Chapter 1

A Photoaffinity Labeling-Based Chemoproteomics Strategy for Unbiased Target Deconvolution of Small Molecule Drug Candidates

Jason R. Thomas, Scott M. Brittain, Jennifer Lipps, Luis Llamas, Rishi K. Jain, and Markus Schirle

Abstract

The combination of photoaffinity labeling (PAL) and quantitative chemoproteomics enables the comprehensive, unbiased determination of protein interaction profiles to support target identification of bioactive small molecules. This approach is amenable to cells in culture and compatible with pharmacologically relevant transmembrane target classes like G-protein coupled receptors and ions channels which have been notoriously hard to access by conventional chemoproteomics approaches. Here, we describe a strategy that combines PAL probe titration and competition with excess parental compounds with the goal of enabling the identification of specific interactors as well as assessing the functional relevance of a binding event for the phenotype under investigation.

Key words Photoaffinity labeling, Chemoproteomics, Quantitative proteomics, Target identification, Isobaric mass tags, G-protein coupled receptor

1 Introduction

Phenotypic screening is an approach to both drug and biological discovery that utilizes screenable cellular models or model organisms that reflect a given aspect of a biological pathway or disease state. These models are then used to screen collections of small molecules to identify compounds that modulate the pathway or disease state in the desired manner in a target-agnostic manner. In part reflecting the recent resurgence of phenotype-based drug discovery [1], a large number of complementary approaches have been introduced to support follow-up studies to identify the efficacy target, i.e., the protein through which the compound elicits its phenotypic effect via direct binding and modulation (*see* ref. 2 for a

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recent review). Among these approaches, chemoproteomic strategies that generate efficacy target hypotheses by defining the cellular protein interactome of a compound by coupling an affinity enrichment step to quantitative mass spectrometry are among the most popular and powerful [3]. Most chemoproteomic strategies are based on affinity tool compounds, biologically active derivatives of the compound of interest that have been designed to allow for immobilization and subsequent enrichment of protein interactors. In photoaffinity labeling (PAL), the compound is derivatized to contain (a) an ultraviolet (UV) light-activated photocrosslinking moiety and (b) an enrichment handle such as biotin. Upon UV activation, a highly reactive intermediate is produced allowing the probe to irreversibly react with its target; the enrichment handle facilitates enrichment of probe-labeled proteins. These probes can be used in whole cell lysates or applied directly to cells in culture. For the latter scenario, the enrichment handle is often installed only after cell lysis using bioorthogonal chemistry to avoid potential issues with cell permeability. Through its ability to interrogate binding events in live cells, PAL has emerged as an attractive strategy for target deconvolution under in situ conditions particularly because it allows access to pharmacologically relevant target classes that are frequently missed by the noncovalent, lysate-based chemoproteomics approaches. On the other hand, there are known challenges with PAL which may reduce its overall effectiveness, in particular when applied to unbiased de novo target deconvolution as opposed to direct target hypothesis testing. First, the crosslinking efficiency is notoriously low, even when using purified proteins. Second, there is a significant degree of nonspecific labeling which can be at least in part explained by the proximity-based nature of the crosslinking step.

Here, we describe a quantitative mass spectrometry-based PAL strategy (Fig. 1) that addresses a key challenge in PAL, namely low overall signal-to-noise, to facilitate the identification of specific interactors of a small molecule of interest, as well as provides a first step toward assessing if an interactor is the potential efficacy target. In order to discriminate specific from nonspecific binding, a combination of titrating probe concentration and competition with excess parental compound is performed. Proteins that bind specifically to the PAL probe are defined by the ability of a protein to bind to the PAL probe in a dose-responsive and saturable manner (Fig. 1a). Proteins that bind to the parent compound are determined by the ability of the excess parental compound to compete PAL probe labeling. After the identification of proteins that bind specifically, the relevance of the binding event to the phenotype under investigation can then be determined through competition with a series of structurally related compound with varying degrees of phenotypic activity (structure activity relationship, SAR). The goal here is to determine if a correlation can be established between

binding and phenotypic activity (Fig. 1b). Competition experiments in general also provide a means to deprioritize proteins that preferentially interact with the pharmacologically irrelevant photocrosslinker portion of the PAL probe: These proteins will show specific enrichment in the probe titration but should not show significant competition by a competition compound that lacks the photocrosslinker moiety. With regard to proteomic readouts, several variants of quantitative mass spectrometry are available [4, 5],

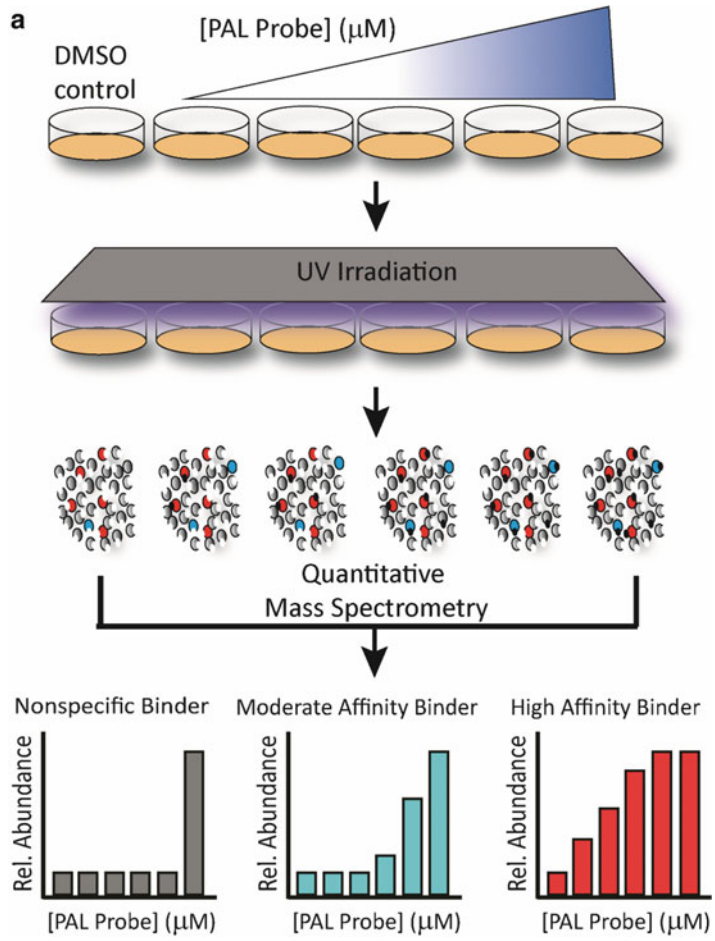


Fig. 1 (**a**) Overview of the PAL probe titration workflow: key goal for this workflow is the identification of specific binders showing dose-responsive, saturable binding to differentiate from nonspecific background. (**b**) Overview of the compound competition workflow with constant probe concentration and single dose competition with a series of structurally related compounds that differ in their activity in the phenotypic assay (SAR series): key goal for this workflow is the identification of specific binders to the pharmacophore that are relevant for the phenotype under investigation. Workflows are depicted separately for clarity and with six conditions (TMT Sixplex™ reagent), but it should be noted that the multiplexing capability of TMT reagents for up to ten conditions (TMT 10plex™) allows for mixed workflows, e.g., inclusion of a competition channel in a probe titration experiment as in the presented study

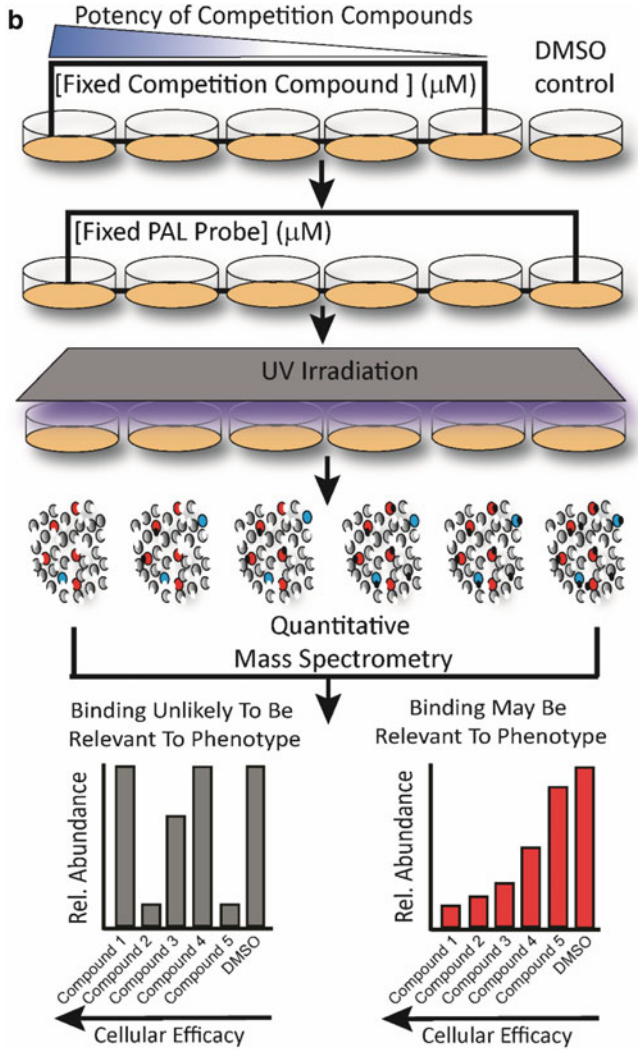


Fig. 1 (continued)

but the flexibility and multiplexing capabilities of isobaric mass tags such as tandem mass tags (TMT^{TM}) make them attractive options for complex workflows such as the extended PAL probe titration and SAR correlation experiments described here.

In the presented example, we apply this workflow in the context of a previous study that had determined that expression of the G-protein coupled receptor (GPCR) GPR39 is required for the inhibitory activity of the cyclohexyl-methyl aminopyrimidine (CMAP) chemotype on Hedgehog (Hh) signaling [6]. Noncovalent, lysate-based chemoproteomics had failed to provide proof of direct interaction which suggested a target that may require an intact cellular environment for binding capability including integral transmembrane target families such as GPCR and ion channels. We were able

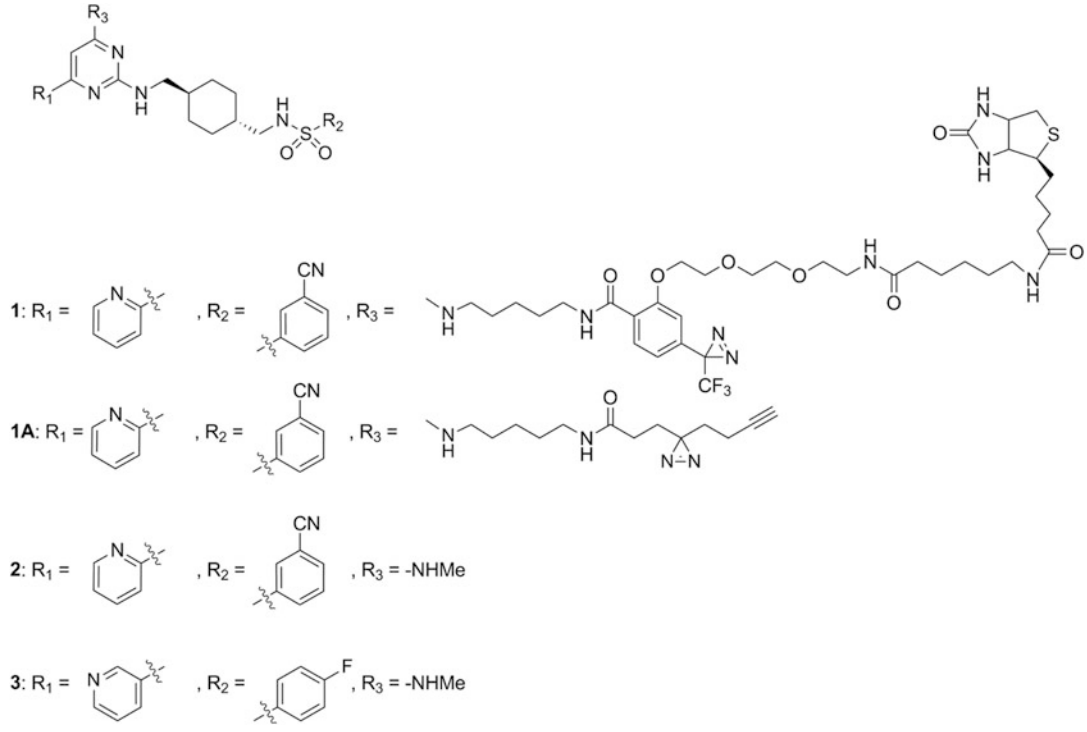


Fig. 2 PAL probe **1** and the competition compounds **2, 3** used in this study as well as an alternative PAL probe **1A**. Hedgehog pathway activities (Gliluc) are 0.04 μM (compound **1**), 0.015 μM (**2**) and 4.5 μM (**3**)

to generate a PAL probe **1** that retained comparable activity (40 nM) to the active parent compound **2** (15 nM) in a luciferase-based Hedgehog reporter gene assay (Gliluc) in murine TM3 cells. Using the comprehensive PAL-based strategy described above and HEK293T cells expressing murine GPR39 (mGpr39) as a model system (the CMAP scaffold does not show appreciable activity in human cells), we are able to provide evidence for direct interaction of the CMAP-based PAL probe with mGpr39 by probe titration as well as competition by compound **2** (*see* Fig. 2 for compounds, Fig. 3a, b for data). In addition, we illustrate how the dual criteria of saturable labeling and SAR-dependent competition narrows down the list of protein interactors to those that are likely functionally relevant to Hedgehog signaling. This also further corroborates mGpr39 as an efficacy target of this chemotype (Fig. 3c). While the presented data are based on probe **1** which contains a preinstalled biotin enrichment handle, we have also included the experimental steps for the introduction of the biotin moiety after cell lysis to an exemplary alkyne-bearing probe **1A** using copper-based click chemistry [7]. We routinely use the general experimental strategy described here for unbiased target deconvolution for small molecule hits from phenotypic screens.

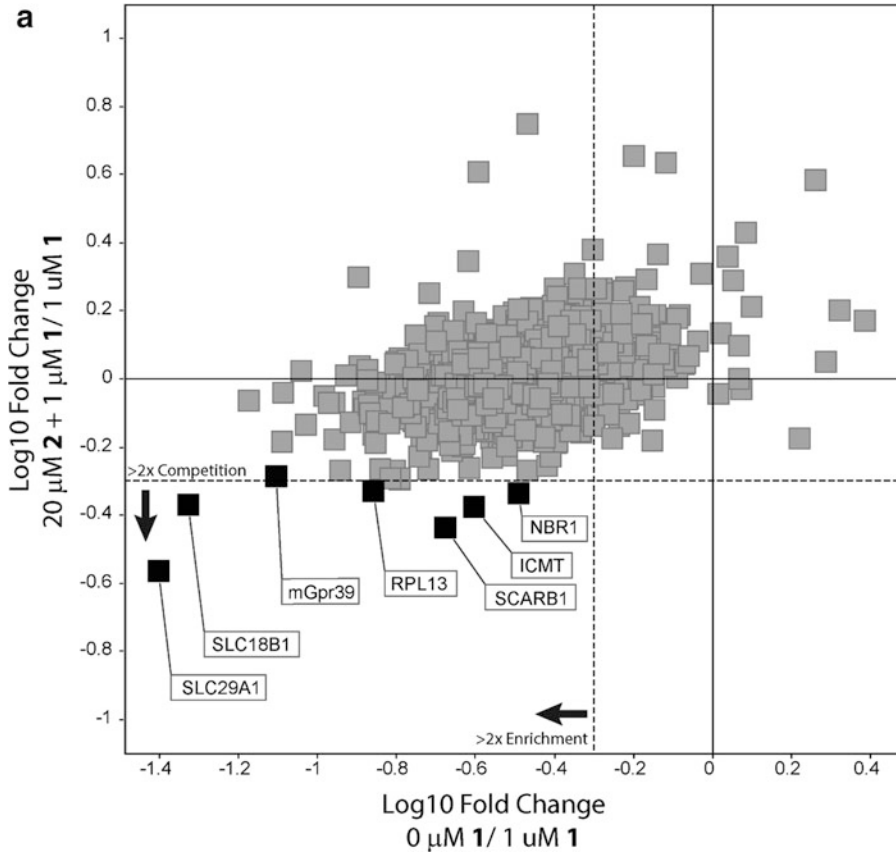
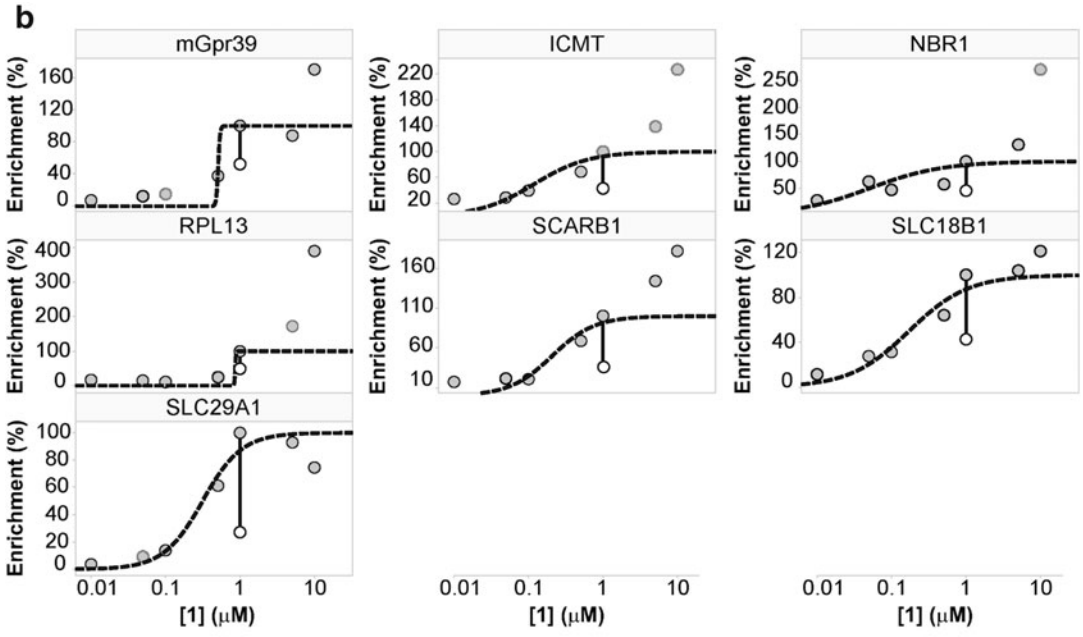


Fig. 3 (**a**) Scatter plot showing protein enrichment by probe **1** on the X-axis (depicted as log10 fold change for 0 μM probe **1** over 1 μM probe **1**) and competition by 20 μM **2** at 1 μM probe **1** on the Y-axis (depicted as log10 fold change over DMSO at 1 μM probe **1**). Several proteins, including murine GPR39 (mGpr39), show significant enrichment as well as competition with compound **2** indicative of specific binding to the pharmacophore. (**b**) Representative full quantitative profiles for selected proteins demonstrating the combination of probe titration curves (0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 μM PAL probe, *gray circles*) with competition by 20 μM compound **2** at 1 μM probe **1** (*white circle*). mGpr39, SLC18B1, and SLC29A1 show saturable labeling already at low PAL probe concentrations as well as competition by excess compound, indicative of specific binding to the pharmacophore. This is in contrast to ICMT, NBR1, RPL13, and SCARB1 which do not exhibit saturable labeling by probe **1**. Given the presence of proteins that exhibit saturable labeling, these latter proteins could be deprioritized as putative efficacy targets. (**c**) Table showing residual labeling for selected protein hits from Fig. 1a upon competition with 20 μM of compounds **2** and **3** as representative competitor compounds with high and significantly lower activity in the Gliluc assay (1 μM PAL probe in both cases). mGpr39 competition correlates with activity, in line with this being a compound efficacy target. Unlike mGpr39, SLC18B1 exhibits similar degree of competition upon treatment with compound **2** or **3**. The fact that SLC18B1 binds with comparable affinity to each compound despite the large difference in cellular activity would suggest that SLC18B1 is not responsible for the observed activity on Hedgehog signaling. Similar to mGpr39, SLC29A1 shows a competition profile that correlates with activity on Hedgehog signaling. For further hit triaging in such cases, an extended SAR series would typically be used



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	2 *(highly active)*	3 *(less active)*
mGRP39	52%	78%
ICMT	42%	77%
NBR1	46%	107%
RPL13	47%	58%
SCARB1	37%	62%
SLC18B1	43%	45%
SLC29A1	27%	55%

Fig. 3 (continued)

2 Materials

Use only high purity reagents and prepare all solutions using distilled, deionized water. All organic solvents should be HPLC grade or higher.

2.1 Cell Culture, Compound Treatment, and Irradiation

1. HEK293T (ATCC) cells engineered to stably express V5-tagged mGpr39.
2. Gibco Dulbecco's phosphate buffered saline (DPBS) (Thermo Fisher Scientific).
3. Gibco Opti-MEM™ reduced serum media, no phenol red (Thermo Fisher Scientific).
4. Compound 1: 10 mM PAL probe in DMSO (*see* **Note 1**).

5. Compound 2 (competition compound with potent cellular activity): 10 mM in DMSO.
6. Compound 3 (competition compound with weak cellular activity): 10 mM in DMSO.
7. Blak-Ray XX-40BLB UV bench lamp, 40 Watt (UVP).
8. Blak-Ray UV intensity meter Model J-221 (UVP).
9. Cell culture dish 140 × 20 mm.
10. Conical centrifuge tubes (5 mL).

2.2 Cell Lysis

1. HEPES buffer stock solution: 50 mM, pH 7.5.
2. Cell lysis buffer: 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol. Immediately before use, add 0.8% NP40 and Halt protease inhibitor cocktail (Thermo Fisher Scientific).
3. Barocycler NEP2320, FT500 PULSE tubes and tube tool (Pressure Biosciences, Inc.).

2.3 Click Reaction

This step is only required if the PAL probe does not already contain a biotin affinity handle.

1. Sodium dodecyl sulfate (SDS) solution: 10% in water (w/v).
2. Biotin-PEG3-Azide (Click Chemistry Tools, Scottsdale, AZ): 5 mM in DMSO.
3. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) solution: 9 mg/mL in butanol:DMSO (4:1, v/v).
4. Cu(II)SO_4 solution: 50 mM in water.
5. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution: 50 mM in water.
6. Base buffer: 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol (v/v).
7. Heating block shaker for microcentrifuge tubes (e.g., Eppendorf thermomixer).
8. Sonicator (e.g., Q700, Qsonica Llc).

2.4 Affinity Enrichment

1. Pierce NeutrAvidin Agarose beads, 50% aqueous slurry (Thermo Fisher Scientific).
2. Wash buffer I: 1 mM DTT, 0.4% NP40 in base buffer (*see* Subheading 2.3, **item 6**). Prepare fresh before use.
3. Wash buffer II: 1 mM DTT in base buffer (*see* Subheading 2.3, **item 6**). Prepare fresh before use.
4. Pierce 660 nm protein assay, ionic detergent compatibility reagent (Thermo Fisher Scientific).
5. Microcentrifuge tubes (5 mL).

6. Chromatography spin columns, 1 mL, with 90 μm pore size frit and Luer-lock connector (e.g., Mobicol, MoBiTec, Germany).
7. NuPAGE LDS (Lithium dodecyl sulfate) sample buffer (4×) (Thermo Fisher Scientific).

2.5 Sample Preparation, Visualization, and Proteolysis

1. Iodoacetamide solution: 200 mg/mL in water. Prepare fresh before use and protect from light.
2. Criterion™ XT Bis-Tris Gel Pierce Silver Stain Kit, 4–12% (Bio-Rad).
3. Trypsin protease, MS-grade (Thermo Fisher Scientific).
4. Detergent removal spin columns (Thermo Fisher Scientific).

2.6 Isobaric Labeling

1. TMT Sixplex™ or 10plex™ reagent kit (Thermo Fisher Scientific).
2. Triethylammonium bicarbonate (TEAB) solution: 1 M in water.
3. SpeedVac (e.g., HT-4X Evaporator, Genevac Inc.).

2.7 High pH Reversed Phase Separation

1. Dionex UltiMate™ 3000 HPLC system with fraction collection operating Chromeleon v. 6.8 (Thermo Fisher Scientific) or similar performance HPLC system.
2. Waters Xbridge™ C18 3.5 μm 2.1 × 150 mm HPLC column (Waters Corporation) or equivalent C18 HPLC column.
3. Low volume autosampler vials and septa.
4. U-bottom 96-well plate.
5. V-bottom 96-well plates.
6. High pH mobile phase A: water (HPLC grade).
7. High pH mobile phase B: acetonitrile (HPLC grade).
8. High pH mobile phase C: 200 mM ammonium acetate, pH 10.
9. Trifluoro-acetic acid (TFA) solution: 0.5% in water (v/v).

2.8 Protein Identification and Quantitation by nanoLC-MS/MS

1. Q Exactive mass spectrometer (Thermo Fisher Scientific; Xcalibur 3.0.63, Tune 2.5 SP1 software) equipped with Picoview 550 Nanospray source (New Objective).
2. EasyLC 1000 HPLC system (Thermo Fisher Scientific), equipped with a 20 μL loop, set up for 96-well plates, or similar performance HPLC system.
3. Spraying capillary: fused silica capillary (75 μm ID × 360 μm OD) pulled to a tip diameter of approximately 8–10 μm using a P-2000 capillary puller (Sutter Instruments) and packed, e.g., with ReproSil-Pur 120 C18-AQ, 3 μm material, 160 mm bed length (Dr. Maisch GmbH, Germany).

4. Kasil-fritted trapping column (75 μm ID) packed with ReproSil-Pur 120 C18-AQ, 5 μm material (15 mm bed length), or equivalent trapping column (*see* **Note 2**).
5. Mobile phase A: 0.1% formic acid/acetonitrile (98:2, v/v).
6. Mobile phase B: 0.1% formic acid/98% acetonitrile (2:98, v/v).

2.9 Data Processing and Analysis

1. Mascot v 2.5.1 (Matrix Science).
2. Transproteomic pipeline (TPP) v3.3sqall, available on the Internet from the Seattle Proteome Center at the Institute of System Biology (Seattle, USA).
3. Uniprot protein database (canonical human sequences, EBI, Cambridge, UK) supplemented with sequences of mGpr39, common contaminants as well as reversed entries for all forward entries.
4. In-house developed software for parsing and uploading of information from TPP-derived xml files and Mascot result files to a relational database (Oracle 10 g; Oracle).
5. Spotfire 6.5.3 software for data visualization and analysis (TIBCO Software Inc.).

3 Methods

3.1 Cell Culture, Compound Treatment, and Irradiation

1. Seed 20×10^6 HEK293T cells stably expressing V5-tagged mGpr39 into a 140 × 20 mm cell culture dish and allow cells to recover for 12–16 h at 37 °C with 5% CO_2. Ten plates in total will be needed (*see* **Note 3**).
2. Prepare DMSO-treated media by adding 10 mL Opti-MEM™ (37 °C) with 0% FBS and 20 μL DMSO to 8–15 mL tubes. Incubate tubes at 37 °C until ready for use (*see* **Note 4**).
3. Prepare highly active competition compound-treated media by adding 10 mL Opti-MEM™ (37 °C) with 0% FBS and 20 μL competition compound **2** (10 mM) to a 15 mL tube. Incubate tubes at 37 °C until ready for use.
4. Prepare less active competition compound-treated media by adding 10 mL Opti-MEM™ (37 °C) with 0% FBS and 20 μL competition compound **3** (10 mM) to a 15 mL tube. Incubate tubes at 37 °C until ready for use.
5. Prepare a serial dilution of the PAL probe (compound **1)** from 10 mM to 0.01 mM and from 5 mM to 0.05 mM in DMSO. Each dilution should have a final volume of 25 μL.
6. Wash each plate twice with 10 mL DPBS (37 °C).
7. To one plate of cells add compound **2**-treated media, to another plate of cells add compound **3**-treated media, and to

the remaining plates add DMSO-treated media. Incubate the cells for 60 min at 37 °C.

8. To one plate containing DMSO-treated media add 10 μL DMSO.
9. To one plate containing DMSO-treated media add 10 μL compound **1** (0.01 mM).
10. To one plate containing DMSO-treated media add 10 μL compound **1** (0.05 mM).
11. To one plate containing DMSO-treated media add 10 μL compound **1** (0.1 mM).
12. To one plate containing DMSO-treated media add 10 μL compound **1** (0.5 mM).
13. To one plate containing DMSO-treated media add 10 μL compound **1** (1 mM).
14. To one plate containing DMSO-treated media add 10 μL compound **1** (5 mM).
15. To one plate containing DMSO-treated media add 10 μL compound **1** (10 mM).
16. To the plate containing compound **2**-treated media add 10 μL compound **1** (1 mM).
17. To the plate containing compound **3**-treated media add 10 μL compound **1** (1 mM).
18. Incubate cells for 60 min at 37 °C (*see* **Note 5**).
19. During 60 min incubation turn on the UV bench lamp (*see* **Notes 6** and **7**).
20. Remove media via aspiration (leave ~5 mL behind).
21. Irradiate (368 nm) cells at 4 °C for 20 min.
22. Harvest cells by scraping, transfer to individual 15 mL tubes, and spin in a prechilled centrifuge at 200 × *g* for 5 min at 4 °C.
23. Remove the supernatant via aspiration and store treated cell pellets at −80 °C.

3.2 Cell Lysis

1. Suspend cell pellet in 1 mL of chilled lysis buffer and transfer to a PULSE tube.
2. Add 400 μL of chilled lysis buffer to each PULSE tube (final volume for each cell pellet should be 1.4 mL).
3. Lyse cells in a chilled Barocycler using 5 cycles of 35 kpsi for 20 s followed by 20 s at atmospheric pressure. Transfer lysed cells to individual 1.5 mL tubes.
4. Spin samples at 800 × *g* in a prechilled centrifuge for 20 min and collect supernatant ("lysate").

3.3 Click Reaction

This step is only required if the PAL probe does not already contain a biotin affinity handle.

1. Aliquot 975 μL lysate from each sample in a 1.5 mL tube.
2. Add 125 μL 10% SDS solution.
3. Add 25 μL of 5 mM Biotin-PEG3-Azide in DMSO.
4. Prepare Catalyst Mix by combining 1.35 mL of butanol: DMSO (4:1, v/v), 150 μL of TBTA stock, 0.5 mL 50 mM Cu(II)SO_4 solution, and 0.5 mL 50 mM TCEP.
5. Vortex Catalyst Mix and add 125 μL to each sample.
6. Vortex each reaction vigorously.
7. Using an Eppendorf thermomixer, incubate for 2 h at 37 °C with gentle agitation (300 rpm).
8. During incubation, add 6 mL cold acetone to individual 15 mL tubes. Store at −20 °C until ready for use.
9. After the 2 h incubation, transfer the entire contents of each click reaction to individual 15 mL tubes containing 6 mL cold acetone.
10. Incubate for 4–16 h at −20 °C.
11. Spin precipitated protein in a prechilled centrifuge at 1250 × *g* for 10 min.
12. Remove excess acetone by aspiration and reconstitute protein pellet in 1 mL base buffer containing 1% SDS by probe sonication using 20 consecutive 1 s pulses (12 W).

3.4 Affinity Enrichment (See Note 8)

1. Aliquot 100 μL of the 50% NeutrAvidin agarose bead slurry, wash agarose three times with 1 mL of wash buffer I and three times with 1 mL of wash buffer II, and transfer the aliquots to 5 mL tubes. After each addition of buffer the beads are spun at 90 × *g* for 2 min.
2. Determine total protein concentration for each sample using Pierce 660 nm protein assay and adjust to a protein concentration of 5 mg/mL.
3. For each sample, add 4 mL of base buffer to the 50 μL of NeutrAvidin agarose bead bed in the 5 mL tubes, and then add 1 mL of sample per tube.
4. Incubate samples on agarose beads overnight on a rocking platform at room temperature.
5. Spin samples at 90 × *g* for 2 min at room temperature.
6. Remove the supernatant, leave ~1 mL supernatant above the resin bed.
7. Resuspend agarose beads in residual supernatant and transfer samples to Mobicol spin columns.

8. Wash beads with 3 mL wash buffer I.
9. Wash beads with 3 mL wash buffer II.
10. Spin samples at 100 × *g* for 30 s, place plug in the bottom of the Mobicol spin column, and replace the Luer-lock lid with the screw cap lid.
11. Add 72 μL 2× LDS sample buffer to each sample and heat samples to 95 °C for 5 min (*see* **Note 9**).
12. Remove bottom plug and spin samples at 14,000 × *g* for 2 min to collect eluent.

3.5 Sample Preparation, Visualization, and Proteolysis

1. To alkylate the cysteine sulfhydryls, add 5.5 μL of 200 mg/mL iodoacetamide solution per 50 μL of collected eluent and vortex.
2. Incubate samples for 30 min at room temperature in the dark.
3. Load 10 μL of each sample onto a 4–12% Bis-Tris NuPAGE gel, run gel at 100 V until the ladder begins to separate and run gel at 160 V for ~45 min or until desired resolution is achieved. Prepare gel for silver staining to assess total protein levels using the Pierce Silver Stain Kit (*see* **Note 10**).
4. Remove bottom stopper from detergent removal spin columns.
5. Use 2 mL collection tubes to wash the columns; spin the storage tubes for 1 min at 1500 × *g* to remove the storage buffer.
6. Wash each column with 400 μL 10 mM HEPES, pH 7.8 and spin for 1 min at 1500 × *g*.
7. Discard eluate and repeat **step 6** three times.
8. Carefully add alkylated eluents without touching the resin of the column and incubate for 2 min.
9. Centrifuge for 2 min at 1500 and then add 4 mL of base buffer to each tube and spin for 2 min at 1500 × *g* to collect detergent-free sample.
10. Add 135 μL of 10 mM HEPES buffer, pH 7.8, and wash the resin by centrifuging for 2 min at 1500 × *g*.
11. Add 1 mL 10 mM HEPES buffer, pH 7.8, to 20 μg trypsin (*see* **Note 11**).
12. Add 5 μL trypsin solution to each detergent-free sample and allow digestion to proceed overnight at 37 °C.

3.6 Isobaric Labeling (See Note 12)

1. Selecting a different TMT reagent for each condition, allow one vial of each TMT reagent to come to room temperature, add 200 μL of acetonitrile to each vial, and vortex several times for 30–60 s over a 5–10 min period.

2. Add 30 μL 1 M TEAB solution directly to each digested sample and vortex.
3. Add 450 μL acetonitrile directly to each digested sample and vortex.
4. Add 100 μL TMT reagent to each sample, and incubate at room temperature for 1 h.
5. Dry samples in SpeedVac, then reconstitute each sample in 50 μL of a 0.1% acetic acid/acetonitrile (30:70, v/v) solution.
6. Combine all the reconstituted samples into one vial and dry samples in SpeedVac.
7. Reconstitute consolidated sample in 100 μL of a 0.1% trifluoroacetic acid/acetonitrile (98:2, v/v) solution for off-line high-pH reversed phase separation.

3.7 High-pH Reversed Phase Separation (See Note 13)

1. Add consolidated TMT-labeled sample to low-volume autosampler vial and place in HLPC autosampler.
2. Peptide separation is achieved using a flow rate of 250 μL/min and the following ternary gradient: high-pH mobile phase C is held at 10% throughout gradient; starting conditions are 89% high-pH A and 1% high-pH mobile phase B, ramping high-pH mobile phase B to 50% over 65 min, and returning to 1% B from 65 to 75 min.
3. Add 20 μL of 0.5% TFA to each well of the collection plate prior to collection
4. Inject 50 μL of sample and collect 1 min fractions in a U-bottom 96-well plate from 15 through 73 min.
5. Dry U-bottom well plate with collected fractions in SpeedVac.
6. Pool every ~3 fractions with 40 μL mobile phase A to achieve 16 fractions and transfer into a V-bottom 96-well plate for nanoLC-MS/MS analysis.

3.8 Peptide Identification and Quantitation by nanoLC-MS/MS

1. Load 18 μL of sample on the trapping column using mobile phase A at a flow rate of 2.5 μL/min. Peptides are eluted using an 80 min gradient (2% mobile phase B for 5 min, 2–40% B from 5 to 65 min, followed by 70% B from 65 to 70 min, then returning to 2% B from 70 to 80 min) at a flowrate of 250 nL/min on the capillary separation column with direct spraying into the mass spectrometer.
2. Acquire LC-MS/MS data by operating the Q-Exactive mass spectrometer in a data-dependent mode, typical settings are: MS/MS are collected for the 12 most intense ions within a mass-to-charge ratio *(m/z)* range of 300–1250 detected in the full MS survey scan event. MS scans are acquired at 35,000 mass resolution (R) at m/z 200, using a target value of 1×10^6 ions and a maximum fill time of 100 ms. MS/MS scans are

acquired using a target value of 5×10^4 ions, maximum fill time of 100 ms, and isolation window of 2 Da. MS intensity threshold for ions to be selected for MS/MS is 1×10^5 ions and normalized collision energy is set to 28%. Further criteria for ions to be selected for MS/MS are charge state z of 2–4, and a typical exclusion time of 30 s before ions can be selected again for MS/MS. Spray voltage is set to 2.0 kV, heated capillary temperature is 250 °C, and S-lens RF level is 50. No sheath or auxiliary gas flow is applied.

3.9 Data Processing and Analysis

1. Conversion of Xcalibur Rawfiles to Mascot-compatible mgf-files as well as subsequent generation of mzXML, pepXML, protXML and Interact files are done using the corresponding TPP-modules.
2. Mgf-files are searched against the protein sequence database using Mascot with peptide mass tolerance set to 10 ppm, fragment tolerance set to 0.1 Da, and trypsin cleavage specificity (cleavage at K, R except if followed by P) allowing for two missed cleavages. Carbamidomethylation of cysteine is set as fixed modification, methionine oxidation, and TMT-modification of N-termini and lysine residues are set as variable modifications.
3. Data validation of peptide and protein identifications is done at the level of the complete dataset consisting of combined Mascot search results for all individual samples per experiment via the PeptideProphet and ProteinProphet modules in TPP using the decoy database function, nonparametric model, and the standard peptide probability threshold of 0.05.
4. TPP output (Interact) files containing peptide and protein information and corresponding TMT reporter ion peak intensity information for each peptide-to-spectrum match (as parsed from the Mascot result files) are uploaded to Oracle using in-house software.
5. For each peptide sequence and modification state, reporter ion signal intensities from all spectral matches are summed for each reporter ion type to account for lower precision of reporter ion ratios at low absolute signal intensities. Summed intensities are corrected according to the isotope correction factors given by the manufacturer to account for isotope impurities. Only peptide-to-spectrum matches that are unique assignments to a given identified protein within the total dataset are considered for protein quantitation.
6. High confidence protein identifications are reported based on protein probability cut-off values based on a false positive prediction of $<1\%$ by ProteinProphet.

7. For each protein in the high confidence dataset, individual peptide fold changes are calculated by normalizing each channel to the control channel (here: 1 μM probe, no competition compound, *see* **Notes 14** and **15**).
8. Protein fold changes over control are derived as median peptide fold change per protein.
9. Data for proteins with ≥2 quantified peptides are visualized in Spotfire (*see* the scatter plot in Fig. 3a) and the curve fitting function in Spotfire is used for the generation of probe titration curves and determination of concentrations where 50% enrichment is observed (Fig. 3b).

4 Notes

1. We have found that the different PAL moieties are quite stable under general laboratory conditions and handling. In an abundance of caution, we do recommend storing DMSO solutions protected from light; that is, in amber vials/tubes or wrapped with protective foil. Extreme precautions, such as working in as little as possible light, have not made a difference in our hands either with regard to compound stability or assay performance.
2. To generate Kasil fritted capillaries, mix 450 μL Kasil®1624 potassium silicate and 88 μL formamide in a glass vial and vortex. Dip fused silica capillary into the mixture for a few seconds to draw the mixture into capillary. Polymerize at 100 °C for 1 h and trim the frit to 1 mm.
3. The exact number of plates needed for assessing phenotypic relevance of specific binders will depend on the number of compounds in the SAR series. One plate of cells will be needed for each compound to be assessed; additionally, one plate for a DMSO control and one plate for a no PAL probe control will also be needed.
4. The HEK293T cells stably expressing V5-tagged mGpr39 used here can be incubated in media with 0% FBS without any apparent deleterious effects; e.g., cells lifting from the plate or change in cell morphology. However, other cell lines may require the presence of FBS to remain healthy. The concentration of FBS should be kept as low as possible to reduce nonspecific labeling of serum proteins by the PAL probe.
5. For de novo target deconvolution, we have found 60 min preincubation with competition compound followed by 60 min photoaffinity labeling probe treatment to be a good general starting point.
6. The UV bench lamp is generally allowed to warm up for at least 20 min prior to use. We have found it useful to monitor the UV

bench lamp performance by checking the UV intensity prior to irradiating cells using a Blak-Ray UV intensity Meter (Model J-221). Under optimal conditions the UV intensity will be out of range for the J-221 even at the highest setting. If the UV intensity falls within the range of the J-221 the UV bulbs are replaced immediately.

7. While there is no Federal or State regulatory limit for ultraviolet (UV) radiation exposure in the United States, it is worth emphasizing the importance of wearing full personal protection gear (lab coat, gloves, and protective eyewear) while working with the UV bench lamp. Operating the UV bench lamp in a cold room helps to keep the cells cold during the irradiation process and keeps UV exposure limited to samples.
8. Keratins from hair and skin are frequent contaminants in proteomics samples. Use gloves, tie back hair, and wear full-length lab coat during all the following procedures and sample handling. Use fresh buffers whenever possible.
9. Incubating the samples at 95 °C for 5 min creates a significant amount of pressure in the Mobicol columns. It is important to loosen the screw caps (~1/4 turn) before removing the bottom plug.
10. For probe titration experiments, due to contributions from nonspecific background, the total protein levels should generally increase with increasing probe concentrations. For competition experiments, the total protein levels should generally be consistent, i.e., independent of the concentration of competitor compound used in the experiment.
11. For improved coverage of transmembrane proteins that often yield few tryptic peptides in the optimal *m/z* range for MS-based peptide sequencing, we have found that a double digestion strategy using a chymotrypsin predigestion step prior to standard trypsin digestion can be beneficial. However, it should be noted that due to lower reporter ion yield, chymotryptic peptides in a predominantly tryptic peptide background can show exacerbated fold change compression.
12. It is recommended to check TMT labeling efficiencies prior to further sample separation by analyzing aliquots of individual TMT reactions by LC-MS/MS, and comparing signal intensities (or peptide-to-spectrum matches) for the fully labeled peptide species to the corresponding non/under-labeled species. If low labeling efficiency is observed (<95%), check that lyophilized TMT reagents are fully solubilized and that the pH is ~8 throughout the reaction.
13. The benefits of this step are two-fold. Beyond increasing the analytical depth by reducing complexity of individual fractions, it reduces fold change compression observed in all isobaric

labeling strategies including TMT [8]. Alternatively, fold change compression can be addressed by MS3-based approaches to peptide quantitation as available, e.g., on Thermo Orbitrap Fusion Lumos mass spectrometers or to some extent computationally (e.g., [8]).

14. For competition experiments it is preferable to subsequently renormalize data using the median fold change of all quantified peptides or proteins to compensate for differences in total protein yield for each individual affinity purification.
15. We have found that a final concentration of 1 μM PAL probe is a good general probe concentration to use in competition experiments; however, in select cases, we have used the data from the PAL probe titration experiment to identify a more optimal PAL probe concentration. For example, if the target(s) of interest show (near-)saturable binding for a full log unit at 1 μM PAL probe, then a lower concentration of PAL probe could be more ideal.

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