



HIGH THROUGHPUT PROTEOME AND PHOSPHOPROTEOME SAMPLE PROCESSING COUPLED TO FAST GRADIENT DIA

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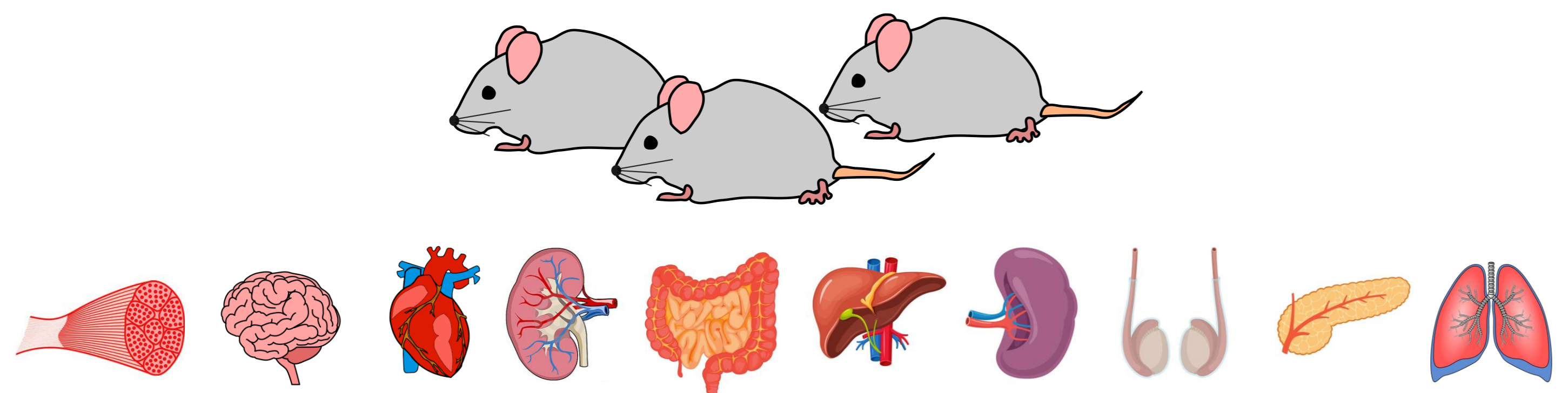
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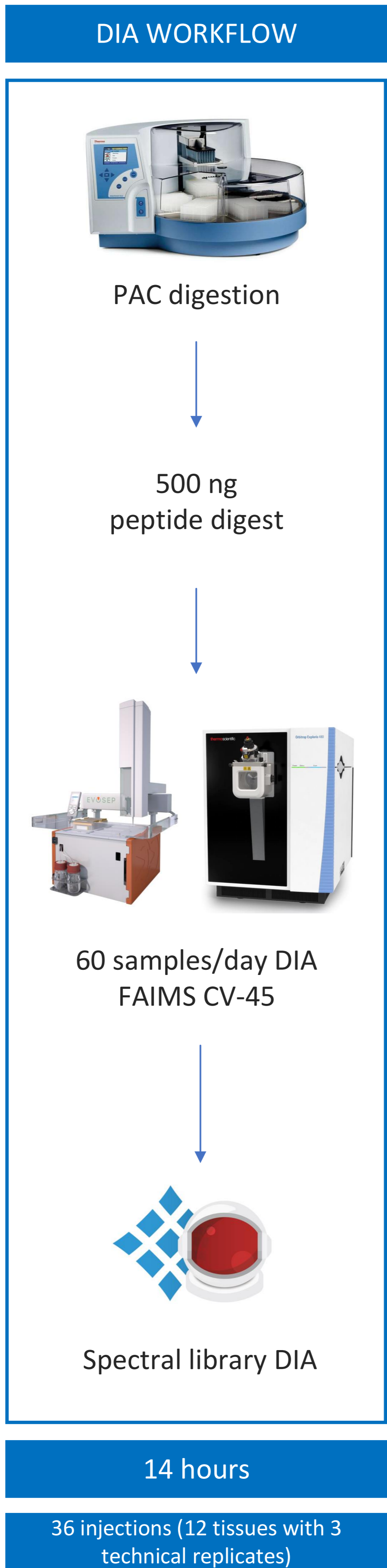
INTRODUCTION

The requirement for robust and routine high throughput sample preparation workflows has become a necessity as clinical proteomics reaches maturity. The workflows will enable processing of large sample cohorts with the throughput, robustness and reproducibility required for a routinized laboratory setting. In this study we illustrate an automated workflow for proteome and phosphoproteome profiling coupled to fast gradient liquid chromatography (LC) and data-independent mass spectrometry analysis. Automation of sample preparation increased throughput and reproducibility covering all steps from protein capture, clean-up, digestion and phosphopeptide enrichment to mass spectrometry analysis, allowing for parallel processing of up to 96 samples in less than 6 hours (excluding digest time). Magnetic beads are considered desirable since these are easy to handle, simple to automate, linearly scalable, and high throughput compatible on a range of magnetic bead handling stations. The automation of phosphopeptide enrichment was originally illustrated by Tape *et al* in 2014, and coupling to automated upfront clean-up and digestion was recently reported by Leutert *et al*, 2019. The current workflow adapts the protein-aggregation-capture (PAC) method described by Baath *et al* in 2019 to automation on a KingFisher™ Flex magnetic bead handling system, and couples it to phosphopeptide enrichment using new prototype Ti-IMAC and Zr-IMAC HP (high performance) magnetic beads, and data analysed by Spectronaut™ (Biognosys).

OVERVIEW



Tissue heat inactivation & homogenization of 12 tissues from 3 replicates



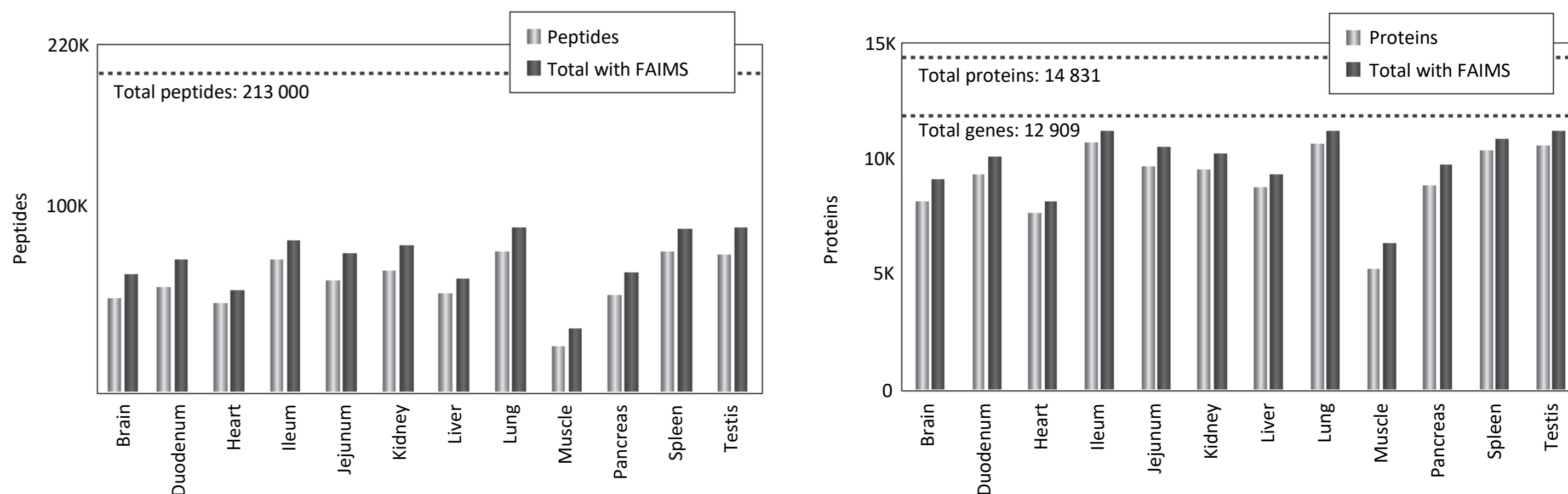
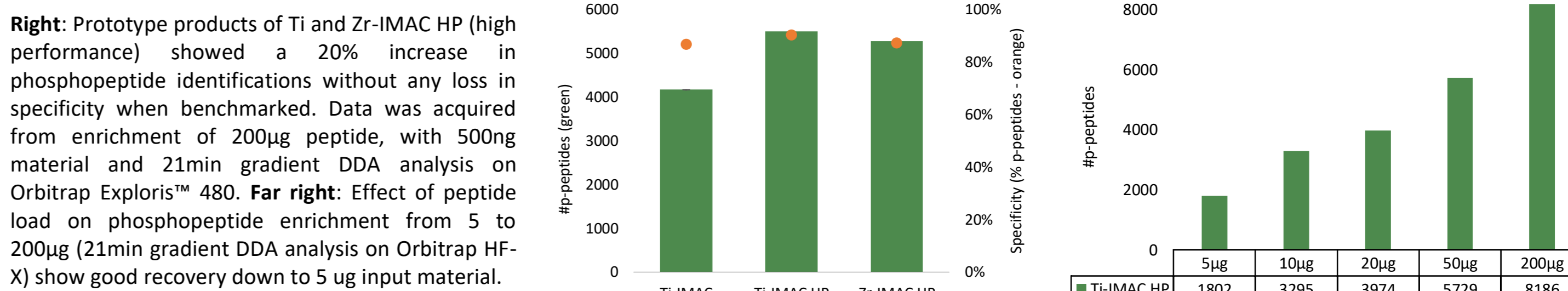
DETAILED WORKFLOWS

| AUTOMATED PAC WORKFLOW | | | |
|------------------------|--|---|-------------------|
| Procedure | Steps | Details | Time (96 samples) |
| Protein capture | Add beads | MagReSyn® Amine (20% ACN)* | 25 min |
| | Add protein extract | 5 to 1000 µg in 5% SDS 50mM Tris pH 8 (1:5 protein to bead ratio) | |
| | Add ACN | Final concentration of 70% | |
| | Mix | 1 min | |
| | Pause | 10 min | |
| | Repeat Mix & Pause cycle | 1 x cycle | |
| Sample Washing | Collect | Bead-protein aggregate captured | 12.5 min |
| | Transfer bead-protein aggregate to wash plate | 1 ml 95% ACN (3 plates) OR 70% EtOH (2 plates) | |
| | Mix gently with bead-protein aggregate on magnet | 2.5 min | |
| | Cycle | 5 x wash cycles | |
| Digest | Transfer aggregate to digest plate and release | 250 µl 50mM AmmBic and digest enzymes | 60-720 min |
| | Incubation (37-47°C, 1-12 hrs) | Loop: mix 15sec, pause 2 min 15 sec for intermittent mixing | |
| | Quench digest and mix | 1% TFA | |
| | Collect beads on magnetic | | |
| | Transfer beads to storage | 500 µl 70% ACN | |
| Bead storage | Release beads from magnet | | 1 min |

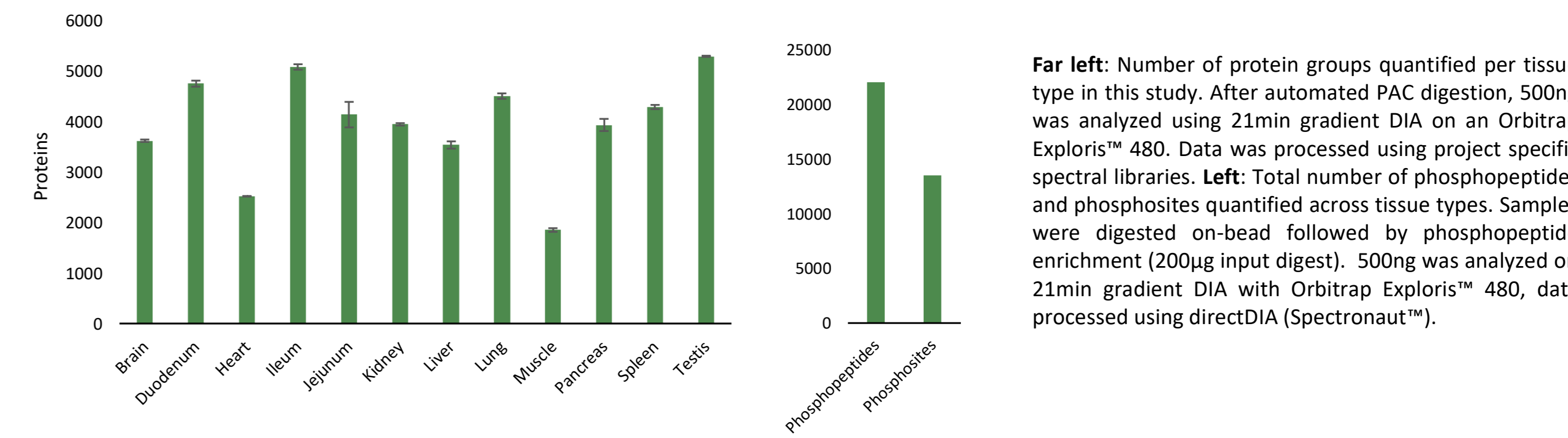
* alternate aggregation nucleus optimized for PAC is available, please inquire.

| AUTOMATED PHOSPHOPEPTIDE ENRICHMENT WORKFLOW | | | |
|--|--|--|-------------------|
| Procedure | Steps | Details | Time (96 samples) |
| Bead Equilibration | Aliquot beads | MagReSyn® Ti-IMAC or Zr-IMAC, 40 µl (800 µg) | 5 min |
| | Collect beads to transfer beads to equilibration plate | 500 µl 80% ACN, 5% TFA, 0.1-1M Glycolic acid | |
| Phosphopeptide Binding | Transfer beads to desalted digest | 200 µg in 80% ACN, 5% TFA, 0.1-1M KF medium speed | 20 min |
| | Mix gently to ensure peptide sample interaction | | |
| Sample Washing | Transfer beads with bound phosphopeptides to wash plate 1 | Wash 1: 500 µl 80% ACN, 5% TFA, 0.1-1M Glycolic acid | 6 min |
| | Mix | 2 min | |
| | Transfer beads with bound phosphopeptides to wash plate 2 | Wash 2: 500 µl 80% ACN, 1% TFA | |
| | Mix | 2 min | |
| | Transfer beads with bound phosphopeptides to wash plate 3 | Wash 3: 500 µl 10% ACN, 0.1% TFA | |
| | Mix | 2 min | |
| Elution | Capture beads with phosphopeptides and transfer to elution plate | 200 µl 1% NH ₄ OH | 10 min |
| | Transfer beads to storage | | |
| Bead storage | Release beads in storage buffer | 20% Ethanol | 1 min |

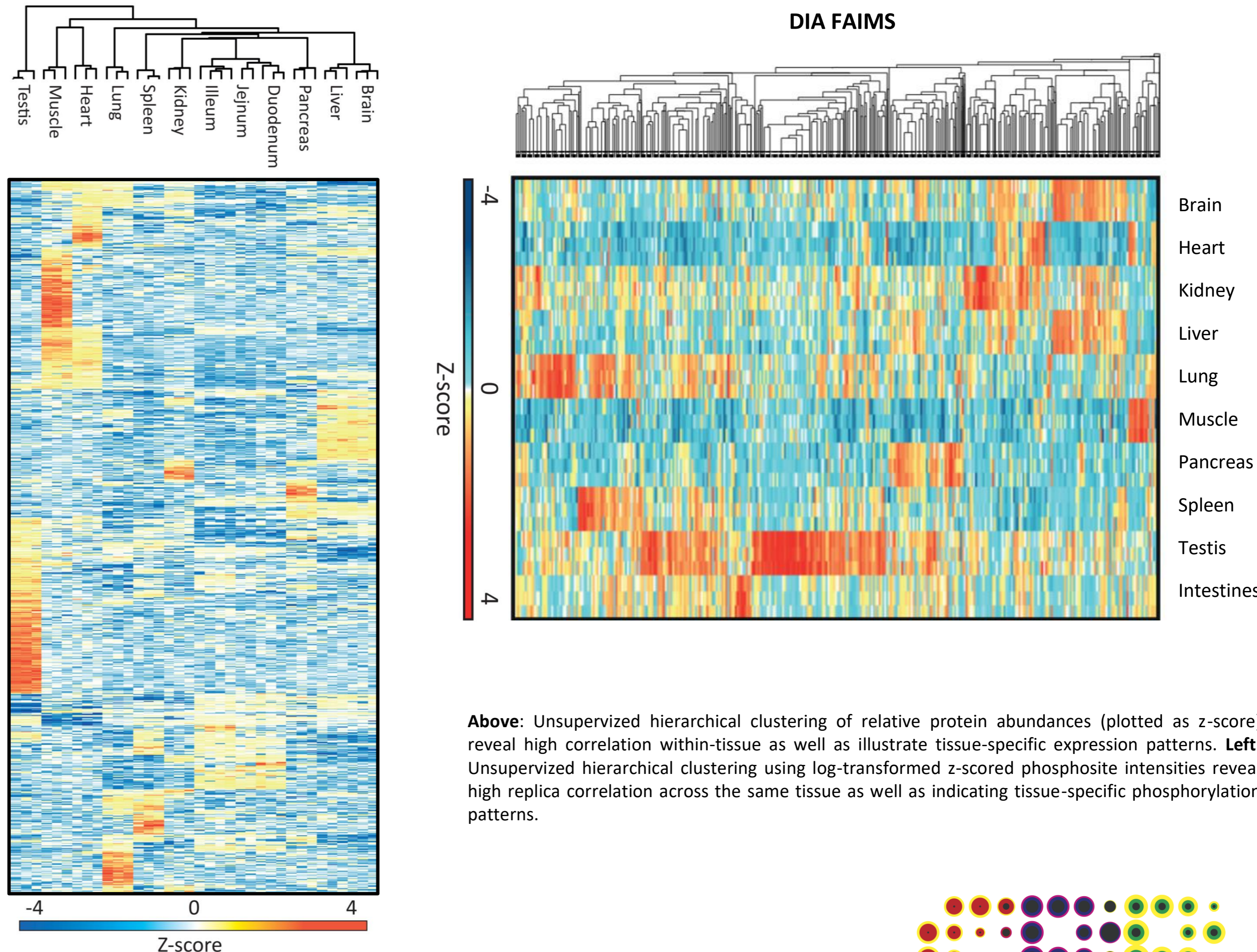
RESULTS



Above images reproduced from Bekker-Jensen *et al*, 2020. Number of peptides (above left) and proteins (above right) quantified from each tissue library. 46 off-line high pH fractions per tissue were acquired using 250ng (5min gradient) without (light grey) and with FAIMS (dark grey) on an Orbitrap HF-X and Lumos respectively. The total number of peptides and proteins in the complete library (from 12 tissues with and without FAIMS) are indicated by the dashed lines.



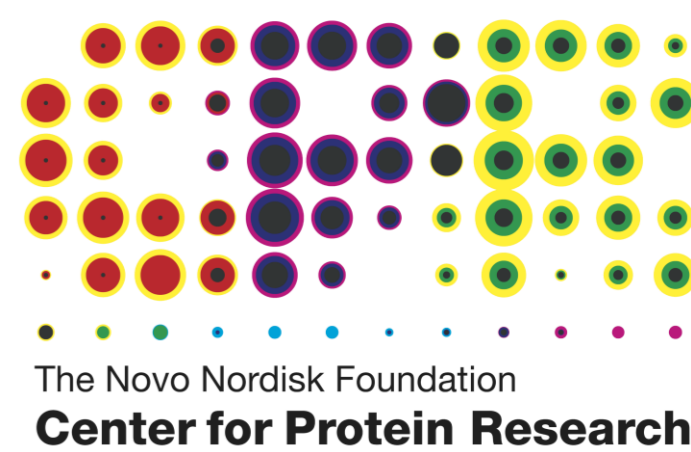
Far left: Number of protein groups quantified per tissue type in this study. After automated PAC digestion, 500ng was analyzed using 21min gradient DIA on an Orbitrap Exploris™ 480. Data was processed using project specific spectral libraries. **Left:** Total number of phosphopeptides and phosphosites quantified across tissue types. Samples were digested on-bead followed by phosphopeptide enrichment (200µg input digest). 500ng was analyzed on 21min gradient DIA with Orbitrap Exploris™ 480, data processed using directDIA (Spectronaut™).



Above: Unsupervised hierarchical clustering of relative protein abundances (plotted as z-score) reveal high correlation within-tissue as well as illustrate tissue-specific expression patterns. **Left:** Unsupervised hierarchical clustering using log-transformed z-scored phosphosite intensities reveal high replica correlation across the same tissue as well as indicating tissue-specific phosphorylation patterns.

CONCLUSIONS

- We demonstrate an automated workflow for global proteome and phosphoproteome profiling suitable for a range of tissues by coupling PAC to phosphopeptide enrichment
- Automation enables high throughput sample preparation for 96 samples in less than 6 hours, allowing short gradient DIA analysis of 60 to 100 samples per day using an Evosep 1 LC system coupled to a ThermoFisher Exploris™ 480.
- This approach can quantify up to 5100 mammalian proteins in a short 21 minute gradient, allowing for up to 60 samples to be measured within 24 hours.
- New high performance prototype variants of Titanium and Zirconium IMAC improved phosphopeptide coverage of samples, without reduction in specificity, showing good recovery for low peptide inputs.
- We intend to further evaluate the selectivity of these new prototypes in future studies to ensure optimal sample coverage.



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