Tracking Transient Protein-Protein Interactions in Targeted Protein Degradation using Proximity-Dependent Labeling Coupled to Mass Spectrometry

Shu You, Kristina Ivanov, Duc Tran, Clark Peterson, Craig Stumpf, Peggy Thompson, Alex Campos

Plexium, San Diego, CA

Shu.you@plexium.com

Abstract

Introduction. Protein-protein interactions (PPI) play an important role in virtually all cellular processes. Detecting changes in these interaction networks is critical to understand biological processes and drug mechanism of actions. Emerging technology, such as proximity labeling using TurboID, can capture weak and transient interactions in the context of live cells, making it a powerful tool to study drug-induced protein-protein interactions. To enable the study of PPIs driven by Targeted Protein Degradation (TPD), we developed an automated sample preparation workflow using the AssayMap Bravo platform to identify the interactions of specific E3 ligases following cell treatment with monovalent degraders.

Novel aspect. An automated 96-well plate proteomics sample preparation workflow for biotinylated protein samples using Agilent AssayMap was developed to identify PPI's innate/endogenous and induced by small molecules. Our workflow allows the sample preparation and LC-MS analysis of up to 96 proximity labeled samples in less than 2 days.

Automated proteomics workflow for proximity labeling samples using Bravo AssayMap Platform, Evosep and TimsTOF technology

1. Cell preparation and lysis:

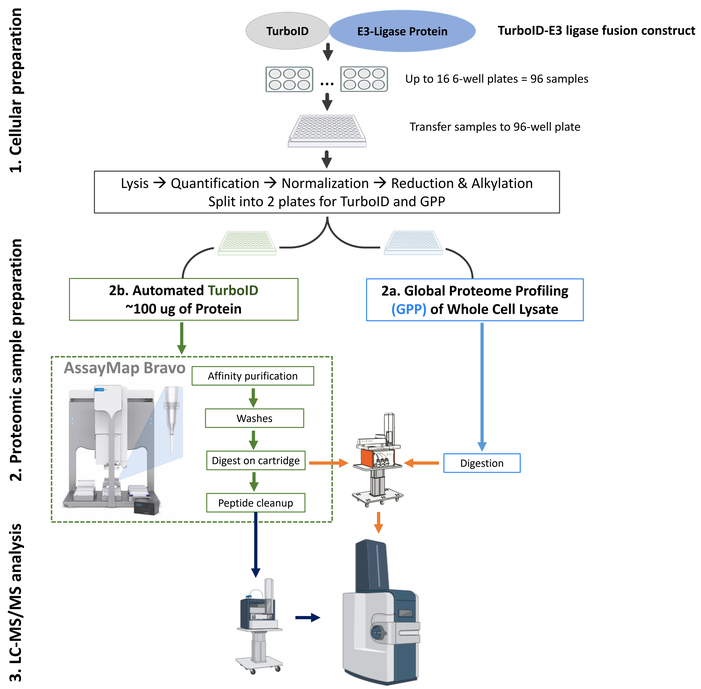
- Cells are seeded on 6-well plates and transfected with an E3 ligase-TurboID fusion construct
- Treat cells with monovalent degraders or DMSO control plus bortezomib for an appropriate incubation time
- Incubate with biotin for 1 hour to allow labeling of proteins proximal to the E3 ligase-TurboID proximity
- Cells are then lysed (8M Urea, 50mM AMBIC, 200U/mL Benzonase)
- Protein disulfide bonds are reduced (5mM TCEP, 60 min, 30°C) and alkylated (15mM IAA, 30 min, RT)
- Resulting lysates are subjected to 2 parallel proteomic sample preparation workflows below

2. Proteomic sample preparation

- **2a.** ~10% of each lysate was subjected to a standard **G**lobal **P**roteome **P**rofiling (GPP) workflow in which expression level of the TurboID-E3 Ligase construct was confirmed and the effect of monovalent degraders on the whole proteome was assessed.
- **2b.** The majority of each lysate (100µg) was subjected to an automated TurboID Sample Preparation Workflow where biotinylated proteins are pulled down using a streptavidin cartridge on the AssayMap Bravo Platform and directly digested. The resulting peptide digest was desalted on the same AssayMap platform or using Evotips.

3. LC MS/MS analysis

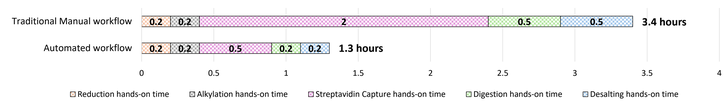
- Desalted peptides were analyzed by LC-MS/MS using diaPASEF on an Evosep/nanoElute liquid chromatography coupled to a Bruker TimsTOF HT mass spectrometer. All data analysis was performed with DIANN software (v 1.8.1) and in-house R-based statistical test pipeline and Metascape web tool (metascape.org).



Automated workflow significantly reduces operator's hands-on time required for sample preparation

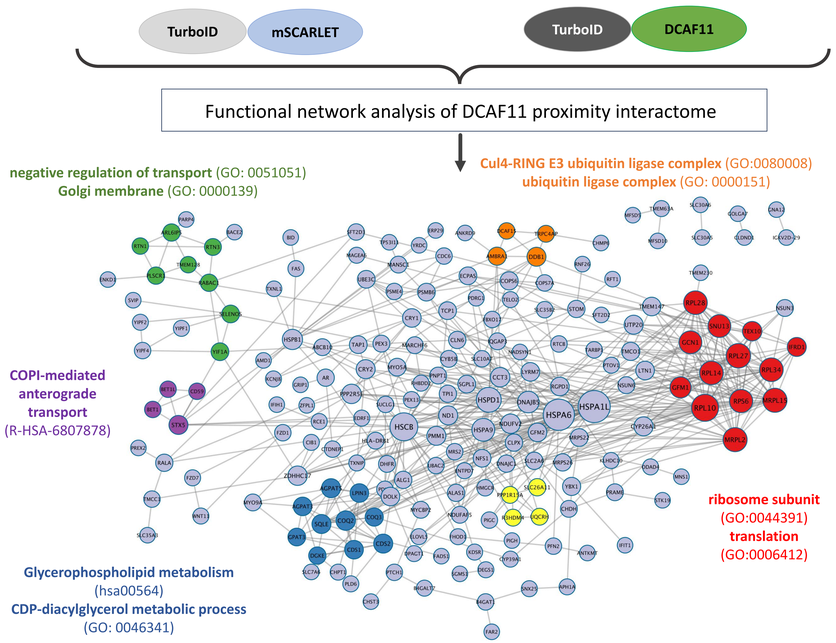
- In the traditional manual workflow, samples are incubated with streptavidin beads for 3 hours, including the wash steps before and after incubation, then cysteine disulfide bonds are reduced for 1 hour, alkylated for 30min, followed by on-bead protein digestion overnight. Peptides are then desalted before being subjected to LC-MS analysis. This workflow requires roughly ***2 days***.
- With our automated workflow presented here, cysteine-reduced and alkylated protein samples are loaded onto the streptavidin cartridges using the Bravo AssayMap system. Captured biotinylated proteins are then directly digested while bound to streptavidin. The resulting peptide digest can be desalted on the same platform or loaded directly to Evotip cartridges for LC-MS analysis.
- Our automated workflow requires approximately 1.3 hours of operator's hands-on time to set up the process, allowing preparation of up to 96 samples for LC-MS analysis within a working day (less than 8 hours including instrument and incubation time).

Operator's hands-on time required for sample preparation in Traditional vs. Automated workflow (in hours)



Proximity labeling TurboID technology unveils novel potential substrates of the DCAF11 E3 ligase

We have used our workflow to explore the endogenous substrates of E3 ligases of interest for TPD. Here, we show potential interactions of DCAF11; as a background negative control we used mScarlet-TurboID construct. Cells were seeded in a 6-well plate, transfected with either DCAF11 or mScarlet fused with TurboID, then treated with bortezomib for 4 hours and biotin for an additional hour before being lysed and subjected to our automated sample process workflow. Analysis of biotinylated proteins significantly enriched in DCAF11 compared to mScarlet identified numerous potential interactors. Using Metascape we carried out PPI enrichment analysis of all significantly enriched proteins against STRING, BioGrid, OmniPath, and InWeb_IM databases. The network below shows the subset of proteins that form physical interactions with at least one other member in the list. Using Molecular Complex Detection algorithm, we identified a number of densely connected network components (highlighted below in different colors) such as Cul4-RING E3 ubiquitin ligase complex (e.g., DDB1, TRPC4AP, AMBRA1, DCAF15), COPI-mediated anterograde transport (e.g., BET1, BET1L, CD59, STX5), Golgi membrane proteins that play a role in regulation of transport (e.g., RTN3, YIF1A, RBAC1), and COP9 signalosome (e.g., HSPA1L, HSPA6, COPS6, COPS7A).



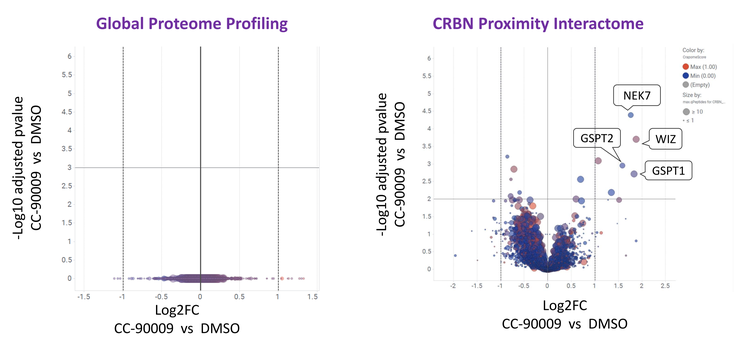
Automated workflow offers high accuracy and sensitivity to detect drug-induced protein-protein interactions

To demonstrate the potential of the proximity labeling workflow to profile drug-induced protein interactions with E3 ligases of interest such as CRBN and DCAF11, we profiled two selective and potent degraders, GSPT1 degrader eragidomide (CC-90009) and Plexium's BRD4 monovalent degrader PLX-3618.

Cereblon-TurboID

First, we used cereblon (CRBN)-TurboID system (HEK293) with the molecular glue eragidomide (CC-90009), a known GSPT1 degrader. Cells were seeded in a 6-well plate, transfected with CRBN-TurboID construct, treated 1 hour with bortezomib, followed by 1µM eragidomide for 4 hours. The samples were then treated with 100mM biotin for an additional hour, lysed and subjected to our automated sample process workflow. At the global proteome level, there was no statistically significant regulation of protein abundance driven by drug treatment as shown in the volcano plot below (left side). For the CRBN proximity interactome (volcano plot below, right side), we observed statistically significant increased proximity for the known neosubstrate, GSPT1, as well as other previously reported neosubstrates such as GSPT2, NEK7 and WIZ.

Global Proteome Profiling | **CRBN Proximity Interactome**

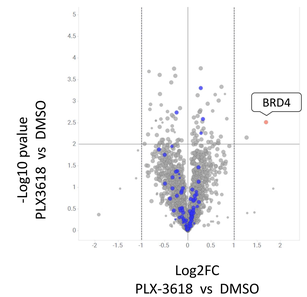


Volcano plot of CC-90009 vs DMSO at the total proteome (left side) and proximity interactome (right side) levels. The number of peptides used for the statistical test is coded by the size of each data point; proteins with 10 or more peptides are represented by the large points and proteins with few peptides are represented by small data points.

DCAF11-TurboID

To test our workflow with an alternative E3 ligase, we transfected HEK293 cells with DCAF11-TurboID construct and treated with Plexium's BRD4-selective monovalent degrader (1µM for 4 hours followed by 100mM biotin treatment for 1 hour). The volcano plot below shows a highly selective PLX-3618-induced enrichment of BRD4 over other bromodomain and extraterminal domain (BET) family members.

To further investigate the selectivity of PLX-3618 and the robustness of the proximity labeling platform to detect compound-induced interactions, we highlighted (colored in blue) on the volcano plot all proteins previously described to interact with BRD4 (from BioPlex or STRING databases). Interestingly, most known BRD4 protein interactions show no increase in biotinylation, suggesting that PLX-3618 mediates a selective interaction between DCAF11 and BRD4.



Conclusions

- Presented here is an automated proximity labeling sample preparation workflow for LC-MS/MS analysis using the Agilent AssayMap platform. The workflow requires only ~100µg of lysate input (~20x less than recommended in traditional protocols).
- The entire sample preparation process from cells to MS analysis takes as little as 8 hours (with less than 1.5 hours of hands-on operator) and a throughput of 96 samples per day. In addition, using Evosep system coupled to the TimsTOF MS allowed data acquisition for 96 samples in 24 hours using the 100 SPD Evosep method.
- Altogether, this data validates the automated and streamlined proximity labeling workflow that can be utilized to identify both the endogenous and compound-induced protein-protein interactome with E3 ligases of interest.