Direct quantification of cell type-specific proteins using Luminex assays with TurboID-labeled cells and tissues

Authors: Sara Bitarafan[#,1,2], Brendan Tobin[#,1,3], Claudia Espinosa-Garcia[4], Srikant Rangaraju[4], Levi B. Wood[1,2,5]

[1]Parker H. Petit Institute for Bioengineering, Georgia Institute of Technology, Atlanta, GA, USA
[2]George W. Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA, USA
[3]School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, USA
[4]Department of Neurology, Yale University, New Heaven, CT, USA
[5]Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA, USA

[#]Equally contributed as first authors

Abstract: Cell type-specific proteome labeling provides enhanced understanding of cellular function and structure within tissues by tagging proteins during translation, while cells and tissues are in their "native" states. TurboID is a methodology to enable rapid and efficient biotinylation of proteins. New TurboID viral constructs and a Cre-mediated TurboID transgenic mouse line enable cell type-specific proteomic investigations in cell culture and *in vivo* settings. Together, these new tools enable diverse studies designed to interrogate individual cell type contributions within complex multi-cellular systems. While biotin-based labeling enables enrichment of the labeled proteome via immunoprecipitation, it is also compatible with biotin/streptavidin-based immunoassays, including the Luminex xMAP multiplexed immunoassay platform. Here, we detail protocols to utilize existing commercially available Luminex kits to directly detect TurboID-biotinylated (and therefore cell type-specific) proteins of interest from cell culture and bulk tissue samples. Luminex immunoassays have multiple advantages compared to other methodologies, including 1) requiring small sample volumes/masses, 2) direct immuno-reaction-based quantification from a target sample without intermediate processing steps, 3) reduced costs and 4) direct readout. Below, we describe an adapted Luminex xMAP protocol to quantify phospho-proteins and cytokine from TurboID-labeled cells or tissues with cell type-specificity.

Keywords: TurboID, Proteomics, Bead-based multiplex immunoassay, Luminex, Cell type-specific proximity labeling

Methods:

I. **Methods to detect biotinylated phospho-proteins from tissue and cell lysates.**
II. **Methods to detect biotinylated cytokines from tissue lysates and cultured cells.**

I. Methods for quantification of biotinylated phospho-proteins using adapted Luminex xMAP assays from tissues and cells

Definition: Protein phosphorylation is a post-translational modification, and a key regulator of protein function within cellular systems (1, 2). Therefore, quantification of phospho-proteins is essential to understand how signaling cascades evoke cellular activity and phenotypes during physiological and pathophysiological states. Single cell transcriptomic analyses of human and mouse tissues reveal distinct transcriptional signatures associated with the unique functions of different cell types (3-9). Because transcriptional changes do not reflect all protein-level changes, phospho-proteomic profiling of specific cell types enables an explicit understanding of a cellular mechanism under physiological or pathophysiological states. An adapted Luminex assay provides a powerful tool to quantify cell type-specific phospho-proteins in TurboID-labeled tissues and cells (**Fig. 1**).

Rationale: The adapted Luminex xMAP assay can be used with TurboID to quantify the cell type-specific quantification of phospo-proteins from both *in-vivo* and *in-vitro* systems. The standard Luminex xMAP technology utilizes fluorescent magnetic beads conjugated with capture antibodies and biotinylated detection antibodies, both specific to each analyte (14, 15). Streptavidin-phycoerythrin is then coupled to the biotinylated detection antibodies and produces a fluorescent readout proportional to analyte concentration. In the TurboID system, a promiscuous biotin ligase, TurboID, ligates biotin onto proteins (12). Therefore, the biotinylated detection antibodies are omitted in this adapted xMAP protocol and SAPE will bind to the endogenously biotinylated proteins. Since TurboID can be designed to only be expressed in a specific cell type, this assay enables cell type-specific resolution. When detecting intracellular proteins, such as phospho-proteins, in *in-vitro* systems, cell lysates commonly serves as the starting material, and in *in-vivo* systems, tissue lysate is commonly the starting material (**Fig. 1**)

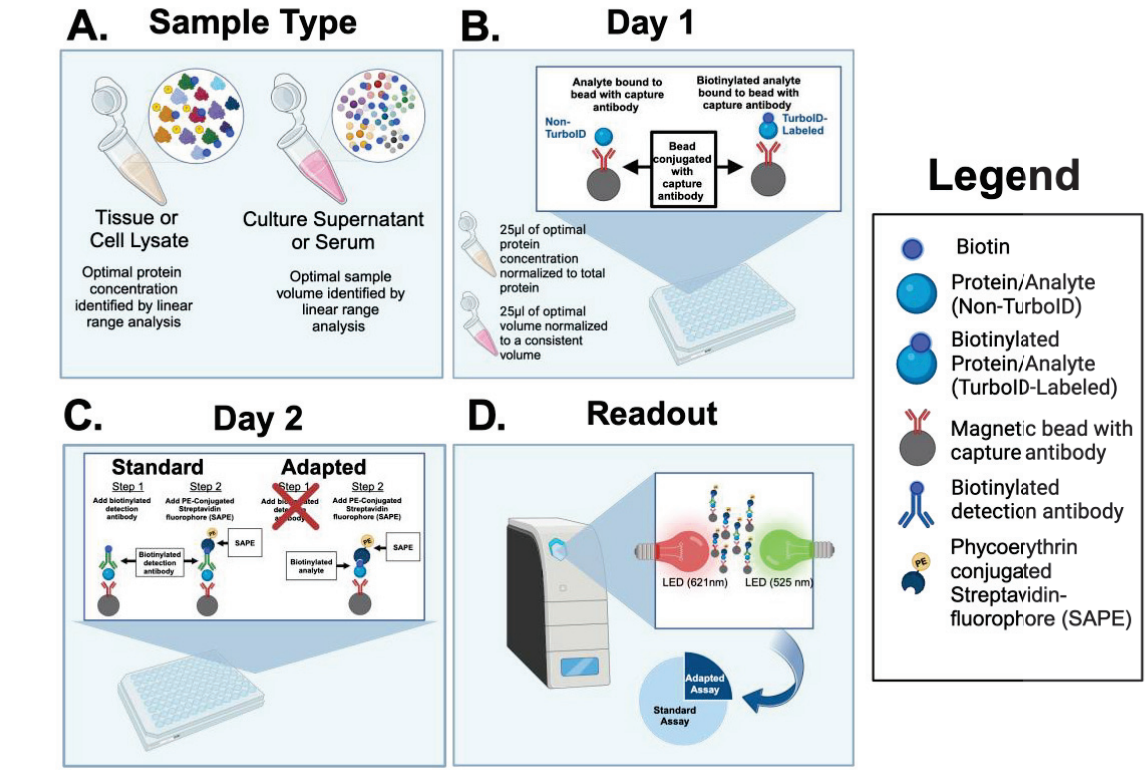

Figure 1. Detection of TurboID-labeled proteins using Luminex xMAP assay. (**A**) Fresh or flash frozen tissue/cell lysates, serum and culture supernatants are all compatible with the Luminex xMAP adapted assay. Optimal loading concentrations for all sample types are identified by linear range analysis (16). (**B**) 25µL of lysates are loaded at optimal protein concentrations while serum and culture supernatant are loaded at optimal volume. Magnetic bead with capture antibody captures both TurboID-labeled and non-labeled proteins. (**C**) In a standard Luminex xMAP assay, biotinylated detection antibody is added, followed by SAPE. In contrast, in the Luminex adapted assay, the biotinylated detection antibody is omitted because the protein is natively biotinylated by TurboID. (**D**) Plates are read on a Luminex MAGPIX (or similar) instrument per manufacturer protocol. While the standard assay detects all proteins from all cell types, the adapted assay only labels cell type-specific TurboID-labeled proteins/analytes. Created in BioRender. Wood, L. (2024) BioRender.com/d78q702.

Reagents:

- xMAP phospho-protein detection kit (e.g. Millipore Sigma MILLIPLEX MAPK/SAPK Signaling 10-Plex Kit, Cat No. 48-660MAG)
- Luminex magnetic beads conjugated with capture antibody
- Streptavidin-phycoerythrin (SAPE)

- Assay buffer
- 0.1% phosphate buffered saline (PBS) – Tween 20 (PBST)
- MAGPIX drive fluid (or sheath fluid for certain Luminex instruments)

Equipment:

- Luminex MAGPIX instrument (or other compatible Luminex instruments)
- Laboratory vortex mixer
- Adjustable pipettes with tips capable of delivering 1 µL to 1000 µL
- Multichannel pipettes capable of delivering 1 µL to 200 µL
- Reagent reservoirs
- Polypropylene microfuge tubes
- Aluminum foil
- Orbital plate shaker
- Magnetic separation plate
- Thin Bottom 96 well plate
- PCR plate sealer

Protocol:

Preparation for two-day assay: Phospho-protein detection protocols are the same for tissue or cell homogenates, except for sample collection and protein extraction steps, which should be selected based on the sample type (17). Using 8M urea lysis buffer with proteinase/phosphatase inhibitors (urea lysis buffer cocktail) (18) is recommended because it is compatible with protein affinity purification experiments, such as immunoprecipitation (IP), but other buffers, such as RIPA, may also work. Please note, this protocol was established based upon the Millipore Sigma MILLIPLEX workflow but is expected to be compatible with most bead-based multiplex immuno-assays across vendors.

Day 1:

Preparation of reagents and plates

1. Prepare 1x wash buffer solution by mixing 0.1% Tween 20 in 1x PBS.
2. Wash thin bottom 96 well plate by adding 200 µL of wash buffer into each well, mixing on plate shaker for 10 min at 750 rpm, then decant all well contents.
3. Allow all xMAP kit reagents to warm to room temperature.

Preparation of tissue or cell lysates

1. If frozen, thaw lysates on ice, then centrifuge samples (4°C, 10 min, 13,000 rpm) to pellet precipitate.
2. Prepare 25 µL of sample at the optimal protein concentration normalized to total protein using BCA or similar assay. To normalize total volume across samples, dilute samples in lysis buffer cocktail.

Note: Optimal protein concentration should be identified by linear range analysis (16).

Adapted immunoassay procedure for capturing biotinylated phospho-proteins

1. Add 25 µL assay buffer to all wells.
2. Add 25 µL of urea lysis buffer cocktail to background wells. Background wells are used to define background level of fluorescent intensity read by instrument without presence of sample.
3. Add 25 µL of diluted samples to sample wells.
4. Add 25 µL of 1x multiplex magnetic beads to all wells.
   Note: The Millipore Luminex phospho-protein kits provide multiplex magnetic beads at 20x stock solution. Vortex 20x stock multiplex magnetic bead solution for 2 min, and then dilute it in assay buffer to 1x solution. Vortex 1x multiplex magnetic bead for 1 min before adding to wells.
5. Seal the plate with a plate sealer and cover the plate with aluminum foil to protect beads from light exposure. Incubate on a shaker at 750 rpm, overnight (16-20 hours) at 2-8 °C.

Day 2:

Adapted immunoassay procedure for detection of biotinylated phospho-proteins

1. Place 96 well plate on magnetic separator for 2 min. Decant well contents while the plate is still attached to the magnetic separator.
2. Wash the 96 well plate 2x times as follows:
   a. Add 200 µL wash buffer (0.1% Tween 20 in PBS)
   b. Place the plate on plate shaker (750 rpm) for 30 seconds at room temperature
   c. Place the plate on the magnetic separator for 2 min at room temperature
   d. Decant well contents while well plate is still attached to magnetic separator.
3. Dilute 25x stock streptavidin-phycoerythrin in assay buffer to 1x. Add 25 µL 1x streptavidin-phycoerythrin to each well. Cover the plate with foil to protect light from exposure. Incubate for 15 min on plate shaker (750 rpm) at room temperature.
4. Add 25 µL of 1x amplification buffer to each well. Cover with foil. Incubate for 15 min on plate shaker (750 rpm) at room temperature.
5. Place the well plate on the magnetic separator for 2 min. Decant well contents, detach from magnetic separator.
6. Add 150 µL Luminex drive fluid. Re-suspend beads on plate shaker (750 rpm) for 10 min at room temperature.
7. Read on Luminex MAGPIX instrument per manufacturer protocol.

II. Adapted Luminex xMAP assay for quantification of biotinylated cytokine/chemokine proteins in cells and tissues

Definition: Cytokines and chemokines produced by cells in response to stimuli have significant immunomodulatory potential. These proteins are produced and then secreted by cells in response to an immune stimulus and trigger anti- or pro-inflammatory responses (19). Cytokines have been implicated in various inflammatory diseases and some are the target of approved therapeutics. Therefore, quantifying their expression serves immense value in characterizing immune physiology. TurboID biotin proximity-dependent labeling provides cell type-specific cytokine labeling, both within tissues and secreted into the culture medium of cultured cells. A modified Luminex assay provides a powerful tool to quantify cell type-specific cytokine labeling in TurboID-labeled tissues and cells (**Fig. 1**).

Rationale: The adapted Luminex xMAP assay can be used with TurboID to quantify the cell type-specific expression of cytokines from both *in-vivo* and *in-vitro* systems. The standard Luminex xMAP technology utilizes fluorescent magnetic beads conjugated with capture antibodies and biotinylated detection antibodies, both specific to each analyte (14, 15). Streptavidin-phycoerythrin is then coupled to the biotinylated detection antibodies and produces a fluorescent readout proportional to analyte concentration. In the TurboID system, a promiscuous biotin ligase, TurboID, ligates biotin onto proteins (12). Therefore, the biotinylated detection antibodies are omitted in this adapted xMAP protocol and Streptavidin-phycoerythrin (SAPE) will bind to the endogenously biotinylated proteins. Since TurboID can be designed to only be expressed in a specific cell type, this assay enables cell type-specific resolution. When detecting extracellular proteins such as cytokines and chemokines in *in-vitro* systems, cell culture medium commonly serves as the starting material, and in *in-vivo* systems, tissue lysate is commonly the starting material (**Fig. 1**)

Reagents:

- xMAP cytokine detection kit (e.g. Millipore Sigma MILLIPLEX Human Cytokine/Chemokine/Growth Factor Panel A – Immunology Multiplex Assay, Cat No. HCYTA-60K)
- Luminex magnetic beads conjugated with capture antibody
- Streptavidin-phycoerythrin (SAPE)
- Assay buffer
- 0.1% Phosphate Buffered Saline – Tween 20 (PBST)
- MAGPIX drive fluid (or sheath fluid for certain Luminex instruments)

Equipment:

- Luminex MAGPIX instrument (or other compatible Luminex instruments)
- Laboratory vortex mixer
- Adjustable pipettes with tips capable of delivering 1 µL to 1000 µL

- Multichannel pipettes capable of delivering 1 µL to 200 µL
- Reagent reservoirs
- Polypropylene microfuge tubes
- Aluminum foil
- Orbital plate shaker
- Magnetic separation plate
- Thin bottom 96 well plate
- PCR plate sealer

Protocol:

Preparation for two-day assay: Like for phospho-proteins, cytokine and chemokine detection protocols are the same for tissue homogenates and cell homogenates/media, except for sample collection and protein extraction steps, which should be selected based on the sample type (17). Using 8M urea lysis buffer with proteinase/phosphatase inhibitors (urea lysis buffer cocktail) (18) is recommended because it is compatible with protein affinity purification experiments, such as immunoprecipitation (IP), but other buffers, such as RIPA, may also work. Please note, this protocol was established based upon the Millipore Sigma MILLIPLEX workflow but is expected to be compatible with most bead-based multiplex immuno-assays across vendors.

Day 1:

Preparation of reagents and plates

1. Prepare 1x wash buffer solution by mixing 0.1% Tween 20 in 1x PBS.
2. Wash thin bottom 96 well plate by adding 200 µL of wash buffer into each well, mixing on plate shaker for 10 min at 750 rpm, then decant all well contents.
3. Allow all xMAP kit reagents to warm to room temperature.

Preparation of lysates and culture medium:

1. If frozen, allow frozen tissue lysate or culture medium to thaw on ice then centrifuge samples (4°C, 10 min, 13,000 rpm) to remove precipitate.
2. **Tissue Lysate**: Prepare 25 µL of sample at the optimal protein concentration normalized to total protein using BCA or similar assays. To normalize total volume across samples, dilute samples in lysis buffer cocktail.
   Note: Optimal protein concentration should be determined by linear range analysis to identify a linear range between the amount of sample loaded and resulting instrument readout (16).
   Cell Medium: Prepare 25 µL of sample at the optimal volume normalized to total volume. To normalize total volume across samples, dilute samples in fresh untreated culture medium or assay buffer.
   Note: For medium and serum, samples should be normalized to equal sample volume loaded. Optimal loading should be determined using a linear range procedure (16).

Adapted immunoassay procedure for capturing biotinylated cytokines

1. Add 25 µL assay buffer to all wells.
2. **Tissue Lysates**: Add 25 µL of urea lysis buffer cocktail (or any lysis buffer used for tissue lysing) to background wells. Background wells are used to define background level of fluorescent intensity read by instrument without presence of sample.
   Cell Medium: Add 25 µL of fresh untreated culture medium or assay buffer to background wells. Background wells are used to define background level of fluorescent intensity read by instrument without presence of sample.
3. Add 25 µL of diluted samples to sample wells.
4. Add 25 µL of 1x premixed multiplex magnetic beads to all wells. Note: Vortex 1x multiplex magnetic bead for 1 min before adding to wells.
5. Seal the plate with a plate sealer and cover the plate with aluminum foil to protect beads from light exposure. Incubate on a shaker at 750 rpm, overnight (16-20 hours) at 2-8 °C.

Day 2:

Adapted immunoassay procedure for detection of biotinylated cytokines

1. Place 96 well plate on magnetic separator for 2 min. Decant well contents while the plate is still attached to the magnetic separator.
2. Wash the 96 well plate 2x times as follows:
   a. Add 200 µL wash buffer (0.1% Tween 20 in PBS)
   b. Place the plate on shaker for 30 seconds at room temperature
   c. Place the plate on the magnetic separator for 2 min at room temperature
   d. Decant well contents while well plate is still attached to magnetic separator.
3. Add 25 µL of 1x SAPE and 25 µL of assay buffer to each well. Wrap the plate in foil and incubate for 30 min on plate shaker (750 rpm) at room temperature.
4. Place the well plate on the magnetic separator for 2 min. Decant well contents, detach from magnetic separator.
5. Wash the 96 well plate 2x times as follows:
   a. Add 200 µL wash buffer (0.1% Tween 20 in PBS)
   b. Place the plate on shaker for 30 seconds at room temperature
   c. Place the plate on the magnetic separator for 2 min at room temperature
   d. Decant well contents while well plate is still attached to magnetic separator.
6. Add 150 µL Luminex drive fluid. Re-suspend beads on plate shaker for 10 min at room temperature at 750 rpm.
7. Read on Luminex MAGPIX instrument per manufacturer protocol.

Note: The protocol for phospho-proteins, and cytokines and chemokines are highly similar. The main distinctions lie in the sample sourcing and loading and in Day 2 of the protocol. Given the extracellular nature of cytokines and chemokines, they are commonly isolated from cell media rather than cell lysate and are thus loaded by volume rather than protein mass. On Day 2, the cytokine kits require that the SAPE be incubated with the sample for 30 minutes before decanting, washing with PBST and adding drive fluid. Alternatively, the sensitivity of the phospho-proteins requires the addition of amplification buffer for 15 minutes after the SAPE has been incubating for 15 minutes, the cytokine kit does not require this reagent. Otherwise, the adapted protocol for each is the same.

Analysis and statistics: Cell type-specific biotinylated phospho-protein and cytokine levels are quantified via adapted Luminex immunoassay in TurboID lysates. The output from the Luminex MAGPIX instrument provides fluorescent intensity for all measured analytes across samples and backgrounds measured in arbitrary units. To prepare the data for further analysis, the mean signal of each analyte measured from background wells should be subtracted from each analyte across all samples. Various statistical methods can be used to identify analyte differences between groups using Luminex readouts. For example, **Fig. 2** shows a partial least squares discriminant analysis (PLSDA) using published adapted Luminex data (12) from TurboID labeled brain lysates. The specific TurboID mouse strains used were *Rosa26*$^{TurboID/wt}$/Camk2a and *Rosa26*$^{TurboID/wt}$/Aldh1l1, which enabled the specific biotinylation of proteins expressed in either Camk2a neurons or Aldh1l1 astrocytes. An adapted Luminex assay was performed to quantify 21 phospho-proteins in the MAPK and PI3K/Akt pathways following the adapted protocol detailed herein with brain tissues lysed in 8M urea. The PLSDA identifies a latent variable (LV1) that separates the Aldh1l1 astrocyte-labeled proteins (negative) and Camk2 neuron-labeled proteins (positive) (**Fig. 2A**). LV1 consists of a profile of phospho-proteins (**Fig. 2B**) that are correlated with astrocytes (negative) or with neurons (positive). Error bars represent standard deviation in a leave one out cross validation (LOOCV), wherein each sample is iteratively left out, and provide an indication of the variability within each signal among the PLSDA models generated. Multivariate analyses, such as PLSDA, may be accompanied by standard univariate methods. These data highlight the ability of TurboID native state labeling to identify differences in signaling between cell types.

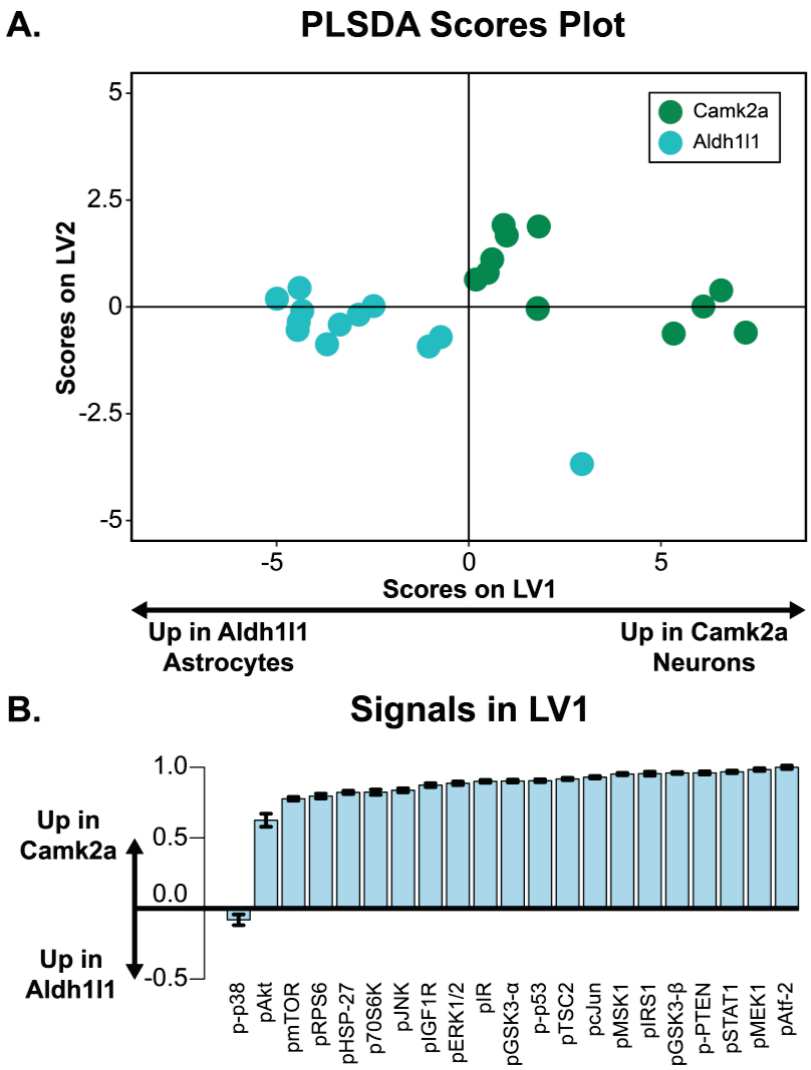

Figure 2. Example of cell type-specific analysis using TurboID system with adapted Luminex assay: (**A**) Partial least squares discriminant analysis of phosphorylated PI3K/Akt and MAPK proteins separated astrocyte-labeled proteins (left) from neuron-labeled proteins (right). (**B**) LV1 is composed of a profile of phospho-proteins that are elevated in either neurons (positive) or astrocytes (negative) (mean±SD in leave-one-out cross validation).

Related techniques: There are multiple alternative approaches to measure proteins in TurboID-labeled brain homogenates. Some of the techniques are low throughput, such as Western blot analysis or sandwich ELISA, following biotin immunoprecipitation (IP). Alternatively, high throughput assays, such as mass spectrometry (MS) coupled with biotin IP, can be used as an approach for proteome quantification. MS-based phospho-proteomics offer a broader range of proteins detected compared to Luminex-based assays. However, the Luminex-based immune assay has multiple advantages, including 1) requiring small sample volumes/masses, 2) direct immuno-reaction-based quantification from a target sample without intermediate processing

steps, 3) reduced costs, and 4) direct readout. Collectively, adapted Luminex assays offer an efficient, reliable, and sensitive protein quantification method for TurboID-labeled samples.

Pros and cons of adapted Luminex assay:

Pros	Cons
Similar workflow to traditional ELISA	Labels proximal proteins, but not specifically interacting proteins
Direct quantification from bulk homogenate or culture medium	Biotinylation may affect cellular functions
Low cost compared to MS	Luminex assay have limit of detection for some analytes
High sensitivity to detect phospho-protein compared to MS	
Easily interpretable	
Rapid preparation	
Very small amount of sample protein is needed compared to other techniques	
Capable of detecting low abundance proteins	

Alternative methods/procedures:

Cell type-specific protein expression can be characterized using other approaches that do not rely on TurboID. One method includes dissociating tissue and isolating cells of interest using fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) to sort cells based on cell markers. The proteome of the purified cell population can then be profiled using LC-MS (20) or other molecular approach. Another method includes using methionyl-tRNA synthetase^{L274G} (MetRS*)-based azidonorleucine (Anl) labeling for analyzing intercellular interactions in complex multi-cellular systems. However, this method does not give high protein yield limiting the number of proteins can be used for in-depth analysis. Alternatively, standard protocols for immunohistochemistry or immunocytochemistry to co-label proteins of interest and cell markers in tissue sections or cultured cells can be used to assess cell type type-specific expression. However, these approaches are difficult to quantify and have limited utility for identifying the source of extracellular/secreted proteins.

Troubleshooting and optimization:

Problem	Solution
Noisy readout	Repeat linear range analysis
Non-specific binding or high background signal	Increase washes at the start of day 2
Hook effect (antigen excess)	Further dilute samples

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