Simultaneous targeted and discovery phosphoproteomics of cell signaling pathways using novel hybrid-DIA acquisition strategy

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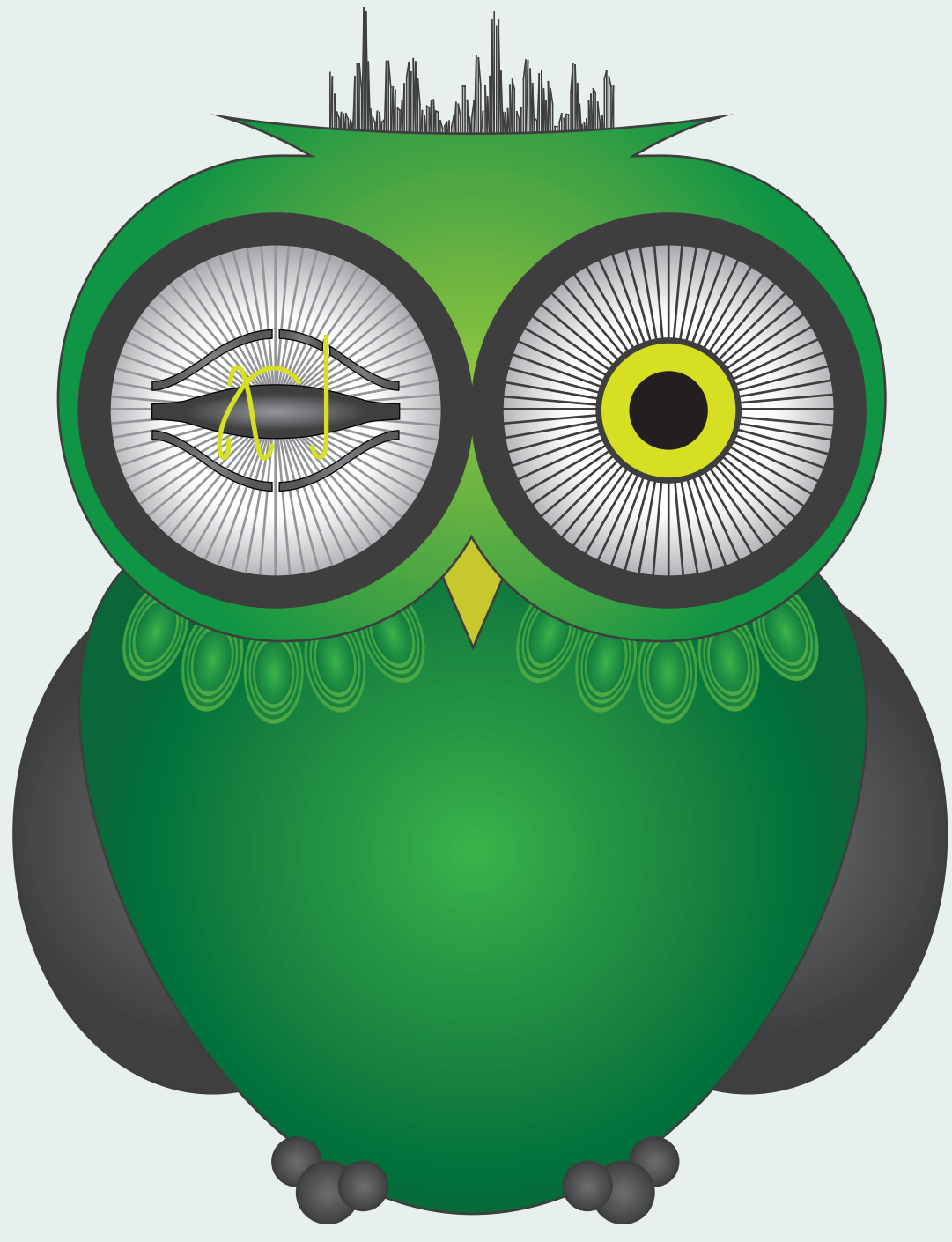
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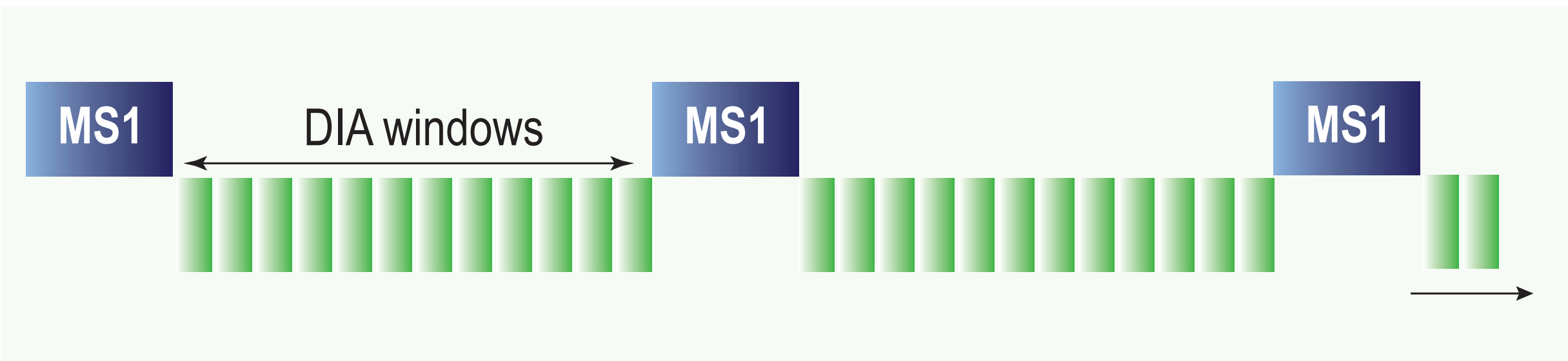
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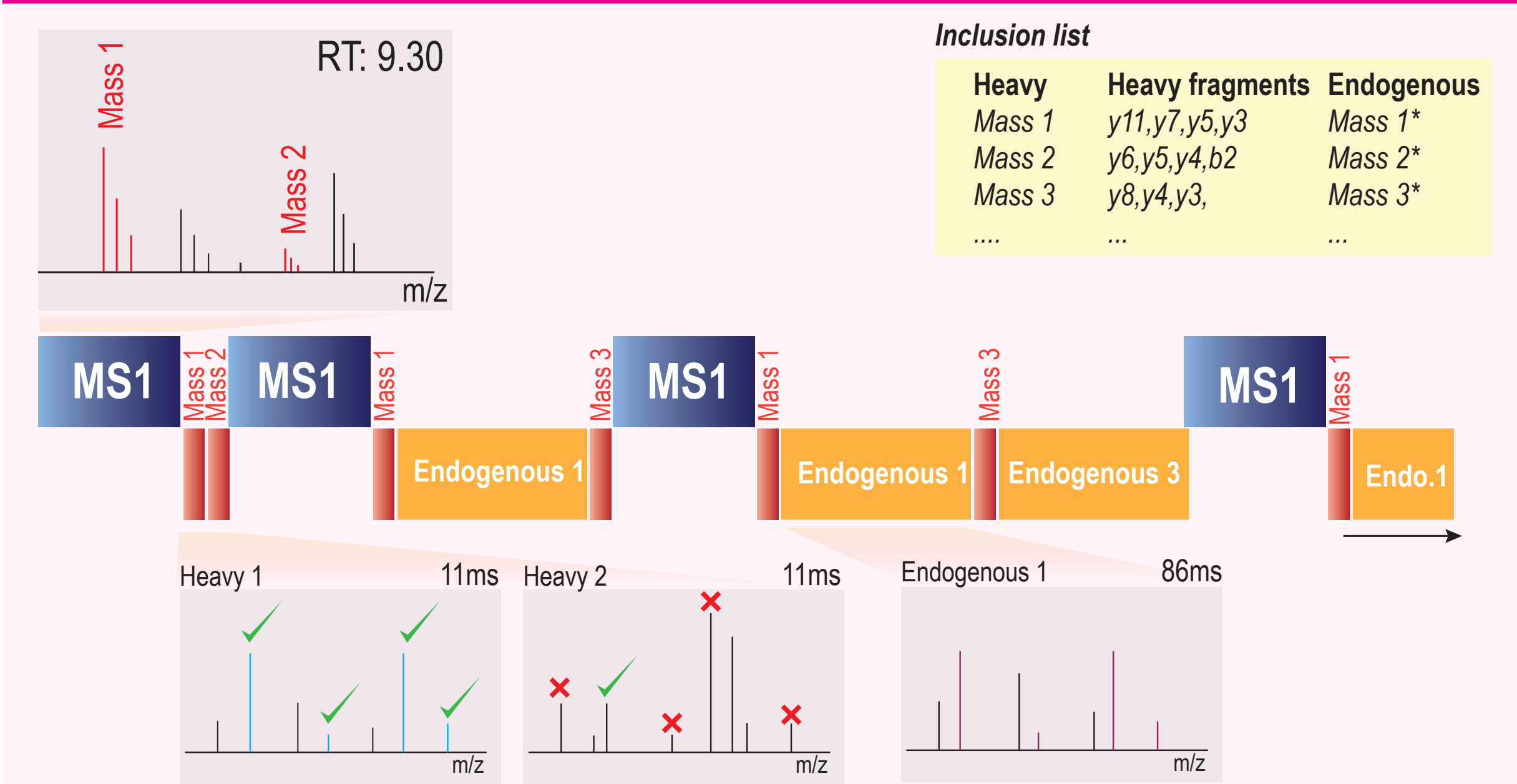
▶ **Hybrid DIA** is based on an **Application Programming Interface**, which enables the ability to **combine targeted and discovery proteomics**, by intercalating DIA scans with accurate triggering of predefined peptide targets.

▶ We have **benchmarked the sensitivity** of hybrid DIA against purely targeted methods, such as SureQuant.

▶ We have evaluated the utility of this data acquisition strategy to study changes in phosphorylation levels of 131 relevant peptides together with a comprehensive analysis of the phospho-proteome.

Overview of Data Acquisition Methods
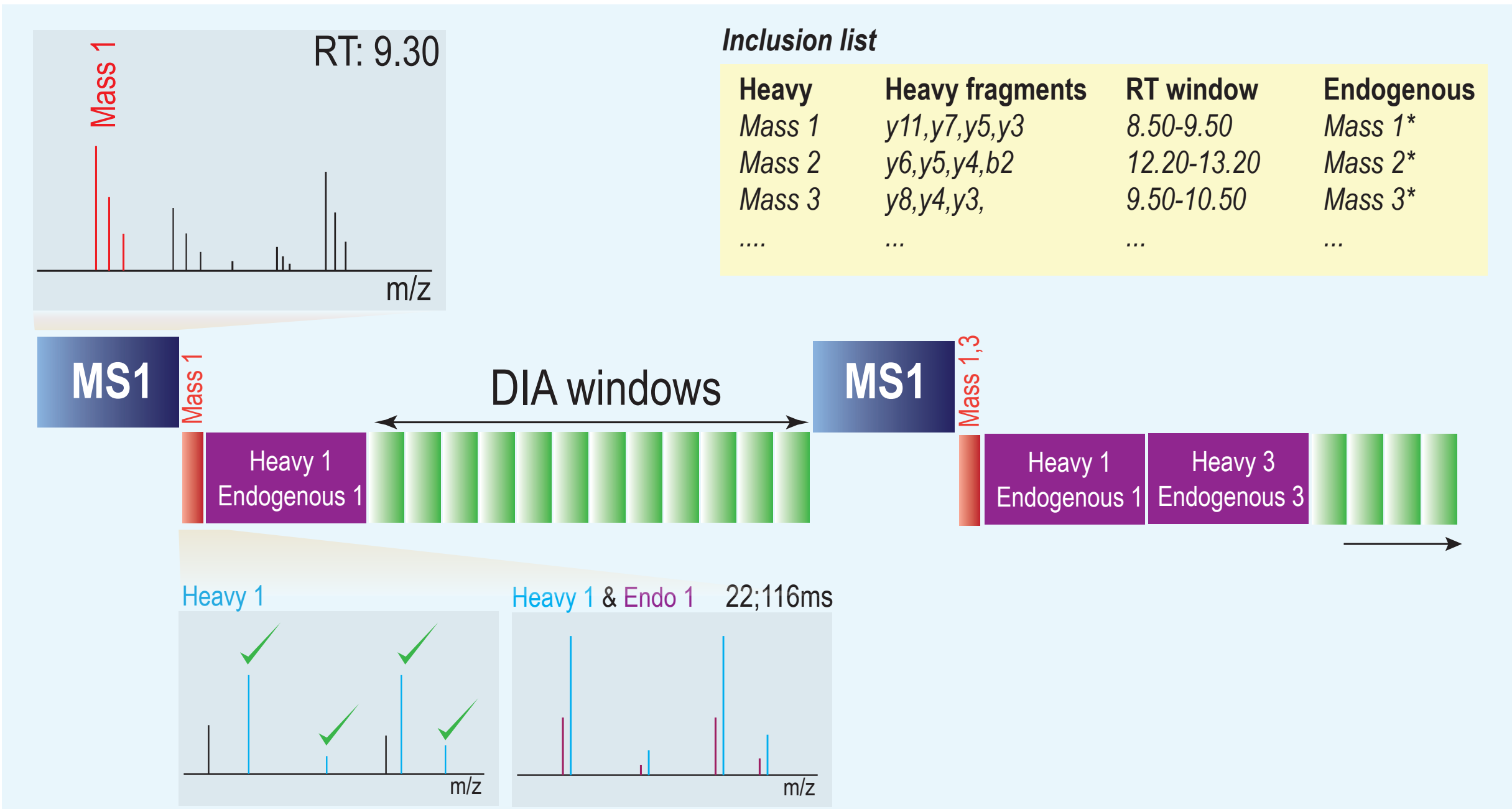
Conventional DIA
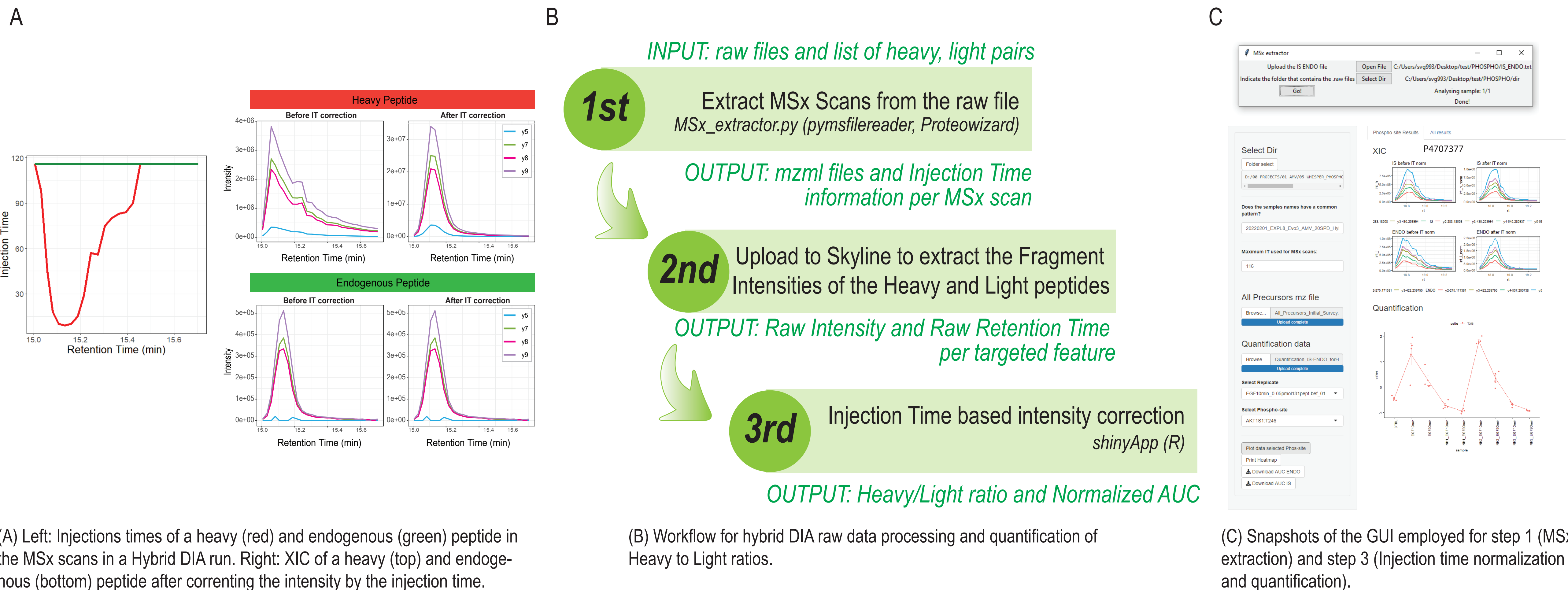
Targeted method (i.e. SureQuant)
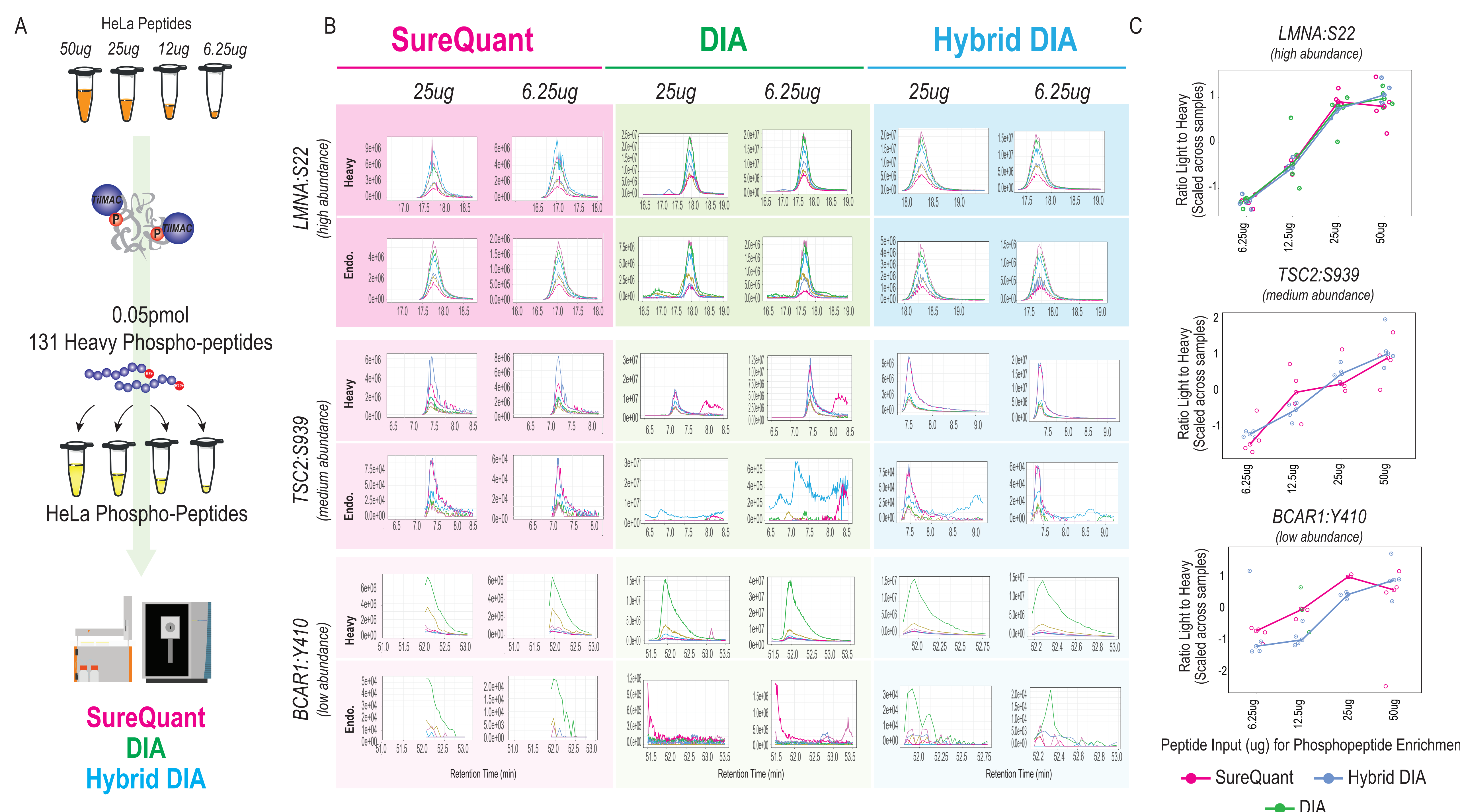
Hybrid DIA

Data Analysis Pipeline

1st INPUT: raw files and list of heavy, light pairs
Extract MSx Scans from the raw file
MSx_extractor.py (pymsfilereader, Proteowizard)
OUTPUT: mzml files and Injection Time information per MSx scan

2nd Upload to Skyline to extract the Fragment Intensities of the Heavy and Light peptides
OUTPUT: Raw Intensity and Raw Retention Time per targeted feature

3rd Injection Time based intensity correction
shinyApp (R)
OUTPUT: Heavy/Light ratio and Normalized AUC

(A) Left: Injections times of a heavy (red) and endogenous (green) peptide in the MSx scans in a Hybrid DIA run. Right: XIC of a heavy (top) and endogenous (bottom) peptide after correcting the intensity by the injection time.
(B) Workflow for hybrid DIA raw data processing and quantification of Heavy to Light ratios.
(C) Snapshots of the GUI employed for step 1 (MSx extraction) and step 3 (Injection time normalization and quantification).

Improved limit of detection

HeLa Peptides: 50ug, 25ug, 12ug, 6.25ug
0.05pmol 131 Heavy Phospho-peptides
HeLa Phospho-Peptides
SureQuant / DIA / Hybrid DIA

SureQuant — **DIA** — **Hybrid DIA**
25ug / 6.25ug

LMNA:S22 (high abundance)
TSC2:S939 (medium abundance)
BCAR1:Y410 (low abundance)

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Peptide Input (ug) for Phosphopeptide Enrichment

— SureQuant — Hybrid DIA — DIA

Targeted and discovery analysis of EGF stimulation

(A) Overview EGFR signaling pathway with indication of the kinase inhibitors used in the experiment.
(B) Experimental workflow for phospho-proteomics analysis of EGFR signaling using the SureQuant™ Multipathway Phosphopeptide Standard.

(C) Phospho-sites from the Multipathway Phosphorylation Kit idenfied by HybridDIA targeted scans or SureQuant that are differentially regulated by EGF after 10 minutes stimulation and that show differentially regulation by the different kinases inhibited.
(D) Profile plot showing the ration Light to Heavy measured in HybridDIA or SureQuant for some relevant EGFR targets.

— SureQuant — HybridDIA

Full phosphoproteome analysis: Spectronaut directDIA results

5,055 Phospho-sites identified (Loc. Prob >0.75)
3,611 Phospho-sites identified quantified in at least 75% of the replicates.
1,214 Phospho-sites differential in total (ANOVA FDR<0.05%)
224 Phospho-sites activated due to EGF stimulation (10 minutes)
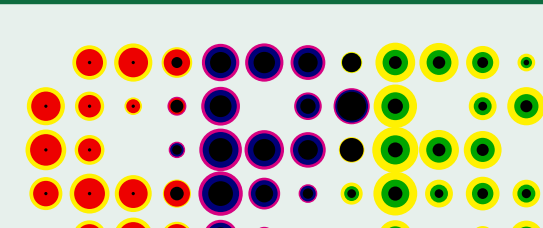
(E) Full phosphoproteome results obtained from a library-free search of the HybridDIA files.
(F) Kinase activity inferred from the full phospho-proteome profiles in the different experimental condition calculated with RoKAI App (v2.1.3).