Background: Proteomics with multifunctionalized lipid probes

**This repository is still under construction, please forgive us for incomplete sections as we build features!**

## Interactomics Experimental schema

In this overview, we aim to explain the fundamental procedures of utilizing lipid probes in proteomics analysis. [Figure 1](#fig-ExperimentalSchema) depicts the steps described below

|  |
| --- |
| Figure 1 |

### Cell treatment

As has been reported previously by Höglinger et al. (2017), Muller et al. (2021), Farley et al. (2024), Farley et al. (2024), and Thomas et al. (2025), lipid probes are readily taken up by cell membranes within minutes upon treatment with lipid probes at concentrations ranging from 2 (as for trifunctional sphingosine and sphinganine) to 5 (as for trifunctional phosphatidic acid and phosphatidylethanolamine) to 100 (as for trifunctional diacylglycerol)1–5. These studies utilized the inherent fluorescence of the coumarin cage to validate the uptake and wide intercalation of the lipid probes throughout the cell6.

### Uncaging

Irradiation with 405 nm wavelength light induces the release of the coumarin photocage and thus allows for the recognition and metabolism of the headgroup of the lipid probe (See our [Coumarin overview](../Background/MultifunctionalLipidProbesOverview.qmd#sec-Coumarin) for more background on using the coumarin photocage).

Substantial work has validated that these lipid probes are recognized by the cell as their native counterparts, as the remaining functional groups have minimal effect on the probes’ biophysical properties. This has been validated in numerous studies, including Höglinger et al. (2015) and Höglinger et al. (2017), Muller et al. (2020) and Muller et al. (2020), and Farley et al. (2024) and Farley et al. (2024)1,2,7,8.

### Crosslinking

Irradiation with 355 nm wavelength light induces the production of a reactive carbene which readily inserts itself into any nearby biochemical bond (See our [Diazirine overview](../Background/MultifunctionalLipidProbesOverview.qmd#sec-Diazirine) for more background on diazirine crosslinking). Notably, the majority of probes will likely crosslink with either water or with neighboring lipids. Quantitative proteomics analysis will fully ignore these conjugates, as only enriched peptides will be measured in the final mass spectrometry analysis (described below in our [Mass spectrometry overview](@sec-MassSpectrometryAnalysis)).

The covalently bound lipid protein conjugates which form after crosslinking are highly stable and thus are ideally amenable toward stringent enrichment and washing, producing a high-quality sample with few non-specific proteins tagging along.

### Cell lysis and sample collection

Depending on application, may want to perform a membrane prep or otherwise isolate an organelle of interest (e.g. mitochondrial enrichment, if one is seeking mitochondrial interactions)

Due to the high stability of the diazirine crosslinking bond, no extra steps need to be taken to ensure that interactions are maintained – lysis can be as aggressive as one wishes.

In general, samples due for mass spectrometry analysis should be kept with as little detergent as possible to protect the instrument – however, because the crosslinking bond is covalent and the downstream affinity purification is covalent, these samples may be washed very stringently and thus remove effectively all contaminating detergents. Thus, a mild detergent such as NP-40 can be included in the lysis buffer of these samples.

### Affinity purification of probe-conjugated proteins

(See our [Alkyne overview](../Background/MultifunctionalLipidProbesOverview.qmd#sec-Alkyne) for more background on alkyne click chemistry)

* Example using azide beads: Perform click chemistry reaction in presence of beads coated with picolyl-azide functional groups for covalent capture
* Conjugates of protein-lipid-bead are covalently bound and can thus be stringently washed with detergent solutions to remove non-specific proteins. Detergent can then be removed with repeated high volume washes of 8 M urea and 20 % acetonitrile.
* Captured proteins are then reduced and alkylated on-bead.
* Peptides are digested off the protein-lipid-bead conjugates via tryptic digest. Digested peptides are then desalted to remove any remaining contaminants using reverse phase desalting columns.

### Mass spectrometry analysis

Broadly, all mass spectrometry techniques for proteomic identification of differentially-enriched proteins will involve injection into a mass spectrometer via liquid chromatography. Within the mass spectrometer, individual peptides can be isolated and fragmented to determine their sequence.

There are several methodologies commonly used to quantify mass spectrometry data. At the highest level, these may be separated into label-free and label-based quantification techniques. Both categories are valid for reporting differential enrichment, and each has pros and cons – discussed in detail below.

#### Label-Free Quantification

Label-free quantification (LFQ) is a widely used mass spectrometry-based proteomics approach that estimates protein abundance without chemical labeling. Instead, it relies on comparing the summed intensity of detected peptides across different samples analyzed under identical conditions.

##### How It Works

1. **Peptide Detection (MS1):** Peptides are isolated and detected based on their mass-to-charge ratio (m/z).
2. **Peptide Sequencing (MS2):** Selected peptide ions are fragmented, and the resulting spectra determine peptide sequences.
3. **Quantification:** The total signal intensity of all identified peptides corresponding to a protein is summed as a proxy for its abundance.
4. **Comparison Across Samples:** Samples prepared and analyzed using the same LC-MS/MS protocol can be compared using peptide-spectrum match (PSM) counts as an abundance proxy.

##### Advantages of Label-Free Quantification

* **Cost-Effective & Simple Sample Preparation:** Unlike labeling methods (e.g., TMT), LFQ does not require expensive reagents or complex modifications, making it more accessible.
* **Flexible & Widely Used:** Compatible with various experimental designs and allows for comparisons across many samples without multiplexing constraints.

##### Challenges & Considerations

* **Potential Bias from Co-Elution:** High-abundance peptides can overshadow low-abundance ones, reducing the number of reliably quantified peptides.
* **Run-to-Run Variability:** LFQ requires separate MS runs for each sample, making it susceptible to differences in instrument calibration, chromatography conditions, or sample handling. Careful quality control is necessary to minimize artificial differences.

##### Conclusion

Label-free quantification is a practical and widely used method for proteomics research. While cost-effective and flexible, its accuracy depends on careful experimental design and quality control to reduce variability.

#### Isotopic Label-based quantification

All label-based mass spectrometry quantification methods use stable isotopes to distinguish the source of proteins within a pooled collective of several experimental samples. Previously, the technique known as Stable Isotope Labeling with Amino acids in Cell Culture (SILAC) was common, though this has fallen out of use due to significant cost and difficult sample preparation.

An early (and still used) form of isotopic labeling was known as Stable Isotope Labeling with Amino acids in Cell culture (SILAC). This method allows for quantification of the relative protein abundances between two or three conditions in cell culture.

Modern isotopic label-based techniques such as iTRAQ (isotopic Tagging for Relative and Absolute Quantification) and TMT (Tandem Mass Tagging) have become the norm. Isotopic labeling is a powerful quantitative mass spectrometry technique that enables the simultaneous analysis of multiple samples in a single MS run. This approach improves efficiency and allows for precise relative quantification of proteins across experimental conditions. For the purposes of this overview, we will focus on TMT as it has greater capacity for samples per experiment (16+ as opposed to 8), and the techniques are fundamentally similar.

##### How SILAC Works:

1. **Isotopic Labeling:** Independent cell culturing conditions are pre-treated with media containing with differing amino acid composition, encoding the peptides of each condition with either “heavy” arginine and lysine residues (containing C13 isotopes) or “light” (containing a standard proportion of C12 isotopes). Media treatments are typically for several days or weeks to allow for substantial incorporation of heavy/light labeled isotopes into cells (this is typically dependent on the rate of cell division).
2. **Experimental conditions:** After isotopic labeling, the experimental conditions can be applied. In the case of lipid probe interactions studies, one would treat the cells with probe, and either uncage via UV irradiation or leave caged for control. The cells are then collected, lysed and protein is collected.
3. **Sample pooling:** After protein collection, samples can be pooled, digested, and desalted.
4. **Mass Spectrometry Analysis:**

* **MS1 (Peptide Isolation):** Heavy and Light peptides will differ in m/z depending on their composition of lysine and arginine (a heavy peptide with a single lysine residue will be 6 daltons heavier than its light counterpart). These Heavy/Light peptide pairs will be isolated independently for MS2 analysis.

- \*\*MS2 (Peptide Sequencing & Tag Fragmentation):\*\* Peptides are fragmented for sequence identification.

1. **Quantification:** The relative abundance of a given peptide can be calculated from the Heavy/Light MS1 ratio – thus, the quantification data is collected at the MS1 level, and is limited by the MS1 duty cycle. For this reason, SILAC can have a high sensitivity to abundant peptides “swamping” the signal of less abundant peptides. A high degree of fractionation is frequently needed to maximize protein identifications.
2. **Comparison and Statistics:** The fold-change in protein abundance is directly encoded in the Heavy/Light ratio of SILAC peptide pairs. Typically a Student’s t-test can be used to calculate the statistical significance of each protein.

##### Advantages of SILAC

* **Simple and Robust:** Because the isotopic labeling is accomplished via media feeding, there are no challenging chemical reactions and a high degree of incorporation can be achieved reliably *in vitro*. Similarly, the sample pooling occurs at a very early stage in the sample preparation process, limiting any biasing losses of one sample or its control.

##### Disadvantages/Limitations of SILAC

* **Long Label Incorporation Time:** Because the rate of label incorporation is dependent on the cell division rate, slowly-dividing cells (e.g. neurons, organoids) can be prohibitively expensive to perform.
* **Low Factor of Multiplexing:** As of this writing, only two or three conditions can be compared within a single multiplex cassette.
* **MS1 Quantification Sacrifices Protein IDs:** Because SILAC quantification is at the MS1 level, one can only acquire data for half as many unique peptide IDs (or one third, in the case of Triple SILAC).
* **Limited to Laboratory Samples:** Because the samples must be pre-treated with heavy or light media, only cell cultures or small lab animals are conducive to SILAC analysis. It is impossible (or prohibitively expensive) to investigate biopsied human samples or larger tissues using SILAC.

##### SILAC Conclusion

While SILAC is a powerful technique, its limitations have resulted in a general reduction in its use in interaction experiments. Among the datasets presented in this repository at the time of writing, only the dataset presented in [Chiu et al. 2025](../IndividualStudies/DCC_2025.qmd) was prepared using SILAC9.

##### How TMT & iTRAQ Work:

1. **Chemical Labeling:** Each sample is chemically tagged with a unique TMT or iTRAQ label, which consists of a reactive group binding to peptides and a mass reporter for sample differentiation.
2. **Sample Pooling:** Labeled samples are combined into a single pooled group, reducing the need for separate MS runs.
3. **Mass Spectrometry Analysis:**
   * **MS1 (Peptide Isolation):** Peptides are separated and detected based on their mass-to-charge ratio (m/z).
   * **MS2 (Peptide Sequencing & Tag Fragmentation):** Peptides are fragmented for sequence identification, and TMT/iTRAQ mass tags fragment to generate reporter ions.
4. **Quantification:** The intensity of reporter ions reveals the relative abundance of peptides across the original samples.
5. **Comparison Across Samples:** Within a TMT cassette (typically 16 samples), relative protein abundance is calculated from the reporter ion intensities. A pooled control channel enables comparison across cassettes, allowing many samples to be analyzed in a single experiment or large-scale study. iTRAQ has fallen out of favor somewhat because it is limited to 4 samples per cassette, though it is still commonly used.

##### Advantages of TMT/iTRAQ Labeling

* **Increased Throughput & Efficiency:** Enables multiplexing of many samples (e.g., 16 or more), reducing the number of separate MS runs.
* **Deeper Proteome Coverage:** Pooled samples can be fractionated (e.g., by cation exchange chromatography), enhancing detection of low-abundance peptides.
* **Improved Quantification Accuracy:** Since all samples are analyzed in the same MS run, technical variability between runs is minimized.

##### Challenges & Considerations

* **Higher Cost:** TMT reagents and high-end mass spectrometers add to the expense.
* **Complex Sample Preparation:** Requires additional steps for labeling, pooling, and fractionation, increasing the risk of sample handling variability.
* **Normalization Requirement:** Accurate quantification requires equal protein input across samples to avoid artificial balancing biases.
* **Demanding Instrumentation & Expertise:** Requires high-resolution mass spectrometers, often necessitating specialized facilities.

##### Conclusion

TMT- or iTRAQ based mass spectrometry is a valuable tool for large-scale proteomics, offering high-throughput, precise, and deep proteome coverage. While it requires advanced instrumentation and careful sample preparation, its ability to multiplex multiple samples in a single run makes it an attractive option for high-confidence protein quantification.

1. Höglinger, D. *et al.* [Trifunctional lipid probes for comprehensive studies of single lipid species in living cells](https://doi.org/10.1073/pnas.1611096114). *Proceedings of the National Academy of Sciences* **114**, 1566–1571 (2017).

2. Müller, R., Kojic, A., Citir, M. & Schultz, C. [Synthesis and Cellular Labeling of Multifunctional Phosphatidylinositol Bis- and Trisphosphate Derivatives](https://doi.org/10.1002/anie.202103599). *Angewandte Chemie International Edition* **60**, 19759–19765 (2021).

3. Farley, S. E. *et al.* [Trifunctional fatty acid derivatives: The impact of diazirine placement](https://doi.org/10.1039/D4CC00974F). *Chemical Communications* **60**, 6651–6654 (2024).

4. Farley, S., Stein, F., Haberkant, P., Tafesse, F. G. & Schultz, C. [Trifunctional Sphinganine: A New Tool to Dissect Sphingolipid Function](https://doi.org/10.1021/acschembio.3c00554). *ACS Chemical Biology* **19**, 336–347 (2024).

5. Thomas, A. *et al.* Trifunctional lipid derivatives: PE’s mitochondrial interactome. *Chemical Communications* 10.1039.D4CC03599B (2025) doi:[10.1039/D4CC03599B](https://doi.org/10.1039/D4CC03599B).

6. Schultz, C. [Chemical Tools for Lipid Cell Biology](https://doi.org/10.1021/acs.accounts.2c00851). *Accounts of Chemical Research* **56**, 1168–1177 (2023).

7. Höglinger, D. *et al.* [Intracellular sphingosine releases calcium from lysosomes](https://doi.org/10.7554/eLife.10616). *eLife* **4**, (2015).

8. Müller, R., Citir, M., Hauke, S. & Schultz, C. [Synthesis and Cellular Labeling of Caged Phosphatidylinositol Derivatives](https://doi.org/10.1002/chem.201903704). *Chemistry – A European Journal* **26**, 384–389 (2020).

9. Chiu, D.-C., Lin, H. & Baskin, J. M. Photoaffinity Labeling Reveals a Role for the Unusual Triply Acylated Phospholipid N-Acylphosphatidylethanolamine in Lactate Homeostasis. (2025) doi:[10.26434/chemrxiv-2025-j8nqz](https://doi.org/10.26434/chemrxiv-2025-j8nqz).