, and continue to fraction collection. Membrane fractions collected postflotation may also be purified by centrifugation at 100,000 × *g* for 50 min at 4 °C following a 10× dilution in LHB or HHB.

19. Centrifugal filters have various starting volumes. A 4–15 mL volume is recommended for this technique. 3 g liver tissue will result in around 8 mL of PMS, which is concentrated 6× to achieve a final volume of approximately 1.4 mL. Centrifugation times will vary depending on the preparation, so this should be continually monitored to prevent drying of the filter membrane.
20. Sharp interfaces will make the membranes easier to collect.
21. Top up the centrifuge tube to a point 1 mm below the top edge with LHB, as tubes have no caps, they will warp if not completely filled.
22. It is critical that the layers are not disturbed by sudden acceleration/deceleration so these must be set to a minimum, e.g., deceleration = no brake/coast.
23. This step can be performed at any point on day 1.
24. Antibody concentration will depend on the quality of the antibody, how well it detects antigens in the IP environment, and expression level of the bait protein. An appropriate antibody:bead ratio will have to be empirically determined for each antibody.
25. As an extra negative control for MS/MS prepare beads coated with the same concentration of an isotype-specific IgG antibody.
26. If the bait protein is very close in size to 25 kDa (light chain IgG) or 50 kDa (heavy chain IgG), its identification may be masked on a silver stained gel by the eluted immunoglobulins. DMP can be used to chemically couple the antibodies to the beads reducing this background signal. Following overnight incubation with the primary antibody, cross-link the antibodies to the beads as described in [7].
27. An appropriate detergent will have to be experimentally determined for your membrane protein and tissue of interest. Nonionic detergents such as NP-40 and Triton X-100 are the most gentle, and at 1–2% will solubilize most membrane domains while maintaining protein structure and protein–protein interactions. Digitonin is another mild nonionic detergent, very effective at solubilizing membrane proteins that should be evaluated during the optimization stage of IP or co-IP and may permit identification of different protein interactions compared to NP-40/TritonX-100. CHAPS is a zwitterionic detergent that is harsher than the nonionic detergents and very effective at solubilizing membranes. Some protein

interactions are preserved with CHAPS, as it is less harsh than ionic detergents like SDS or sodium deoxycholate (DOC). Only extremely stable protein interactions will be preserved using RIPA buffer containing 0.1% SDS, 1% DOC, and 1% NP-40. Ionic detergents are not recommended for IP or co-IP. When optimizing the IP, start by using NP-40, Triton X-100, or Digitonin at 1% and increase stringency if the bait protein is not recovered from membranes. Increase the stringency by increasing the nonionic detergent concentration to 2% or use CHAPS at 1–2%. If very few proteins are found to interact with the bait protein, decrease the stringency of the solubilization to ensure you can detect some reported protein interactions. Pay close attention to the temperature of the reactions at all times to maintain 0–4 °C, as the critical micelle concentration (CMC) of nonionic detergents is variable across temperature ranges, and slight fluctuations will alter the reproducibility of results [17].

28. Immunoblot analysis should be performed on both WT and KO fractions to ensure the gradients were similarly harvested.
29. Integral membrane proteins are preferred as fractionation markers; however, organelle activity and integrity is preserved during this isosmotic procedure, allowing for the identification and enzyme assay of soluble, organelle matrix proteins.
30. Buffer replacement is required, as high concentrations of OP may impede the diffusion of larger protein complexes and the formation of antibody–antigen interactions.
31. At this point the protein concentration of the lysates should be equal between the 2 genotypes (volume between 1 and 12.5 mL).
32. The required mass of IP material must be determined experimentally; however, ~5 mg of starting material (enriched gap junction fraction lysate) is a recommended minimum.
33. This step removes any protein from the lysate that nonspecifically binds to the bead matrix.
34. Alternate bead washing techniques. Depending on the stringency of the detergent, the nature of the tissue, proteins may nonspecifically bind to the beads and to the bait protein. If there are many nonspecific proteins eluting in the KO purification or in the control isotype purification, wash the beads more, or reevaluate the detergent choice. To modify the washes, try adding a detergent wash and further PBS washes. Do not use a detergent more stringent than used during the IP. The salt concentration in the wash buffer may also be titrated to a maximum stringency of 1000 mM. Increasing the bead wash stringency can reduce false-positive interactions but may prevent the identification of transient or weak protein interactions.

35. If the antibodies were not coupled to the bead matrix using DMP and there is no centrifugal vacuum concentrator available, proteins may be eluted from the beads using 1–2 bead volumes 2× SDS sample buffer containing 10% BME. Boil the beads in sample buffer for 5 min and centrifuge for 1 min at 1000 × *g*. Reserve the bead supernatant.
36. Reserve 5–10% of resuspended IP product for verification of IP success by immunoblotting.
37. Anything that touches the gel at this point must be wiped clean with 70% ethanol and care must be taken to ensure that no keratin or dust is introduced to the gel environment. Limit handling of the gel if at all possible, even with gloves.
38. Wipe the blade with 70% ethanol before use and change the blade between each band.
39. It is crucial that mass spectrometry identification of protein interactions from replicate IPs be confirmed biochemically to limit false-positive reports. Prioritize the biochemical validation of protein identities assigned from 1 or more unique peptides, and identities assigned from peptides that cover the largest % of the protein sequence, with the highest Mascot ion scores (probability that the MS/MS spectrum matches to the stated peptide). Mascot scores depend on the type and quality of the dataset. However, mean scores representing correct protein identities are usually around ~100 [18]. For large datasets, application of a 1% false-discovery rate (FDR) calculation should be applied to limit false-positive protein identity assignments [19, 20]. As proof that the experiment is able to replicate the literature, prioritize the validation of previously identified interacting proteins, in addition to validating novel proteins identified in the screen.
40. A positive reciprocal co-IP does not prove that proteins directly interact, but is a strong piece of evidence that the proteins are contained within the same complex. A validated reciprocal co-IP occurs if an IP for bait protein identifies interacting protein in the lysate and IP for interacting protein identifies bait protein in the lysate (by immunoblotting). Three main reactions should be performed: (1) Positive reaction: agarose beads coupled to primary antibody of choice with WT lysate applied; (2) negative reaction: agarose beads coupled to primary antibody of choice with KO lysate applied; and (3) control reaction: agarose beads coupled to an isotype-matched IgG antibody with WT lysate applied (e.g., mouse IgG antibody added to the agarose beads at the same concentration/bead ratio as the anti-mouse primary Cx32 antibody).

41. Scale each reaction down to 25–50 μL of beads and 250 μg of total protein. Maintain the same antibody:bead ratio as optimized in the original experiment.
42. The same floated membrane fractions prepared for the MS/MS experiment may also be used for the validation co-IP experiment.

Acknowledgements

This work was supported by the Canadian Institutes of Health Research (CIHR) AWT143081 and Canadian Institutes of Health Research (CIHR) Training Program in Neurodegenerative Lipidomics (CTPNL) (TGF-96121 to SALB). SF received scholarships from CTPNL, CIHR (Banting and Best Ph.D. Scholarship), Ontario Graduate Scholarship (OGS) Queen Elizabeth II Graduate Scholarship in Science and Technology.

References

1. Decrock E, De Bock M, Wang N et al (2015) Connexin and pannexin signaling pathways, an architectural blueprint for CNS physiology and pathology? Cell Mol Life Sci 72:2823–2851
2. Herve JC, Derangeon M, Sarrouilhe D et al (2012) Gap junctional channels are parts of multiprotein complexes. Biochim Biophys Acta 1818:1844–1865
3. Zhou JZ, Jiang JX (2014) Gap junction and hemichannel-independent actions of connexins on cell and tissue functions--an update. FEBS Lett 588:1186–1192
4. Dbouk HA, Mroue RM, El-Sabban ME et al (2009) Connexins: a myriad of functions extending beyond assembly of gap junction channels. Cell Commun Signal. doi:10.1186/1478-811X-7-4
5. Herve JC, Bourmeyster N, Sarrouilhe D (2004) Diversity in protein-protein interactions of connexins: emerging roles. Biochim Biophys Acta 1662:22–41
6. Giepmans BN (2004) Gap junctions and connexin-interacting proteins. Cardiovasc Res 62:233–245
7. Fowler SL, Akins M, Zhou H et al (2013) The liver connexin32 interactome is a novel plasma membrane-mitochondrial signaling nexus. J Proteome Res 12:2597–2610
8. Kanter HL, Laing JG, Beyer EC et al (1993) Multiple connexins colocalize in canine ventricular myocyte gap junctions. Circ Res 73:344–350
9. Altevogt BM, Paul DL (2004) Four classes of intercellular channels between glial cells in the CNS. J Neurosci 24:4313–4323
10. Berggard T, Linse S, James P (2007) Methods for the detection and analysis of protein-protein interactions. Proteomics 7:2833–2842
11. Lalonde S, Ehrhardt DW, Loque D et al (2008) Molecular and cellular approaches for the detection of protein-protein interactions: latest techniques and current limitations. Plant J 53:610–635
12. Jarvik JW, Telmer CA (1998) Epitope tagging. Annu Rev Genet 32:601–618
13. Laird DW, Jordan K, Thomas T et al (2001) Comparative analysis and application of fluorescent protein-tagged connexins. Microsc Res Tech 52:263–272
14. Lagny TJ, Bassereau P (2015) Bioinspired membrane-based systems for a physical approach of cell organization and dynamics: usefulness and limitations. Interface Focus. doi:10.1098/rsfs.2015.0038
15. Wieckowski MR, Giorgi C, Lebiedzinska M et al (2009) Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells. Nat Protoc 4:1582–1590
16. Cox B, Emili A (2006) Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics. Nat Protoc 1:1872–1878

17. Chen L-J, Lin S-Y, Huang C-C et al (1998) Temperature dependence of critical micelle concentration of polyoxyethylenated non-ionic surfactants. Colloids Surf B Physiochem Eng Aspects 135:175–181
18. Stead DA, Preece A, Brown AJ (2006) Universal metrics for quality assessment of protein identifications by mass spectrometry. Mol Cell Proteomics 5:1205–1211
19. Elias JE, Haas W, Faherty BK et al (2005) Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. Nat Methods 2: 667–675
20. Wang G, Wu WW, Zhang Z et al (2009) Decoy methods for assessing false positives and false discovery rates in shotgun proteomics. Anal Chem 81:146–159