Chapter 23

APEX Proximity Labeling of Stress Granule Proteins

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Abstract

Ascorbate peroxidase (APEX)-catalyzed proximity labeling has been recently established as a robust approach to uncover localized protein environments and transient protein-protein interactions occurring across mammalian cells. This molecular tool enables improved identification of individual proteins localized to and involved in specific cellular and subcellular pathways and functions. Engineering of an APEX2 fusion protein into the endogenous loci of proteins of interest enables directed biotinylation of neighboring polypeptides and mRNAs. This results in identification of subcellular and context-dependent proteomes or transcriptomes via quantitative mass spectrometry or RNA sequencing, respectively. Here, we describe the utility of APEX-mediated proximity labeling to recover components of stress granules (SGs) by endogenous tagging of well-established SG-associated proteins.

Key words APEX2, Proximity labeling, Stress granule, RNA binding protein, Biotinylation, Affinity purification, Tandem mass tag, Quantitative mass spectrometry

1 Introduction

RNA processing is essential for regulation of gene expression and is tightly orchestrated in space and time. RNA binding proteins (RBPs) and their RNA substrates are frequently organized into discrete areas of high local concentration, often referred to as RNA granules or membraneless organelles [1, 2]. RNA granules are highly dynamic and can respond rapidly to changes in cellular metabolism or external stimuli. A prominent example of dynamic RNA granules are stress granules (SGs), which rapidly assemble in the cytoplasm under conditions of cellular stress and disassemble once the stressor has been removed [3, 4]. In recent years, SGs have been the focus of intense research due to an emerging link between the biology of SGs and the insoluble protein aggregates that are the hallmarks of numerous late-onset neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) [5, 6]. Importantly, several SG-associated proteins have been identified as potential therapeutic targets in

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neurodegenerative disorders [7–9]. SGs have been found to contain hundreds of different proteins [7, 10–12], but for most SG-associated proteins it is unclear if and how they contribute to SG formation and/or disassembly. Apart from a defined set of proteins that consistently associate with SGs under a broad range of conditions, SG composition can vary significantly between cell types and different stress conditions [7, 13]. In addition, SGs interact with numerous different cellular structures and machineries, including the endoplasmic reticulum [14], molecular chaperones [15], RNA helicases [12], proteasomes [16], autophagy [17], lysosomes [18], and the nucleocytoplasmic transport (NCT) machinery [19]. Furthermore, SG composition and behavior is regulated by posttranslational modifications, including ubiquitylation, SUMOylation, and arginine methylation [20, 21]. To elucidate how all these different factors shape the molecular interactions within SGs and to identify key regulators of SG biology, it is critically important to be able to profile dynamic changes in SG protein composition with high spatial and temporal resolution.

Proximity labeling of proteins with a covalent tag coupled with affinity purification and quantitative mass spectrometry has been developed in recent years to characterize protein landscapes of defined subcellular compartments. While there are several different methods for protein proximity labeling, the engineered peroxidase APEX2 has emerged as the most suitable enzyme for the characterization of dynamic RNA granules due to its rapid labeling kinetics [7, 10]. Upon activation with hydrogen peroxide (H_2O_2) for a period of only 30–60 s, APEX2 catalyzes the conversion of a biotin phenol substrate into short-lived and highly reactive phenoxyl radicals, which covalently biotinylate immediately proximal proteins within a very small diffusion radius [22–25]. By fusing APEX2 to strongly partitioned canonical SG proteins, it is thus possible to label and capture snapshots of the spatially restricted SG-associated proteome under a wide range of conditions. This approach is generalizable to other dynamic RNA granules, as long as APEX2 can be fused to a core component with a sufficiently high-partitioning coefficient.

We initially demonstrated the utility of proximity labeling combined with quantitative mass spectrometry to determine the composition of stress granules by using a G3BP1-APEX2-GFP fusion protein to simultaneously visualize SGs and biotinylate SG-proximal proteins in live cells. It is important to ensure that APEX2-fusion proteins are expressed at near-endogenous levels to avoid granulation artefacts that can arise from overexpression of RNA granule proteins such as G3BP1. We achieved this by tagging the endogenous G3BP1 locus with an APEX2-GFP fusion protein using CRISPR/Cas9 genome engineering in HEK293T cells and human iPSCs [7]. An alternative approach is to drive expression of a protein of interest (POI) fused to APEX2 from an inducible

promoter that can be tuned to achieve near-endogenous expression levels [10].

Here, we provide a protocol for APEX proximity labeling of stress granule proteins based on our previous work using engineered HEK293T G3BP1-APEX2-GFP cells (Fig. 1). While our original work used a SILAC labeling approach for quantitative proteomics, the protocol presented here is compatible with a range of proteomics strategies that have been used in the context of APEX proximity labeling, including label-free proteomics and isobaric labeling methods such as TMT. It can also be easily adapted for proximity labeling of other types of RNA granules, provided that cell lines expressing suitable APEX2 fusion proteins are available.

2 Materials

2.1 APEX-Expressing Cell Line Culture

1. G3BP1-APEX2-GFP-expressing HEK293T cell line (*see* **Note 1**): single frozen vial usually contains 1×10^6 cells in 1 mL 10% DMSO freezing medium.
2. HEK293T Culture Medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) (optional). Sterile filter and store at 4 °C. Prewarm in a 37 °C water bath prior to use on cells (*see* **Note 2**).
3. *Optional (For SILAC labeling).*
   (a) *SILAC DMEM deficient in both arginine and lysine.*
   (b) *Dialyzed FBS.*
   (c) *L-arginine–HCl (MW 210.7 g/mol).*
   (d) *L-lysine–HCl (MW 182.7 g/mol).*
   (e) *L-arginine–HCl* $^{13}C_6$, $^{15}N_4$ *(MW 220.6 g/mol).*
   (f) *L-lysine–2HCl* $^{13}C_6$, $^{15}N_2$ *(MW 227.1 g/mol).*

   Prepare medium from SILAC DMEM deficient in both arginine and lysine. Supplement with 10% dialyzed FBS. For "light" medium, add L-arginine to 0.4 mM and L-lysine to 0.8 mM. For "heavy" medium, add L-arginine $^{13}C_6$, $^{15}N_4$ *at 0.5 mM and L-lysine* $^{13}C_6$, $^{15}N_2$ *at 0.8 mM (see* **Note 3***). Supplement both light and heavy medium with L-proline at 200 mg/mL (see* **Note 4***).*
4. 10 cm plastic tissue-culture dishes (*see* **Note 5**).
5. Dulbecco's phosphate-buffered saline (DPBS).
6. TrypLE Express Enzyme.

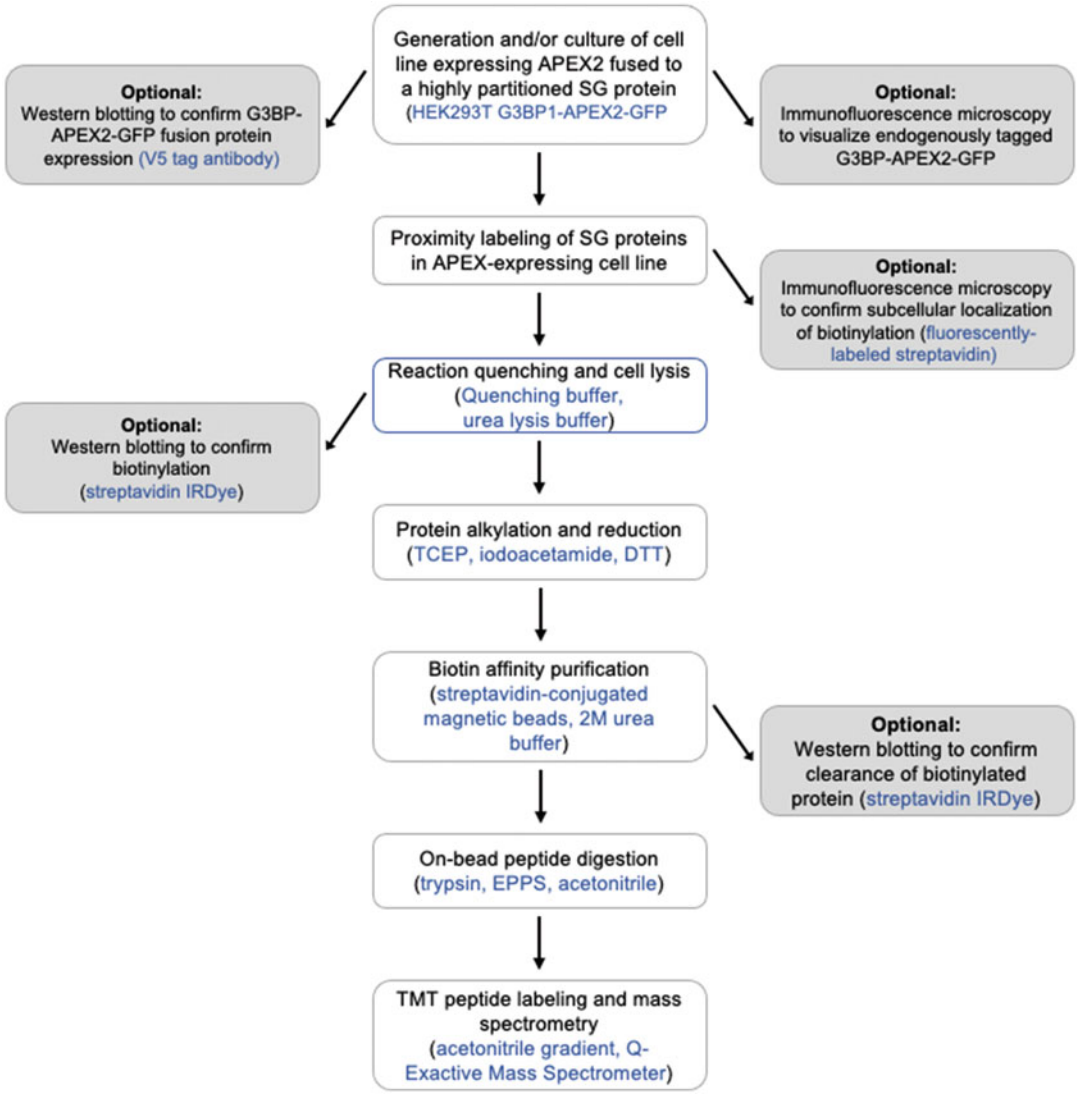

Fig. 1 Schematic outlining major steps for APEX proximity labeling with the end goal of mass spectrometry to identify stress granule associated proteins

2.2 Optional Validation of Cell Lines

1. Radioimmunoprecipitation assay (RIPA) lysis and extraction buffer.
2. BCA protein assay.
3. 4× lithium dodecyl sulfate (LDS) sample buffer.
4. DL-dithiothreitol (DTT) reducing agent.
5. 4–12% gradient bis-tris gel (NuPAGE Novex NP0321BOX).
6. 1× MOPS Running Buffer: dilute 50 mL of concentrated 20× MOPS (NuPAGE NP0001) with 950 mL deionized water to

make 1 L stock in an autoclaved glass bottle. Store at room temperature.

7. Broad range protein ladder (e.g., Spectra Broad Range, ThermoFisher).
8. PVDF membranes.
9. 1× Transfer buffer: dilute 50 mL of concentrated 20× transfer buffer (NuPAGE NP0006-1) with 950 mL deionized water to make 1 L stock in an autoclaved glass bottle. Store at 4 °C.
10. TBST: dilute 100 mL of 10× TBS and 1 mL Tween® 20 detergent in 900 mL of deionized water.
11. Membrane blocking buffer: 5% milk (w/v) in TBST.
12. V5 Tag antibody (37-7500-A488). (Alternatives: GFP antibody, G3BP1 antibody).
13. Chamber slides.
14. Paraformaldehyde (PFA): dilute 2 mL of 20% stock PFA in 8 mL DPBS for a 4% working solution.
15. IF wash buffer: dilute Triton X-100 to 0.01% (v/v) in DPBS.
16. IF blocking buffer: dilute Triton X-100 to 0.1% (v/v) and serum (of the same species as secondary antibody) to 5% (v/v) in DPBS.

2.3 APEX-Mediated Biotinylation

1. Biotin phenol ($C_{18}H_{25}N_3O_3S$), also sold as biotinyl-tyramide.
2. 0.05 mol/l sodium arsenite ($NaAsO_2$) solution (*see* **Note 6**).
3. 30% hydrogen peroxide solution (*see* **Note 7**).
4. Quenching solution.
   (a) Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid). Prepare a 500 mM stock solution by resuspending 1 g of Trolox in 8 mL of DMSO. Aliquot and store at −20 °C or −80 °C.
   (b) Sodium ascorbate ($C_6H_7NaO_6$). Prepare a 1 M stock solution by resuspending 1 g of sodium ascorbate in 5 mL of PBS. Prepare fresh immediately prior to each experiment.
   (c) Sodium azide. Prepare a 1 M stock solution by resuspending 650 mg in 10 mL of H_2O. Aliquot and store at room temperature protected from light (*see* **Note 8**).

   The labeling reaction is stopped by adding Trolox, sodium ascorbate and sodium azide to final concentrations of 5 mM, 10 mM and 10 mM, respectively. Prepare a 2× quenching solution containing 10 mM Trolox and 20 mM each of sodium ascorbate and sodium azide in ice-cold PBS (*see* **Note 9**).
5. Urea Lysis buffer: 8 M urea, 150 mM NaCl, 20 mM Tris pH 8.0 supplemented with protease inhibitors (e.g., Protease

Inhibitor Cocktail Set III, EDTA-Free, EMD Millipore) (*see* **Note 10**).

6. Dilution buffer: 150 mM NaCl, 20 mM Tris pH 8.0 supplemented with protease inhibitors.
7. Optional: Amine-free dilution buffer: 150 mM NaCl, 50 mM EPPS pH 8.5, supplemented with protease inhibitors (*see* **Note 10**).

2.4 Validation of Biotinylation by Western Blotting and/or Immunocytochemistry

1. 660 nm protein assay (Pierce).
2. IRDye 800CW Streptavidin antibody diluted 1:1000 in TBST.
3. Fluorescently labeled streptavidin, for example, streptavidin-Alexa488 (Thermo Fisher).

2.5 Streptavidin Affinity Purification and Preparation for Mass Spectrometry

1. Streptavidin-conjugated magnetic beads (Pierce).
2. Tris(2-carboxyethyl)phosphine (TCEP) reducing agent.
3. 2-Iodoacetamide alkylating agent.
4. Wash buffer: 2 M urea, 150 mM NaCl, 20 mM Tris pH 8.0 (alternatively use 50 mM EPPS pH 8.5, *see* **Note 10**).
5. Sequencing Grade Modified Trypsin (Promega).
6. 100% HPLC grade acetonitrile.
7. Quantitative Colorimetric Peptide Assay (Pierce).

3 Methods

3.1 Experimental Design Considerations

For successful identification of proteins associated with a particular type of RNA granule it is critical to (a) choose a primary bait that is highly partitioned into the RNA granule of interest, and (b) choose appropriate control conditions to calculate enrichment in granules vs background interaction with the bait.

To illustrate, for a comprehensive characterization of SG-associated proteins we performed 4 different pairwise enrichment analyses (Fig. 2) [1]. These included a comparison with a negative control (no biotin phenol substrate and no H_2O_2) to ensure that known SG proteins could be successfully enriched using the system. In addition, we analyzed the interactomes of the core SG protein G3BP1 under both stressed and unstressed conditions to determine dynamic changes in the G3BP1 interactome and to identify proteins that interact with G3BP1 only in the context of SGs. However, this comparison alone may miss substantial numbers of proteins that already interact with G3BP1 under unstressed conditions. To capture these G3BP1 interactors and to determine whether they maintain or lose their proximity to G3BP1 under stress conditions, we additionally compared enriched

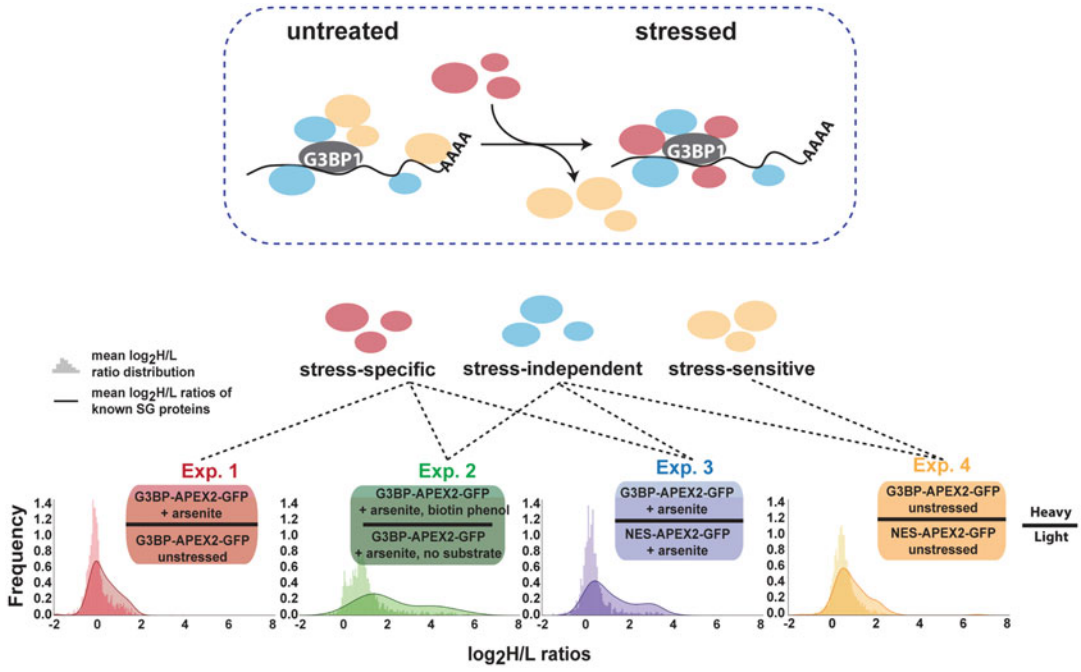

Fig. 2 Schematic of G3BP1 interactome changes upon stress and outlined experimental designs for detecting the G3BP1 interactome changes under different conditions, including log2 H/L ratio distributions of all proteins detected, overlaid with log2 H/L ratio distributions of known SG proteins. (Adapted with permission from Markmiller et al., Cell 2018)

proteins from G3BP1-APEX2-GFP fusions vs an NES-APEX2-GFP protein under both stressed and unstressed conditions. Ideally the control should consist of APEX2 being expressed at levels very similar to the bait protein and reflecting the subcellular distribution of the bait protein population that is not partitioned into the RNA granule of interest as closely as possible. The diffuse cytoplasmic localization of NES-APEX2-GFP closely mirrors the subcellular distribution of G3BP1-APEX2-GFP in the absence of stress and thus makes this a suitable control. Other types of RNA granules and bait proteins may require different controls, and it may be necessary to include more than one control condition in some cases.

In addition to careful selection of experimental and control conditions, it can be helpful to perform proximity labeling with additional bait proteins that partition strongly into the RNA granule of interest. The utility of this approach was recently demonstrated by Marmor-Kollet et al. (2020), who used three different SG-associated bait proteins to gain high-confidence sets of SG-associated proteins under various experimental conditions [2].

Lastly, the choice of mass spectrometry method is another important factor in experimental design. Early APEX proximity labeling studies, including our own work, used SILAC-based quantitative proteomics. This approach is robust and can be

advantageous in cases where additional experimental sample manipulations after biotinylation (e.g., partial biochemical enrichment of subcellular compartments prior to affinity purification) might introduce sources of variability. However, for most protein proximity labeling applications other quantitative approaches such as TMT labeling have proven to be more versatile and easier to analyze.

3.2 Culture of APEX-Expressing Cell Lines

1. Remove 1 frozen vial each containing approximately 1×10^6 HEK293T G3BP1-APEX2-GFP cells and HEK293T NES-APEX2-GFP cells from liquid nitrogen storage.
2. Quickly thaw cells by placing vial into a 37 °C water bath for 1.5 to 2 min until about 90% thawed.
3. Slowly pipette 1 mL of cell suspension into a 15 mL conical tube.
4. Slowly add 9 mL of HEK293T culture medium.
5. Centrifuge at 200 × *g* for 3 min to pellet cells.
6. Remove the supernatant and resuspend cells in 10 mL of prewarmed HEK293T culture medium.
7. Slowly pipette 10 mL of cell suspension into a 10 cm tissue culture dish and gently rock side to side in all directions to ensure even coverage and cell dispersion.
8. Place cells into a humidified incubator at 37 °C and 5% CO_2 and allow them to adhere overnight.
9. Replace spent medium with fresh, prewarmed every 2–3 days, dependent upon cell growth rate.
10. Once 80–90% confluency is reached, passage or expand cells at ratios between 1:10 to 1:20 to reach original confluency in approximately 3–5 days respectively (*see* **Note 11**).
11. Remove spent medium and rinse once with 5 mL of DPBS to remove debris before passaging cells.
12. Add 2 mL of TrypLE per 10 cm dish and place back into the incubator until cells begin to lift (*see* **Note 12**).
13. Triturate cells with pipette to further remove from the dish and transfer to a 15 mL conical tube.
14. Centrifuge at 200 × *g* for 3 min to pellet cells.
15. Remove the supernatant, resuspend cells in 10 mL of HEK293T culture medium, and add 1 mL of cell suspension per 10 cm dish containing 9 mL of fresh culture medium.
16. Return the cells to a humidified incubator at 37 °C and 5% CO_2 and exchange spent medium every 2–3 days as needed.
17. Passage cells at minimum 2–3 times after thawing before utilizing in the final experiment.

18. *Optional: If a SILAC-based quantitative mass spectrometry approach will be pursued, split cells into two separate cultures immediately upon thawing and maintain in "light" and "heavy" SILAC medium (see* Subheading 2.1, **item 3** *and* **Notes 2–4***).* Perform SILAC label incorporation test after 4–5 passages to confirm >95% label incorporation (*see* **Note 13**).

3.3 Cell Line Validation

We have extensively characterized the HEK293T G3BP1-APEX2-GFGP cells described in Markmiller et al. (2018) and found them to be stable and to maintain robust expression of the G3BP1-APEX2-GFP fusion protein (Fig. 3). For less well-characterized or novel cell lines or for troubleshooting purposes we include several optional cell line validation steps below.

3.3.1 Fluorescence Microscopy to Visualize SG Localization of G3BP-APEX2-GFP

1. To visualize G3BP-GFP expression of cells directly via immunocytochemistry, seed 5-10 × 10^4 cells into each well of an 8-well chamber slide in ~250 μL of HEK293T culture medium.
2. After overnight incubation, add an equal volume of 1 mM arsenite to half of the chamber wells for a final concentration of 500 μM.
3. Incubate at 37 °C and 5% CO_2 for 1 h.
4. Remove medium (*see* **Note 6**), rinse once with DPBS and add 200 μL of 4% PFA to each chamber well.
5. Fix cells by incubating for 15–30 min at room temperature.
6. Remove PFA (*see* **Note 14**) and rinse cells twice with DPBS.
7. Simultaneously permeabilize and block cells by incubating in IF blocking buffer for 45 min at room temperature.
8. Rinse wells once with IF wash buffer.
9. Wash with DAPI for 15 min at room temperature.
10. Wash once with wash buffer.
11. Preserve in 50% glycerol/DPBS solution or mount and coverslip slides, drying overnight prior to imaging endogenous GFP signal.

3.3.2 Western Blotting to Confirm V5 Tag Expression of G3BP-GFP-APEX Fusion Protein

1. In 10 cm tissue culture dishes, seed 8 × 10^6 HEK293T G3BP1-APEX2-GFP cells in 8 mL of HEK293T culture medium.
2. After overnight incubation, aspirate spent medium and lyse the cells with 500 μL of cold RIPA buffer.
3. Harvest the lysed cells immediately using cell scrapers while keeping the plates on ice and transfer cell lysates to microcentrifuge tubes.

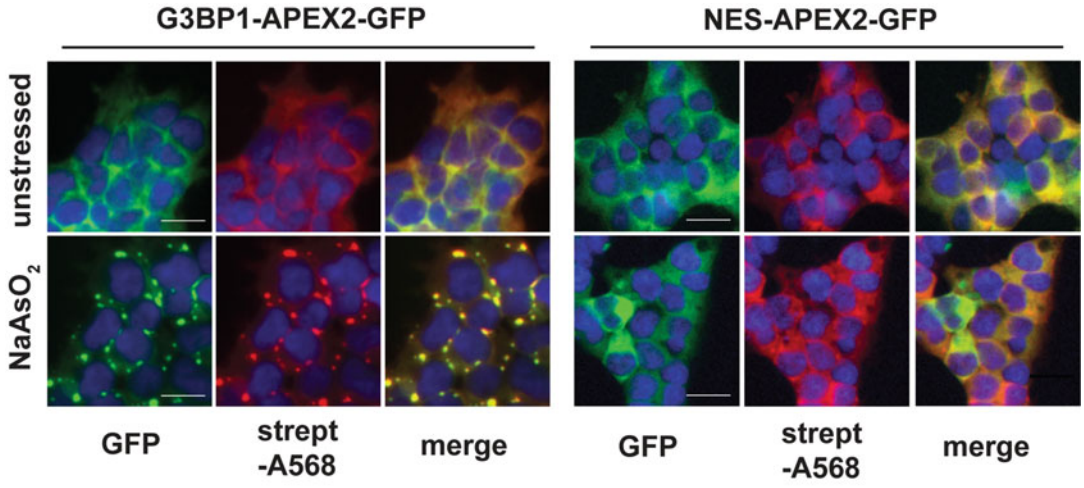

Fig. 3 Streptavidin staining of unstressed and $NaAsO_2$-treated HEK293T G3BP1-APEX2-GFP and hPGK-NES-APEX2-GFP cells. (Adapted with permission from Markmiller et al., Cell 2018)

4. Sonicate the samples for 5 cycles with 30 s on/off and at high intensity (*see* **Note 15**).
5. Centrifuge at (13,500 × *g*) for 10 min at 4 °C to clear the lysates and transfer the supernatant to a new microcentrifuge tube.
6. Determine the total protein concentration of each sample using the BCA protein assay.
7. Run a western blot using approximately 15 μL of each lysate, adjusted for equivalent protein concentration.
8. Add 4× LDS buffer at 4:1 v/v and DTT to a final concentration between 50 and 100 mM to each lysate sample. Pipette up and down to mix well. Briefly centrifuge tubes for 5–10 s.
9. Boil samples on a heat block at 95 °C for 5 min. Briefly centrifuge tubes and return samples to ice.
10. Load samples on a 4–12% gradient Bis-Tris gel with 1× MOPS running buffer. Buffer should be filled enough to submerge the sample wells completely as well as the electrode strips at the bottom of the gels.
11. Load 5 μL of protein ladder on both end wells.
12. Run the gel at 60 V for 20 min and then 150 V for 80 min.
13. Transfer gel onto methanol-activated PVDF membrane at 100 V for 2.5 h in 1× transfer buffer.
14. Block membrane in 5% milk in TBST for 1 h at room temperature.
15. Wash membrane for 10 min with TBST buffer. Repeat wash three times.

16. Probe with V5 tag fluorescent antibody diluted 1:500 in TBST for 1 h at room temperature or 4 °C overnight on a shaker. (Alternatively, probe with GFP fluorescent antibody diluted 1:2000 or G3BP1 antibody diluted 1:5000 to assess difference in size of the protein.)
17. Use a gel imager to image membrane for fluorescent signal at the appropriate channel (i.e., V5 Tag Antibody 37-7500-A488 is green).

3.4 APEX-Mediated Biotinylation of SG Proximal Proteins

1. In 10 cm tissue culture dishes, seed 8 × 10^6 HEK293T G3BP1-APEX2-GFP cells in 8 mL of HEK293T culture medium.
2. After overnight incubation, aspirate spent medium and add 4 mL of fresh medium containing biotin phenol (BP) at a final concentration of 500 μM (*see* **Note 2**).
3. For half of the plates, also add sodium arsenite ($NaAsO_2$) at a final concentration of 500 μM (*see* **Note 6**).
4. Incubate for 1 h at 37 °C.
5. Add 2 mL of DPBS containing 3 mM hydrogen peroxide (H_2O_2) for a final concentration of 1 mM and immediately mix by swirling the plate for 15–20 s.
6. After exactly 60 s, quench the reaction by adding one volume (6 mL) of ice cold 2× quenching solution (*see* **Notes 16** and **17**) and transferring the plate onto ice.
7. Aspirate quenched medium and rinse once with 4 mL of ice-cold 1× quenching solution.
8. Aspirate quenching solution and add 500 μL of ice-cold urea lysis buffer supplemented with protease inhibitors, 5 mM Trolox and 10 mM each of sodium ascorbate and sodium azide.
9. Harvest cells using a cell scraper and transfer to microcentrifuge tubes.
10. Complete cell lysis by sonicating the samples for 5 cycles of 30 s on/off at high intensity (*see* **Note 15**).
11. Centrifuge at (13,500 × *g*) for 10 min at 4 °C to clear the lysates and transfer the supernatant to a new microcentrifuge tube.
12. Determine the total protein concentration of each sample using the 660 nm protein assay (*see* **Note 18**).

3.5 Validation of APEX Labeling: Streptavidin Western Blotting and Immunocytochemistry

1. To confirm biotinylation, run a western blot using approximately 10 μL of each lysate, adjusted for equivalent protein concentration (Fig. 4).
2. Refer to **steps 8–15** under Subheading 3.3.2.
3. Probe with streptavidin IRDye diluted 1:1000 in TBST for 1 h at room temperature or 4 °C overnight.

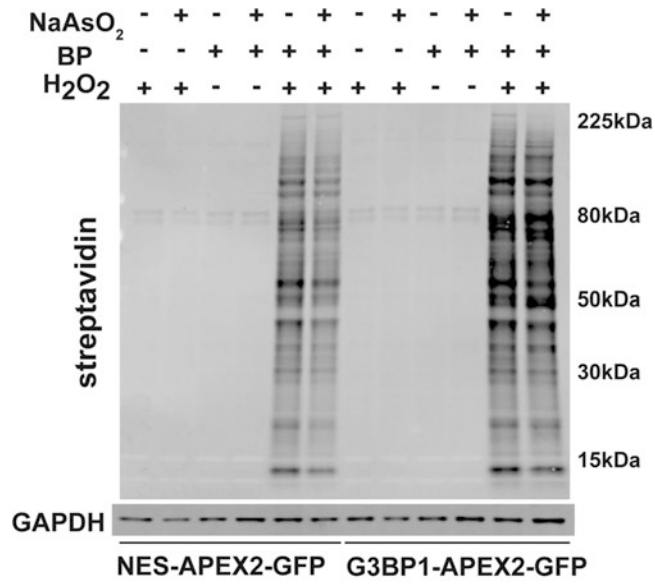

Fig. 4 Streptavidin-HRP western blot analysis of induced protein biotinylation in lysates from NES-APEX2-GFP and G3BP1-APEX2-GFP cells. (Adapted with permission from Markmiller et al., Cell 2018)

4. Use a gel imager to image membrane for fluorescent signal at the appropriate channel.
5. To visualize biotinylation of cells directly via immunocytochemistry (Fig. 3), seed 0.03×10^6 cells into each well of an 8-well chamber slide in 200 μL of HEK293T culture medium.
6. Repeat **steps 2**–7 under Subheading 3.4. Do not lyse cells.
7. Wash with DPBS twice and fix in 150 μL of 4% PFA for 20 min at room temperature.
8. Refer to **steps 6**–**8** under Subheading 3.3.1.
9. Incubate in fluorescently labeled streptavidin diluted 1:1000 in IF wash buffer with 1% BSA (w/v) overnight at 4 °C or 1–2 h at room temperature.
10. Aspirate antibody and wash chamber wells with IF wash buffer twice.
11. Refer to **steps 9**–**11** under Subheading 3.3.1.

3.6 Affinity Purification of Biotinylated Samples and On-Bead Peptide Digestion

1. Add TCEP to a final concentration of 10 mM to each lysate and incubate at room temperature for 30 min (*see* **Note 19**).
2. Add freshly prepared iodoacetamide to a final concentration of 15 mM to each lysate and incubate in the dark at room temperature for 45 min (*see* **Note 19**).

3. Add DTT to a final concentration of 10 mM to each lysate and incubate in the dark at room temperature for 15 min (*see* **Note 19**).
4. Dilute 450 μL of each lysate with 1350 μL of EPPS buffer (150 mM NaCl, 50 mM EPPS pH 8.5) for a final urea concentration of 2 M.
5. Equilibrate 125 μL of streptavidin-conjugated magnetic beads in EPPS buffer containing 2 M urea (*see* **Note 20**).
6. Incubate samples with rotation at room temperature for 2 h at room temperature or overnight at 4 °C.
7. Briefly centrifuge to remove material from the inside of the lid and place tube on magnetic separation rack.
8. Wait until beads have completely separated and transfer the supernatant to a new microcentrifuge tube. Supernatant can be flash-frozen, stored at −20 °C or −80 °C, and analyzed to confirm complete clearance of biotinylated material during the affinity purification (*see* **Notes 21** and **22**).
9. Wash remaining streptavidin bead samples 6 times with 1 mL of cold EPPS buffer containing 1 M urea for 5 min per wash while rotating at 4 °C. Use a magnetic separation rack to collect streptavidin beads between each wash and remove the buffer with a pipette, being careful not to remove beads. After the final wash, remove as much supernatant as possible.
10. Add 1 μg of trypsin diluted in 50 mM EPPS pH 8.5 per sample. Use double volume or 250 μL trypsin per 125 μL of beads for each sample (*see* **Note 23**).
11. Incubate the trypsin digestion for 6 h at 37 °C with gentle shaking (*see* **Note 24**).
12. Add 100% HPLC grade acetonitrile at a final concentration of 10% (*see* **Note 25**).
13. Determine the peptide concentration of each sample using a quantitative colorimetric peptide assay. Subtract the trypsin concentration from the final peptide concentration to determine the accurate concentration of nontrypsin peptides.
14. Proceed to TMT labeling steps based on the amount of total peptides per sample.

4 Notes

1. In principle, APEX-mediated proximity labeling can be conducted with any cell type modified to express APEX2 fused to a protein of interest (POI) with a specific subcellular localization. For stress granules, APEX2 has been fused to several different

proteins, including G3BP1, FMR1 and FXR1 [7, 10]. We have had good results with CRISPR/Cas9-mediated insertion of APEX2 into the endogenous G3BP1 locus in HEK293T cells and induced pluripotent stem cells [7]. The homologous recombination donor plasmid for endogenous tagging is available from Addgene (Plasmid #105284) and the cell lines are available upon request. However, successful APEX labeling has also been performed in cells expressing POI-APEX2 fusion proteins from constitutive or tetracycline-inducible promoters upon either transient or stable transfection.

2. We have found APEX labeling to work well in a range of different cell culture media. However, we have repeatedly experienced poor labeling in mTeSR1 stem cell medium. We recommend substituting DMEM/F12 basal medium for the biotin phenol incubation period.
3. Amino acid concentrations given here are for DMEM but can vary depending on the cell culture medium used. We routinely prepare stock solutions of amino acids to supplement media as needed. For example, 0.4 mM of "heavy" L-arginine–HCl (MW 220.6 g/mol) = 88.24 mg/L. Thus, a 1000x stock solution of 88.24 mg/mL for DMEM can be stored at 4 °C or −20 °C and volumes can easily be adjusted to supplement other types of media (e.g., DMEM/F12 at 0.7 mM).
4. When grown in SILAC medium, many cell lines can convert substantial amounts of isotope-labeled arginine into proline. This isotope-labeled proline is then incorporated into newly synthesized proteins and impairs the accurate identification and quantification of heavy vs light peptides. This conversion can be prevented by supplementing the medium with excess proline at 200 mg/L [26]. We initially found this conversion to be a substantial issue in HEK293T cells and now routinely complement both heavy and light SILAC media with proline.
5. HEK293T cells are maintained on 10 cm plastic tissue-culture dishes throughout for the end goal of mass spectrometry. One 10 cm dish of ~80–90% confluent cells is used per final condition per replicate. This can be scaled up or scaled down based on alternative endpoints which require different amounts of starting material (e.g., different mass spectrometry techniques, RNA sequencing, immunocytochemistry, western blotting).
6. Sodium arsenite is toxic and potentially dangerous. Consult Material Safety Data Sheet and appropriate local laboratory safety guidelines for handling and waste disposal.
7. Once opened, hydrogen peroxide solution will lose activity within 3–6 months. Keep stock bottle closed and at 4 °C when not in use and keep track of the opening date.

8. Sodium azide is extremely toxic and must be handled accordingly. Consult Material Safety Data Sheet and appropriate local laboratory safety guidelines for handling and waste disposal.
9. Solubility of Trolox in aqueous solutions is low and Trolox may begin to precipitate in concentrations above the 10 mM used in 2× quenching solution.
10. For downstream applications that are not compatible with primary amine buffers such as Tris (e.g., TMT labeling), 50 mM EPPS buffer pH 8.5 (e.g., Fisher Scientific catalog no. AAJ61476AE) can be used instead of Tris.
11. For best results and reproducibility, it is critical that cells are cultured consistently under appropriate conditions. Do not allow cells to become overconfluent before passaging. We routinely use HEK293T cells for no more than 5–10 passages of thawing from a general frozen stock to maintain consistency between replicates and different experiments.
12. We routinely culture HEK293T cells without coating of tissue culture vessels. As a result, cells usually detach within 2–3 min of TrypLE treatment. Under some circumstances it may be desirable or necessary to grow HEK293T cells on dishes coated with poly-L-lysine. In this case, incubation for 5–10 min or longer may be necessary to detach cells for passaging.
13. *Optional (if performing SILAC labeling): In rapidly proliferating cells such as HEK293T, we generally observe near-complete label incorporation after 3–5 passages. By contrast, in other cell types with slower protein turnover* (e.g., *postmitotic neurons), extended SILAC labeling may be required for sufficient label incorporation.*
14. PFA is toxic and potentially carcinogenic. Consult Material Safety Data Sheet and appropriate local laboratory safety guidelines for handling and waste disposal.
15. We routinely use sonication to achieve complete sample lysis and nucleic acid fragmentation, but other physical disruption methods, including liquid homogenization or shearing through a small gauge needle are also acceptable. Complete homogenization and clearance of samples is important to avoid sample aggregation during streptavidin affinity purification.
16. Since MS/MS experiments are eventually done in triplicate samples and the APEX labeling requires several conditions for comparison (stressed and unstressed, with and without BP substrate), many plates must be handled at one time. The SG proteome is highly context dependent so slight variations in APEX labeling treatment times may lead to varying results across replicates. It is recommended to stagger the treatment times for BP and stressors in order to allow for a few minutes between each plate to perform the subsequent H_2O_2 treatment

followed by quenching and lysis. It is important to keep the BP and stressor treatment time (1 h) consistent across samples as well as the H_2O_2 treatment time precise (60 s) across samples to limit variability as much as possible.

17. Using a 2× quenching solution reduces hands-on time at the end of the 60 s incubation and allows for better control of the labeling time. It is also possible to aspirate the H_2O_2-containing medium and quench the reaction with ice-cold 1× quenching solution instead.
18. Urea lysis buffer supplemented with antioxidants (Trolox and sodium ascorbate) is not compatible with most other methods used to determine protein concentration (e.g., BCA assay).
19. Alkylation and reduction treatments with TCEP, iodoacetamide, and DTT can be done either predigestion at the protein level or postdigestion at the peptide level. Either TCEP or DTT can be used for the reduction step to expose free cysteine residues. Iodoacetamide then alkylates the free cysteines for easier detection via tandem mass spectrometry. The last DTT step inactivates any excess iodoacetamide.
20. We determined the appropriate amount of streptavidin beads per sample by titration and analyzing the amount of biotinylated proteins left behind in the supernatant after affinity purification. This amount may need to be adjusted depending on binding capacity of the specific beads used and the density, total protein content, and biotinylation efficiency of the cells that are being used.
21. If not proceeding directly to biotin affinity purification, lysates can be stored at −80 °C by first snap freezing the tubes in liquid nitrogen or ethanol over dry ice directly after clearing by centrifugation. Additionally, partial aliquots of each sample can be saved at this step for downstream protein quantification and streptavidin western blotting to confirm successful biotinylation of associated proteins.
22. Supernatants collected after the streptavidin bead incubation can be used to determine the efficacy of the biotin enrichment and streptavidin immunoprecipitation. Run 10 μL of each supernatant on a western blot and probe with streptavidin antibody. A successful enrichment of biotinylated proteins captured by the streptavidin beads would result in very little or no signal on the supernatant western blot. If a strong biotin signal is observed, the enrichment must be repeated and further optimized before proceeding.
23. Trypsin should be at a 1:100 enzyme to substrate ratio. However, the amount of protein captured by the beads will not be known at the time. Therefore, although 1 μg of trypsin per sample should be sufficient, further optimization may be

required and dependent on the abundance and interactions of the SG protein of interest.

24. The 6 h trypsin digestion at 37 °C can also be done overnight instead. The minimum digestion time suggested is 6 h and it is not recommended to go shorter.
25. Handle 100% HPLC grade acetonitrile carefully and only in a fume hood as it is highly flammable and toxic.

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Competing interests: G.W.Y. is on the Scientific Advisory Board of Jumpcode Genomics, and a cofounder, member of the Board of Directors, on the Scientific Advisory Board, equity holder and paid consultant for Locanabio and Eclipse BioInnovations. G.W.Y. is a visiting professor at the National University of Singapore. G.W.Y.'s interests have been reviewed and approved by the University of California San Diego, in accordance with its conflict-of-interest policies. The authors declare no other competing interests.

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