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

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| 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.

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 It Works

1. **Chemical Labeling:** Each sample is chemically tagged with a unique TMT 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 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.

##### Advantages of TMT 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-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). *Angew Chem Int Ed* **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). *Chem. Commun.* **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 Chem. Biol.* **19**, 336–347 (2024).

5. Thomas, A. *et al.* Trifunctional lipid derivatives: PE’s mitochondrial interactome. *Chem. Commun.* 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). *Acc. Chem. Res.* **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 J* **26**, 384–389 (2020).