nature portfolio

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Last updated by author(s): Jun 2, 2022

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐	☒ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	All Q-exactive data were collected by Xcalibur™, which is commercial software by Thermo Fisher Scientific. S/N ratios from RAW files was extracted by ThermoRawFileParser (https://github.com/compomics/ThermoRawFileParser/pull/137). All timsTOF SCP data were collected by timsControl 3.1, which is commercial software by Bruker.
Data analysis	Raw mass-spec data were searched by DIA-NN (version 1.8.1 beta 16) The output of DIA-NN was further analyzed by a pipeline using the R programming language, which is available at https://github.com/SlavovLab/plexDIA and https://scp.slavovlab.net/plexDIA

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The raw data and search results are available at MassIVE: MSV000089093 Processed data and metadata are available at: scp.slavovlab.net/Derks_et_al_2022

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Bulk plexDIA and LF-DIA benchmarking involved using 3 samples with different compositions of mixed-species proteomes. We chose to use 3 samples in this demonstration because commercially available mTRAQ is currently limited to 3 non-isobaric, isotopologous labels. These 3 unique samples are sufficient to benchmark quantitative accuracy, proteomic coverage, and other metrics relevant to proteomic analysis. The samples were analyzed in triplicate, each with two different acquisition methods.
Data exclusions	The 3rd replicate of Sample C LF-DIA using MS2-optimized data acquisition was excluded from analysis due to technical reasons that resulted in unrepresentatively poor performance. It's exclusion only affects a subset of results shown in Figure S1 and does not change any core findings of the paper.
Replication	Benchmarking data was acquired in triplicate (technical replicates) for plexDIA and LF-DIA studies, for MS1- and MS2-optimized data acquisition methods. The replicates for plexDIA were reproducible; however, for LF-DIA, the 3rd replicate of Sample C using MS2-optimized data acquisition was excluded from analysis due to technical reasons that resulted in unrepresentatively poor performance. It's exclusion only affects a subset of results shown in Figure S1 and does not change any core findings of the paper.
Randomization	Sample A, B, and C used for benchmarking in figure 3 were labeled with mTRAQ-140, mTRAQ-144, and mTRAQ-148 respectively. We also acquired data from all possible permutations between the these 3 bulk samples and the labels used to investigate whether there are labeling-associated artifacts. The single cells were randomized with respect to the mTRAQ mass tag used.
Blinding	Blinding is not relevant to this study because having prior knowledge of which samples are being tested cannot change the raw data or the down-stream results.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	U-937, Jurkat, and HPAF-II cells pancreatic ductal adenocarcinoma (PDAC) cells purchased from ATCC. Melanoma cells (WM989-A6-G3, a kind gift from Arjun Raj, University of Pennsylvania)
Authentication	We relied on the authentication performed by ATCC and did not perform additional authentication.
Mycoplasma contamination	The cells were not tested for mycoplasma contamination
Commonly misidentified lines (See ICLAC register)	No commonly misidentified lines were used in our study.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	U-937 monocytes were harvested and aliquoted to a final 1 mL suspension of approximately 1 × 10^6 cells in RPMI-1640 Medium. Then DNA was stained by incubating the cells with Vybrant DyeCycle Violet Stain (Invitrogen, V35003) at a final concentration of 5 µM in the dark for 30 minutes at 37C, as per the manufacturer's instructions. Next, the cells were centrifuged, then resuspended in PBS to a density of 1 × 10^6 cells/mL. The cell suspension was stored on ice and protected from light until sorting began.
Instrument	The cells were then sorted with a Beckman CytoFLEX SRT.
Software	CytExpert was used to collect data on the Beckman CytoFLEX SRT. FCS Express 7 was used to plot of a density histogram for the cell cycle populations.
Cell population abundance	The 3 different cell cycle phases were sorted into their own respective tubes, i.e. not mixed. Given that cell-cycle phases fall along a continuum, there may be some impurity in the sorted populations. However, the distribution of cells in G1, S, and G2/M cell-cycle phase was in line with expectations suggesting the DNA-staining was successful and the populations are reasonably pure.
Gating strategy	The population of U-937s was gated to select singlets based on FSC-A and FSC-H, this population of singlets was then subgated based on DNA content using the PB-450 laser (ex = 405 nm / em = 450 nm) to select G1, S, and G2/M phase cells as shown in Figure 4a.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.