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TITLE:

Purification of Endogenous *Drosophila* Transient Receptor Potential Channels

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SUMMARY:

Based on the assembling mechanism of the INAD protein complex, in this protocol, a modified affinity purification plus competition strategy was developed to purify the endogenous *Drosophila* TRP channel.

ABSTRACT:

Drosophila phototransduction is one of the fastest known G protein-coupled signaling pathways. To ensure the specificity and efficiency of this cascade, the calcium (Ca^{2+})-permeable cation channel, transient receptor potential (TRP), binds tightly to the scaffold protein, inactivation-no-after-potential D (INAD), and forms a large signaling protein complex with eye-specific protein kinase C (ePKC) and phospholipase Cβ/No receptor potential A (PLCβ/NORPA). However, the biochemical properties of the *Drosophila* TRP channel remain unclear. Based on the assembling mechanism of INAD protein complex, a modified affinity purification plus competition strategy was developed to purify the endogenous TRP channel. First, the purified histidine (His)-tagged NORPA 863-1095 fragment was bound to Ni-beads and used as bait to pull down the endogenous INAD

protein complex from *Drosophila* head homogenates. Then, excessive purified glutathione S-transferase (GST)-tagged TRP 1261-1275 fragment was added to the Ni-beads to compete with the TRP channel. Finally, the TRP channel in the supernatant was separated from the excessive TRP 1261-1275 peptide by size-exclusion chromatography. This method makes it possible to study the gating mechanism of the *Drosophila* TRP channel from both biochemical and structural angles. The electrophysiology properties of purified *Drosophila* TRP channels can also be measured in the future.

INTRODUCTION:

Phototransduction is a process where absorbed photons are converted into electrical codes of neurons. It exclusively relays opsins and the following G protein-coupled signaling cascade in both vertebrates and invertebrates. In *Drosophila,* by using its five PDZ domains, scaffold protein inactivation-no-after-potential D (INAD) organizes a supramolecular signaling complex, which consists of a transient receptor potential (TRP) channel, phospholipase Cβ/No receptor potential A (PLCβ/NORPA), and eye-specific protein kinase C (ePKC)[1]. The formation of this supramolecular signaling complex guarantees the correct subcellular localization, high efficiency, and specificity of *Drosophila* phototransduction machinery. In this complex, light-sensitive TRP channels act as downstream effectors of NORPA and mediate calcium influx and the depolarization of photoreceptors. Previous studies showed that the opening of the *Drosophila* TRP channel is mediated by protons, disruption of the local lipid environment, or mechanical force[2–4]. The *Drosophila* TRP channel also interacts with calmodulin[5] and is modulated by calcium by both positive and negative feedback[6–8].

So far, electrophysiology studies on the gating mechanism of *Drosophila* TRP and TRP-like (TRPL) channels were based on excised membrane patches, whole-cell recordings from dissociated wild-type *Drosophila* photoreceptors, and hetero-expressed channels in S2, SF9, or HEK cells[2,9–13], but not on purified channels. The structural information of the full-length *Drosophila* TRP channel also remains unclear. In order to study the electrophysiological properties of purified protein in a reconstituted membrane environment and to gain structural information of the full-length *Drosophila* TRP channel, obtaining a purified full-length TRP channel is the necessary first step, similar to the methodologies used in mammalian TRP channel studies[14–17].

Recently, based on the assembling mechanism of INAD protein complex[18–20], an affinity purification plus competition strategy was first developed to purify the TRP channel from *Drosophila* head homogenates by streptavidin beads[5]. Considering the low capacity and expensive cost of streptavidin beads, an improved purification protocol is introduced here that uses His-tagged bait protein and corresponding low-cost Ni-beads with much higher capacity. The proposed method will help to study the gating mechanism of the TRP channel from structural angles and to measure the electrophysiological properties of the TRP channel with purified proteins.

PROTOCOL:

1. Purification of GST-tagged TRP and His-tagged NORPA fragment

1.1 Purify GST-tagged TRP 1261-1275 fragment

1.1.1 Transform the pGEX 4T-1 TRP 1261-1275 plasmid[10] into *Escherichia coli (E. coli)* BL21 (DE3) cells using the $CaCl_2$ heat-shock transformation method[21]. Inoculate a single colony in 10 mL of Luria Bertani (LB) medium and grow overnight at 37 °C. Then, amplify the 10 mL of seeding culture in 1 L of LB medium at 37 °C.

1.1.2 After the optical density (OD_{600}) of the cells reaches 0.5, cool down the cells to 16 °C and add 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration) to induce the overexpression of the target protein and incubate at 16 °C for 18 h.

1.1.3 After overexpression, pellet 1 L of cultured cells by centrifugation at 6,000 × *g* and resuspend in 40 mL of phosphate-buffered saline (PBS) buffer.

1.1.4 Load the resuspended cells in a high-pressure homogenizer pre-cooled at 4 °C. Slowly increase the homogenizer pressure to 800 bar. Open the inlet tap and let the resuspended cells circularly pass through a valve with very narrow slits.

NOTE: The cells are homogenized by the high shearing forces caused by a large pressure drop and cavitation.

1.1.5 Load 5 mL of glutathione beads to a gravity flow column and wash the beads with 50 mL of PBS buffer for a total of three times.

1.1.6 Centrifuge the cell lysate from the high-pressure homogenizer at 48,384 x *g*. Add the supernatant of the centrifuged cell lysate (40 mL) to the equilibrated glutathione beads in the gravity flow column and incubate for 30 min at 4 °C. Resuspend the glutathione beads every 10 min.

1.1.7 After 30 min of incubation, open the column outlet tap to separate the beads and flow-through fraction. Discard the flow-through fraction. Rinse the remaining glutathione beads twice with 50 mL of PBS buffer.

1.1.8 Add 15 mL of elution buffer to the glutathione beads and incubate for 30 min. Resuspend the beads every 10 min.

1.1.9 After 30 min of incubation, elute the GST-tagged TRP 1261-1275 fragment in a 50 mL conical tube and load in a size-exclusion column (preparation grade), which is equilibrated using 50 mM Tris (pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT) buffer.

1.1.10 Keep the elution flow rate of the size-exclusion column to 3 mL/min. Collect the eluted proteins at the rate of 5 mL/tube.

1.1.11 Identify the peak of the target protein in the size-exclusion column by analyzing the UV absorption signals at 280 nm and verify by SDS-PAGE gel analysis (electrophoresis parameters: 150 V for the stacking gel; 200 V for the resolving gel). Stain the gel with Coomassie blue R250.

1.1.12 Concentrate the purified GST-tagged TRP 1261-1275 fragment from the size-exclusion column to 1 mL using a 15 mL ultrafiltration spin column centrifuged at 3,000 x *g* at 4 °C in a desk-top refrigerated centrifuge.

1.1.13 Determine the concentration of concentrated protein using the Beer-Lambert Law. Measure the UV absorption of GST-tagged TRP 1261-1275 fragment at 280 nm using a spectrophotometer.

1.1.14 Obtain the extinction coefficient at 280 nm by importing the protein sequences into the Protparam program (https://web.expasy.org/protparam/). Typically, 1 L culture of GST-tagged TRP 1261-1275 yields 1 mL of 600 µM of protein (6 x 10^{-7} mol). See **Table 1** for materials needed.

1.2 Purification of His-tagged NORPA 863-1095 fragment

1.2.1 Transform the pETM.3C NORPA 863-1095 plasmid[18–20] into *E. coli* BL21 (DE3) cells using the $CaCl_2$ heat-shock transformation method[21]. Inoculate a single colony in 10 mL of LB medium and grow overnight at 37 °C. Then, amplify the 10 mL seeding culture in 1 L of LB medium at 37 °C.

1.2.2 After the OD_{600} of the cells reaches 0.5, cool down the cells to 16 °C and add 0.1 mM IPTG (final concentration) to induce the overexpression of target protein and incubate at 16 °C for 18 h.

1.2.3 After overexpression, pellet 1 L of cultured cells by centrifugation at 6,000 x *g* and resuspend in 40 mL of binding buffer. Next, lyse the resuspended cells in a high-pressure homogenizer at 4 °C as described in step 1.1.5.

1.2.4 Load 5 mL of Ni-beads into a gravity flow column and wash three times with 50 mL of binding buffer.

1.2.5 Centrifuge the cell lysate from the high-pressure homogenizer at 48,384 x *g*. Add the supernatant of the centrifuged cell lysate to the equilibrated Ni-beads in the gravity flow column and incubate for 30 min at 4 °C. Resuspend the Ni-beads every 10 min.

1.2.6 After 30 min of incubation, open the column outlet tap to separate the beads and flow-through fraction. Discard the flow-through fraction and wash the remaining Ni-beads twice with 50 mL of binding buffer.

1.2.7 Add 15 mL of elution buffer to the Ni-beads and incubate for 30 min. Resuspend the Ni-beads every 10 min.

1.2.8 After 30 min of incubation, collect the eluted His-tagged NORPA 863-1095 fragment in a 50 mL conical tube and load into a size-exclusion column (preparation grade), which is equilibrated using 50 mM Tris (pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT).

1.2.9 Keep the elution flow rate of the size-exclusion column to 3 mL/min. Collect the eluted protein at the rate of 5 mL/tube.

1.2.10 Identify the peak of the target protein in the size-exclusion column by analyzing the UV absorption signals at 280 nm and verify by SDS-PAGE gel analysis (electrophoresis parameters: 150 V for the stacking gel; 200 V for the resolving gel). Stain the gel using Coomassie blue R250.

1.2.11 Concentrate the purified His-tagged NORPA 863-1095 fragment from the size-exclusion column to 1 mL using a 15 mL ultrafiltration spin column centrifuged at 3,000 x *g* at 4 °C in a desk-top refrigerated centrifuge.

1.2.12 Determine the concentration of concentrated protein using the Beer-Lambert Law. Measure the UV absorption of His-tagged NORPA 863-1095 fragment at 280 nm using a spectrophotometer.

1.2.13 Obtain the extinction coefficient at 280 nm by importing the protein sequences into the Protparam program (https://web.expasy.org/protparam/). Typically, 1 L culture of His-tagged NORPA 863-1095 fragment yields 1 mL of 600 µM of protein (6 x 10^{-7} mol). See **Table 2** for materials needed.

2. Preparation of *Drosophila* heads

2.1 Collect adult flies in 50 mL conical centrifugation tubes using the CO_2 anaesthetization method[22,23]; immediately freeze in liquid nitrogen for 10 min and store in a -80°C freezer.

2.2 After collecting a sufficient number of flies, vigorously shake the frozen 50 mL conical tubes by hand to separate the flies' legs, heads, wings, and bodies. Transfer the mixture to three sequentially stacked pre-cooled stainless-steel sieves (20/30/40 mesh size, respectively) and shake the sieves.

2.3 Next, since the heads cannot pass through the 40-mesh sieve, use a brush to sweep the fly heads off the 40-mesh sieve, transfer them into 50 mL conical tubes, and store them at −80 °C.

2.4 Continuously collect the flies and their heads and store them in a -80 °C freezer until they reach the required amount needed for experimentation (0.5 g). Typically, to collect 0.5 g of heads, 35 mL of flies in a 50 mL conical tube are needed. See **Table 3** for materials needed.

3. ***Drosophila*** **TRP channel purification**

3.1 Weigh a total of 0.5 g of heads and completely homogenize in liquid nitrogen using a pre-cooled mortar-pestle. Dissolve the homogenized heads in 10x v/w lysis buffer (5 mL), incubate in a shaker at 4 °C for 20 min, and then centrifuge at 20,817 x *g* for 20 min at 4 °C.

3.2 Collect the spin-down supernatant ("20817 g S", **Figure 4**) and further centrifuge it at 100,000 x *g* for 60 min at 4 °C. Use the spin-down supernatant ("100,000 g S", **Figure 4**) for the following pull-down assay.

3.3 Add 1 mL of Ni-beads into the gravity flow column and wash the beads with 10 mL of double-distilled H_2O (ddH_2O) at 4 °C for a total of three times. Equilibrate the beads with 10 column volumes of lysis buffer three times at 4 °C.

3.4 Add 500 µL of 600 µM purified His-tagged NORPA 863-1095 protein (3×10^{-7} mol) into the Ni-column and incubate for 30 min at 4 °C. Resuspend the beads every 10 min.

3.5 Open the column outlet tap to separate the beads and flow-through fraction. Take the flow-through fraction for SDS-PAGE analysis (NORPA F, **Figure 4**). In this section, the bait proteins are immobilized on the Ni-beads.

3.6 Wash the Ni-beads with 10 column volumes of lysis buffer (10 mL) at 4 °C and keep the washing fraction for SDS-PAGE analysis (Wash 1, **Figure 4A**). Repeat the above steps and keep the sample for SDS-PAGE analysis (Wash 2, **Figure 4A**). In this section, the excessive bait proteins on the Ni-beads are removed.

3.7 Add the supernatant of *Drosophila* head homogenate after 100,000 x *g* centrifugation into the Ni-column at 4 °C, where the His-tagged NORPA 863-1095 fragment has been immobilized.

3.8 Incubate the supernatant with the Ni-beads at 4 °C for 30 min. Resuspend the beads every 10 min. Then, open the column outlet tap to separate the beads and flow-through fraction.

3.9 Collect the supernatant for SDS-PAGE analysis (Dro head lysis F, **Figure 4A**). In this section, the INAD protein complexes (INAD/TRP/ePKC) in the head homogenates are captured by the immobilized NORPA 863-1095 fragments on the Ni-beads.

3.12 Wash the Ni-beads with 10 column volumes of lysis buffer (10 mL) at 4 °C and keep the supernatant from gravity precipitation for SDS-PAGE analysis (Wash 3, **Figure 4A**). Repeat the above steps and collect the supernatant for SDS-PAGE analysis (Wash 4, **Figure 4A**). In this section, the unbound proteins on the Ni-beads are removed.

3.13 Add 500 µL of 600 µM of GST-tagged TRP 1261-1275 protein (3 x 10^{-7} mol) into the Ni-beads and incubate for 20 min at 4 °C. Resuspend the beads every 10 min.

3.14 Collect the eluted fraction from the gravity column (TRP E1, **Figure 4B**), which contains the endogenous *Drosophila* TRP channel. Repeat the above steps and collect the elution fraction (TRP E2, **Figure 4B**). In this step, by using the GST-tagged TRP 1261-1275 fragments as the competitor, the TRP channels are eluted from the captured INAD protein complexes (INAD/TRP/ePKC) on the Ni-beads.

3.15 Wash the Ni-beads with 10 column volumes of binding buffer (10 mL; **Table 1**) at 4 °C and collect the washing fraction for SDS-PAGE analysis (Wash 5, **Figure 4B**).

3.16 Add 500 µL of elution buffer (**Table 1**) into the Ni-beads and incubate for 20 min at 4 °C. Collect the elution fraction from the gravity flow column (NORPA E1, **Figure 4B**). Repeat the above steps, and collect the elution fraction (NORPA E2, **Figure 4B**).

3.17 Using the elution buffer, elute the His-tagged NORPA 863-1095 fragment accompanied with the INAD/ePKC protein complexes. Next, resuspend the Ni-beads in 500 µL of binding buffer.

3.18 Take the resuspended Ni-beads to run the SDS-PAGE (stained by Coomassie blue R250) to analyze the efficiency of the elution and evaluate whether the elute buffer works (beads, **Figure 4B**). See **Table 4** for materials needed.

4. Size-exclusion column purification of *Drosophila* TRP channel

4.1 Install a size-exclusion column (analytical grade) on the protein purification system. Equilibrate the column with the column buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM DTT, 0.75 mM DDM), which is filtered by a 0.45 µm filter.

4.2 Concentrate the TRP E1 and E2 fraction from step 3.14 using a 4 mL ultrafiltration spin column, centrifuged at 3,000 x *g* at 4 °C in a refrigerated centrifuge.

4.3 Rinse the sample loop with the column buffer and load the sample into the sample loop. Inject the sample into the size-exclusion column and elute the proteins with a proper flow rate (0.5 mL/min).

4.4 Identify the peak of the target protein by absorption at 280 nm and run an SDS-PAGE gel to detect the purified endogenous *Drosophila* TRP channel (**Figure 5**). See **Table 5** for materials needed.

REPRESENTATIVE RESULTS:

In this article, a protein purification method is demonstrated to purify endogenous *Drosophila* TRP channel (**Figure 1**).

First, recombinant protein expression and purification are applied to obtain the bait and competitor proteins. Then, a GST-tagged TRP 1261-1275 fragment is expressed in *E. coli* BL21 (DE3) cells in LB medium and purified using glutathione beads and a size-exclusion column (**Figure 2**). The samples were verified using SDS-PAGE analysis with Coomassie blue R250 staining. In the SDS-PAGE sample preparation process, 30 µL of protein sample is mixed with 10 µL of 4x loading dye and boiled at 100 °C for 10 min. Then, 15 µL of boiled sample is individually loaded into each well. The His-tagged NORPA 863-1095 fragment is also similarly expressed in *E. coli* BL21 (DE3) cells in LB medium and purified by Ni-beads and size-exclusion column (**Figure 3**). The purified GST-tagged TRP 1261-1275 and His-tagged NORPA 863-1095 are concentrated for the purification of the endogenous *Drosophila* TRP channel.

Second, *Drosophila* heads are collected and homogenized in liquid nitrogen using a pre-cooled mortar-pestle, and then dissolved in 10x v/w lysis buffer (**Table 4**). The dissolved head homogenate is incubated in a shaker at 4 °C for 20 min and centrifuged at 20,817 x *g* for 20 min at 4 °C. The spin-down supernatant (20817 g S, **Figure 4A**) is collected and further centrifuged at 100,000 x *g* for 60 min at 4 °C. The second spin-down supernatant (100,000 g S, **Figure 4A**) is used for the subsequent pull-down assay.

Finally, based on the principles of pull-down and competition assay, the affinity purification plus competition strategy is used to purify the endogenous TRP channel. The purified His-tagged NORPA 863-1095 fragment is bound to Ni-beads and used as the bait to pull down the endogenous INAD protein complexes from *Drosophila* head homogenates. Then,

excessive purified GST-tagged TRP 1261-1275 fragment is added to compete for the TRP channel from the captured INAD complexes on the Ni-beads (TRP E1, TRP E2, **Figure 4B**). In the end, the eluted TRP channel is separated from the excessive GST-tagged TRP 1261-1275 peptide by size-exclusion chromatography (**Figure 5**). In the SDS-PAGE sample preparation process, 30 µL of protein sample is mixed with 10 µL of 4x loading dye and boiled at 100 °C for 10 min. Then, the 15 µL of sample is individually loaded into each well. As a byproduct, the INAD-ePKC-NORPA 863-1095 complexes can also be obtained by eluting the Ni-beads after TRP 1261-1275 peptide competition (NORPA E1, NORAP E2, **Figure 4B**). Using this method, the typical yield of the final purified *Drosophila* TRP channel from 0.5 g fly heads is 50 µL of 3 µM TRP protein (1.5 x 10^{-10} mol). If more purified TRP channels are needed, scale up the amount of fly heads, Ni-beads, bait protein, and competitor correspondingly.

FIGURE AND TABLE LEGENDS:

Figure 1: The schematic diagram for purification of endogenous *Drosophila* TRP channel. (**A**) Purified His-tagged NORPA 863-1095 proteins are immobilized on the Ni-beads. (**B**) *Drosophila* heads are homogenized and the spin-down supernatant after 100,000 x *g* centrifugation is added to the NORPA-bound Ni-beads, where the NORPA 863-1095 protein acts as the bait to capture the endogenous INAD protein complexes (INAD/TRP/ePKC). (**C**) The GST-tagged TRP 1261-1275 fragment is added to compete for the endogenous *Drosophila* TRP channel from the captured INAD protein complexes. (**D**) The eluted TRP protein is further purified by a size-exclusion column to separate the excessive GST-tagged TRP 1261-1275 peptide fragment. The red arrows highlight the elution positions of the TRP channel and GST-tagged TRP 1261-1275 fragment, respectively.

Figure 2: Purification of GST-tagged TRP-CT 1261-1275 protein by glutathione beads and size-exclusion chromatography. (**A**) Purification profile of GST-tagged TRP-CT 1261-1275 protein in a size-exclusion column (preparation grade). The fractions are collected at 5 mL/tube. The fractions at the arrow position (tubes 44–48) are collected and concentrated for the following purification of the endogenous TRP channel. (**B**) Coomassie blue R250 stained SDS-PAGE gel showing the GST-tagged TRP 1261-1275 fragment in the Glutathione-beads affinity purification and subsequent size-exclusion column purification. The arrow highlights the position of the GST-tagged TRP 1261-1275 fragment in the SDS-PAGE gel. Abbreviations: P: pellet from *E. coli.* BL21 (DE3) cell lysate after homogenization in PBS buffer and centrifugation at 48,384 x *g*; S: supernatant from *E. coli.* BL21 (DE3) cell lysate after homogenization and centrifugation at 48,384 x *g*; F: flow-through fraction after previous S fraction incubated with glutathione beads for 30 min at 4 °C; W1 and W2: the first and second washing fraction by 10 column volumes of PBS buffer; B: Un-eluted protein on the resuspended glutathione beads is analyzed by SDS-PAGE gel to evaluate the elution efficiency; E: elution fraction from glutathione beads by elution buffer. The buffer recipe for the GST-tagged protein purification is described in **Table 1**.

Figure 3: Purification of His-tagged NORPA 863-1095 protein by Ni-beads and size-exclusion chromatography. (**A**) Purification profile of His-tagged NORPA 863-1095 protein in a size-exclusion column. Flow rate = 3 mL/min. The fractions are collected at 5 mL/tube. The fractions at the arrow position (tubes 44–49) are collected and concentrated for the following purification of the endogenous TRP channel. (**B**) Coomassie blue R250 stained SDS-PAGE gel showing the His-tagged NORPA 863-1095 protein in Ni-column purification and subsequent size-exclusion column purification. The arrow highlights the position of His-tagged NORPA 863-1095 protein in the SDS-PAGE gel. Abbreviations: P: pellet from *E. coli.* BL21 (DE3) cell lysate after homogenization in binding buffer and centrifugation at 48,384 x *g*; S: supernatant fraction from *E. coli.* BL21 (DE3) cell lysate after homogenization and centrifugation at 48,384 x *g*; F: flow-through fraction after the previous S fraction is incubated with Ni-beads for 30 min at 4 °C; W1 and W2: the first and second washing fraction by 10 column volumes of wash buffer; B: Un-eluted protein on the resuspended Ni-beads after elution; E: elution fractions from Ni-beads by the elution buffer. The buffer recipe for the His-tagged protein purification is listed in **Table 2**.

Figure 4: Purification of endogenous *Drosophila* TRP channel. The collected samples from every step are analyzed by SDS-PAGE and stained with Coomassie blue R-250 dye. (**A**) 20817 g S: supernatant fraction of head homogenates after 20,817 x *g* centrifugation; NORPA F: flow-through fraction of Ni-beads after His-tagged NORPA 863-1095 fragment binding; Wash1 and Wash2: the first and second washing fractions of Ni-beads by lysis buffer after His-tagged NORPA 863-1095 binding; 100,000 g S: the previous 20,817 g S supernatant is further centrifuged at 100,000 x *g* and the supernatant is collected for SDS-PAGE; Dro head lysis F: flow-through fraction of Ni-beads after incubation with the 100,000 g S sample; Wash3 and Wash4: washing fractions of Ni-beads by lysis buffer after incubation with 100,000 g S sample. (**B**) TRP E1 and E2: the first and second eluted TRP channel fractions by GST-tagged TRP 1261-1275 fragment; Wash5: washing fractions of Ni-beads by binding buffer after competition by GST-tagged TRP 1261-1275; NORPA E1 and E2: the first and second elution fraction of His-tagged NORPA 863-1095 fragments with captured INAD/ePKC complexes; beads: un-eluted protein staying in the resuspended Ni-beads after elution buffer treatment. The buffer recipe for endogenous *Drosophila* TRP channel purification is described in **Table 4**.

Figure 5: Purification of endogenous *Drosophila* TRP channel protein by size-exclusion chromatography. (**A**) Purification profile of endogenous *Drosophila* TRP channel protein in size-exclusion column. Flow rate = 0.75 mL/min. The fractions were collected at 0.5 mL/tube. The fractions at the arrow position (1E8–1F2) were collected and concentrated. (**B**) Coomassie blue R-250 stained SDS-PAGE gel showing the endogenous *Drosophila* TRP channel protein after size-exclusion column purification. The position of purified endogenous *Drosophila* TRP channel protein is highlighted by the red arrow.

Table 1: Materials needed for the purification of the GST-tagged TRP 1261-1275 fragment.

Table 2: Materials needed for the purification of the His-tagged NORPA 863-1095 fragment.

Table 3: Materials needed for the preparation of *Drosophila* heads.

Table 4: Materials needed for the purification of *Drosophila* TRP channel.

Table 5: Materials needed for the size-exclusion column purification of the *Drosophila* TRP channel.

DISCUSSION:

INAD, which contains five PDZ domains, is the core organizer of *Drosophila* phototransduction machinery. Previous studies showed that INAD PDZ3 binds to the TRP channel C-terminal tail with exquisite specificity (K_D = 0.3 μM)[18]. INAD PDZ45 tandem interacts with NORPA 863-1095 fragment with an extremely high binding affinity (K_D = 30 nM). These findings provide a solid biochemical basis to design the affinity purification plus competition strategy, which enables the NORPA CC-PBM fragment to be used as the pulldown bait, while the TRP C-terminal tail (fragment 1261-1275) functions as a competitive reagent. Therefore, the first critical point for this method is to understand the assembling mechanism of the INAD complex and obtain enough NORPA and TRP fragments. At the same time, since the TRP channel is the membrane protein that needs to be extracted from the membrane and stabilized in solution, the usage of detergent is the second critical point of this method. As a popular detergent for structural and functional studies of TRP channels[24,25], n-Dodecyl-B-D-Maltoside (DDM) is used in this method. If the purification results are unsatisfactory, the qualities of the bait protein, competitor protein, and the detergent need to be checked carefully. In addition, the extraction efficiency of the TRP channels can be traced by western blot using the TRP antibody.

In a previous study[5], expensive streptavidin beads were used to purify the TRP channel from fly head extracts, which limits routine purification in the lab. Therefore, the method was improved by using a His-tagged NORPA 863-1095 fragment coupled with Ni-beads to reduce cost and increase yield. Currently, the yields of the purified TRP channel in the improved method are sufficient to conduct a transmission electron microscope (TEM) negative staining experiment, in which the purified TRP channels form tetramers (data not shown), indicating the purification process does not disrupt the tetramer formation of TRP channels. Therefore, this protocol will be potentially suitable for future cryo-EM and electrophysiology experiments.

However, since the competitors used in the experiments (NORPA 863-1095 fragment, TRP 1261-1275 fragment) have similar binding affinities with wild-type proteins, the limitation of this method is that massive competitive proteins and beads must be used to pull down

the target protein. It will be not convenient for labs that cannot purify the bait on a large scale.

A potential future application of this method will be to study the structural information of the *Drosophila* TRP channel using Cryo-EM techniques. In addition, measuring the electrophysiological properties of purified endogenous TRP channels in the artificial bilayer lipid membrane is also feasible. Moreover, in this reconstituted model system, it will be interesting to characterize the electrophysiological properties of purified endogenous TRP channels by modulating the INAD complex composition and lipid composition. Finally, combined with structural information and electrophysiological properties, the gating and regulation mechanisms of the TRP channel can be carefully examined in the future.

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DISCLOSURES:

The authors have nothing to disclose.

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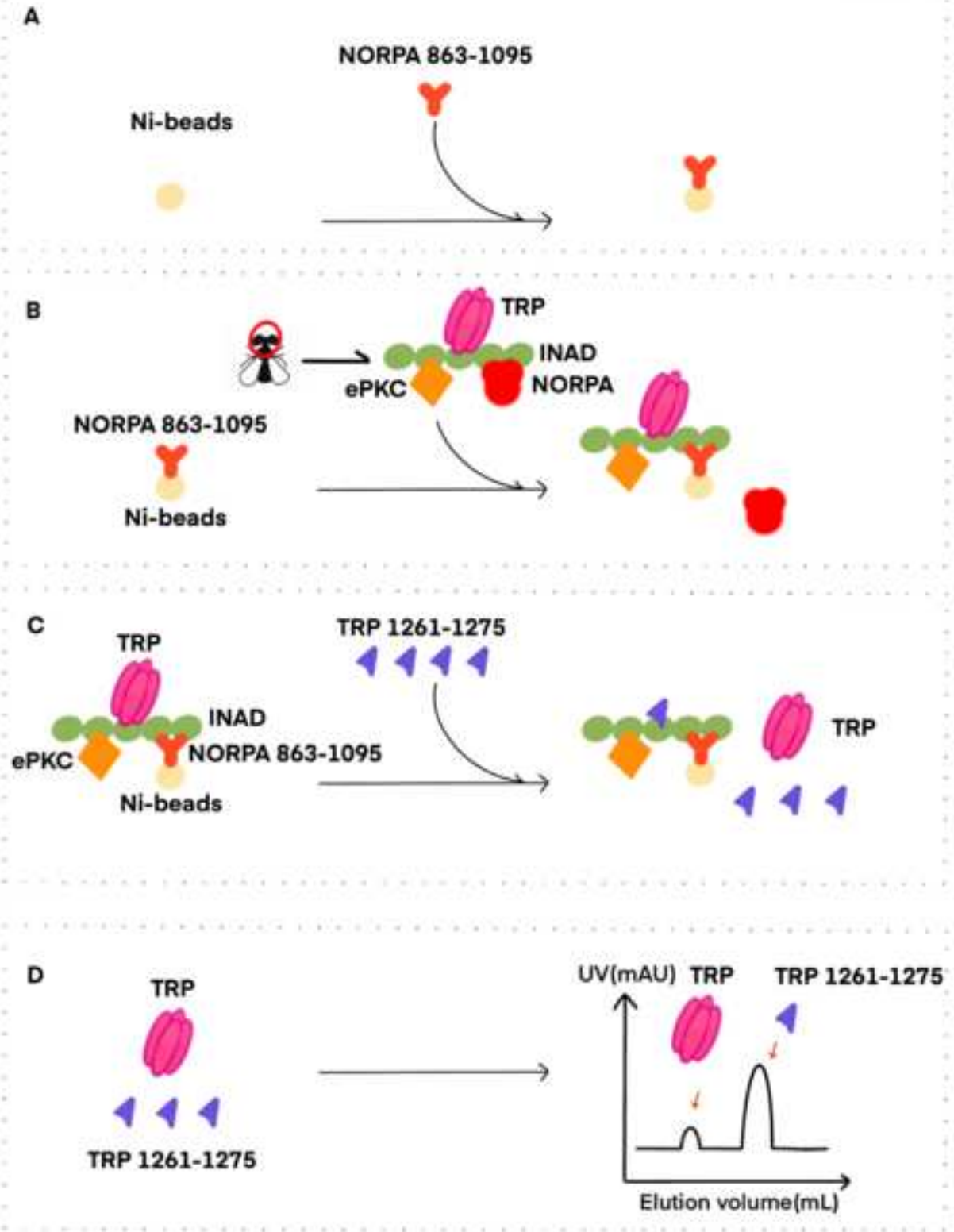
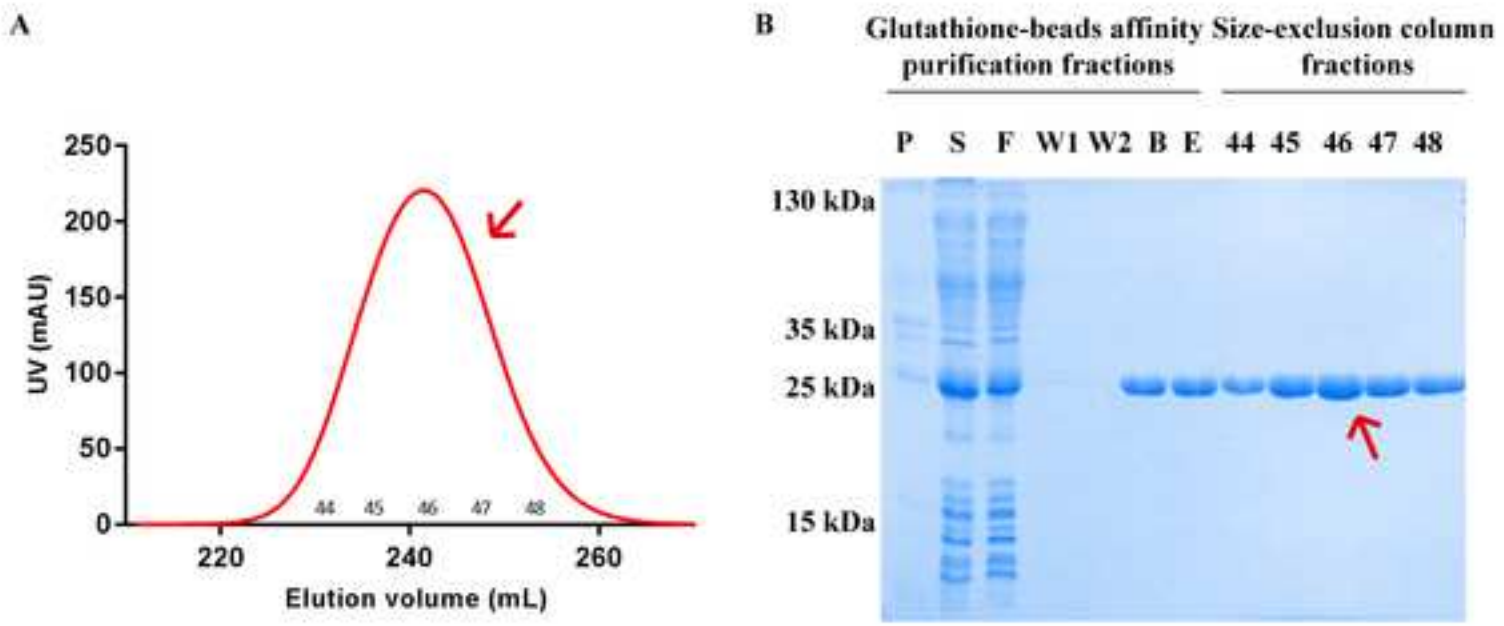
Figure

Click here to access/download;Figure;Figure 1 11-18.tiff



Figure



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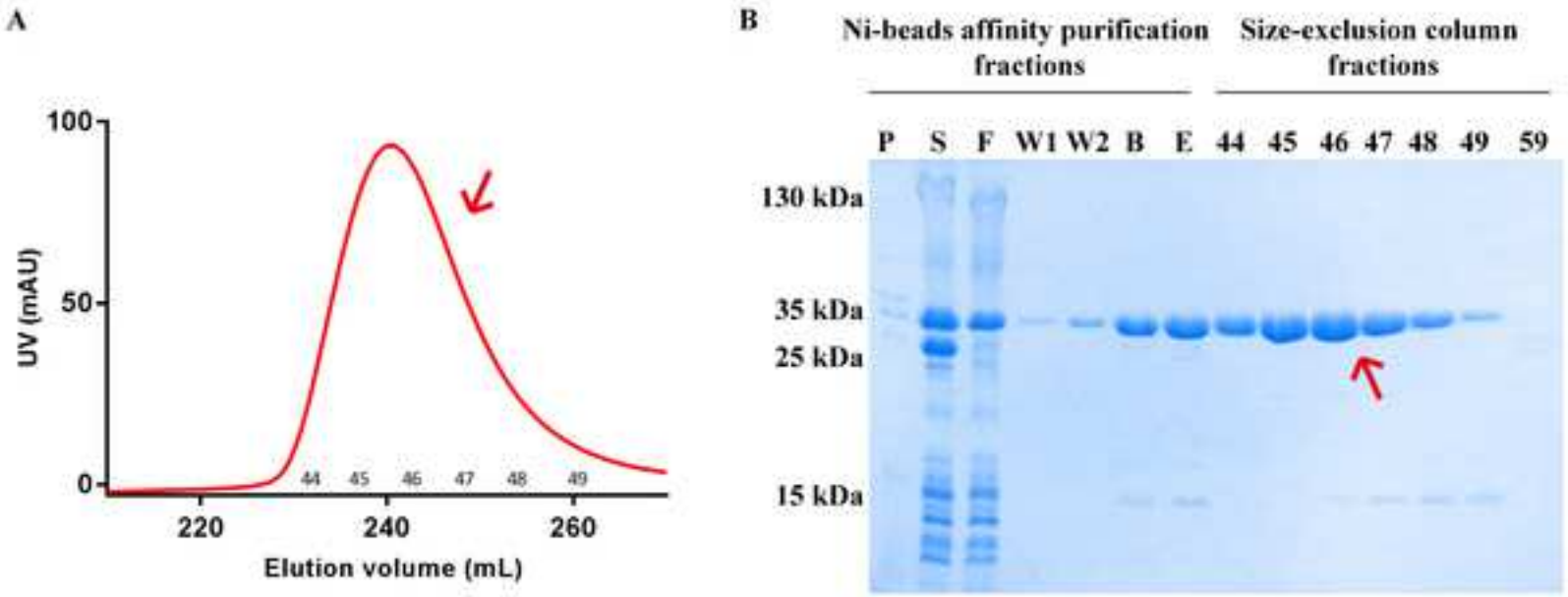
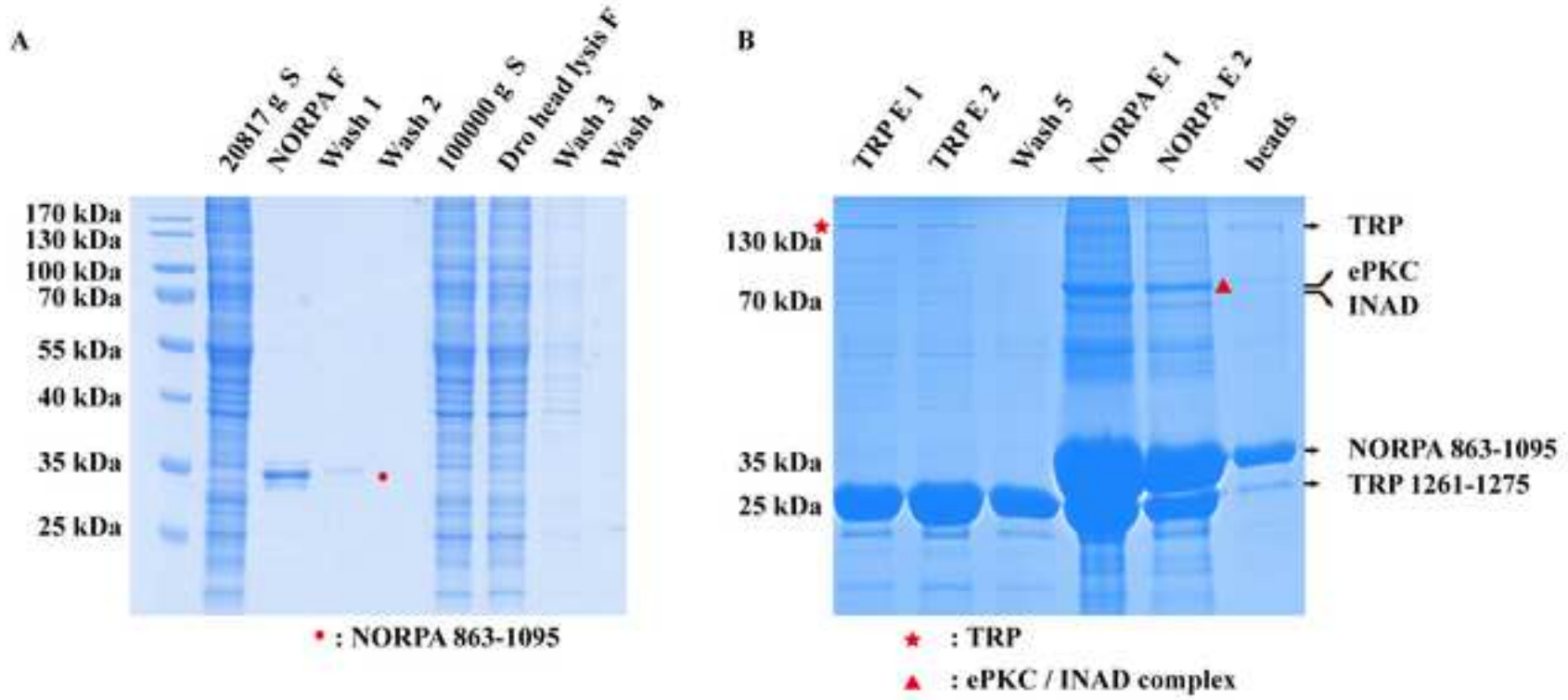
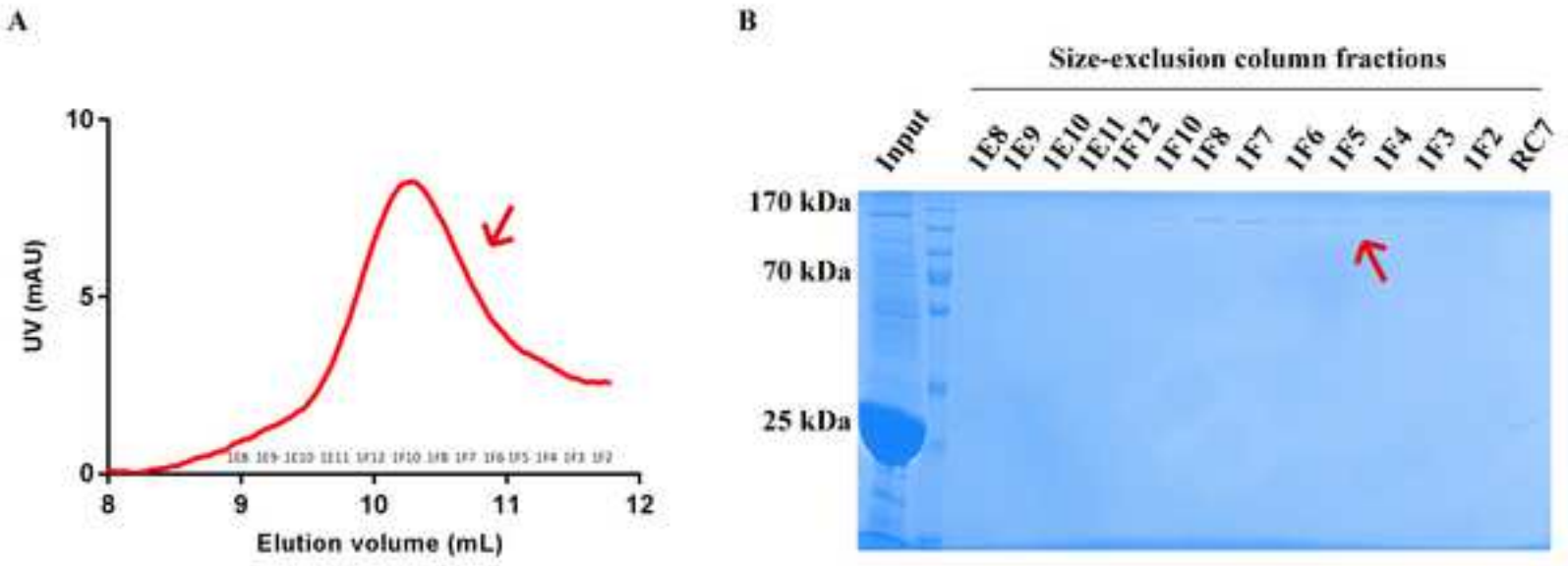


Figure



Figure



Table

Name of Material/ Equipment	Company	Catalog Number
Bacterial strains		
E.coli. BL21(DE3) Competent Cells	Novagen	69450
Material		
30% Acrylamide-N,N′-Methylenebisacrylamide(29:1)	Lablead	A3291
Ammonium Persulfate	Invitrogen	HC2005
Coomassie brilliant blue R-250	Sangon Biotech	A100472-0025
DL-Dithiothreitol (DTT)	Sangon Biotech	A620058-0100
Ethylenediaminetetraacetic acid disodium salt (EDTA)	Sangon Biotech	A500838-0500
Glycine	Sangon Biotech	A610235-0005
Glutathione Sepharose 4 Fast Flow beads	Cytiva	17513202
Isopropyl-beta-D-thiogalactopyranoside (IPTG)	Sangon Biotech	A600168-0025
LB Broth Powder	Sangon Biotech	A507002-0250
L-Glutathione reduced (GSH)	Sigma-aldrich	G4251-100G
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-aldrich	T9281-100ML
PBS	Sangon Biotech	E607008-0500
PMSF	Lablead	P0754-25G
Prestained protein marker	Thermo Scientific	26619/26616
Sodium chloride	Sangon Biotech	A501218-0001
Size exclusion column (preparation grade)	Cytiva	28989336
Sodium dodecyl sulfate (SDS)	Sangon Biotech	A500228-0001
Tris base	Sigma-aldrich	T1503-10KG
Ultrafiltration spin column	Millipore	UFC901096
Equipment		
Desk-top high-speed refrigerated centrifuge for 15mL and 50mL conical centrifugation tubes	Eppendorf	5810R
Empty gravity flow column (Inner Diameter=2.5cm)	Bio-Rad	738-0017
Gel Documentation System	Bio-Rad	Universal Hood II Gel Doc XR System
High-speed refrigerated centrifuge	Beckman coulter	Avanti J-26 XP
High pressure homogenizer	UNION-BIOTECH	UH-05
Protein purification system	Cytiva	AKTA purifier
Spectrophotometer	Thermo Scientific	NanoDrop 2000c
Spectrophotometer	MAPADA	UV-1200
Buffer		**R**
Binding buffer	PBS: 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KC	
Wash buffer	PBS: 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 pH 7.4, 137 mM NaCl, 2.7	
Elution buffer	20 mM GSH, 100 mM Tris-HCl (pH 8.0), 500 mM NaCl	
Size-exclusion column buffer	50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT	

Comments/Description
Protein overexpression
SDS-PAGE gel preparation
SDS-PAGE gel preparation
SDS-PAGE gel staining
Size-exclusion column buffer preparation
Size-exclusion column buffer preparation
SDS-PAGE buffer preparation
Affinity chromatography
Induction of protein overexpression
E.coli. cell culture
Glutathione beads elution buffer preparation
SDS-PAGE gel preparation
Homogenization buffer for *E.coli.* cells
Protease inhibitor
Prestained protein ladder
Protein purification buffer preparation
HiLoad 26/60 Superdex 200 PG column
SDS-PAGE gel/buffer preparation
Protein purification buffer preparation
Protein concentration
Protein concentration
Competitor protein purification
SDS-PAGE imaging
Centrifugation of *E.coli.* cells/cell lysate
Homogenization of *E.coli.* cells
Protein purification
Determination of protein concentration
OD_{600} measurement of *E.coli.* cells
ecipe
:l (pH 7.4)
mM KCl(pH 7.4)

Name of Material/ Equipment	Company	Catalog Number
Bacterial strains		
E.coli. BL21(DE3) Competent Cells	Novagen	69450
Material		
30% Acrylamide-N,N′-Methylenebisacrylamide(29:1)	Lablead	A3291
Ammonium Persulfate	Invitrogen	HC2005
Coomassie brilliant blue R-250	Sangon Biotech	A100472-0025
DL-Dithiothreitol (DTT)	Sangon Biotech	A620058-0100
Ethylenediaminetetraacetic acid disodium salt (EDTA)	Sangon Biotech	A500838-0500
Glycine	Sangon Biotech	A610235-0005
Isopropyl-beta-D-thiogalactopyranoside(IPTG)	Sangon Biotech	A600168-0025
Imidazole	Sangon Biotech	A500529-0001
LB Broth Powder	Sangon Biotech	A507002-0250
Ni-Sepharose excel beads	Cytiva	17371202
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-aldrich	T9281-100ML
PMSF	Lablead	P0754-25G
Prestained protein marker	Thermo Scientific	26619/26616
Sodium chloride	Sangon Biotech	A501218-0001
Size exclusion column (preparation grade)	Cytiva	28989336
Sodium dodecyl sulfate (SDS)	Sangon Biotech	A500228-0001
Tris base	Sigma-aldrich	T1503-10KG
Ultrafiltration spin column	Millipore	UFC901096
Equipment		
Desk-top high-speed refrigerated centrifuge for 15mL and 50mL conical centrifugation tubes	Eppendorf	5810R
Empty gravity flow column (Inner Diameter=2.5cm)	Bio-Rad	738-0017
Gel Documentation System	Bio-Rad	Universal Hood II Gel Doc XR System
High-speed refrigerated centrifuge	Beckman coulter	Avanti J-26 XP
High pressure homogenizer	UNION-BIOTECH	UH-05
Protein purification system	Cytiva	AKTA purifier
Spectrophotometer	Thermo Scientific	NanoDrop 2000c
Spectrophotometer	MAPADA	UV-1200
Buffer		**R**
Binding buffer	20mM Tris-HCl (pH 8.0), 500mM NaCl, 5mM Imidazole	
Wash buffer	20mM Tris-HCl (pH 8.0), 500mM NaCl, 40mM Imidazole	
Elution buffer	20mM Tris-HCl (pH 8.0), 500mM NaCl, 500mM Imidazole	
Size-exclusion column buffer	50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT	

Comments/Description
Protein overexpression
SDS-PAGE gel preparation
SDS-PAGE gel preparation
SDS-PAGE gel staining
Size-exclusion column buffer preparation
Size-exclusion column buffer preparation
SDS-PAGE buffer preparation
Induction of protein overexpression
Elution buffer preparation
E.coli. cell culture
Affinity chromatography
SDS-PAGE gel preparation
Protease inhibitor
Prestained protein ladder in SDS-PAGE
Protein purification buffer preparation
HiLoad 26/60 Superdex 200 PG column
SDS-PAGE gel/buffer preparation
Protein purification buffer preparation
Protein concentration
Protein concentration
Bait protein purification
SDS-PAGE imaging
E.coli. cell centrifugation/cell lysate centrifugation
Homogenization of *E.coli.* Cells
Protein purification
Determination of protein concentration
OD_{600} measurement of *E.coli.* cells
ecipe

Table

Name of Material/ Equipment	Company	Catalog Number
Experiment models		
D.melanogaster: W^{1118} strain	Bloomington *Drosophila* Stock Center	BDSC:3605
Material		
20/30/40 mesh stainless-steel sieves	Jiufeng metal mesh company	GB/T6003.1
Equipment		
Analytical Balance	DENVER	APX-60
Liquid nitrogen tank	Taylor-Wharton	CX-100
Refrigerator (-80℃)	Thermo	900GP

Comments/Description
Drosophila head preparation
Drosophila head preparation
Metage of Drosophila head
Drosophila head preparation
Drosophila head preparation

Table

Name of Material/ Equipment	Company	Catalog Number
Material		
30% Acrylamide-N,N′ -Methylenebisacrylamide(29:1)	Lablead	A3291
Ammonium Persulfate	Invitrogen	HC2005
Cocktail protease inhibitor	Roche	05892953001
Coomassie brilliant blue R-250	Sangon Biotech	A100472-0025
DL-Dithiothreitol (DTT)	Sangon Biotech	A620058-0100
Ethylenediaminetetraacetic acid disodium salt (EDTA)	Sangon Biotech	A500838-0500
Glycine	Sangon Biotech	A610235-0005
Ni-Sepharose excel beads	Cytiva	17371202
N-Dodecyl beta-D-maltoside (DDM)	Sangon Biotech	A610424-001
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-aldrich	T9281-100ML
PMSF	Lablead	P0754-25G
Prestained protein marker	Thermo Scientific	26619/26616
Size exclusion column (analytical grade)	Cytiva	29091596
Sodium dodecyl sulfate (SDS)	Sangon Biotech	A500228-0001
Tris base	Sigma-aldrich	T1503-10KG
Ultrafiltration spin column	Millipore	UFC801096
Equipment		
Desk-top high-speed refrigerated centrifuge 1.5mL centrifugation tubes	Eppendorf	5417R
Desk-top high-speed refrigerated centrifuge for 15mL and 50mL conical centrifugation tubes	Eppendorf	5810R
Empty gravity flow column (Inner Diameter=1.0cm)	Bio-Rad	738-0015
Gel Documentation System	Bio-Rad	Universal Hood II Gel Doc XR System
Protein purification system	Cytiva	AKTA purifier
Spectrophotometer	Thermo Scientific	NanoDrop 2000c
Ultracentrifuge	Beckman coulter	Optima XPN-100 Ultracentrifuge
Buffer		**R**
Binding buffer	50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM Imidazole	
Elution buffer	50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 500 mM Imidazole	
Lysis buffer	50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 6 mM DDM,	

Comments/Description
SDS-PAGE gel preparation
SDS-PAGE gel preparation
Protease inhibitor
SDS-PAGE gel staining
Protein purification buffer preparation
Size-exclusion column buffer preparation
SDS-PAGE buffer preparation
Affinity chromatography
Detergent for protein purification
SDS-PAGE gel preparation
Protease inhibitor
Prestained protein ladder
Superose 6 Increase 10/300 GL column
SDS-PAGE gel/buffer preparation
Protein purification buffer preparation
Protein concentration
Centrifugation of *Drosophila* head lysate after homogenization
Protein concentration
TRP protein purification
SDS-PAGE imaging
Protein purification
Determination of protein concentration
Ultracentrifugation
ecipe
1× cocktail protease inhibitor (Roche)

Table

Name of Material/ Equipment	Company	Catalog Number
Material		
30% Acrylamide-N,N′-Methylenebisacrylamide(29:1)	Lablead	A3291
Ammonium Persulfate	Invitrogen	HC2005
Coomassie brilliant blue R-250	Sangon Biotech	A100472-0025
DL-Dithiothreitol (DTT)	Sangon Biotech	A620058-0100
Ethylenediaminetetraacetic acid disodium salt (EDTA)	Sangon Biotech	A500838-0500
Glycine	Sangon Biotech	A610235-0005
N-Dodecyl beta-D-maltoside (DDM)	Sangon Biotech	A610424-001
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-aldrich	T9281-100ML
Prestained protein marker	Thermo Scientific	26619/26616
Size exclusion column (analytical grade)	Cytiva	29091596
Sodium dodecyl sulfate (SDS)	Sangon Biotech	A500228-0001
Tris base	Sigma-aldrich	T1503-10KG
Ultrafiltration spin column	Millipore	UFC801096
Equipment		
Desk-top high-speed refrigerated centrifuge for 15mL and 50mL conical centrifugation tubes	Eppendorf	5810R
Gel Documentation System	Bio-Rad	Universal Hood II Gel Doc XR System
Protein purification system	Cytiva	AKTA purifier
Spectrophotometer	Thermo Scientific	NanoDrop 2000c
Buffer		**R**
Size-exclusion column buffer	50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM DTT, 0.75 mM	

Comments/Description
SDS-PAGE gel preparation
SDS-PAGE gel preparation
SDS-PAGE gel staining
Size-exclusion column buffer preparation
Size-exclusion column buffer preparation
SDS-PAGE buffer preparation
Detergent for protein purification
SDS-PAGE gel preparation
Prestained protein ladder
Superose 6 Increase 10/300 GL column
SDS-PAGE gel/buffer preparation
Protein purification buffer preparation
Protein concentration
Protein concentration
SDS-PAGE imaging
Protein purification
Determination of protein concentration
ecipe
1 DDM

Dear editor and reviewers:

Thank you very much for your warm comments and constructive suggestions on our paper (entitled: Purification of Endogenous Drosophila TRP Channels). We have revised the manuscript carefully and HIGHLIGHT the changes by using the TRACK CHANGES mode in MS Word.

The following are the point-by-point responses to the comments by the editor and reviewers, and the corresponding revisions we made in the paper (in blue).

The language in the manuscript is not publication grade. Please employ professional copy-editing services.

--We have employed a professional copy-editing service (www.letpub.com) for its linguistic assistance.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns.

--We have revised the manuscript to thoroughly address the reviewers' concerns.

Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

--We have described the changes that have been made.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

--We have thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Line 50-51: Please remove the lines.

--We have removed the lines.

3. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

--We made a clear statement of the overall goal of this method. Please refer to lines 70-74 in the revised manuscript.

b) The rationale behind the development and/or use of this technique

--The reasons that we develop and/or use this technique have been presented in lines 66-70 in revised manuscript.

c) The advantages over alternative techniques with applicable references to previous studies

-- So far, electrophysiology studies on the gating mechanisms of *Drosophila* TRP were based on excised membrane patches, whole-cell recordings from dissociated wild-type Drosophila photoreceptors, and hetero-expressed channels in S2, SF9, or HEK cells, but not based on purified TRP channels. The only known method for purifying *Drosophila* TRP channels depends on expensive streptavidin beads with low capacity. So the advantage of our

method is that we provide a low-cost method to purify TRP channels for the electrophysiologists and structural biologists (lines 66-70/76-81) .

d) A description of the context of the technique in the wider body of literature

-- We descripted the context of the technique by citing the similar methodologies used in mammalian TRP channel studies (line 74, references 14-17,).

e) Information to help readers to determine whether the method is appropriate for their application

-- We have provided the information that helps the readers to determine whether the method is suitable for their application (line 81-83).

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

-- We have revised the text and deleted all the personal pronouns.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

-- We have removed all commercial language from the manuscript and use generic terms instead. All commercial products have been sufficiently referenced in the Table of Materials.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

-- We have revised the manuscript as requested.

7. For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2

-- We have revised the manuscript as requested.

8. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

-- We have revised the manuscript as requested.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your

protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

-- We have revised the manuscript as requested.

10. Line 86-87/109-110: How was it transformed in to E.coli?

-- We have revised the manuscript by adding the transformation method ("$CaCl_2$ heat-shock transformation") and the reference (lines 90-91/132-133).

11. Line 103-104/126-127: How is the fragment concentrated. What are the steps involved?

-- The fragment is concentrated by an ultrafiltration spin column at 3000 × g at 4°C in a desk-top refrigerated centrifuge. We have revised the manuscript correspondingly in lines 120-122/162-164.

12. Line 135: What is 20/30/40? Mesh size? Please specify the units.

We have revised the manuscript as requested (line 180).

13. Please specify the conditions/parameters for SDS- PAGE.

We have specified the parameters for SDS-PAGE (lines 118-119/160-161).

14. Pleas ensure that the Figures are described in the Representative results section in the order of appearance.

--We confirmed that the Figures are described in the Representative results section in the order of appearance.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

--We have highlighted the critical steps in the discussion part (lines 393-399).

b) Any modifications and troubleshooting of the technique

--Modifications of the technique are discussed by comparing the previous developed method (lines 404-411). The troubleshooting is mentioned in the lines 399-402.

c) Any limitations of the technique

--The limitations of the technique have been discussed in lines 416-420.

d) The significance with respect to existing methods

--The significance with respect to existing methods has been discussed in lines 404-411.

e) Any future applications of the technique

--The future applications of the technique are discussed in lines 420-428.

16. Please do not use the &-sign or the word "and" when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

--We have revised the references as requested.

17. Figure 1: Please label the figure to make it more informative.

--We have re-drawn the Figure 1 to make it more informative.

18. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

--We have revised the table as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The paper describes a protocol for the purification of a Drosophila Ca channel - TRP. I think this is useful, although very specialised and I imagine the interest will not be widespread. I recommend for publication and I list below some aspects that could be corrected

Minor Concerns:

- l 37 - are instead of is

--We thanks for the kind reminder of the reviewer. We have corrected it.

- Please explain better the reason for using TRP 1261-1275 and NORPA 863-1095 in the protocol.

--We have explained the reasons and effects of using TRP 1261-1275 and NORPA 863-1095 in the protocol (lines 214-216/226-228/278-282).

- Better to use present tense in the protocol step by step instructions.

--We have used present tense in the protocol instructions.

- l 132 - flies instead of files (and as is, the sentence is odd)

--We have corrected the typo error.

- l 132 - Maybe better "Add 500 uL of 600 uM purified ..."

--We have edited the text as requested (line 200).

- Fig 1 is pixelated (maybe because of pdf compression). It is hard to understand which components are in the columns, despite the fig legend.

--We have redrawn the Figure 1.

- l 326 - this sentence is self defeating. If this is the case maybe better to fully optimize the protocol before publishing in Jove...

--We expect that the purification method of TRP channels from fly head can be further optimized in the future. In this manuscript, we share the methods that we are using now. We have deleted this sentence to avoid the self-defeating.

Reviewer #2:

Manuscript Summary:

In their manuscript titled "Purification of Endogenous Drosophila TRP Channel," Liu et al. present an affinity-competition protocol to purify the TRP channel from Drosophila heads. In the first step, they discuss purification of a GST-tagged TRP fragment. Next, they purify a NORPA fragment. These basically serve as "bait" and "competitor" in later steps. The third step is generation of lysate from adult fly heads, binding of the endogenous TRP-containing complex to NORPA bound on beads, and then competition with the TRP fragment to elute endogenous TRP. Size

difference between the TRP fragment and the endogenous channel allow a final step of gel filtration to obtain a pure channel. This protocol would be of relevance to the fly neuroscience community and potentially TRP researchers. While the protocol has potential, there are multiple small details that are missing from the text that would make it difficult to repeat in another lab without extensive hands-on knowledge of this specific purification. Further, the introduction, results and discussion need to be improved and better place this protocol in the existing literature. My comments on how to improve the manuscript are below.

--We have carefully revised the manuscript and provided the necessary information to the introduction, results and discussion parts as requested.

Major Concerns:

1. There are many small details that are left-out throughout the protocol. These need to be included, to enable someone else to repeat this purification based on the provided protocol. For example, in Step 1.1.3, how much PBS buffer is used here? This is important in 1.1.4 to know how much supernatant to expect to have to add to the beads. Other instances are noted below.

--We have revised the protocol as requested (lines 98/103).

2. Step 1.1.4 and 1.2.4: should read "cell lysate" instead of "cell lysis"

--We have revised the text as requested (lines 102/144).

3. Step 1.1.4 and 1.2.4: the equilibration of the Sepharose beads in PBS for 3x should be a separate step at the appropriate point in the protocol

-- We have revised the text as requested (lines 100-101/142-143).

4. The use of "supernatant" and "flow-through fraction" is confusing throughout the protocol section. I have a hard time following where the supernatant is being removed, the flow-through is being collected or a fraction is being isolated from the AKTA. For example (but not a complete list) Step 1.1.5, 1.2.5, 3.3, 3.4. It might help to include what kind of column or conical tube is being used in each section or each step, for example in Steps 1.1.3 or 1.2.3. This way the reader can follow it the sample is supernatant collected after spin-down, from gravity precipitation or as flow-through from an AKTA column.

---- We have revised the manuscript by specifying the sources of different supernatants in the protocol: "The supernatant of the centrifuged cell lysate", "The spin-down supernatant"," the supernatant from gravity precipitation", etc. (lines 103/145/194/195/218).

5. It would help to have a "materials needed" section introduced for 1.1, 1.2, 1.3 etc., so necessary consumables and equipment for each part of the protocol is clear.

--The "materials needed" information for each section has been created as requested and can be found in the Table 1-5.

6. Step 1.1.8 and 1.2.8, line 212: How do you do the concentration? Dialysis? There are many instances throughout the manuscript where the authors mention that the purified protein is concentrated, without explicitly mentioning how they do this.

We have revised the manuscript as requested by introducing how do we concentrate the proteins and estimate their concentrations (lines 120-122/162-

164).

7. What is the expected yield or range of yields for GST-tagged TRP 1261-1275 in 1.1? Or for NORPA 863-1095 or even for the endogenous TRP channel?

--We have provided the typical yields of GST-tagged TRP 1261-1275, NORPA 863-1095 from a 1 L culture and the endogenous TRP channels from 0.5g heads (lines 127-128/169-170/255-256).

8. Step 2.1 - Why do the flies need to be 2-weeks old? Would younger flies also work? How many flies do you need to collect here? 50 mL? Can you freeze flies at -80 or in LN2 until you have collected a sufficient amount?

-- The flies do not need to be 2-weeks old. Adult flies can be used to collect the heads. To avoid misleading, we used "Adult flies" instead of "2-weeks old adult flies". To obtain 0.5g heads, we need to collect 35 mL of flies in a 50 mL conical tube. The flies and their heads are continuously collected and stored in a -80°C freezer until they reach a sufficient amount. We have revised the text correspondingly (lines 174/184-186).

9. Step 2.2 - Does vigorous shaking mean by hand or using a vortex? If a vortex, what setting? What are the heads collected into when you remove them from the sieve? An Eppendorf tube or another 50 mL conical? Do you dump out of the sieve, use a funnel or remove the heads using a spoon?

--The frozen 50 mL conical tubes are shaken vigorously by hand. We use a brush to sweep the fly heads off the 40 mesh, transfer them into 50 mL conical tubes, and store at -80°C. We have revised the text as requested (lines 178/181-183).

10. Step 2.3 - How many starting flies do you need to get 0.5 g of heads? About how many uL is this in an Eppendorf tube? What do you do if you do not have 0.5g, so you have more or less? For example, should 2 g of fly heads be split into 0.5 g fractions?

-- To obtain 0.5g heads, we need to collect 35 mL of flies in a 50 mL conical tube (line 184) . We did not measure the volume of 0.5g heads precisely. The volume of 0.5g heads in a 15 mL conical tube is about 1 mL. To purify TRP channels from 0.5g fly heads, 1 mL Ni-beads, 3×10^{-7} mol bait protein and 3×10^{7} mol competitor are needed. If we need to purify more TRP channels, we can scale up the amounts of fly heads, Ni-beads, bait protein and competitor correspondingly (line 255-257).

11. Step 3.1 - It is unclear what column the authors refer to here, and the only columns in the Materials List are for the AKTA. Is this same column maybe also used in 1.1 and 1.2?

-- The size-exclusion column used in step 4.1 is different from 1.1 and 1.2. To avoid misleading and the commercial language, we named the size-exclusion column in step 4.1 as "size-exclusion column (analytical grade)", and the size-exclusion column used in 1.1 and 1.2 as "size-exclusion column (preparation grade)" (lines 113/155/244). The detailed information of these columns can be found in Table1, Table2 and Table5.

12. Step 3.5 & 3.7 - Is this incubation at RT or 4C, and does the sample need

shaking/rocking or just standing on bench? Is the Drosophila lysate being applied to a column with the Ni-Sepharose beads now bound to NORPA 863-1095, or is this in a conical tube with agitation like a normal immunoprecipitation?

-- The incubation is at 4 ℃. The column is in the cold room and stand on a bench. We resuspend the resins every 10 minutes. *Drosophila* head lysate are added to the Ni-column where the NORPA 863-1095 fragment has been immobilized. (line 200-202/211-212) .

13. Step 3.9 - It is unclear what the authors refer to when they say "analyze the un-eluted proteins on the beads."

--As shown in the "beads" fraction in Figure 4B, after elution we normally resuspend the beads and collect the "beads" sample to run SDS-PAGE gel to see whether the bound target protein is thoroughly eluted from the beads. This action is to evaluate the elution efficiency of the beads and judge whether the elution buffer works (lines 237-239).

14. Sentence in lines 322-324 is missing multiple words.

--We have revised the manuscript as requested.

15. Paragraph lines 326-332 - If the "…yields … are still not satisfactory and the purification efficiency is relatively low…", what is the utility of publishing this method? Do the authors rather mean the method can still be optimized to improve yield, but the current protocol can produce protein that is of sufficient quality and quantity to perform experiments?

--We have rephrased this part (lines 407-412). The yield of the TRP channels from 0.5g fly heads are already sufficient to perform the transmission electron microscope (TEM) negative staining experiment, in which the purified TRP channels are shown to form tetramers (data not shown).

16. Please define abbreviations in Figure 1 in the legend. NORPA-CT I presume is NORPA 863-1095 etc. Also, in the last step for AKTA purification, the arrow should come from the flow-through, not both column and flow-through, right?

--We have re-defined the abbreviations in Figure 1 legend. In the last step for AKTA purification in Figure 1, the blue arrows refer to the excessive GST-tagged TRP 1261-1275 fragments, which can be separated from the TRP channel by eluted in different elution volumes by the size-exclusion column, as showed in revised Figure 1D. The red arrows in Figure 1D highlight the elution positions of TRP channel and GST-tagged TRP 1261-1275 fragment respectively.

17. Is Figure 2B Coomassie staining? How otherwise are you visualizing the protein on the SDS-PAGE gel? Also, it is not clear if just fraction 47 was collected and concentrated, or all of fractions 44-48?

--In Figure 2B, the SDS-PAGE gel was stained by Coomassie blue R250 , which has been clarified in line 306. All of the fractions 44-48 were collected and concentrated (lines 309-310).

18. For Figure 2B and 3B, the legend should mention if the arrow points to the same thing as in A, as well as clarify that the numbers in the last columns refer to the

fractions. Also, please state in both Figure 2 and Figure 3 how much protein was loaded in each lane?

-- For Figure 2B and 3B, we have explained the meanings of the arrows (line 308-309/329-331). We also clarified that the numbers in the last columns refer to the fractions in the size-exclusion column in the Figure 2B and 3B. We have stated in both Figure 2 and Figure 3 that how much protein was loaded in each lane (line 310-312/330-330).

19. In Figure 4, is NORPA complex the bands around 50 and 70 kDa? Also, the KD labeling is mislabeled in Figure A, there are two bands labeled 70 kDa.

--In Figure 4, the his-tagged NORPA 863-1095 is around 35 kDa, which is highlighted by a red star in Figure 4A. We have re-labeled the positions of protein ladder in Figure 4A and 4B.

20. Material list is missing items: the stain used for the SDS-PAGE gels, the sieves used to collect Drosophila heads, the columns used for affinity-competition purification. Also, if the Table of Materials will be converted to PDF in the final version, please be aware that there are currently 76 pages of empty cells in that document.

--We have supplemented the missing items mentioned above. We also reformatted the Table of Materials.

21. In the introduction and especially the discussion, please place this study in the context of other published protocols. Such purification is a rather standard approach, and the discussion only mentions two of the authors' own publications.

If the authors have already developed a better purification with strep-tagged TRP channel, as suggested in lines 331-332, what is the value of this protocol?

--We have revised the introduction and discussion part as requested. The description in lines 331-332 is a future plan. We plan to purify the TRP channel by using transgenic flies. But we do not have a better one yet. We have deleted this part in the manuscript.

Minor Concerns:

The text needs to be edited for proper English grammar.

--We have employed a professional copy-editing service (www.letpub.com) for its linguistic assistance.

Certificate of English Language Editing

Manuscript Title:

Purification of Endogenous Drosophila TRP Channels

Date of Revision:

November 11, 2021

Abstract:
Drosophila phototransduction is one of the fastest known G-protein-coupled signaling pathways. To ensure the specificity and efficiency of this cascade, the Ca2+-permeable cation channel transient receptor potential (TRP) binds tightly to scaffold protein inactivation-no-after-potential D (INAD) and forms a large signaling protein complex with eye-specific protein kinase C (ePKC) and phospholipase Cβ/No receptor potential A (PLCβ/NORPA). However, the biochemical properties of the Drosophila TRP channel remain unclear. Based on the assembling mechanisms of INAD protein complex, a modified “affinity purification plus competition” strategy was developed to purify the endogenous TRP channel. First, the purified his-tagged NORPA 863-1095 fragment was bound to Ni-beads and used as the bait to pull down the endogenous INAD protein complex from Drosophila head homogenates. Then, excessive purified GST-tagged TRP 1261-1275 peptide was added to the Ni-beads slurry to compete the TRP channel. Finally, the TRP channel in the supernatant was separated from the excessive TRP 1261-1275 peptide...

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