Post-translational modifications (PTMs) are enzymatically regulated, covalent modifications that change a protein’s structure. These alterations to functional groups on a protein can affect its stability, spatial localization, and binding partners, ultimately regulating its function. Any given protein is likely to be modified by a number of different PTMs, and each specific PTM primes the protein to perform a unique and dynamic function in the cell. Therefore, it is reasonable to hypothesize that PTMs are an omnipresent mechanism regulating many diverse functions of any given protein.

In support of this hypothesis, PTMs such as ubiquitination, glycosylation, acetylation, SUMOylation, and phosphorylation have been shown to regulate nearly every biological process through control of signal transduction, protein turnover, protein recognition, and localization. These PTMs and others vastly increase proteomic complexity by orders of magnitude because each post-translationally modified protein represents a unique protein-form (proteoform). Importantly, characterization of specific proteoforms has resulted in great insight into the target protein’s function (See the last article in this eBook, PTM Functional Characterization and Beyond, page 26, for more information), and a significant number are being actively pursued as promising therapeutic targets or biomarkers (See the previous article in this eBook, PTMs: Functional Regulators of Disease, page 4, for further insight). For example, a particularly promising therapeutic targets the p53 proteoform MDM2 ubiquitinated p53, which has been shown to be deregulated in a wide variety of cancers.

Unraveling mechanistic aspects of PTM proteoforms via molecular biology approaches is technically very challenging. With the exception of phosphoproteins, histones, and several medically relevant proteins such as p53 and Tau, there are few proteoform-specific antibodies in the PTM toolbox, necessitating the use of more indirect analytical techniques. The remainder of this article addresses key technical issues associated with PTM proteoforms as well as useful discovery and validation techniques utilized to overcome them.
Technical issues when studying PTMs

- Low stoichiometry
  - Proteoforms tend to be sub-stoichiometric; any one proteoform accounts for less than 1–5% of a protein’s total population at any given time, making them difficult to detect.

- Highly dynamic
  - Proteoforms are dynamic, often appearing transiently in response to a specific signal.

- Cell or tissue specificity
  - Proteoforms can be cell or tissue type specific.

- Sample preparation: Gain or loss of PTMs
  - PTM profiles can be positively and negatively altered during sample processing.

- Utilizing proper controls
  - Key controls are critical for interpreting results.

The preceding list comprises some of the major technical issues that make detecting PTM proteoforms challenging (see page 26 for specific examples). The following are commonly used techniques that molecular biologists use to investigate novel proteoforms of their target protein.

Enrichment of PTM proteoforms

Enrichment of PTM proteoforms is necessary due to stoichiometry issues and is often achieved by immunoprecipitation (IP) techniques. Enrichment with IP utilizes antibodies, protein-binding domains, metal ion affinity, and other specific binding molecules that are attached to a solid support matrix (such as agarose resin). These affinity matrices bind to the target protein or PTM, while non-targeted proteins in the complex lysate are not captured and removed through wash steps. The enriched PTM proteoforms are then detached and isolated from the support matrix using a concentrated volume of elution buffer. The isolated population is analyzed in downstream applications like western blotting or mass spectrometry to determine if a target protein is post-translationally modified.

Two approaches are possible for endogenous PTM proteoform enrichment; one approach uses an affinity matrix that recognizes the target protein, while the other uses an affinity matrix specific for the PTM of interest. An affinity matrix against the target protein of interest may immunoprecipitate potentially all proteoforms of that specific protein, while IP with a PTM-specific affinity matrix may immunoprecipitate nearly all proteoforms modified by that PTM.

Western blotting analysis

Western blotting is a standard molecular biology approach used to identify a specific protein of interest from a complex protein mixture or lysate. In the case of PTM proteoform analysis, the enriched sample is mixed with SDS, separated to specific regions in an acrylamide gel based on size (SDS-PAGE), transferred to a blotting membrane, and specifically identified by antibody recognition.

Figure 1A shows an example where ubiquitinated EGFR was enriched with an ubiquitin affinity reagent and visualized with an EGFR antibody. The western blot results show unambiguous identification of ubiquitinated EGFR. These data are supported by the loss in the ubiquitinated EGFR band when the deubiquitinase inhibitor NEM was removed from
the lysis buffer, thus allowing deubiquitination to occur (Figure 1A). The reciprocal experiment was performed using an EGFR antibody for IP enrichment. The enriched proteins were then visualized by probing with a pan-ubiquitin antibody. Figure 1B shows minimal ubiquitinated EGFR was detected with this approach. One possible explanation for this stark difference could be due to the bound-ubiquitin protein blocking the EGFR antibody recognition site (Figure 1C and D). Previous research has shown that some PTM modifications may block the antibody-binding site on a target protein and prevent interaction with the antibody—thus producing a false negative result.11

Mass spectrometry analysis

Mass spectrometry (MS) is an analytical method that can identify a PTM proteoform based on its mass-to-charge ratio. Two distinct MS approaches are used to investigate PTM proteoforms, bottom-up and top-down analysis (which are described in more detail in Mass Spectrometry and Post-Translational Modifications, page 22). Benefits of mass spectrometry are its potentially unbiased approach, PTM site specificity, and independence from antibodies for detection. Technical challenges when utilizing MS include protein abundance bias,12 method sensitivity13 and methodological expertise, such as sample preparation, digestion strategies, fractionation approaches, and other considerations.14 Investigating PTM proteoforms with MS requires both a comprehensive understanding of the biological model and technical expertise with the instrumentation/analysis; thus, we have found that successful PTM proteoform investigation with this method requires strong collaboration between molecular biologists and MS experts.

Overexpression and mutagenesis

Overexpression is a well-established system where a plasmid of a tagged version of a target protein is transfected into cells, which usually results in high expression levels. The expressed protein normally contains a tag that is recognized by a well-characterized antibody (e.g., His). The increased expression and optimized enrichment antibody improves the chance of identifying the PTM proteoform and is commonly analyzed by western blot. The overexpression system is particularly important when a proteoform is challenging to study endogenously because it allows investigators to control expression, localization, and other factors.

Figure 1. Detection of EGFR ubiquitination. Serum-restricted A431 cells were either unstimulated (−) or stimulated with EGF (+) for 15 minutes prior to lysis with BlastR lysis buffer with or without NEM. WCL was analyzed for EGFR levels (Input). (A) Lysates were incubated with UBA01 ubiquitin affinity beads and analyzed for ubiquitinated EGFR with an anti-EGFR antibody. (B) Lysates were incubated with anti-EGFR antibody and protein-G beads. Captured proteins were analyzed for ubiquitinated EGFR with an anti-Ub-HRP conjugated antibody.
through mutagenesis approaches. Importantly, mutagenesis approaches enable site-specific PTM investigation and characterization. Due to the overexpressed nature, any critical identification should be validated by additional methods, and the final article in this eBook, page 26, provides examples where overexpression studies were performed during PTM proteoform characterization.

**In vitro biochemical assay**

Biochemical assays utilize purified or *in vitro* translated versions of a target protein to determine if it can be modified by a specific PTM. The purified protein is added to a test tube with specific enzymes (e.g., E1, E2, E3 ubiquitin ligase) and the appropriate substrate (e.g., ubiquitin), co-factors, and energy sources. After incubation, the sample is then analyzed by western blot analysis. It is important to note that *in vitro* biochemical analysis is not available for all types of PTMs; however, it is routinely performed to investigate phosphorylation, ubiquitination, SUMOylation, and other PTM modifications. A limiting step in performing *in vitro* biochemical assays is obtaining purified versions of the target protein and modifying enzymes.

**Proximity ligation assay**

Proximity ligation assay (PLA) is a novel immunoassay technology that can be used to study protein interactions and PTMs. PLA is unique in its ability to identify proteoforms in fixed tissues and cells. The principle of PLA-PTM works by utilizing two antibodies; one targets a PTM of interest while the other binds a specific protein of interest. The initial steps are similar to standard immunofluorescence staining where the primary antibodies bind the epitopes of interest, and the secondary antibodies recognize their respective primary antibodies. The difference with PLA is that these secondary antibodies have short DNA strands covalently attached to them (these antibody–DNA complexes are called PLA probes). If the two PLA probes are in close enough proximity, presumably because the two antibodies are bound to a proteoform, they will form circularized DNA. PCR amplification of circularized DNA is performed, and fluorescently labeled complementary DNA probes are added for visualization. Due to the significant DNA amplification, which can be up to several hundred-fold, the fluorescent signal from very few molecules will be visible by microscopy.

**Specialized techniques**

An emerging technology that will have profound effects on the PTM field is genetic code expansion. This technology allows researchers to specifically add a PTM of interest, homogenously onto a target protein without a requirement for the specific modifying enzyme. Having homogenous purified versions of the PTM proteoform will be critical for structural studies, *in vitro* functional studies, and others. There are numerous other techniques available that are outside the scope of this article, such as NMR spectroscopy for site specificity and structural insight of proteoforms, thermal dissociation assays for detection of enzymatic PTM removal, and Nanopore technology for label-free PTM detection. Additionally, for each specific PTM there are highly specialized methods that have been developed. For example, investigators are using Hotspot Thermal Profiling of native proteins in live cells to identify which site-specific phosphorylation events alter protein stability. Other investigators are utilizing novel ubiquitin clipping mechanisms to gain insight into polyubiquitin chains and architecture. Furthermore, novel approaches have been developed to quantitate kinetic parameters of
Table 1. Pros and cons of PTM enrichment and detection strategies

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<th>Enrichment Strategies</th>
<th>Detection Strategies</th>
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<td>PTM Antibody Immunoprecipitation</td>
<td>Protein Antibody Immunoprecipitation</td>
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<td><strong>Pros:</strong>&lt;br&gt;• May be able to study endogenous PTM changes&lt;br&gt;• Kits are available: minimal IP optimization&lt;br&gt;• No PTM interference with PTM affinity reagents&lt;br&gt;• No specialized tools required&lt;br&gt;• No specialized expertise required&lt;br&gt;• Familiarity with target protein antibody for western application</td>
<td><strong>Pros:</strong>&lt;br&gt;• Familiarity with target protein antibody&lt;br&gt;• May be able to study endogenous PTM changes&lt;br&gt;• No specialized tools required&lt;br&gt;• No specialized expertise required</td>
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<td><strong>Cons:</strong>&lt;br&gt;• Identification of PTM IP antibody, bead conjugation, IP optimization (if kits are not available)&lt;br&gt;• False negatives are possible if lower abundance PRM-modified proteins are not captured&lt;br&gt;• No site specificity</td>
<td><strong>Cons:</strong>&lt;br&gt;• Must validate target protein antibody works in IP applications&lt;br&gt;• PTM modification may block binding site of antibody against target protein; thus, false negatives are possible&lt;br&gt;• Heavy and light chain contamination, unless additional bead conjugation steps are performed&lt;br&gt;• Requires optimization of IP methodology&lt;br&gt;• Must identify PTM antibody for western blotting&lt;br&gt;• No site specificity</td>
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<tr>
<th>Western Blotting</th>
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<th>In Vitro Biochemical Analysis</th>
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<td><strong>Pros:</strong>&lt;br&gt;• Familiarity with target protein antibody for western application&lt;br&gt;• Familiarity with western methodology&lt;br&gt;• No specialized tools required&lt;br&gt;• No specialized expertise required</td>
<td><strong>Pros:</strong>&lt;br&gt;• Broad spectrum analysis of proteins modified by PTM&lt;br&gt;• Broad spectrum analysis of PTMs that modify target protein&lt;br&gt;• Unbiased approach to identify novel PTM modified proteins&lt;br&gt;• Identify site specific modifications&lt;br&gt;• Cores may be available to perform mass spectrometry analysis (for a fee)</td>
<td><strong>Pros:</strong>&lt;br&gt;• Determine if target protein is modified by specific PTM&lt;br&gt;• Useful tool to identify regulatory enzymes for PTM modification of specific POI&lt;br&gt;• Good validation tool to confirm PTM modification of target POI</td>
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<td><strong>Cons:</strong>&lt;br&gt;• No site specificity&lt;br&gt;• Western protocol may alter some modification like oxidation&lt;br&gt;• False negatives are possible if lower abundance PTM-modified proteins are not captured</td>
<td><strong>Cons:</strong>&lt;br&gt;• Requires mass spectrometer&lt;br&gt;• Requires mass spectrometry expertise to run samples and analyze large data sets&lt;br&gt;• Significant mass spectrometry and sample preparation optimization&lt;br&gt;• False negatives are possible due to mass spectrometry limitations and bias</td>
<td><strong>Cons:</strong>&lt;br&gt;• Proteins, enzymes, and substrates are not a physiologic concentrations, thus, false positive results may be possible&lt;br&gt;• Requires obtaining purified or translated POI as well as enzymes required for specific PTM modification&lt;br&gt;• May not be representative of what occurs in cells and animals</td>
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regulatory enzymes such as lysine acetyl transferases using reverse-phase HPLC.  

Conclusions

Due to a PTM proteoform’s biological nature, (i.e., post-translationally modified proteins are present at low stoichiometric levels), identification of a specific proteoform often goes undetected via standard protein detection methods like western blotting and immunofluorescence staining. Table 1 highlights the pros and cons of several enrichment and detection approaches that may be used for preliminary investigation. The PTM-specific IP approach is particularly beneficial, as kits are available in this format that allow investigators to bypass extensive optimization issues. A recent review identified Signal-Seeker kits as a novel tool to investigate PTMs of target proteins. More information is provided in the next article, Signal-Seeker PTM Discovery Toolkits: Utilities and Applications, page 17. Better tools and approaches to efficiently detect these important modifications will undoubtedly facilitate the PTM proteoform discovery and validation process and, when used in combination with other PTM investigation tools, will allow for successful functional characterization of a PTM proteoform.

About the authors

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References


Simple Technology, Powerful Results

**Signal-Seeker™ Detection Kits**

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- Analyse with standard western immunoblotting

**Powerful**
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- Study multiple PTMs using the same system

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- IP control Beads
- Universal Lysis System
- Key Inhibitors
- Wash Reagents
- Elution Reagents
- Spin Columns
- Protein Quantitation System
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