Targeted phosphoproteomics to reveal mTOR pathway signalling dynamics in zebrafish

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Cells employ reversible protein phosphorylation as a means to transmit information in response to external factors like nutrient levels or stress. Monitoring the activity of molecular signalling networks holds potential for predictive ecotoxicology, for example in assessing the effects of chronic chemical exposure on fish growth. To overcome the limitations of traditional antibody-based methods, we sought to develop a targeted mass spectrometry-based (phospho)proteomics approach to study phosphorylation-based signalling networks. This novel approach enables the simultaneous quantification of phosphorylation and abundance for multiple protein targets, thus eliminating the need to develop and utilize multiple antibodies, which may be lacking for non-mammalian species. We used the zebrafish (*Danio rerio*) embryonic cell line PAC2 as a model, and focused on the mTOR pathway, a central signalling pathway involved in the regulation of cell growth and proliferation. The developed workflow involves a fast cell lysis using 5% SDS for 1 minute, followed by in-column protein trapping and digestion using S-Trap^{TM}. Heavy-labelled synthetic peptides of the endogenous peptide targets are added after the digestion step to determine the recovery after desalting and subsequent enrichment of the phosphopeptides. This allowed us to compare the peptide recovery in the bound and unbound fractions of the enrichment step, for 48 phosphopeptide targets and two enrichment methods. The average recovery was found to be only 14% for TiO_2-beads, but reached 87 ± 9% with Fe^{3+}-NTA beads. Only the latter enrichment method effectively removed interferences and thus improved the detection sensitivity for the targeted phosphopeptides via multiple reaction monitoring (MRM). We applied our method to investigate the mTOR pathway phosphorylation dynamics in the PAC2 cells (i) at different growth stages in cell culture, (ii) after nutrient deprivation, and (iii) after exposure to chemicals, including mTOR inhibitors and chemicals known to impact fish growth *in vivo*. Time-resolved analysis of protein phosphorylation responses within the zebrafish mTOR pathway provides insights on various checkpoints associated with the regulation of cell growth and proliferation.