Protein phosphorylation is a main regulatory switch in the cell, controlling processes such as cell growth, proliferation, differentiation and survival. This control is performed by intricate signaling networks, which are capable of altering protein activities and rapidly communicating messages from different extracellular or internal cues to ultimately promote adequate cell readjustments. Numerous studies have addressed protein phosphorylation over the past decades, often on a single protein/pathway level. However, the global picture of signaling events can only be accomplished from comprehensive studies, which are becoming attainable by mass spectrometry (MS)-based proteomics.

The main difficulty in MS large-scale phosphorylation studies is the limitation in detecting phosphorylated species within complex sample mixtures due to their low abundance. However, the past five years have seen a steady improvement in phosphopeptide enrichment and MS data acquisition methods along with the development of computational tools for data analysis and validation, allowing us to routinely identify thousands of phosphorylation events from a single experiment. An overview of such efforts and the current status of technologies to profile the phosphoproteome will be given.

Furthermore, these strategies have been combined with metabolic labeling using stable isotopes (SILAC) for quantitative studies where two cell populations are compared. One of the most challenging problems in signal transduction is establishing kinase-substrate relationships. We have combined chemical genetics and large-scale quantitative phosphoproteomics to identify substrates of the master mitotic kinase Cdk1 at different stages of the cell cycle, and meiotic kinase Ime2 in the budding yeast *Saccharomyces cerevisiae*. Using this approach, we expanded the number of known *bona fide* substrates to ~400 for each kinase, and pinpointed the precise sites of phosphorylation. We observed that substrates for Cdk1 and Ime2 vastly overlap; however the sites targeted seem to differ, which provides an example on how different layers of regulation are assembled in complex systems.