

## Hybrid-DIA: Intelligent Data Acquisition for Online Targeted and Discovery Proteomics Applied to Phosphoprotein signaling in Colorectal Cancer

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Achieving sufficient coverage of regulatory phosphorylation sites by mass spectrometry (MS)-based phosphoproteomics for signaling pathway reconstitution is challenging, especially when analyzing tiny sample amounts. To address this, we present an innovative hybrid data-independent acquisition (DIA) strategy (hybrid-DIA) that combines targeted and discovery proteomics through an Application Programming Interface (API) to dynamically intercalate DIA scans with accurate triggering of multiplexed tandem mass spectrometry (MSx) scans of predefined (phospho)peptide targets. By spiking-in heavy stable isotope labeled phosphopeptide standards covering seven major signaling pathways, we benchmarked hybrid-DIA against state-of-the-art targeted MS methods (i.e. SureQuant) using EGF-stimulated HeLa cells and found the quantitative accuracy and sensitivity to be comparable while hybrid-DIA also profiled the global phosphoproteome. To demonstrate the robustness, sensitivity and biomedical potential of hybrid-DIA, we profiled chemotherapeutic agents in single colon carcinoma multicellular spheroids and evaluated the phospho-signaling difference of cancer cells in 2D vs 3D culture. Finally, we show the applicability of this methodology to find novel kinase targets in clinical samples.

