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TECHNICAL BRIEF

Reliability of protein abundance and synthesis measurements in human skeletal muscle

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Deuterium oxide; heavy water; fractional synthesis rate; biosynthetic labelling; protein turnover; proteome dynamics; repeatability; precision; coefficient of variation

Abbreviations:

^2H_2O, deuterium oxide; ABD, abundance; AU, arbitrary unit; CI, confidence interval; DPP, dynamic proteome profiling; FSR, fractional synthesis rate; LFQ, label-free quantitation; RMA, reduced major axis.

Abstract

We investigated the repeatability of dynamic proteome profiling (DPP), which is a novel technique for measuring the relative abundance (ABD) and fractional synthesis rate (FSR) of proteins in humans. LC-MS analysis was performed on muscle samples taken from male participants (*n* = 4) that consumed 4x 50 ml doses of deuterium oxide (^2H_2O) per day for 14 d. ABD was measured by label-free quantitation and FSR was calculated from time-dependent changes in peptide mass isotopomer abundances. One-hundred and one proteins had at least 1 unique peptide and were used in the assessment of protein ABD. Fifty-four of these proteins met more stringent criteria and were used in the assessment of FSR data. The median (M), lower- (Q_1) and upper-quartile (Q_3) values for protein FSR (%/d) were M = 1.63, Q_1 = 1.07, Q_3 = 3.24. The technical CV of ABD data had a median value of 3.6 % (Q_1 1.7 % - Q_3 6.7 %), whereas the median CV of FSR data was 10.1 % (Q_1 3.5 % - Q_3 16.5 %). These values compare favorably against other assessments of technical repeatability of proteomics data, which often set a CV of 20 % as the upper bound of acceptability.

Skeletal muscle is an accessible tissue in humans and offers a unique opportunity to study complex human physiology, including ageing, polygenic disease and adaptations to exercise, which can be challenging to reproduce in animal or cell models. Proteomic analysis of muscle is particularly relevant because the proteome is the interface between gene-environment interactions that underpin the current functional state of a tissue. Proteomic studies of human muscle have provided insight by associating patterns of protein abundance or post-translational modification with different functional states (reviewed in [1] amongst others). However, this static information does not capture dynamic aspects of the proteome such as turnover or adaptation. Static information, even when collected in a time-series, cannot give insight to protein turnover or the relative contributions that synthesis and degradation make to changes in protein abundance. Proteins within human muscle exhibit a broad range of different turnover rates and changes to both synthesis and degradation contribute to adaptations in protein abundance [2]. We [3] recently developed the new technique of dynamic proteome profiling (DPP) that can measure both the abundance and synthesis rate of individual proteins in human muscle. DPP combines deuterium oxide (^2H_2O)-labelling with peptide MS and offers the first insight to dynamic aspects of the human proteome *in vivo*. To further establish DPP it is important to investigate the reliability of the technique and estimate the sensitivity of DPP to detect biologically meaningful changes in relative protein abundance (ABD) and fractional synthesis rate (FSR).

We report the repeatability of protein ABD and FSR data in replicate analysis of muscle samples from 4 sedentary men (age = 38 ± 7 y; body mass = 76 ± 4 Kg). Each volunteer gave their informed consent to the experimental procedures, which were approved (16/WM/0296) by the Black Country NHS Research Ethics Committee (West Midlands, UK) and conformed with the Declaration of Helsinki. Stable isotope labelling of newly synthesised proteins *in vivo* was achieved by oral consumption of ^2H_2O over a 14-day period. Consistent with our previous work [3], participants consumed 50 ml of 99.8 atom % of ^2H_2O four times per day. Venous blood was collected bi-daily, and muscle was collected at baseline (day 0), and after 4, 9, and 14 days of ^2H_2O consumption. Samples (~100 mg) of vastus lateralis were taken using the conchotome technique after administration of local anaesthetic (0.5 % Marcaine). Two biopsies were taken from each leg in alternate order and all samples were obtained after an overnight fast.

Body water enrichment of ^2H was measured in plasma samples against external standards by gas chromatography-mass spectrometry [4]. Soluble proteins were extracted from muscle samples as previously described [3]. Tryptic digestion was performed using filter-aided sample preparation [5]. Digests containing 4 µg of peptides were de-salted using C_{18} Zip-tips (Millipore) and analysed by LC-MS consisting of nanoscale reverse-phase ultra-performance LC (NanoAcquity; Waters Corp., Milford, MA) and online ESI QTOF MS/MS (Q-TOF Premier; Waters Corp.). Samples (5 µl corresponding to 1 µg tryptic peptides) were loaded by partial-loop injection on to a 180 µm ID x 20 mm long 100 Å, 5 µm BEH C_{18}

Symmetry trap column (Waters Corp.) at flow rate of 5 µl/ min for 3 min in 2.5 % (v/v) ACN, 0.1% (v/v) FA. Separation was conducted at 35 °C via a 75 µm ID x 250 mm long 130 Å, 1.7 µm BEH C_{18} analytical reverse-phase column (Waters Corp.). Peptides were eluted using a linear gradient that rose to 37.5 % ACN 0.1% (v/v) FA over 75 min at a flow rate of 300 nl/ min. Eluted peptides were sprayed directly in to the MS via a NanoLock Spray source and Picotip emitter (New Objective, Woburn, MA). Additionally, a LockMass reference (100 fmol/ µl Glu-1-fibrinopeptide B) was delivered to the NanoLock Spray source of the MS and was sampled at 240 s intervals. For all measurements, the MS was operated in positive ESI mode at a resolution of 10,000 FWHM. Before analysis, the TOF analyser was calibrated using fragment ions of [Glu-1]-fibrinopeptide B from m/z 50 to 1990. Peptide MS were recorded between 350 and 1600 m/z and muscle samples were analysed in duplicate, in a randomized order interspersed by inter-sample blanks (5 µl 0.1 % FA separated over a 15 min linear gradient). Data-dependent MS/MS spectra were collected from baseline (day 0) samples over the range 50–2000 m/z. The 5 most abundant precursor ions of charge 2+ 3+ or 4+ were selected for fragmentation using an elevated (20–40 eV) collision energy. A 30-s dynamic exclusion window was used to avoid repeated selection of peptides for MS/MS.

Label-free quantitation (LFQ) was performed using Progenesis Quantitative Informatics for Proteomics (Waters Corp.) consistent with our previous work (e.g. [3, 6, 7]). Analytical data were LockMass corrected using the doubly-charged monoisotopic ion of the Glu-1- fibrinopeptide B and prominent ion features were used as vectors to warp each data set to a common reference chromatogram. MS/MS spectra were searched against the Swiss-Prot database (2018.7) restricted to Homo-sapiens (20,272 sequences) using a locally implemented Mascot server (v.2.2.03; www.matrixscience.com). Enzyme specificity was trypsin (allowing 1 missed cleavage), carbamidomethyl modification of cysteine (fixed), and m/z errors of 0.3 Da.

Log-transformed MS data were normalized by inter-sample abundance ratio, and protein relative abundance (ABD) was calculated using nonconflicting peptides. Mass isotopomer abundance data were extracted from MS spectra using Progenesis Quantitative Informatics (Waters Corp.). The abundance of m_0–m_4 mass isotopomers was collected over the entire chromatographic peak for nonconflicting peptides used in LFQ. Incorporation of ^2H into protein is associated with a decrease in the molar fraction of the monoisotopic (m_0) peak. Changes in mass isotopomer distribution follow a nonlinear bi-exponential pattern as a result of the rise-to-plateau kinetics in ^2H enrichment of the body water compartment, and the rise-to-plateau kinetics of ^2H-labelled amino acids in to newly synthesised protein. Data were fitted using the Nelder-Mead simplex method to optimise for the rate of change in the molar fraction of the m_0 peak. The rate constant (k) of change in mass isotopomer distribution is a function of the number of exchangeable H sites and was accounted for by referencing peptide

sequences against the ^{2}H enrichment of amino acids in humans [8]. Peptides were selected for statistical analysis if they were (i) unique to a protein, (ii) fitted well (R^2 >0.85) to the biexponential model, and (iii) were detected in each technical replicate across the entire time series (0, 4, 9 and 14 d) of samples from all 4 participants. Protein FSR was derived from the median *k* of peptides assigned to the protein. All data processing and statistical analyses were performed in *R* version 3.5.2.

The enrichment of ^{2}H in body water increased at a rate of 0.135 ± 0.005 %/d and reached a peak of 2.14 ± 0.08 % on day 14. LFQ encompassed 101 proteins that had at least 1 unique peptide and ABD ranged from 7.75 e^{+01} AU (CISY: citrate synthase) to 2.86 e^{+05} AU (HBA: hemoglobin subunit alpha). Fifty-four proteins passed the more stringent filtering necessary for synthesis calculations and FSR ranged from 0.37 %/d (CASQ1: calsequestrin-1) to 12.90 %/d (APOA1: apolipoprotein A-I). The median (M), lower quartile (Q_1) and upper quartile (Q_3) values for protein FSR (%/d) were M = 1.63, Q_1 = 1.07, Q_3 = 3.24.

The overall repeatability of proteome ABD and FSR data was assessed using reduced major axis (RMA) regression that is appropriate when random error is expected in both *x* and *y* variables. RMA is equivalent to ordinary least products regression [9] and can distinguish between fixed- and proportional-bias. Strong linear relationships (Figure 1 A and D) existed between replicate analyses of ABD (R^2=0.9989.; p = 0.0104) and FSR (R^2=0.9535; p = 0.0104). The 95 % confidence interval (CI) of the intercept and slope were used to assess fixed- and proportional-bias, respectively. ABD data did not exhibit fixed bias (95 % CI of intercept = -68.2 – 38.7) but there was evidence of proportional bias because the 95 % CI for the slope (0.951 – 0.957) did not include 1. The 95 % CI for the slope of FSR data (0.9285-1.001) did span 1 but the 95 % CI for the intercept of FSR data (0.100 – 0.308) did not span zero, which suggests fixed bias exists between replicate analyses. RMA analysis summarises the overall reliability of the proteomic data but each protein exhibits unique technical repeatability. CV is representative of 68 % (1SD) of the likely variation in data and has been a commonly used (e.g. [6, 10, 11]) index in reliability studies. CV was used to illustrate relative differences in the technical repeatability of ABD and FSR data on a protein-by-protein basis (Figure 1B and 1E, respectively). The mean CV of ABD data was 5.5 % (SD = 6.6 %); M = 3.6 % (Q_1 1.7 % - Q_3 6.7 %), the mean CV of FSR data was 14.1 % (SD = 13.6 %); M = 10.1 % (Q_1 3.5 % - Q_3 16.5 %). To give insight to biological variability amongst participants, FSR and ABD data were filtered to exclude proteins with a technical repeatability of >5 %CV. The biological variability of FSR (Figure 1F) was M = 30.4 % (Q_1 17.9 % - Q_3 42.0 %) and was approximately double that of ABD data (Figure 1C; M = 14.6 %; Q_1 7.7 % - Q_3 25.1 %). Ordinary least squares regression found that neither protein ABD, FSR or the number of peptides per protein predicted the level of technical repeatability in FSR (Figure 1 G, H and I). Accordingly, there was no difference (p=0.7511) between the CV of FSR calculated from single peptides versus proteins that had 2 or more peptides.

Lastly, the ability of DPP to replicate a given result (i.e. precision) was defined according to ISO 5725 in which the precision of a method is denoted by its repeatability (*r*)

$$r = 1.96\sqrt{2Sw^2}$$

where Sw is the within-subject standard deviation. The practical interpretation of *r* is "the value below which the difference between two measurements would lie with a probability of 0.95" [12]. Glycolysis was the most dominant functional group amongst the proteins surveyed and the precision of ABD and FSR measurements for enzymes of the glycolytic pathway in human muscle is summarised in Figure 2. Hexokinase was the only enzyme of the glycolytic pathway that was not detected in the current analysis, or in our previous work [6]. This may be an artifact of our standard fractionation procedure or it may relate to the reported differences in subcellular location of hexokinase [13] compared to other glycolytic enzymes [14]. Consistent with [15, 16] enzymes of the second phase of glycolysis were of greater ABD than those belonging to stage 1. Interestingly, the opposite pattern emerged in FSR data and generally the FSR of stage 2 glycolytic enzymes was less than those involved in stage 1.

An understanding of measurement precision is an important aspect of scientific investigation and is prerequisite to proper experimental design. The average CV in ABD data presented here (6 %) compares favorably with 12 % for LFQ in yeast [10], 6 % for SWATH-MS analysis in HEK293 cells [11] and 7 % in our [6] previous assessment of in rat skeletal muscle. Methods for studying the dynamic aspects of the proteome, particularly in humans *in vivo*, are less widely reported than static abundance data. The average technical repeatability (14 %) of protein FSR was remarkably similar to the performance of the more established techniques for measuring relative protein abundance. Especially so, because FSR calculations require time-series analysis encompassing measurements of both precursor enrichment and incorporation of label in to newly synthesised protein. Biological variability in protein ABD and FSR was estimated using proteins with the highest levels of technical repeatability (CV <5 %). The biological variability in FSR was approximately double that of protein ABD. Sample size calculations [17], based on Q_3 biological variation, estimate a required *n* of 6 (ABD) or 15 (FSR) to detect a within-subject change of 50 % (α = 0.05, 80 % power). DPP of human muscle responses to resistance exercise [3] reported changes in FSR that, generally, were of twice the magnitude of changes in ABD. The above observations suggest DPP has an equal ability to detect changes in ABD and FSR in the setting of human exercise physiology.

The current assessment of DPP was limited to a subset of the most abundant muscle cytosolic proteins [16]. While repeatability of protein ABD was similar to more extensive profiling of rat muscle

[6], it remains to been shown whether yet deeper analysis of the muscle proteome would achieve similar levels of technical repeatability in FSR data. Herein, we report high levels of repeatability for the measurement of both ABD and FSR using peptides that were consistently resolved and detected in all samples. However, at a confidence level of 95 %, fixed bias was detected between duplicate analyses of FSR data and proportional bias was detected in the measurement of ABD. In addition, approximately 50 % of proteins were not consistently resolved in all samples and these data were excluded prior to statistical analysis. Increasing the number of proteins eligible for statistical analysis is key to the future development of the DPP technique and could be achieved through the use of more modern instrumentation and orthogonal peptide separation techniques. Whereas, less stringent handling of missing data, for example by inclusion of proteins detected in a subset of participants or samples, should be avoided because this diminishes the technical repeatability of ABD and FSR measurements.

In conclusion, DPP is a robust technique for the assessment of protein abundance and synthesis rates in human skeletal muscle. Peptide MS analysis of $^{2}H_2O$-labelled samples is a burgeoning discipline that has promise in bringing new insight to dynamic aspects of the proteome. The ability of DPP to report abundance and synthesis data on a protein-by-protein basis in humans *in vivo* is unique. In the future DPP of human muscle may help to uncover new information regarding the complex effects of exercise, ageing or chronic diseases on the rate of turnover, as well as abundance, of muscle proteins.

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Figure Legends

Figure 1 – Repeatability of protein abundance and synthesis measurements

Duplicate analysis of (A) normalised protein abundance (ABD) measured by label-free quantitation and (D) fractional synthesis rate (FSR) measured by deuterium oxide labelling *in vivo* and peptide mass isotopomer analysis. Abundance data (AU) are reported for 101 proteins measured in each technical replicate of n = 4 participants at experiment day 0. Synthesis data (%/d) are reported for 54 proteins measured in technical replicates of n = 4 participants in time-series analysis of samples collected at 0, 4, 9 and 14 d of deuterium administration. The line of best fit was calculated by reduced major axis regression and used to investigate fixed- or proportional-bias in ABD or FSR data.

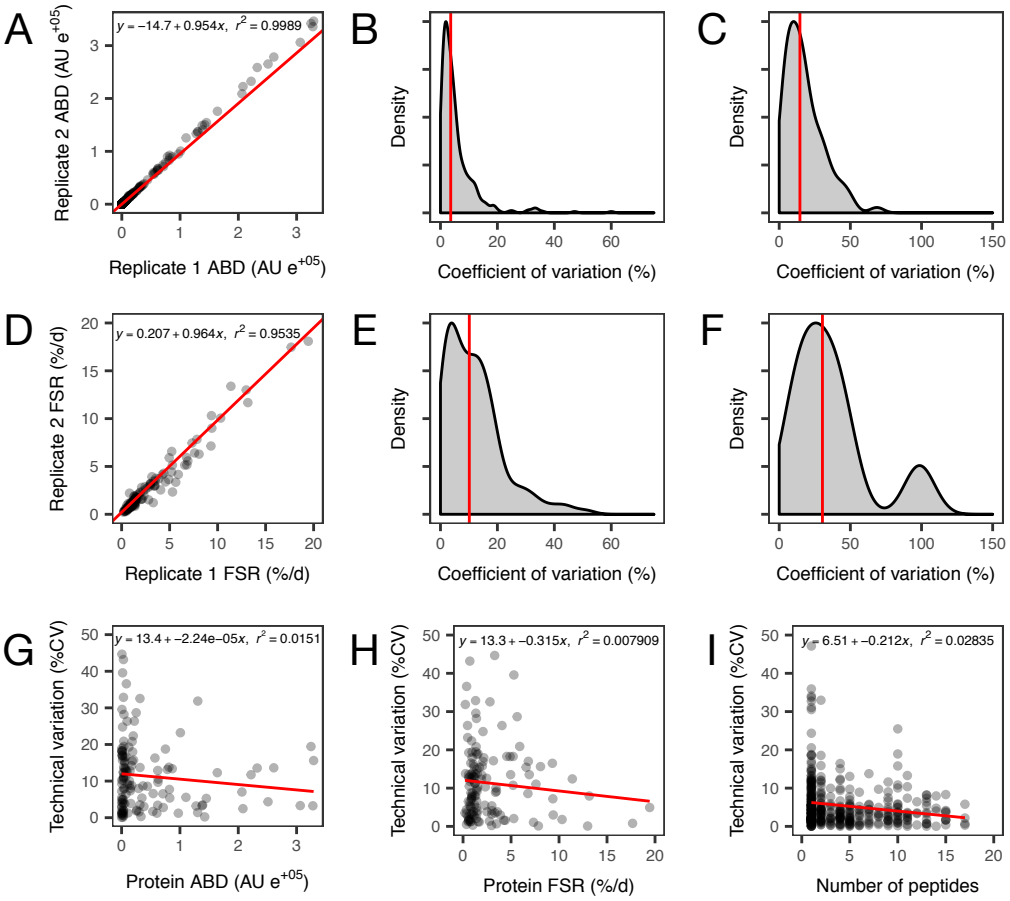
Density plots (B and E) illustrate the distribution and median CV for technical replication of ABD and FSR data. Biological variation (C and F) was illustrated after filtering protein lists to remove proteins with a level of technical CV >5 %.

Scatter plots (G, H and I) illustrate the lack of significant relationship between technical variation in protein FSR (%CV) and (G) protein ABD, (H) protein FSR, or (I) number of peptides per protein.

Figure 2 – Dynamic proteome profiling of glycolytic enzymes in human skeletal muscle

Gray boxes display the common name of each enzyme in the glycolytic pathway, redrawn from the Kyoto Encyclopaedia of Genes and Genomes. Adjacent boxes detail the UniProt protein ID and number of peptides. The median abundance (ABD) and fractional synthesis rate (FSR) of proteins is reported in *n* = 4 participants. Data in parentheses represent the repeatability of ABD and FSR measurement calculated according to ISO 5725 and defined as the maximum difference expected between two measurements in 95 % of cases.

247 Figure 1



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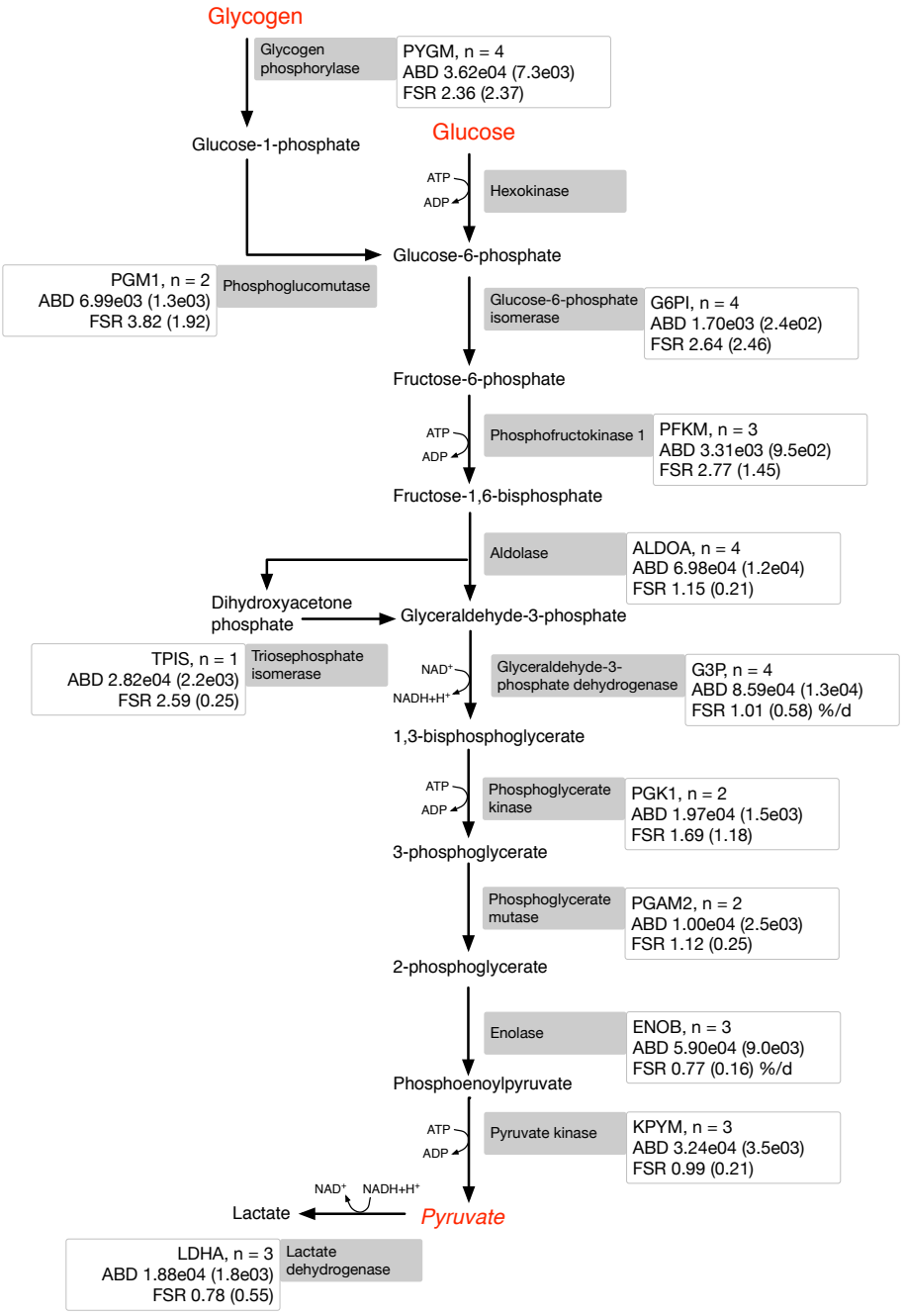
250 Figure 2



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