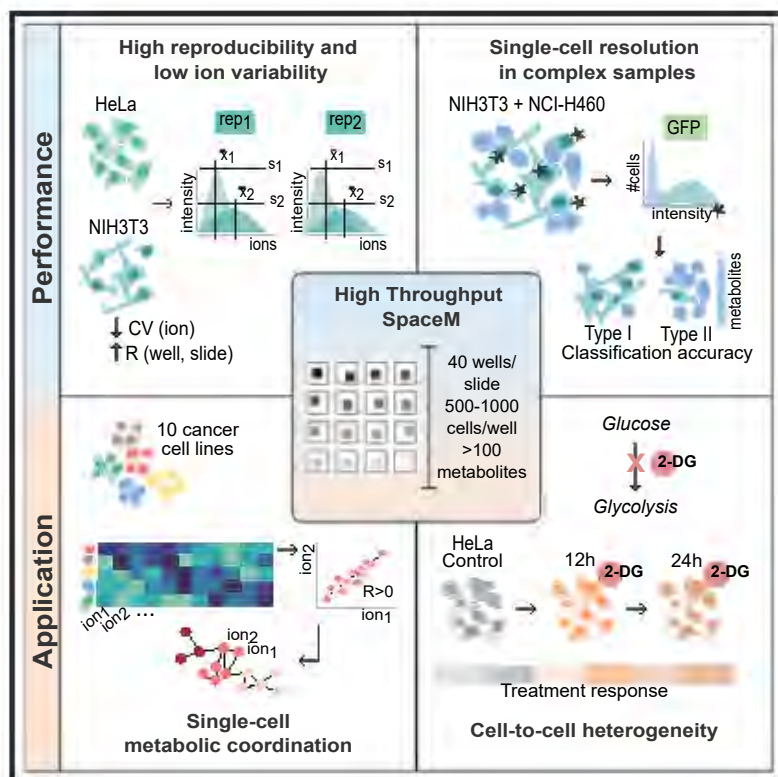


HT SpaceM: A high-throughput and reproducible method for small-molecule single-cell metabolomics

Graphical abstract



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In brief

Single-cell metabolomics (SCM) can probe metabolic heterogeneity but is hindered by low sensitivity for small molecules, limited scalability, and the lack of standardized frameworks for data analysis. HT SpaceM is a high-throughput MALDI-imaging-based SCM method to robustly detect small-molecule metabolites in single cells. Applied to over 140,000 cells across diverse conditions and cancer cell lines, HT SpaceM enabled reproducible metabolic profiling of over 100 small-molecule metabolites, identification of subpopulation-specific markers, and detection of heterogeneity and pathways coordination, thus facilitating scalable and reproducible SCM.

Highlights

- HT SpaceM enables high-throughput single-cell metabolomics across 40 samples per slide
- Detects 100+ small-molecule metabolites per cell from 500 to 1,000 cells per sample
- Achieves high reproducibility between replicates and low variability in ion intensities
- Uncovers metabolic heterogeneity and pathways coordination at single-cell resolution



Article

HT SpaceM: A high-throughput and reproducible method for small-molecule single-cell metabolomics

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SUMMARY

Single-cell metabolomics (SCM) promises to reveal metabolism in its complexity and heterogeneity, yet current methods struggle with detecting small-molecule metabolites, throughput, and reproducibility. Addressing these gaps, we developed HT SpaceM, a high-throughput SCM method combining cell preparation on custom glass slides, small-molecule matrix-assisted laser desorption ionization (MALDI) imaging mass spectrometry (MS), and batch processing. We propose a unified framework covering quality control, characterization, structural validation, and differential and functional analyses. Profiling HeLa and NIH3T3 cells, we detected 73 small-molecule metabolites validated by bulk liquid chromatography tandem MS (LC-MS/MS), achieving high reproducibility and single-cell resolution. Interrogating nine NCI-60 cancer cell lines and HeLa, we identified cell-type markers in subpopulations and metabolic hubs. Upon inhibiting glycolysis in HeLa cells, we observed emerging glucose-centered metabolic coordination and intra-condition heterogeneity. Overall, we demonstrate how HT SpaceM enables robust, large-scale SCM across over 140,000 cells from 132 samples and provide guidance on how to interpret metabolic insights beyond population averages.

INTRODUCTION

Single-cell omics are revolutionizing biology by shedding light on cellular heterogeneity, revealing cell types, functional phenotypes, states, and variability of cellular programs within cell subpopulations.¹ Unraveling this heterogeneity is challenging yet paramount to understanding homeostasis, disease onset, progression, and responses to therapies and environmental stimuli.^{2,3} Single-cell metabolomics (SCM) has emerged as a technology of choice to probe the metabolic underpinnings of this heterogeneity by detecting the end-products of cellular metabolism.^{4–7} In addition to fluorescence dyes and sensors as well as Raman spectroscopy, mass spectrometry (MS) has become the major approach for SCM due to its sensitivity and specificity.⁸ Among various MS methods, matrix-assisted laser desorption ionization (MALDI)-imaging MS, originally developed for spatial metabolomics,^{9,10} is becoming increasingly widespread for SCM due to its commercial availability, rapid techno-

logical progress, speed, sensitivity, and potential to scale to tissue sections.⁶

However, current MALDI-imaging-based SCM methods struggle with detecting small-molecule metabolites, with most reports focusing on highly abundant, easily ionizable lipids, particularly cell membrane phospholipids. Saunders et al.¹¹ in Table 1 allude to SCM laser-based imaging MS only reporting lipids as metabolites. Supporting this, our 2024 literature search (original papers and preprints by October 2024) with the keywords “MALDI” and “single-cell metabolomics” showed five^{12–16} of six publications reporting lipids only. The only exception reporting small-molecule metabolites¹⁷ used a custom MALDI-2 post ionization setup. This reflects the challenges in detecting small-molecule metabolites in single cells due to their low abundance yet lack of amplification, high structural diversity, wide dynamic range, and propensity to cellular leakage during preparation. However, a robust detection of small-molecule metabolites is essential for most metabolism



studies, and their underrepresentation limits the SCM's applicability in biology, medicine, and pharmacology.

The second challenge for SCM is the relatively low throughput in the number of samples and cells and limited reproducibility. Addressing this challenge holds high potential, as SCM has relatively simple sample preparation, high acquisition speed, and low per-sample cost compared with sequencing-, probe-, or antibody-based single-cell omics.¹⁸ Despite MALDI imaging providing the highest detection rate among SCM approaches (see Table 1 in Xu et al.¹⁸), reported cell counts remain relatively low, often below 10,000 cells. Cell throughput is often limited by sample preparation variability, batch effects across multiple slides, and high rates of sample outliers and cell outliers. Among the largest SCM studies, two used MALDI imaging to profile 30,000¹⁹ and 30,584²⁰ cells. Yet, robust high-throughput SCM is needed for interrogating larger numbers of samples and cells, first for increasing the confidence in SCM through replicates and, second, for enabling broader SCM biological and clinical applications.

The third challenge in SCM is the lack of established frameworks and guidelines for data analysis. Specifically, there is unclear guidance on quality control, assessing replicates' reproducibility, or quantifying variations in metabolite levels.^{21,22} Addressing this gap is an important goal toward the maturity of the field.^{6,7}

In this work, we address these challenges by introducing HT SpaceM, an SCM method with an accompanying computational framework for SCM data analysis. HT SpaceM builds on the recently published SpaceM²⁰ and introduces optimized cell preparation for small-molecule metabolites, custom laser-etched glass slides, enhanced image analysis, a MALDI-imaging protocol for small-molecule metabolite detection, and batch processing. Our data analysis framework includes quality control, variability assessment, single-cell characterization, structural validation, and differential and network analyses. We validated HT SpaceM's reproducibility on HeLa and NIH3T3 cells, detecting 135 ions in 78,500 cells across 72 samples on 3 slides, with 73 metabolites validated by bulk liquid chromatography tandem MS (LC-MS/MS). In a co-culture of NIH3T3 and NCI-H460 cells (4,413 cells), we confirmed single-cell resolution of the method. Profiling nine NCI-60 cancer cell lines and HeLa cells (202 ions in 42,153 cells from 40 samples), we identified cell line-specific metabolic markers, co-abundant metabolites, and metabolic hubs. In HeLa cells with glycolysis inhibited by 2-deoxyglucose (2-DG) (15,697 cells), we revealed glucose-centered single-cell metabolic coordination and intra-condition heterogeneity.

RESULTS

HT SpaceM method

We present HT SpaceM, a method for high-throughput small-molecule SCM using MALDI-imaging MS. HT SpaceM can analyze up to 40 samples (different cell types or replicates) on the same glass slide, profiling over 1,000 cells per sample, and is applicable to various cell types. HT SpaceM builds on principles from our previously published SpaceM²⁰ and uses MALDI imaging, similarly to microMS²³ and MAMS.²⁴

The experimental steps begin with laser-etching glass slides with fiducials and well identifiers (Figure 1A). Cells are plated into removable multi-well chambers mounted onto a glass slide, and 40 out of 64 wells fall within the acquisition area of the AP-SMALDI5-imaging system (Figure 1B). After adhesion, cells may be optionally fixed (for lipids only) or stained to improve cell segmentation or phenotyping. Chambers are then removed, cells washed to remove medium, and desiccated under vacuum. Microscopy (bright field and fluorescence) captures cell outlines and phenotypes (Figure 1C). MALDI-imaging MS is performed using a protocol optimized for small-molecule detection. Finally, a second round of bright-field microscopy localizes laser ablation marks. Registration of pre- and post-MALDI images aligns cell outlines with ablation mark positions.

The computational steps of HT SpaceM (Figure 1D) start with stitching microscopy tiles into wide-field images. Using in-house software, an optimal region of interest (ROI) is selected per well based on cell confluency and minimal washing artifacts. MALDI imaging is performed automated across all wells, and the raw file is centroided and split into one *imzML* file per well. Metabolite annotation is performed using METASPACE²⁵ against the Human Metabolome Database (HMDB) v4²⁶ and CoreMetabolome v3 databases.²⁷ Downloaded annotations are compiled into a custom database comprising ions co-localized with cells. Well-dataset reannotation against this custom database on METASPACE reduces dataset-wide dropouts. In parallel, cells are segmented using a Cellpose *cyto2* model²⁸ fine-tuned to our data (Figure 2A). Pixel-cell deconvolution, normalization, ion-cell co-localization, and filtering are performed as in SpaceM,²⁰ delivering single-cell metabolic profiles.

HT SpaceM can analyze 40 samples per slide, five times more than SpaceM. This allows considering more replicates per run, improving reproducibility, and reducing batch effects. Moreover, smaller well sizes better align with the effective MALDI acquisition area and improve cell seeding efficiency. Using laser-etched slides simplified image stitching, facilitated microscopy image registration, and helped select ROI for MALDI analysis. Fine-tuning Cellpose allowed us to reduce the cell segmentation time considerably. Compared with SpaceM, to locate laser ablation mark positions, we fitted a prespecified grid of spots. This method was enabled by the AP-SMALDI5's high-accuracy stage (compared with the AP-SMALDI10 used in SpaceM) and showed to be faster, more robust, and better suited for small MALDI step sizes, which often result in overlapping ablation marks.

Data handling was streamlined by adopting Zarr formats for microscopy and relying on METASPACE for automated metabolite annotation, quality control, data storage, and sharing.

Data analysis framework

We present and showcase a data analysis framework for SCM, covering essential steps (Figure 1E): quality control, unsupervised characterization, reproducibility assessment, quantification of metabolite variability, overrepresentation analysis (ORA), and structural validation of MALDI-annotated metabolites. Moreover, we propose a novel single-cell metabolic network analysis based on calculating metabolite co-abundance across single cells.

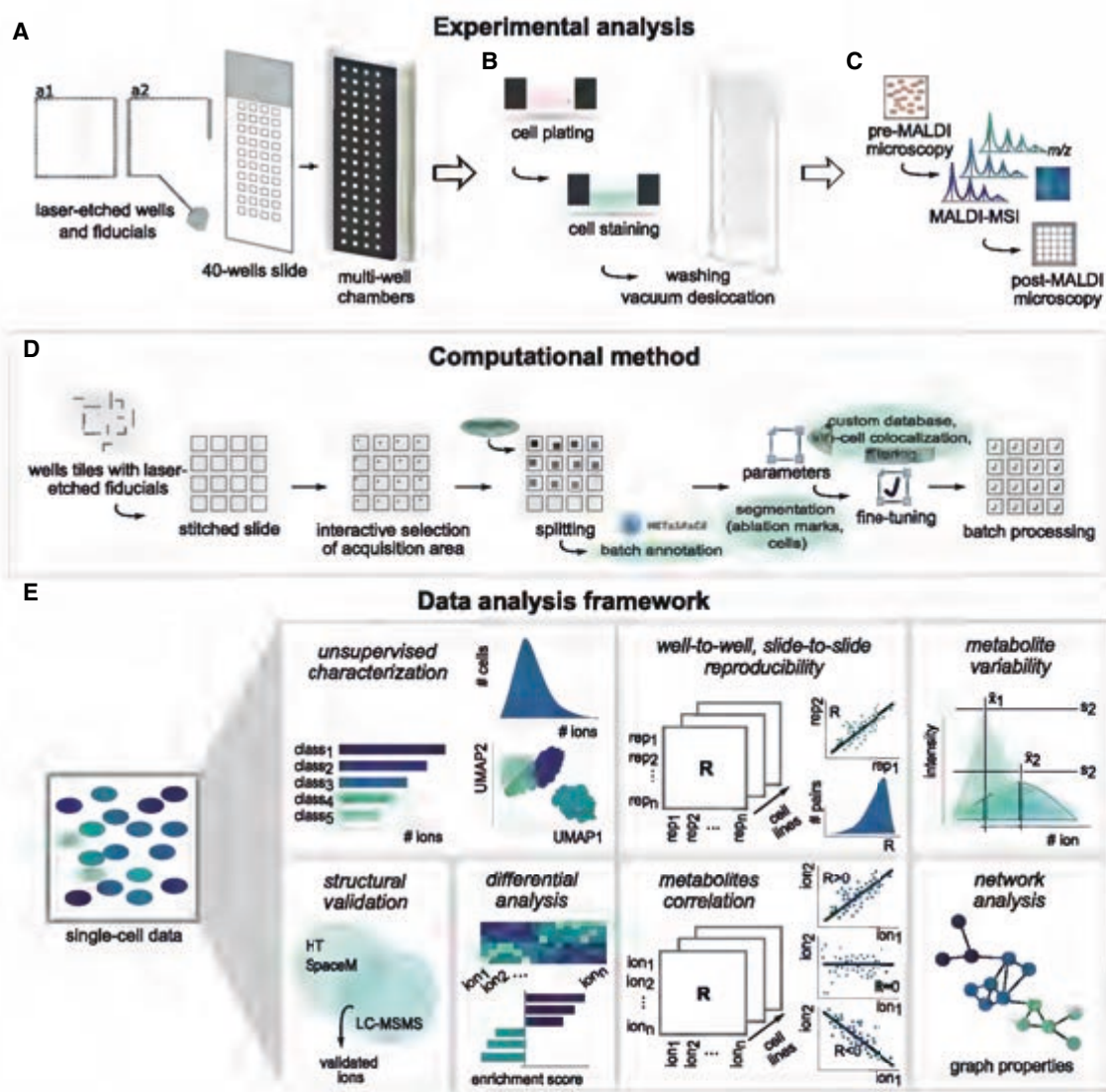


Figure 1. The HT SpaceM workflow and data analysis framework

(A) Laser-etched fiducial marks and well identifiers on a glass slide with removable multi-well chambers.

(B) Cells are plated for adhesion, optionally fixed, stained, and then washed with a volatile solution.

(C) The entire slide is imaged using microscopy, followed by MALDI-imaging MS and another round of microscopy to locate the laser ablation marks.

(D) Fiducials facilitate stitching overlapping microscopy image tiles, registration for pre- and post-MALDI microscopy images, and interactive selection of MALDI-imaging acquisition areas. MALDI-imaging files are batch-uploaded to METASPACE for metabolite annotation. Batch pixel-cell deconvolution is performed using fine-tuned parameters.

(E) Single-cell data analysis framework includes unsupervised characterization, well reproducibility and metabolite variability assessments, structural validation using bulk LC-MS/MS, identification of differentially expressed markers, and single-cell co-abundance analysis through correlation and networks.

HT SpaceM achieves comprehensive small-molecule metabolite coverage across diverse classes and pathways

Despite recent MS advances, detecting small-molecule metabolites in SCM remains challenging due to metabolite loss during sample preparation, low sample volume, limited sensitivity, and ion suppression favoring highly ionizable compounds.¹¹ Many SCM methods, including our original SpaceM, primarily report lipids over small molecules due to lipids' higher abundance and easier detectability. This trend extends beyond MALDI-

based SCM, as ElectroSpray Ionization-based SCM methods also report mainly lipids.^{7,8}

To assess HT SpaceM's small-molecule coverage, we utilized HeLa and NIH3T3 cell lines as models with distinct morphological and metabolic phenotypes. We analyzed 72 wells across three slides: one with all 40 samples and two with only 16 wells each. Consolidating metabolite annotations from all MALDI-imaging datasets, we detected 135 small-molecule ions in negative ion mode using the 2,3-diaminonaphthalene (DAN) matrix, well above the average of 26 metabolites/lipids (at false discovery

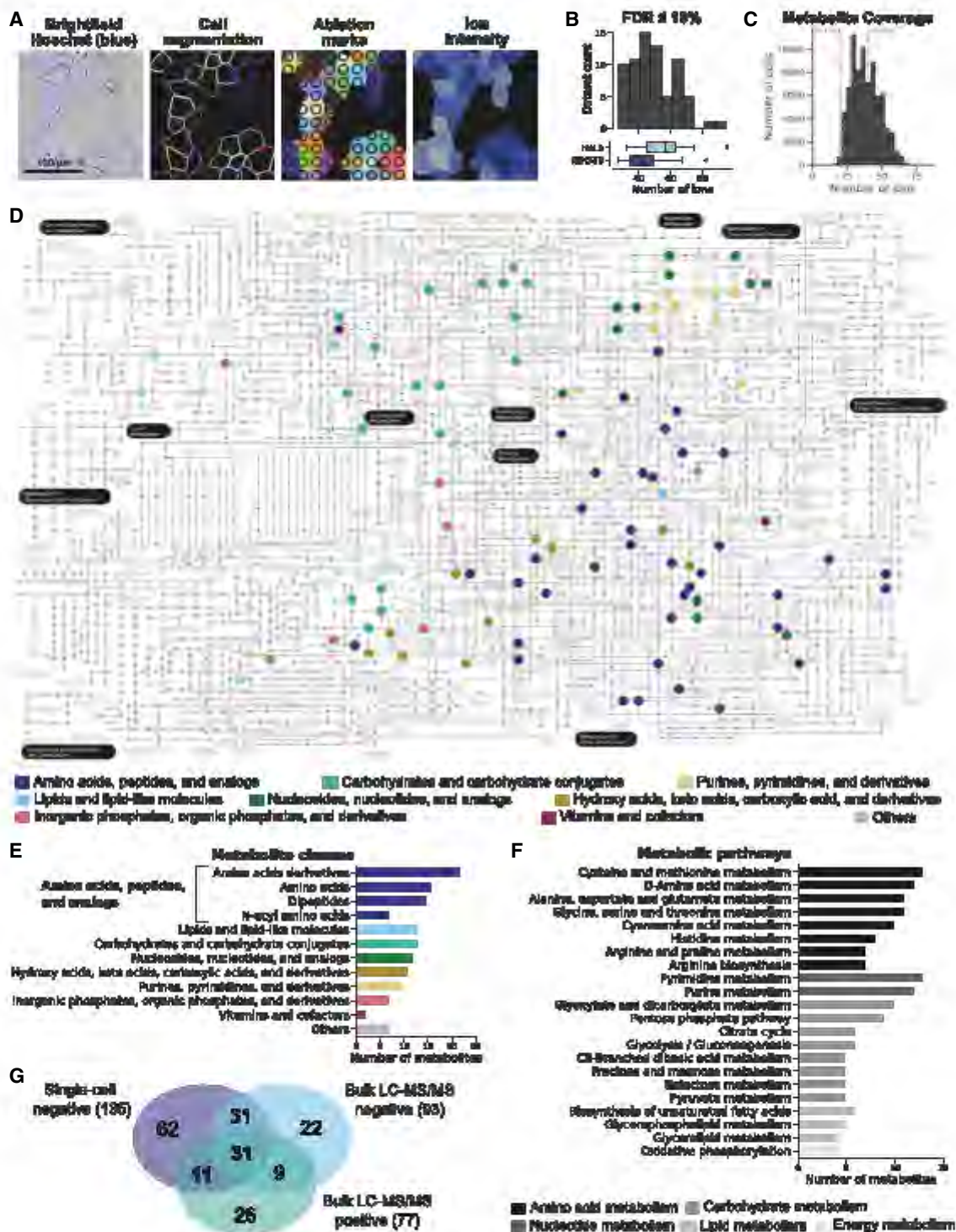


Figure 2. HT SpaceM detects small-molecule metabolites from various classes and pathways

(A) Composite image (bright field and Hoechst, in blue) of HeLa cells showing cell segmentation using Cellpose, the overlay of cells and laser ablation marks, and deconvoluted single-cell intensities (e.g., for N-acetylaspartate [$C_6H_9NO_5$]); scale bar at 100 μm .

(B) Number of ions [$M-H$]⁻ per well at FDR $\leq 10\%$.

(C) Metabolite coverage of single-cell profiles of HeLa (66.2% cells) and NIH3T3 (33.8% cells).

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rate [FDR] $\leq 10\%$) reported in spatial metabolomics studies from the METASPACE knowledge base.³⁰ In either HeLa or NIH3T3 cells, we annotated 30–100 ions per well at the pixel level with FDR $\leq 10\%$ (Figure 2B). HeLa cells consistently yielded more ions than NIH3T3 cells (Figure S1A). At the single-cell level, 20–80 ions per cell were detected with substantial heterogeneity (Figure 2C): only 10% of ions appeared in $>90\%$ of cells, while 47% of ions appeared in $<10\%$ of the HeLa and NIH3T3 cells together.

Overall, we detected 135 [M-H][−] ions co-localized with cells, representing endogenous biologically relevant metabolites. Of these, 101 metabolites matched human metabolic pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database²⁹ (Figure 2D). These metabolites spanned diverse metabolite classes (Figure 2E), with amino acids, peptides, and analogs ($n = 60$) being most frequent. Lipids were mainly fatty acids (FAs) and derivatives, due to the m/z range of 100–400 and ionization in negative ion mode. Importantly, detected metabolite classes align with the expected metabolite detectability of AP-MALDI-imaging MS.³¹ Multiple metabolic pathways are represented, with the most metabolites from cysteine and methionine, pyrimidine, purine, and amino acid metabolism (Figure 2F).

For structural validation, we followed the common approach of using bulk LC-MS/MS of cell lysates, since the limited metabolite abundance in single cells prevents conducting untargeted MS/MS *in situ*. The bulk LC-MS/MS detected 151 metabolites, corresponding to 130 molecular formulas (Figure 2G). About 54% ($n = 73$) of the molecular formulas from SCM were detected by both techniques. LC-MS/MS was conducted in negative and positive ionization modes, and matching MS1-based SCM data with LC-MS/MS confirmed 74 metabolites at level 1 and 17 putatively annotated at level 2 (Table S1), per the Metabolomics Standards Initiative.³² Some metabolites detected only in bulk (e.g., carnitine, creatine, choline, thiamine, and 1-methylnicotinamide) showed higher mean intensities for both HeLa and NIH3T3 cell lines in bulk positive ion mode (Figure S1B). In MALDI these molecules also have a propensity for being much better detected in positive mode (>100 times signal gain; Figure S1C), which can explain why they were not detected by SCM using negative mode.³¹ Overall, metabolites detected by both bulk and single-cell approaches and structurally validated spanned seven of eight main molecular classes (Figure S1D).

Evaluating the throughput, we profiled 78,500 single cells, averaging 1,090 cells per well. The total number of cells varied by cell type, size, and morphology, affecting confluency and cell spread after seeding. HeLa cells, being smaller than NIH3T3 fibroblasts (Figure S1E) and growing in clusters, yielded 52,006 cells, versus 26,494 NIH3T3 cells. This performance exceeds the capacities of SpaceM (with the largest reported number of 30,584 cells²⁰) and microMS (30,000 cells¹⁹).

In summary, HT SpaceM addresses a major gap in SCM by detecting small-molecule metabolites encompassing major classes and pathways, including amino acids, nucleotides, FAs, and carbohydrates.²⁰ The majority of metabolites annotated in HT SpaceM were validated by bulk LC-MS/MS, which detected similar molecular diversity.

HT SpaceM demonstrates high reproducibility across wells and slides with low variability for most of the detected metabolites

Achieving high reproducibility alongside high sensitivity is essential for emerging SCM technologies. However, assessing reproducibility is challenging when constrained by cell numbers and technical replicates. Moreover, there is a lack of established methods for quantifying reproducibility in SCM.^{6,21,33} Here, we propose a data analysis framework to evaluate reproducibility across well and slide replicates and to quantify metabolite variability.

To evaluate well-to-well reproducibility, we computed mean ion intensity per metabolite for each well and calculated Pearson correlation between replicates (Figure 3A). Intra-slide replicates exhibited high reproducibility for both cell lines (Figure S2A), indicated by consistent linear correlation across replicates. Approximately 76% of intra-slide well-well pairs exhibited a high coefficient of correlation ($R \geq 0.97$), and the lowest correlations were observed in HeLa cells on slide 2. As expected, inter-slide correlations were slightly lower but still high ($R = 0.88$ – 0.99), with 61% of pairs having $R \geq 0.97$ (Figure 3B). Reproducibility was consistently high across intra- and inter-slide comparisons, independently of cell line (Figure 3C). Additionally, we evaluated slopes of the best-fit lines between replicates, where a slope near 1 indicates comparable intensities. About 63% of the replicate pairs had slopes ≥ 0.97 (58% inter-slide, 70% intra-slide; Figure S2B). No outliers were found among slide 2 replicates (Figure S2C), and replicates clustered well within each cell line. No batch effects were observed for either the well position (rows A–J; columns 1–4) or MALDI acquisition order. Averaging ion intensities per slide and cell line yielded Pearson R values over 0.98, confirming strong slide-to-slide reproducibility (Figure 3D).

We further examined variability of intensities of individual metabolites by calculating the coefficient of variation (%CV) across replicates. %CVs ranged widely (Figure 3E), spanning from 1.2% (glutamine) to 82.8% (creatinine) in HeLa cells, while the same metabolites displayed different %CVs in NIH3T3 cells, 1.4% and 32.0%, respectively. Glutamine exhibited consistently high intensities across all wells (Figure S2D), leading to low variability, while creatinine showed low or undetectable signals in several replicates, particularly in HeLa, contributing to higher %CV. High-intensity ions tended to have lower variability (Figure 3E), and total ion count (TIC) normalization further reduced ion variability (Figure S2E). For low-abundance metabolites, using the median or the 90% quantile intensities may better reduce

(D) The KEGG map of primary human metabolism with detected endogenous metabolites (nodes) colored by class, generated in iPath3.²⁹ Lines represent metabolic reactions.

(E and F) Bar plots showing the detected metabolite classes and pathways, respectively.

(G) Venn diagram comparing the total number of molecular formulas detected by HT SpaceM (negative ion mode) and bulk LC-MS/MS (negative and positive ion modes).

See also Figure S1.

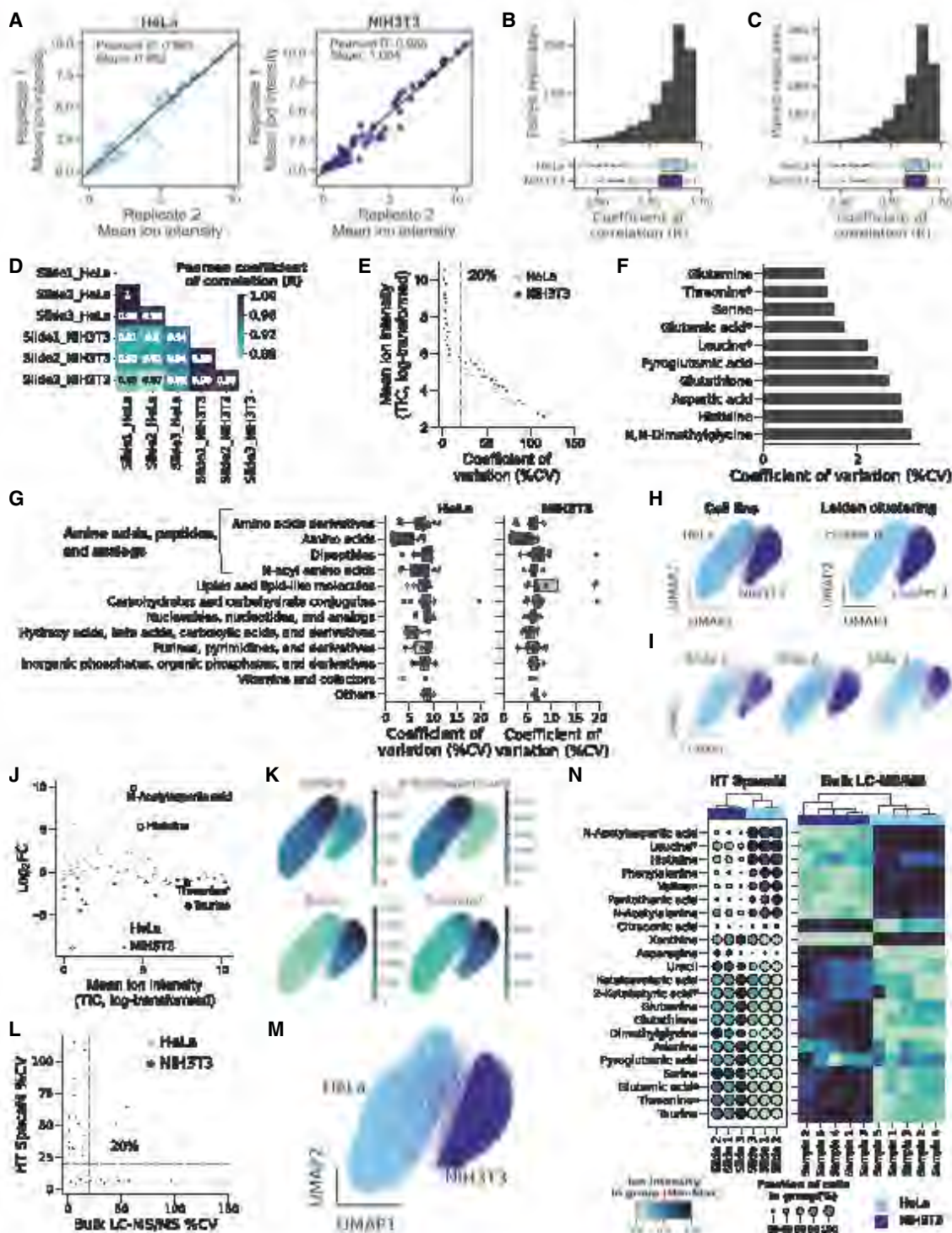


Figure 3. HT SpaceM demonstrates high reproducibility across wells ($n = 72$ wells) and low metabolite variability, delivering metabolite detection comparable to bulk LC-MS/MS

(A) Intra-slide well-to-well reproducibility demonstrated by the Pearson coefficient of correlation (R) and slope calculated for the ion intensities (TIC, log-transformed).
 (B) Distribution of Pearson R calculated between replicates within the cell line (HeLa and NIH3T3) from different slides.
 (C) The same as in (B) but for all well replicates (both inter- and intra-slide).
 (D) Pearson R showing slide-to-slide reproducibility.
 (E) Mean ion intensity within condition (non-zero cell fraction) versus %CV for all detected metabolites.

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sparsity influence on CVs. Overall, most ions demonstrated low variability: 81.48% of the detected ions had %CVs $\leq 20\%$, including 88.17% of HeLa ions and 74.82% of NIH3T3 ions. Comparable %CVs were observed when separating data by slide (Figure S2F), indicating that high %CVs were not linked to batch effects.

Some ions showed consistently low %CVs for both cell lines (Figure 3F), suggesting their variability may be intrinsic to their detectability, i.e., due to abundance or ionization efficiency. Amino acids had the lowest %CVs, with median values $<5\%$ for both HeLa and NIH3T3 (Figure 3G). Overall, variability distributions for metabolites with %CV $\leq 20\%$ were similar between classes, despite cell-specific differences in ion intensities.

In summary, we repurposed correlation, slope, and %CV metrics to assess the technical reproducibility and metabolite variability of HT SpaceM, areas underexplored in SCM. These metrics provided valuable insights into HT SpaceM data quality, highlighting high reproducibility across wells and slides and revealing low variability for most metabolites across diverse metabolic classes.

HT SpaceM achieves molecular coverage and ion variability comparable to bulk LC-MS/MS

While single-cell data capture cell-cell heterogeneity, it is essential to compare conclusions drawn from SCM with those from bulk metabolomics, particularly LC-MS/MS. Moreover, SCM acquired through MALDI imaging requires structural validation, which LC-MS/MS provides. Having this aim in mind, we first compared SCM profiles of HeLa and NIH3T3 cells. Uniform manifold approximation and projection (UMAP) plots showed distinct differences between cell lines, with the unsupervised Leiden clustering reflecting biological differences (Figure 3H), outweighing technical slide-slide variability (Figure 3I). No batch correction was required for integrating data from different slides. Differential analysis revealed that the majority (56%) of metabolites had significant relative intensity differences ($|\text{fold change [FC]}| \geq 1.5$), with most elevated in HeLa cells (Figure 3J). High-intensity metabolites such as histidine, N-acetylaspartic acid, taurine, and threonine had the highest Wilcoxon scores (Figure 3K).

Then, we validated the single-cell profiles using bulk LC-MS/MS in two ways. First, we performed structural validation of the putative metabolite annotations from SCM (Figure 2G). Second, we compared the metabolite intensity variability between

the methods using CVs (Figure 3L). Among shared metabolites, 75% had CV $\leq 20\%$ in both methods (non-zero cell fraction). However, 16.3% showed low variability (CV $\leq 20\%$) in bulk only, while 7.6% showed low variability in single cells only. Detailed CV comparisons are available in Table S1. Single-cell UMAP only for molecular formulas validated with LC-MS/MS and with CV $\leq 20\%$ ($n = 59$) still robustly separated HeLa from NIH3T3 cells (Figure 3M), thus validating the discriminative power of metabolites detected in single cells. Furthermore, differential markers identified at the single-cell level (dot plot) mostly matched the abundance trends in bulk LC-MS/MS (heatmap) (Figure 3N). Among the differentially expressed metabolites ($n = 42$), 85.7% showed similar trends between single-cell and bulk levels in at least one ion mode.

In summary, by comparing against bulk LC-MS/MS, we validated chemical identities and biological relevance of HT SpaceM-detected metabolites.

HT SpaceM distinguishes spatially co-cultured cells based on single-cell metabolic profiles

To evaluate the single-cell resolution of HT SpaceM, we established a spatially heterogeneous co-culture of two cell types, NCI-H460 and NIH3T3. NIH3T3 cells natively express GFP, enabling reliable identification via fluorescence microscopy (Figure 4A). GFP intensities exhibited a clear bimodal distribution, with NCI-H460 cells exhibiting near-zero intensity. Considering all wells together, a threshold of GFP = 10 was set to assign cell types (Figures 4B and S3A).

Cell size and eigenvalues of the inertia tensor provided additional features for distinguishing cell types, as NIH3T3 cells are larger and more elongated than NCI-H460 cells (Figure S4C). Despite reduced size in co-culture, NIH3T3 cells remained visibly larger than NCI-H460 cells, consistent with expectations. Overall, 1,876 cells were assigned as NCI-H460 and 496 cells as NIH3T3.

To test whether single-cell metabolic profiles alone could resolve cell types in the spatially heterogeneous co-culture, we trained and evaluated a random forest classifier using 10-fold cross-validation on a balanced dataset ($n = 496$ cells per class), using the cell-type assignments as the ground truth (Figure 4C). The model achieved an F1 score of 0.81 and an accuracy of 0.81. Among the most informative markers for classification (Figure 4C), FAs (e.g., FA 18:1 and FA 20:4) were enriched in NCI-H460 cells, consistent with monoculture observations (see

(F) Metabolites with the smallest %CV (mean across HeLa and NIH3T3) among all detected ions.

(G) Metabolites %CV for HeLa and NIH3T3 across molecular classes, considering all formulas detected in single-cell data.

(H) UMAP of HeLa and NIH3T3 single cells considering all detected metabolites ($n = 135$). Colored by cell line (left) or Leiden clustering (right).

(I) The same as in (H) but showing slide replicates.

(J) MA plot for the distribution of FC ($\log_2\text{FC}$) and average ion intensity (TIC, log-transformed) for HeLa and NIH3T3. Differential analysis resulted in significant metabolites ($p < 0.05$, FDR-adjusted) with $|\text{FC}| \geq 1.5$ highlighted in light blue if increased in HeLa and dark blue if increased in NIH3T3.

(K) UMAP colored by TIC-normalized intensities of differentially expressed markers ($p < 0.05$, FDR-adjusted).

(L) %CV of HT SpaceM (non-zero cell fraction) and bulk LC-MS/MS for common ions validated at level 1 or 2.

(M) UMAP of the single-cell data but only for ions validated by bulk LC-MS/MS (level 1 or 2) and with %CV $\leq 20\%$ ($n = 59$).

(N) HT SpaceM (dot plot) and bulk LC-MS/MS data (heatmap) for a subset of differentially expressed metabolites ($p < 0.05$, FDR-adjusted). Dot plot grouped by cell line and slide showing the fraction of non-zero ion intensity cells and feature-scaled mean ion intensity. Heatmap showing feature-scaled average metabolite abundance per cell line and replicate. All displayed molecule names were validated in bulk LC-MS/MS at level 1, with (*) denoting the presence of isomers.

See also Figure S2 and Table S1.

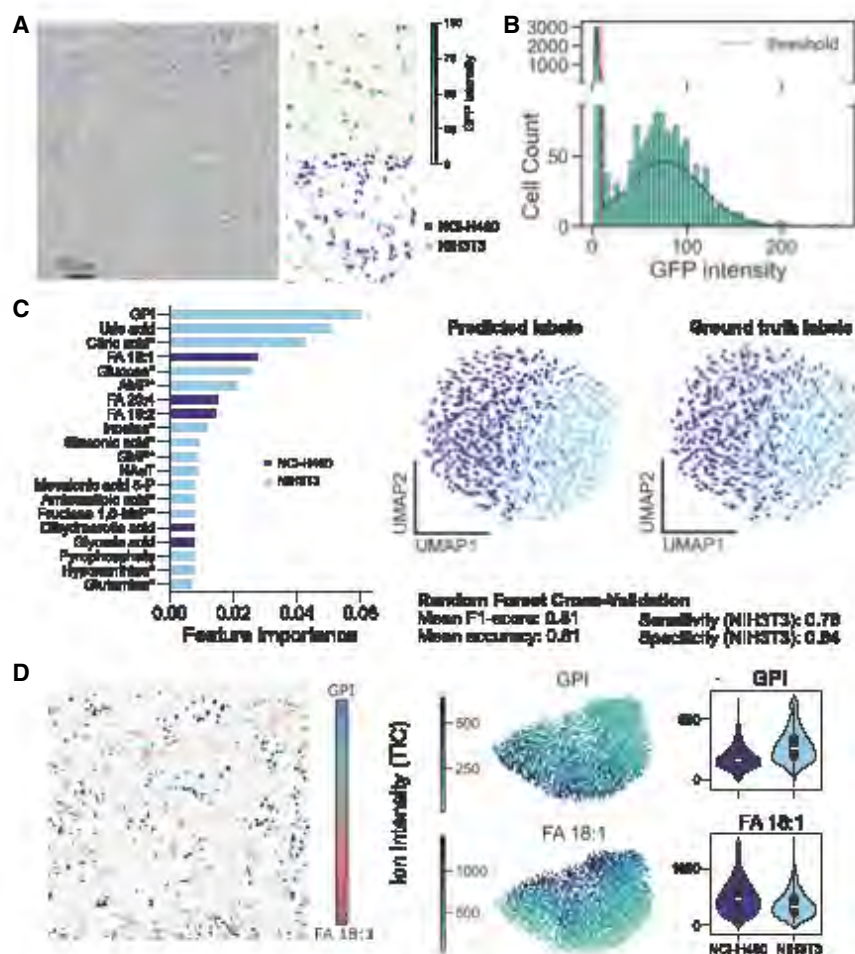


Figure 4. Single-cell metabolic profiles predict cell types

(A) Composite image (bright field and fluorescence) of NCI-H460 and NIH3T3 co-culture; NIH3T3 cells natively express GFP ($\lambda = 609$ nm, green), and all cells' nuclei were stained with Hoechst ($\lambda = 461$ nm, blue), and GFP signal was used for ground-truth cell-type assignment; scale bar at 100 μ m.

(B) GFP intensity bimodality for all replicates indicating cell-type assignment threshold.

(C) Random forest classification (accuracy = 0.80, F1 score = 0.81) based on cell metabolic profiles with the topmost predictive markers shown, and UMAP showing predicted versus ground-truth cell types.

(D) Spatial and intensity (TIC-normalized) distribution of markers (FA 18:1 for NCI-H460; GPI for NIH3T3) across replicates, shown via UMAP and violin plots.

See also Figure S3.

and S4B), with NCI-H460 having the smallest and least variable cell size (median = 346.9 μ m², interquartile range [IQR] = 204.0 μ m²) and BT-549 the largest and most variable (median = 952.6 μ m², IQR = 592.5 μ m²) (Figure S4B). We detected 202 [M-H]⁻ ions co-localized with cells (versus 135 in HeLa/NIH3T3), and 39–147 ions per cell were detected (Figure 5A), with 74.4% of ions present in over 90% of cells. Ion sparsity reflected both biological variability and technical factors. As the number of cell lines increased, the overlap of metabolites detected in all cell lines consistently decreased (Figure S4C). Given the diversity of cells, we investigated how cell size influences metabolite detection. As expected, the number of ablation marks per cell positively correlated with cell size (Figure S4D), with BT-549 overlapping with more ablation marks than NCI-H460. However, metabolite detection did not scale linearly with cell area. For example, NCI-H460, a smaller cell line, showed metabolite counts (mean = 122.0) similar to intermediate-size and larger-size cell lines like BT-549 and HOP-62, respectively (means = 126.6 and 126.3). This suggests that metabolite detection variability is also influenced by intrinsic cell metabolite diversity and abundance. The cell yield at 42,153 cells in 40 wells (39,575 cells of the NCI-60 panel and 2,578 HeLa cells) was comparable to the HeLa/NIH3T3 experiment. Bulk LC-MS/MS data of the same panel revealed 138 metabolites (120 molecular formulas; Figure S4E), with 75 formulas also detected by SCM, comprising 85 level-1 and 5 level-2 metabolite annotations per notation of the Metabolomics Standards Initiative.³²

next section), where FA metabolism was upregulated in this cell line. Error analysis of misclassified cells (Figure S3C) did not reveal any systematic cell-type biases in the classification results.

Finally, spatial mapping of top marker metabolites (e.g., FA 18:1 for NCI-H460 and glycerophosphoinositol [GPI] for NIH3T3) showed that cells with contrasting marker levels were often located in close proximity (Figure 4D). This supports the ability of HT SpaceM to resolve cell-type-specific metabolic profiles, and at the single-cell resolution.

Characterizing cancer cell lines from the NCI-60 panel at the single-cell level

The NCI-60 panel of human tumor cell lines has been pivotal in cancer research, extensively characterized by various omics, including bulk metabolomics.³⁴ However, their single-cell metabolomes remain unexplored. To address this gap and showcase our analysis framework, we applied HT SpaceM to nine NCI-60 cell lines and HeLa, representing diverse tumor origins: breast (BT-549 and HS 578T), cervical (HeLa), colon (HT29), lung (NCI-H460 and HOP-62), ovarian (IGR-OV1 and OVCAR-5), renal cancers (A498), and melanoma (MALME-3M). Cell morphology, e.g., size, varied across cell lines (Figures S4A

and S4B), with NCI-H460 having the smallest and least variable cell size (median = 346.9 μ m², interquartile range [IQR] = 204.0 μ m²) and BT-549 the largest and most variable (median = 952.6 μ m², IQR = 592.5 μ m²) (Figure S4B). We detected 202 [M-H]⁻ ions co-localized with cells (versus 135 in HeLa/NIH3T3), and 39–147 ions per cell were detected (Figure 5A), with 74.4% of ions present in over 90% of cells. Ion sparsity reflected both biological variability and technical factors. As the number of cell lines increased, the overlap of metabolites detected in all cell lines consistently decreased (Figure S4C). Given the diversity of cells, we investigated how cell size influences metabolite detection. As expected, the number of ablation marks per cell positively correlated with cell size (Figure S4D), with BT-549 overlapping with more ablation marks than NCI-H460. However, metabolite detection did not scale linearly with cell area. For example, NCI-H460, a smaller cell line, showed metabolite counts (mean = 122.0) similar to intermediate-size and larger-size cell lines like BT-549 and HOP-62, respectively (means = 126.6 and 126.3). This suggests that metabolite detection variability is also influenced by intrinsic cell metabolite diversity and abundance. The cell yield at 42,153 cells in 40 wells (39,575 cells of the NCI-60 panel and 2,578 HeLa cells) was comparable to the HeLa/NIH3T3 experiment. Bulk LC-MS/MS data of the same panel revealed 138 metabolites (120 molecular formulas; Figure S4E), with 75 formulas also detected by SCM, comprising 85 level-1 and 5 level-2 metabolite annotations per notation of the Metabolomics Standards Initiative.³²

UMAP of single-cell profiles showed clear grouping per cell line (Figure 5B), with high reproducibility between the well replicates (Figure S4F). Unsupervised Leiden clustering showed cell line similarities, e.g., HT29 and IGR-OV1 in cluster 4, and BT-549, OVCAR-5, and HOP-62 in cluster 3 (Figure 5C). Tumor origin did



(B) UMAP of single-cell metabolic profiles from 42,153 cells (39,575 cells from the NCI-60 panel and 2,578 HeLa cells) colored by the cell line, considering 202 ions.

(D) The same as in (B) but colored by tumor origin.

(E) Markers' dot plot showing average metabolite intensity feature-scaled and the fraction of cells expressing the metabolite for each cell line.

(F and G) (F) Overrepresentation enrichment analysis of metabolite classes (HMDB) per cell line ($p < 0.05$). (G) Overrepresentation enrichment analysis of metabolic pathways from the Small Molecule Pathway Database per cell line ($p < 0.1$). (F and G) Color scale corresponds to the enrichment score and dot size to the number of intersected metabolites with the database, and all detected ions in the single-cell analysis were considered for enrichment analysis. Molecule names were validated in bulk LC-MS/MS at level 1, except as indicated by (*). (**) denotes the presence of isomers in the bulk data.

See also [Figure S4](#).

not influence clustering (Figure 5D): NCI-H460 was distinctly separated, including from another lung cancer line, HOP-62. Differential analysis (one versus all) revealed cell line-specific metabolite signatures (Figure 5E). Interestingly, some high-intensity metabolites appeared in <20% of cells (e.g., ribose-phosphate or FA 16:2 in NCI-H460 and FA 20:2 in HeLa), highlighting SCM's capacity to detect rare but biologically relevant events, not attributable to technical dropouts due to sensitivity limitations. Interestingly, NCI-H460 also exhibited the highest cell-cell metabolic heterogeneity as quantified using Shannon's entropy (Figure S4G). Of 70 markers (Figure 5E), 29 were not detected in bulk LC-MS/MS at levels 1 or 2 (marked *), and 10 metabolites presented isomers in bulk data (marked **).

ORA identified enriched classes and pathways. NCI-H460 showed increased FAs (Figure 5F) and FA metabolism (Figure 5G), in line with reported stearyl-coenzyme A (CoA) desaturase upregulation and elevated levels of monounsaturated FAs (FA 16:1 and FA 18:1).³⁵ Conversely, MALME-3M and IGR-OV1 exhibited decreased FA 16:1, FA 18:1, FA 22:6, and FA 22:5 (Figures 5E and 5F), with significant downregulation of omega-3 and omega-6 and alpha-linolenic acid pathways (Figure 5G), despite originating from different tumor types. Additionally, HS 578T showed reduced carbohydrates (Figures 5E and 5F) with altered carbohydrate-related metabolism (Figure 5G), possibly associated with the observed abnormal activity of the Warburg effect (Figure 5G), as breast cancer cells (such as HS 578T) are known for altered glycolysis-related enzymes and transporters.³⁶ By contrast, BT-549 exhibited increased carbohydrate levels (Figure 5E) with positive overrepresentation of this class (Figure 5F). BT-549 presented a trend toward increased pyruvate and citric acid metabolism and glycolysis/gluconeogenesis (Figure 5G).

In summary, HT SpaceM enabled single-cell metabolic characterization of the NCI-60 cancer cell lines, revealing metabolic markers detected even in a small fraction of cells, with ORA providing insights into differential representation of metabolic classes and pathways.

HT SpaceM reveals single-cell metabolic networks of co-abundant metabolites

Through capturing cell-cell heterogeneity, SCM may uncover diverse metabolic programs within cell subpopulations by identifying co-abundant pairs and groups of metabolites within single cells. While co-expression analysis is common in single-cell transcriptomics,³⁷ such exploration has yet to be demonstrated in SCM, likely due to limited small-molecule coverage and high dropout rates. Here, we present an approach for building single-cell metabolic co-abundance networks, showcased for the NCI-60 cancer cell lines.

First, we identified metabolite pairs co-detected across single cells with low dropout mismatch ratios (16.5% of ion pairs) in each cell line (Figure S4H). Only 4.7%–6.7% of all metabolite pairs were substantially co-detected across cell lines. Pearson correlations (R) between co-detected metabolite pairs (Figure S4G) showed that most of them (84.3%–97.0%) had weak linear correlation ($|R| < 0.3$; Figure 6A). Co-abundant metabolites, defined as co-detected pairs with positive or negative linearity ($|R| \geq 0.3$) (Figure S4G), were a minority, with positively co-abundant metabo-

lites more prevalent (2.0%–11.0%) than inversely co-abundant metabolites (0.9%–5.2%). IGR-OV1 and OVCAR-5, two ovarian cancer cell lines, exhibited more positively co-abundant metabolites compared with other cell lines (Figure 6A), suggesting stronger metabolite coordination. HeLa had no strong inversely co-abundant pairs ($R \leq -0.5$). Some metabolite pairs consistently appeared co-abundant across cell lines (Figure 6B), potentially indicating conserved coordination or shared pathways.

For each cell line, we built a network connecting pairs of positively co-abundant metabolites. Using Pagerank weighted by Pearson correlation (Figure 6C), for each network, we ranked node centrality to identify five key “hub” metabolites that were most connected to others (Figure 6D). IGR-OV1 and MALME-3M shared hub metabolites, including glutamic acid, malic acid, and guanine (Figure 6C). Interestingly, these lines also showed similar results in the differential ORA, indicating decreased levels of FAs and conjugates (Figure 5F), along with reduced linoleic acid metabolism and omega FA synthesis (Figure 5G). Additionally, A498 and NCI-H460 shared metabolite hubs: glutamine, oleic acid, taurine, and glutamic acid. High centrality of glutamine and glutamic acid was observed across various cell lines (Figure 6C), with their co-abundance conserved in 9 out of 10 cell lines (Figure 6B), suggesting a mechanism for coordination of these enzymatically related molecules. Some hubs were cell line-specific: BT-549 uniquely presented glycerol 3-phosphate, linking carbohydrate and lipid metabolism. Interestingly, both carbohydrate (glycolysis, gluconeogenesis, citric acid cycle) and lipid (oxidation of branched-chain FAs) metabolic pathways were overrepresented in BT-549 (Figure 5G).

We considered network properties for metabolite classes by calculating class-specific subnetwork node average connectivity (Figure 6E). NCI-H460 exhibited a high lipid connectivity (Figure 6E), aligning with the upregulation of FAs (Figures 5E and 5F) and FA metabolism (Figure 5G), and suggesting single-cell metabolic coordination. Similarly, BT-549 showed the highest connectivity among carbohydrates (Figure 6E), mirroring the upregulation of carbohydrate metabolism (Figures 5E–5G). The ovarian cancer cell lines IGR-OV1 and OVCAR-5 showed similar connectivities for carbohydrates, lipids, and purines/pyrimidines (Figure 6E).

These findings, aligned with the differential ORA, support the utility of the approach of building metabolite co-abundance networks, which can help uncover crucial metabolic hubs or coordinated metabolic programs.

HT SpaceM resolves metabolic coordination and heterogeneity upon glycolysis inhibition

To further demonstrate the applicability of HT SpaceM in a pharmacological setting, we profiled HeLa cells ($n = 15,697$) treated with 2-DG, a glycolytic inhibitor that blocks the first two steps of glycolysis (Figure 7A). Single-cell metabolic profiles and clustering revealed a clear metabolic shift upon 2-DG (12 and 24 h) (Figure 7B), consistent across five replicates (Figure S5A). The markers were in line with the expected effect of 2-DG: glucose accumulation and glycolysis inhibition. Interestingly, citric acid was increased, suggesting compensatory activation of the tricarboxylic acid cycle (TCA) (Figures 7C, S5B, and S5C).

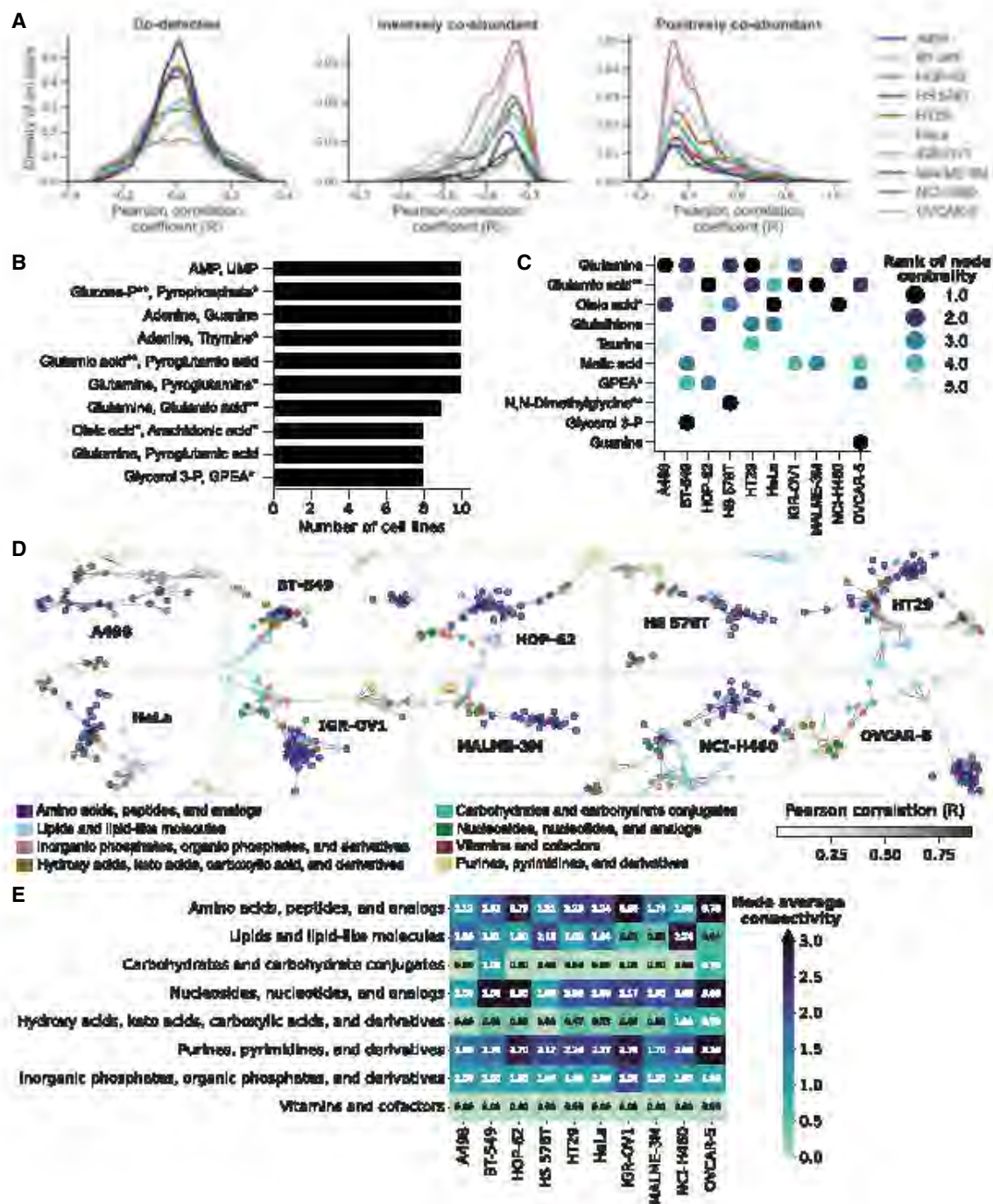


Figure 6. Single-cell metabolite co-abundance networks

(A) Co-detected and co-abundant ion pair distribution across cell lines.

(B) Most conserved co-abundant ion pairs across cell lines.

(C) Dot plot of metabolite hubs (top-ranked by the Pagerank node centrality) in the network of each cell line.

(D) Single-cell metabolite co-abundance networks for each cell line. Node color represents metabolite class, and edge represents the Pearson R, and only edges with $R \geq 0.3$ are shown.

(E) Heatmap of metabolite class-specific node average connectivity per cell line. Molecule names were validated in bulk LC-MS/MS at level 1 or 2, except as indicated by (*). (**) denotes the presence of isomers in bulk data.

See also Figure S4.

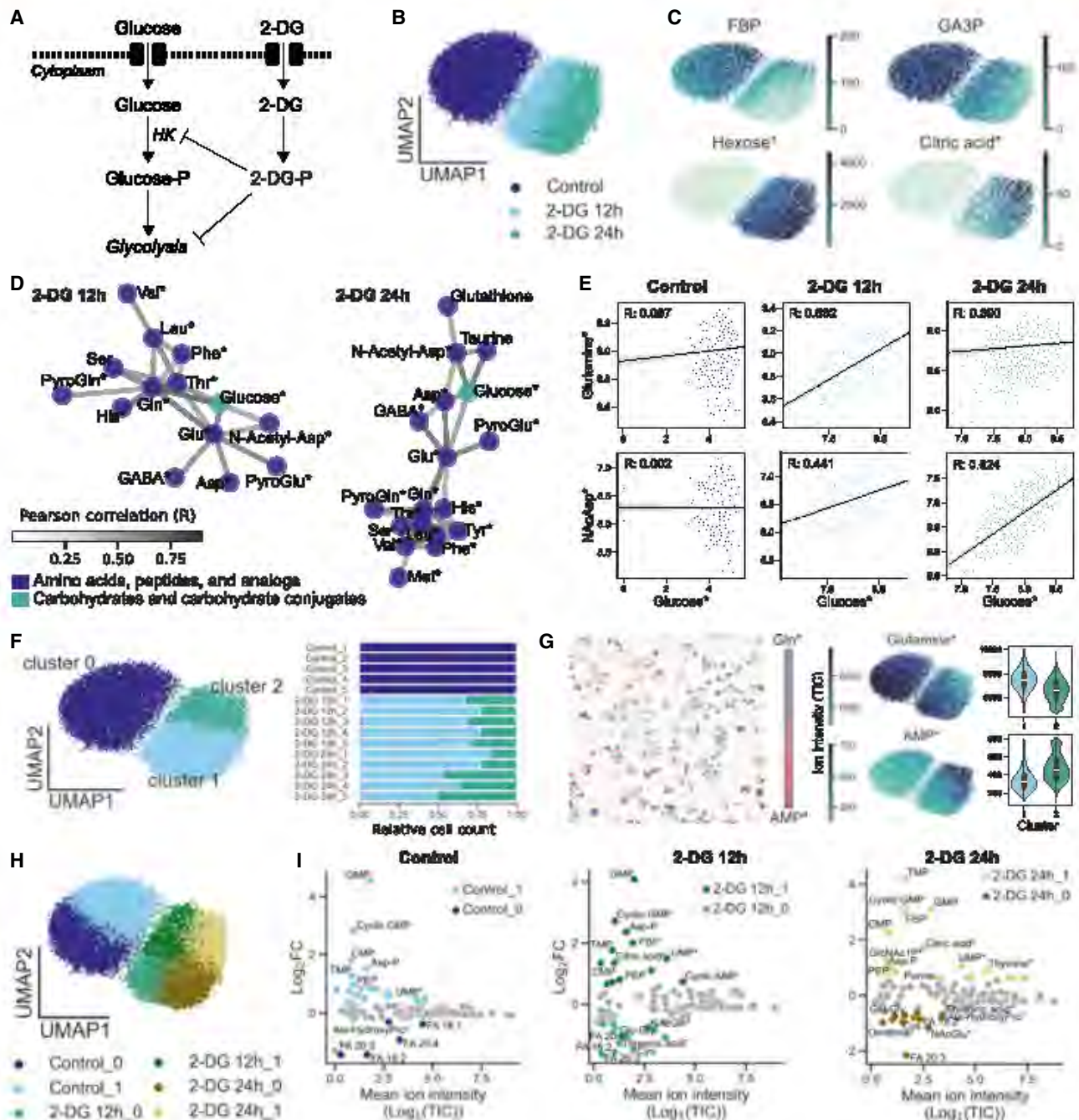


Figure 7. Emerged metabolic coordination and inherent cell-cell heterogeneity upon glycolysis inhibition

(A) Schematic of 2-DG action, inhibiting glycolysis via accumulation of 2-DG-phosphate.

(B) UMAP of HeLa cells under control (n = 8,185), 2-DG 12 h (n = 4,479), and 2-DG 24 h (n = 3,033) conditions.

(C) Metabolic markers' shifts: glucose accumulation, decreased glycolytic intermediates (FBP, glyceraldehyde 3-phosphate [GA3P]), and increased citric acid ion intensities (TIC-normalized).

(D) Co-abundance subnetworks centered on glucose in treated cells highlight altered metabolic coordination. Node color represents metabolite class, and edge represents the Pearson R, and only edges with R ≥ 0.4 are shown.

(E) Representative single-cell correlations between glucose and metabolites (N-acetyl-aspartate [NACAsp], glutamine) illustrating shifts in metabolic correlations. Ion intensities are TIC-normalized and log-transformed.

(F) UMAP showing treatment-specific metabolic states. Bar plots showing relative cell count of each cluster by replicate.

(legend continued on next page)

Single-cell metabolic networks (Figures 7D and S5D) revealed altered metabolic coordination across multiple pathways. Following the 2-DG treatment, glucose emerged as a central metabolite, with an increased number of connected metabolites suggesting a glucose-centered metabolic coordination. From 12 to 24 h, there was a progressive increase in the number of co-abundant metabolites, along with stronger correlations for some metabolites (e.g., N-acetyl-aspartic acid; Figure 7E). Conversely, correlations with some metabolites (e.g., glutamine; Figure 7E) decreased from 12 to 24 h, suggesting that these coordination patterns are, at least in part, transient.

Unsupervised clustering of all cells into three clusters revealed differences among 2-DG-treated cells (Figure 7F). Surprisingly, these differences were more pronounced than the effect of extending 2-DG treatment from 12 to 24 h. This heterogeneity was driven by metabolic markers such as glutamine and AMP, was consistent across all replicates, and was spatially heterogeneous, thus excluding systematic technical biases (Figure 7G).

Independent clustering within each condition (control, 2-DG 12 h, and 2-DG 24 h) (Figure 7H) revealed cluster structures similar to those found in the combined analysis (Figure 7F), suggesting conserved heterogeneity within both control and 2-DG-modulated cells. This interpretation is supported by the consistency of the markers for the clusters (Figure 7I). This observed intra-condition heterogeneity was reproducible across biological replicates (Figure S5E) and was not attributable to technical factors such as variation in cell sampling or differences in the number of ions detected per cell (Figure S5F). Notably, several cluster markers overlapped with metabolites affected by the 2-DG treatment (e.g., fructose 1,6-bisphosphate [FBP] or citric acid), indicating that cells preserve their heterogeneity even under external perturbation. The nature of these markers—FAs and amino acids for cluster 0 versus nucleotides, their metabolic precursors, and energy-related metabolites from glycolysis and the TCA cycle for cluster 1—suggests that cluster 0 may correspond to cells in the G1/S phases of the cell cycle (focused on protein and lipid synthesis for growth and membrane formation), while cluster 1 may represent cells in the G2/M phases (associated with energy-intensive processes of nucleotide synthesis and DNA replication) (Table S2).

DISCUSSION

We have introduced two major advances to increase throughput compared with the original SpaceM method.²⁰ First, we redesigned the experimental setup (Figure 1A) using laser-etched slides with removable well chambers, expanding the well count from 8 to 40. This allowed more efficient use of seeded cells, more technical replicates, and provided experimental flexibility. This versatile setup extends beyond single-cell analysis and

can be applied to diverse biological samples like organoids and spheroids. Second, we restructured the SpaceM processing method (Figure 1D) to streamline analysis: whole-slide microscopy acquisition and Zarr-file format simplified ROI selection and integration of fluorescent markers for better contextualization, multiplexing, and microscopy-based quality control. Streamlined metabolite annotation with METASPACE²⁵ has accelerated data processing and facilitated quality control, data visualization, and sharing. Using fine-tuned Cellpose²⁸ models has improved the cell segmentation, adapting to variations in cell sizes and morphologies. Finally, batch processing across wells minimized user input and increased consistency. By increasing the number of replicates in a single experiment, HT SpaceM achieves the highest sample throughput among the SCM techniques with the highest cell yield.^{6,38,39} Together, these improvements enhanced both experimental and computational efficiency, saving time and increasing control over experimental planning.

The second major advance is the reliable detection of small-molecule metabolites. We demonstrated broad coverage of metabolite classes (Figures 2G and S1D), comparable to spatial metabolomics using MALDI imaging.³¹ While demonstrated for small molecules, HT SpaceM is applicable for single-cell lipidomics by changing the matrix and MALDI-imaging acquisition parameters as SpaceM.²⁰ HT SpaceM revealed coverage and reproducibility comparable to bulk LC-MS/MS yet provided single-cell resolution and detected markers in subpopulations as small as 20% of cells (Figures 2 and 3). Since MALDI imaging used in HT SpaceM does not resolve isomers or isobars, we incorporated an LC-MS/MS-based validation to confirm annotations. We showed ion variability comparable to bulk LC-MS/MS, with multiple metabolites exhibiting %CV $\leq$ 20% (Figure 3L). Low ion variability is essential for bioanalytical method validation, as required by U.S. Food and Drug Administration guidelines, and helps evaluate HT SpaceM performance relative to emerging single-cell technologies. Future improvements in instrument sensitivity and ion yield may further reduce CVs, enabling more robust detection of low-abundance metabolites across replicates of different cell types.

Notably, HT SpaceM requires low amounts of biological material—only about 2,500 cells seeded per replicate—a 400-fold reduction compared with typical LC-MS/MS.⁴⁰ Within each well-replicate, we observed cell-to-cell heterogeneity and metabolic response to stress (Figure 7). Overall, HT SpaceM offers high-throughput, reliable SCM data, making it particularly valuable for limited patient-derived samples, such as circulating tumor cells, immune cells, or biopsies.

Limitations of the study

Despite the demonstrated strengths of HT SpaceM, several limitations remain. First, metabolite identification in MALDI imaging is inherently ambiguous, as it relies on MS1-level

(G) Spatial heterogeneity of clusters and metabolite intensities (TIC-normalized).

(H) Condition-specific clustering recapitulates clusters identified in (F), indicating conserved subpopulation structure.

(I) MA plot of differentially abundant metabolites between clusters ($p < 0.05$, FDR-adjusted, with $|FC| \geq 1.5$ highlighted). Marker metabolites suggest functional divergence between clusters, potentially reflecting cell cycle states. (*) denotes the presence of isomers.

See also Figure S5 and Table S2.

information that cannot resolve structural isomers or isobars. While we employed bulk LC-MS/MS for structural validation—a standard practice—HT SpaceM lacks MS/MS-level annotation at the single-cell level. Emerging techniques like in-source fragmentation (e.g., ms1-id⁴¹) and ion mobility separation may enhance annotation confidence beyond molecular formulas. Second, driven by the low abundance of small molecules and ion suppression, data sparsity and metabolite dropouts remain common challenges in SCM. This leads to high zero-inflation in the data and may require advanced computational strategies, such as imputation and sparsity-aware models. Third, although HT SpaceM detects a broad spectrum of biologically relevant small molecules (Figure 2), it does not capture the full metabolome. Certain metabolites remain difficult to detect due to low intracellular abundance, suboptimal ionization efficiency in MALDI, or the chosen instrument polarity requiring additional experiments. Fourth, chambered well systems pose experimental limitations. Limited surface areas and media volumes can compromise cell adhesion and viability during prolonged incubation or treatments. Mounted chambers may also leak over time. Although formats like LabTeks—used in the original SpaceM—support long-term treatment and small-molecule detection, they divide experiments into smaller batches, reducing throughput and increasing batch effect risk. Fifth, HT SpaceM is optimized for adherent cells. Suspension cells require custom protocols for cell adhesion, such as coating slides with poly-L-lysine or fibronectin. Application to tissue sections would require ~2 μ m spatial resolution to resolve individual cells *in situ*, which exceeds capabilities of commercially available MALDI imaging. Sixth, the higher sample throughput demands greater precision in sample preparation. Crystals from wash buffers can compromise multiple wells. Smaller wells offer fewer options for selecting ROIs for analysis, limiting artifact avoidance. Nonetheless, the increased number of samples per slide mitigates described risks by boosting statistical power and reducing batch effect susceptibility, as demonstrated by the high reproducibility observed in this study (Figures 3C and 3D). Seventh, despite automation and streamlined workflows, HT SpaceM still requires considerable computational expertise for processing and analysis of single-cell omics data. Finally, for future translational or clinical use, additional challenges must be addressed. HT SpaceM currently requires viable cells, necessitating cryopreservation and thawing steps. Patient-specific metabolic heterogeneity may confound analysis unless properly controlled. Integrating protein-based or gene-based phenotyping will be critical to contextualize metabolic data, especially in mixed or poorly defined populations.

Future improvements of HT SpaceM could focus on developing more leakage-resistant setups to simplify cell culture and enable longer incubation. Adapting HT SpaceM to MALDI imaging with a higher spatial resolution could allow tissue and subcellular analysis. Incorporating gene and protein detection from the same cells could further enhance data interpretability. Moving to translational and regulated contexts will require compliance with standards such as good laboratory practice and development of validated clinical workflows. Implementing these advancements, alongside robust data analysis frame-

works, will be crucial for propelling SCM forward and unlocking its potential in both basic and clinical research.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Theodore Alexandrov (talexandrov@health.ucsd.edu).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

- All annotated MALDI-imaging data are available at METASPACE: <https://metaspace2020.org/project/HTSpaceM>. MALDI-imaging acquisition files, processed single-cell data, and LC-MS/MS data are available at BioStudies: <https://www.ebi.ac.uk/biostudies/studies/S-BSST2127> with study identifier S-BSST2127. All data are publicly available as of the date of publication.
- The code used for reproducing figures is available at GitHub: <https://github.com/delafor/HT-SpaceM>.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.A., M.S., J.D., and A.E.; methodology, J.D., M.S., A.E., V.H., A.B., B.W., M.E., A.M., and T.A.; investigation, J.D., M.S., and B.D.; formal analysis, data curation, and visualization, J.D. and T.A.; writing – original draft, J.D., M.S., and T.A.; writing – review and editing, critical input from all authors; and supervision, T.A.

DECLARATION OF INTERESTS

T.A. has a patent on SCM and is a co-founder of DeepCyte, a startup on SCM.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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 - Microscopy imaging
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- Bulk LC-MS/MS analysis
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- Data visualization

● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMEM, high glucose	Gibco™	Cat# 11965118
Penicillin-Streptomycin (10,000 U/mL)	Gibco™	Cat# 15140122
Fetal Bovine Serum (FBS)	Gibco™	Cat# A5670701
Sodium Pyruvate (100 mM)	Gibco™	Cat# 11360070
0.25% trypsin-EDTA	Gibco™	Cat# 25200072
Trypan Blue Solution, 0.4%	Bio-Rad	Cat# 1450021
Hoechst 33342	Thermo Scientific™	Cat# 62249
2-Deoxyglucose	Millipore Sigma	Cat# D6134
Ammonium acetate	Merck Milipore	CAS# 631-61-8
Ammonium formate	Merck Milipore	CAS# 540-69-2
Formic acid	Merck Milipore	CAS# 64-18-6
Ammonium hydroxide	Merck Milipore	CAS# 1336-21-16
1,5-Diaminonaphthalene (DAN)	Sigma-Aldrich	CAS# 2243-62-1
Acetonitrile	Merck Milipore	CAS# 75-05-8
Methanol	Merck Milipore	CAS# 67-56-1
Water, HPLC grade	ChemSolute	Cat# 418
Deposited data		
Mass spectrometry raw and processed files	This study	BioStudies: S-BSST2127 https://www.ebi.ac.uk/biostudies/studies/S-BSST2127
Annotated MALDI imaging data	This study	METASPACE: HTSpaceM http://metaspace2020.org/project/HTSpaceM
Single-cell lipidomics data	Rappez et al. ²⁰	Metabolights: MTBLS78; https://www.ebi.ac.uk/metabolights/editor/MTBLS78/descriptors
Experimental models: Cell lines		
HeLa Kyoto H2B-mCherry	EMBL - Haring Lab	RRID: CVCL_L802
NIH3T3-GFP	EMBL - Mertens Lab	RRID: CVCL_JY97
A498	National Cancer Institute (NCI-NIH)	RRID: CVCL_1056
BT-549	National Cancer Institute (NCI-NIH)	RRID: CVCL_1092
HOP-64	National Cancer Institute (NCI-NIH)	RRID: CVCL_1285
HT29	National Cancer Institute (NCI-NIH)	RRID: CVCL_0320
HS 578T	National Cancer Institute (NCI-NIH)	RRID: CVCL_0332
IGR-OV1	National Cancer Institute (NCI-NIH)	RRID: CVCL_1304
MALME-3M	National Cancer Institute (NCI-NIH)	RRID: CVCL_1438
NCI-H460	National Cancer Institute (NCI-NIH)	RRID: CVCL_0459
OVCAR-5	National Cancer Institute (NCI-NIH)	RRID: CVCL_1628
Software and algorithms		
Data analysis framework - Code	This publication	Github: HT-SpaceM https://github.com/delafor/HT-SpaceM
ImageJ (v1.53)	Fiji	https://github.com/fiji/fiji/
Inkscape (v1.2.1)	Inkscape	www.inkscape.org
iPath (3.0)	EMBL	https://pathways.embl.de/

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
MS-DIAL (4.9)	RIKEN Center for Sustainable Resource Science	https://systemsomicslab.github.io/compms/msdial/main.html
Cellpose	Stringer et al. ²⁸	https://cellpose.readthedocs.io/
BigStitcher	Hörl et al. ⁴²	https://github.com/PreibischLab/BigStitcher
Napari	Sofroniew et al. ⁴³	https://github.com/napari/napari
imzML converter (2.2.1)	Alan Race et al. ⁴⁴	https://github.com/AlanRace/imzMLConverter
Other		
Epredia™ Superfrost™ Plus Adhesion Control Microscope Slide	Fisher Scientific	Cat# 22-042-941
ProPlate® Multi-Well Chambers	Grace Bio-Labs	Cat# 248865
1.0 mm zirconia/glass beads	Biospec Products	Cat# 11079110zx
Atlantis Premier BEH Z-HILIC Column, 1.7 µm, 2.1 mm X 100 mm	Waters™	186009979

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell culture

We cultured 11 cell lines for SCM and bulk LC-MS/MS experiments. These included HeLa Kyoto H2B-mCherry, NIH3T3-GFP, and nine cancer cell lines from the NCI60 panel: A498, BT-549, HOP-62, HT29, HS 578T, IGR-OV1, MALME-3M, NCI-H460, and OVCAR-5. All cell lines were grown separately in 10 cm tissue culture dishes using high glucose DMEM media from Gibco/ThermoFisher Scientific, supplemented with 10% fetal bovine serum (FBS) (ThermoFisher), 100 U/mL penicillin, 100 µg/ml streptomycin, and 1 mM sodium pyruvate (all from Gibco). The cells were maintained at 37°C with 5% CO₂. Cells were split at a 1:10 ratio twice a week using 0.25% trypsin-EDTA (Gibco). Cell counts were determined using Trypan Blue staining and counted with the TC10/TC20 Cell Counter from Bio-Rad Laboratories.

METHOD DETAILS

Laser-etched glass slides

We customized Epredia™ SuperFrost Plus™ glass slides by creating a 40-well layout, arranged in 4 columns and 10 rows, with squares measuring 3 mm x 3 mm. A Zeiss MicroBeam microscope (Carl Zeiss AG) with a 10X objective was used to laser-etch on the top surface of each slide using the PalmRobo software (Figure 1A). Following the etching process, the slides were cleaned with isopropanol and air-dried completely before use.

Cell lines monoculture experiment

Approximately 2,500 cells suspended in 50 µL of DMEM (as for Cell culture) were plated into each 40 wells of a 64-well proplate (Grace BioLabs) mounted onto the customized etched glass slide (Figure 1B). Cell seeding can be adjusted by cell type aiming at a confluency of 60–70%. The wells containing different cell lines were randomized across all experiments. One experiment comprised three slides with monocultures of HeLa and NIH3T3 cells and another experiment used one slide with monocultures of NCI60 cell lines and HeLa, with 4 replicates each. After 24 h of incubation for cell adhesion, the media was removed, cells were stained with Hoeschst 33342 (Thermo Fisher) in DMEM at a final concentration of 10 µg/mL for 15 minutes. Following incubation, culture media was aspirated, and the slides were detached from the proplates. The slides were briefly washed by five sequential dips in 100 mM ammonium acetate. Residual solvent was aspirated, and samples were immediately desiccated under vacuum at –0.08 kPa for 30 minutes at room temperature.

Co-culture experiment

NIH3T3 cells expressing GFP protein and NCI-H460 cells were grown separately (see Cell culture) and mixed 1:1. Approximately 2,500 mixed cells suspended in 50 µL of DMEM were seeded per well. After 24 h of incubation for cell adhesion, the media was removed, cells were stained with Hoeschst 33342 (Thermo Fisher) in DMEM at a final concentration of 10 µg/mL for 15 minutes. Following incubation, culture media was aspirated, and the slides were detached from the proplates. The slides were briefly washed by five sequential dips in 100 mM ammonium acetate. Residual solvent was aspirated, and samples were immediately desiccated under vacuum at –0.08 kPa for 30 minutes at room temperature.

2-DG treatment experiment

Approximately 2,500 HeLa cells suspended in 50 μ L of DMEM were seeded per well. After 24 hours to allow adhesion, media was removed and replaced with fresh DMEM for controls and DMEM with 2-Deoxyglucose (Millipore Sigma) at a final concentration of 25 mM for treated wells. Time points of 12 and 24 h were used for treated wells. Following incubation, culture media was aspirated, and cells were stained with Hoechst 33342 (Thermo Fisher) at a final concentration of 10 μ g/mL for 15 minutes in DMEM or DMEM with 2-Deoxyglucose (as above). After staining, media was aspirated, slides were detached from the proplates and briefly washed by five sequential dips in 100 mM ammonium acetate. Residual solvent was aspirated, and samples were immediately desiccated under vacuum at -0.08 kPa for 30 minutes at room temperature.

Microscopy imaging

Brightfield and fluorescence images ($\lambda = 460$ nm for Hoechst 33342) of the cells were acquired using a Ti-E or Ti2-E inverted microscopes (Nikon Instruments) equipped with a Plan Fluor 10X objective (NA 0.30, Nikon Instruments). For the co-culture experiment GFP fluorescence ($\lambda = 509$ nm) was also acquired. The images were captured with a pixel size of 0.64 μ m (HeLa/NIH3T3 and NCI-60 experiments) or 0.73 μ m (Co-culture and 2-DG experiments) and a 20% overlap between consecutive tiles covering the entire slide using the Nikon NIS Elements software. The same configurations were applied for pre- and post-MALDI images (Figure 1C).

Image processing and analysis

Microscopy tiles were stitched into an image using the Big Stitcher plug-in (Figure 1D).⁴² The resulting images were converted to zarr format. For selecting ROIs for MALDI acquisition, we used a custom interface written in napari.⁴³

For cell segmentation in the pre-MALDI images, we used Cellpose,²⁸ a deep learning-based method. The cyto2 model was fine-tuned on a small number of cells using the brightfield and Hoechst composite images to accommodate variations in cell size and morphology.

For the post-MALDI images, ablation marks were located and segmented using a grid of spots with circular shapes.

MALDI-imaging MS

Before MALDI acquisition, the slides containing cells were sprayed with 1,5-diaminonaphthalene (DAN) (Sigma-Aldrich) prepared at a concentration of 7 mg/mL in 70% acetonitrile (v/v). Spraying was performed in an HTX TM sprayer (HTX Technologies LLC) with the following parameters: temperature at 80°C, 8 passes, a flow rate of 0.05 mL/min, velocity of 1,350 mm/min, track spacing of 3 mm, CC pattern, pressure of 10 psi, gas flow rate of 5 L/min, drying time of 15 seconds, and nozzle height of 41 mm. The estimated matrix density was 0.00069 mg/mm².

For MALDI acquisition, we used a Q-Exactive Plus mass spectrometer (ThermoFisher Scientific) equipped with an AP-SMALDI5 source (Transmit). For the HeLa/NIH3T3 experiment we analyzed one full slide (40 wells) and 2 partial slides (16 wells each), keeping the 1:1 proportion of the number of wells per cell line. For the NCI-60 panel with HeLa, we analyzed a full slide (40 wells) containing 4 wells for each cell line. For the co-culture experiment 5 replicates were acquired. For the experiment of HeLa treated with 2-DG, 5 replicates per condition were acquired. The analysis was performed in negative ion mode with a mass range of m/z 100–400 and a resolving power of 140,000 at m/z 200. The acquisition area of 80 x 80 was selected in each well targeting the best ROI containing cells, and the step size was set to 25 μ m. For HeLa and NIH3T3 experiment MALDI laser attenuator was set to 29°, MS parameters were a S-lens voltage of 50 eV, a capillary temperature of 250°C, and a spray voltage of -3.25 kV. For the NCI-60 panel and HeLa experiment MALDI laser attenuator was set to 29°, MS parameters were a S-lens voltage of 70 eV, a capillary temperature of 350°C, and a spray voltage of 3.1 kV. For the co-culture and HeLa treated with 2-DG experiments, MALDI laser attenuator was set to 30°, and MS parameters were S-lens voltage of 50 eV, a capillary temperature of 350°C, and a spray voltage of 3.25 kV.

Data pre-processing and metabolite annotation

MALDI-imaging acquisition files were split into individual wells, converted to final centroided imzML files using imzMLConverter,⁴⁴ and uploaded to METASPACE in batch mode (Figure 1D). METASPACE, a cloud software (<https://metaspace2020.eu>), performed metabolite annotation using false discovery rate-controlled annotation²⁵ with an m/z accuracy of 3 ppm. Ions were annotated against HMDB (Human Metabolome Database, v4)²⁶ and CoreMetabolome v3²⁷ databases. As part of data quality control and curation, custom databases of molecular formulas were created by selecting ions co-localized with cells (area-Normalized Manders Colocalization Coefficient (NMCC) > 0.7) and datasets were reannotated on METASPACE.

Single-cell data processing

Processing of single-cell data was done as for SpaceM, described earlier.²⁰ Briefly, after all input data was generated (Figure 3D), i.e. pre- and post-MALDI stitched microscopy images, and ion images annotated on METASPACE, the HT SpaceM processing was performed in batch mode to handle the data efficiently. Pre- and post-MALDI images were registered using laser-etched fiducials for improved accuracy and automatization. Segmented cells were aligned to grid-fitted ablation marks, which allowed us to deconvolve pixel-ion intensities provided by METASPACE into single-cell ion intensities. The deconvolution process used the biggest overlap, considering only ablation marks that have at least 30% overlap with cells. Batch processing not only expedites sample processing but guarantees standardization in the use of input data and parameters, reducing processing errors.

LC-MS/MS sample preparation

Each cell line was cultured until reaching confluence (see Cell culture section), yielding approximately 1.5 million cells per replicate ($n=3$ or 5). After aspirating the cell culture media, the cells were washed by pouring phosphate buffered saline (PBS) three times over the dish. Once the PBS was discarded, the dish was placed on ice, and 1 mL of ice-cold 80% (v/v) methanol containing isotope-stable internal standards (0.5% final concentration) was added. The plate was then incubated at -80°C for 20 min. Following the incubation, cells were scraped and transferred, along with the solvent mixture, into small tubes. The cell lysate was vortexed at maximum speed for 5 minutes. Further homogenization was performed on dry ice using a bead beater (FastPrep-24; MP Biomedicals, CA, USA) at 6.0 m/s, with three 30-second bursts and a 5-minute pause in between, using 1.0 mm zirconia/glass beads (Biospec Products, OK, USA). After homogenization, the samples were centrifuged for 10 min at $15,000 \times g$ and 4°C . The supernatants were transferred and dried under a stream of nitrogen (Organomation Microvap, MA, USA). The dried samples were then reconstituted in 100 μL of a solution containing acetonitrile, methanol, and water (2:2:1, v/v), vortexed, and centrifuged under the same conditions. Finally, the supernatants were transferred to silanized glass vials and injected into the analytical system.

Bulk LC-MS/MS analysis

LC-MS/MS analysis was performed according to the EMBL-MCF 2.0 method.⁴⁵ We used a Vanquish UHPLC system coupled with an Orbitrap Exploris 240 high-resolution mass spectrometer (Thermo Fisher Scientific, MA, USA), operating in both negative and positive electrospray ionization (ESI) modes. Chromatographic separation was achieved on an Atlantis Premier BEH Z-HILIC column (Waters, MA, USA; 2.1 mm $\times$ 100 mm, 1.7 μm) at a flow rate of 0.25 mL/min. The mobile phases comprised water (9:1, v/v) for phase A and acetonitrile (9:1, v/v) for phase B, each modified with 10 mM ammonium acetate for negative mode and 10 mM ammonium formate for positive mode. The aqueous components of the mobile phases were pH-adjusted to pH 9.0 (negative mode) with ammonium hydroxide and pH 3.0 (positive mode) with formic acid. The gradient used for separation, including re-equilibration, was as follows: 0 min at 95% B, 2 min at 95% B, 14.5 min at 60% B, 16 min at 60% B, 16.5 min at 95% B, and 20 min at 95% B. The column temperature was maintained at 40°C , the autosampler at 4°C , and the sample injection volume was 5 μL . For MS analysis, full scans were conducted with a mass resolving power of 120,000 over a range of 60–900 m/z , with a scan time of 100 ms and an RF lens setting of 70%. MS/MS fragment spectra were obtained through data-dependent acquisition, with a resolving power of 15,000, a scan time of 22 ms, stepped collision energies of 30%, 50%, and 70%, and a cycle time of 900 ms. Ion source parameters included a spray voltage of 4100 V (positive mode) or -3500 V (negative mode), sheath gas at 30 psi, auxiliary gas at 5 psi, sweep gas at 0 psi, ion transfer tube temperature at 350°C , and vaporizer temperature at 300°C .

Samples were measured in a randomized order. Pooled quality control (QC) samples were prepared by combining equal aliquots from each processed sample type. Multiple QC samples were injected at the beginning of the analysis to equilibrate the analytical system, and a QC sample was analyzed after every fifth experimental sample to monitor instrument performance throughout the sequence. To account for background signals, an additional processed blank sample was recorded. Data processing was performed using MS-DIAL 4.9,⁴⁶ and raw peak intensity data was exported for the annotated molecules and normalized by total ion count (TIC).⁴⁷ Intensities were further log-transformed ($\log(X+1)$).

Single-cell data analysis framework

HT SpaceM processing resulted in spatio-molecular matrices used for single-cell data analysis (Figure 1E). After evaluation of ion-cell distribution, essential steps in data quality control to mitigate experimental variability consisted in filtering the data to include only deprotonated ions $[\text{M}-\text{H}]^{-}$, retaining cells with at least 20 ions and ions present in at least 50 cells. This filtering resulted in a final matrix of 135 ions $\times$ 78,500 cells for the HeLa/NIH3T3 dataset and 202 ions $\times$ 42,153 cells for the dataset composed of NCI-60 cell lines and HeLa. Stricter filterings were applied to the co-culture (min_ions = 40, min_cells = 300) and 2-DG-treated cells datasets (min_ions = 75, min_cell = 2000), where minimum cell sampling proportion of 0.4 was also considered. This resulted in a total of 142 ions $\times$ 4,413 cells for co-culture and 111 ions $\times$ 15,697 cells for treated cells experiment.

Ion intensities were TIC-normalized, excluding highly expressed ions accounting for more than 5% of the total ion count in a cell. Intensities were scaled to 10,000 during normalization and transformed using the natural logarithm ($\log(X+1)$) before analysis.

Unsupervised and supervised characterization

Principal Component Analysis (PCA) was performed for dimensionality reduction. Using the top 50 PCs, we built a nearest neighbors graph ($n_neighbors = 15$, metric = 'euclidean') and visualized it through UMAPs colored by cell line, Leiden clustering (unsupervised), replicates and/or tumor origin. No batch correction method was applied to integrate samples from different slides. These processing steps and analyses were conducted using the Scanpy v1.9.1 package.⁴⁸

Annotated ions were matched to HMDB (www.hmdb.ca) and Kegg (Kyoto Encyclopedia of Genes and Genomes) (www.genome.jp/kegg/) identifiers to evaluate metabolite classes and pathways.

For the co-culture experiment, first we assigned cell labels based on the combination of the combination of GFP, eigenvalues of inertia tensor and cell size thresholds. Second, we balanced the dataset by randomly subsampling NCI-H460 cells to match the number of NIH3T3 cells ($n = 496$). Then, a Random Forest classifier was trained on cell metabolic profiles considering those labels as ground-truth and using a 10-fold stratified cross-validation approach. For each fold, model performance was evaluated using accuracy, F1-score, specificity and sensitivity, considering the correctness of the predicted labels. To identify the most informative metabolites, a final Random Forest classifier was trained on the full dataset, and feature importances computed.

Differential analysis and enrichment analysis

Single-cell differential analysis considering all annotated metabolites was performed using the Wilcoxon test, with a significant p -value < 0.05 (FDR-adjusted, two-tailed). Metabolite enrichment analysis was conducted with the R package S2IsoMer.⁴⁹ We used the simple ORA function considering differentially expressed markers ($|FC| > 1.5$ and p -value < 0.05) for one cell line versus all. We set the custom_universe parameter as all molecules detected in our analysis, with min_intersection = 2 and alpha_cutoff = 1 for the matching between the selected markers and the metabolites and metabolic pathways of SMPDB background (Small Molecule Pathway Database (www.smpdb.ca)).

Structural validation

Bulk LC-MS/MS data was annotated against our recently published metabolomics library (EMBL-MCF 2.0).⁴⁵ Level 1 feature identification used accurate mass, isotope pattern, retention time and MS/MS fragmentation with a minimum matching score of 80%. Metabolites were considered Level 2 annotations when MS/MS matching scores were less than 80%.

A metabolite was structurally validated when its SCM molecular formula matched with a molecular formula confirmed by bulk LC-MS/MS in the negative or positive ionization modes. In cases where two or more metabolites were identified in bulk analysis for one molecular formula, single-cell intensity values for that molecular formula were replicated. The ion intensity of a molecule detected at Level 1 and/or in the negative mode was preferably used for comparison to single-cell metabolite intensity.

Reproducibility and metabolite variability

Reproducibility across wells was evaluated through their correlation. First, we averaged the intensities of each ion across cells within a well-replicate, considering both zero and non-zero intensity cells. Then, we calculated the Pearson correlation coefficient (R) between wells of the same condition within the same slide (intra-slide) and between wells from different slides (inter-slide). We also calculated the slope of the best-fitting line for the data between two wells in the same manner. For slide-to-slide correlations, we averaged the ion intensities of wells from the same slide for each condition before computing R across slides.

Single-cell ion variability was assessed using the coefficient of variation (%CV) metric. We first averaged the intensities of each ion across cells within a replicate, considering only cells with non-zero intensity values. After obtaining the mean intensity per ion per replicate, we calculated the mean (M) and standard deviation (SD) across well-replicates. For bulk data, we calculated M by averaging the intensities of sample replicates within the cell line and computed the respective SD . Metabolite %CV was calculated as $(SD/M) \times 100$. In cases where two or more metabolites were identified in bulk LC-MS/MS for one molecular formula, %CV value for that molecular formula in the single-cell experiment was replicated.

To assess metabolite sparsity across different cell lines, we performed a combinatorial analysis of shared metabolites. For each possible combination of n cell lines (where n ranged from 2 to 10 cell lines of the NCI-60 panel), we identified the set of metabolites detected in $\geq 99\%$ of single cells within each cell line in the group. Then, we computed the intersection of these metabolite sets to determine the percentage of metabolites consistently detected across all cell lines in that combination. This analysis was repeated across all possible combinations of cell lines for each value of n , and the resulting percentages were summarized as boxplots.

Metabolite variability within the cell line was used as a proxy of cell-to-cell heterogeneity. Condition metabolic heterogeneity was calculated by Shannon's entropy for each metabolite across single cells. Entropy values were aggregated per condition and visualized as boxplots, ordered by group-level median entropy. Single-cell lipidomics data were obtained from SpaceM.²⁰

Metabolites correlation and network analysis

Using the single-cell data, we determined within each cell line which metabolites were found co-detected, i.e. metabolites simultaneously detected in a single cell with a low mismatch dropout rate. We selected ion pairs with less than 5% of the cells having zero intensity values simultaneously and more than 60% of cells having non-zero values simultaneously. For those co-detected ions, we computed the Pearson R across metabolite pairs within a cell line, observing if they were positively co-abundant ($R > 0$) or inversely co-abundant ($R < 0$). Network analysis and associated metrics were generated using NetworkX v2.8.6 functions.⁵⁰ We retained metabolite pairs with positive correlation ($R \geq 0.3$ for NCI-60 cell lines and $R \geq 0.4$ for HeLa experiment with 2-DG), to generate network graphs for each condition, where metabolites (nodes) were colored by metabolite class and the connection between two nodes (edges) represented R . We computed PageRank node centrality considering R as weight for the edges and calculated the average node connectivity within metabolite class for each cell line graph.

Data visualization

For data visualization, we used the Python packages Scanpy v1.9.1, Seaborn v0.13.2, and Matplotlib v3.5.3. Cell images were processed using ImageJ 1.53q (FIJI).⁵¹ Pathway integrated visualization was performed using iPath3.0.²⁹ Illustrations and multi-panel figures were created with Inkscape v1.2.1 (www.inkscape.org).

QUANTIFICATION AND STATISTICAL ANALYSIS

For statistical analyses and metrics, we used the Python packages pandas v1.4.3, numpy v1.26.4, scipy v1.12.0, and scikit-learn v.0.0.post1. The statistical approach for each experiment is described in the corresponding STAR Methods section and in the figure legends.

Supplemental figures

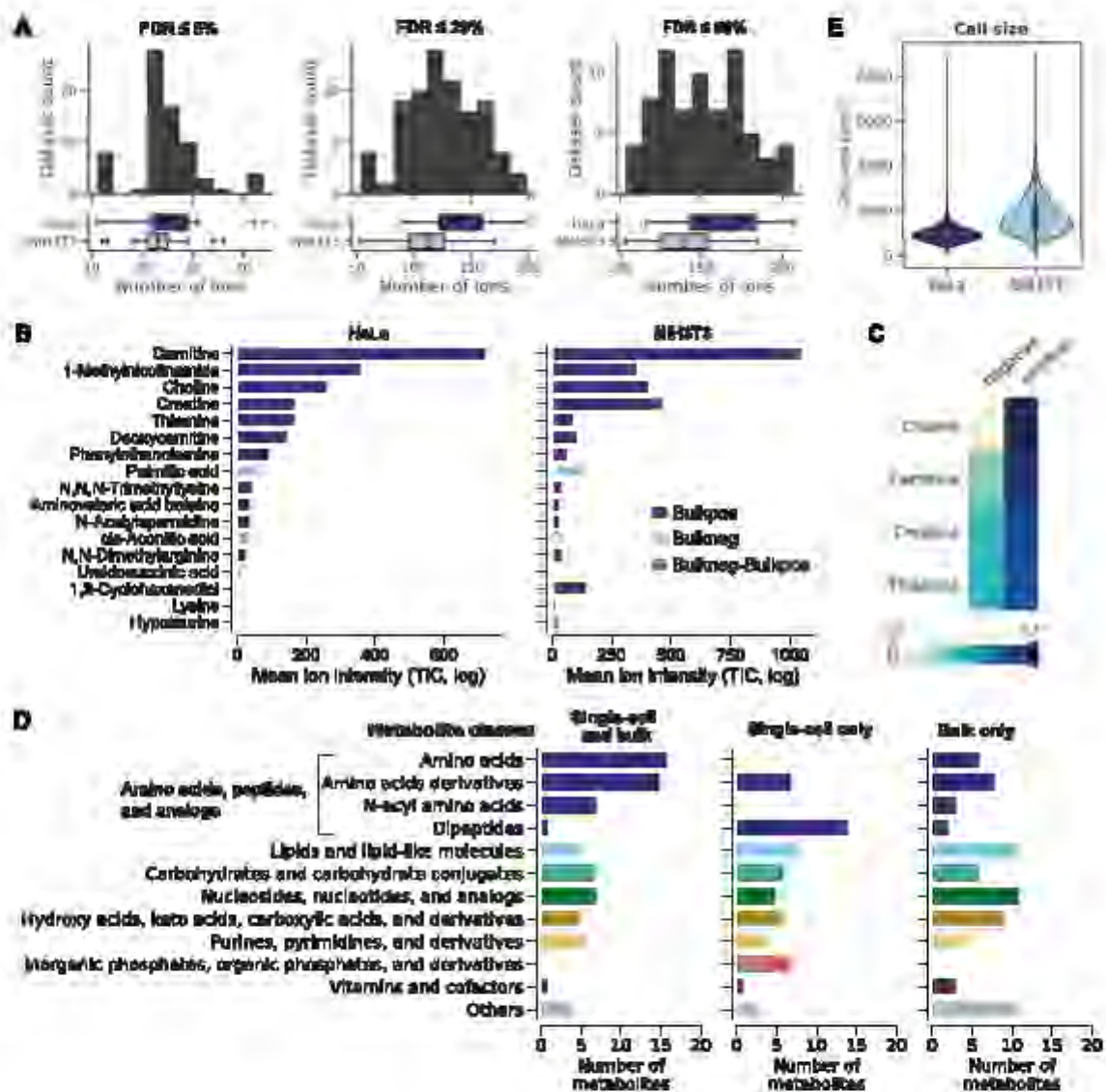


Figure S1. SCM and bulk-LC-MS/MS small-molecule coverage, related to Figure 2

(A) Number of ions $[M-H]^+$ per well at FDR 5%, 20%, and 50%.

(B) High-intensity metabolites detected only in bulk LC-MS/MS.

(C) MALDI detectability of the metabolites detected in bulk LC-MS/MS in positive mode but not detected by HT SpaceM using negative mode, as exported from the METASPACE detectability web application.³¹

(D) Number of metabolites per class detected in both LC-MS/MS and HT SpaceM, only in single-cell data and only in bulk data.

(E) Cell size distribution per cell line.

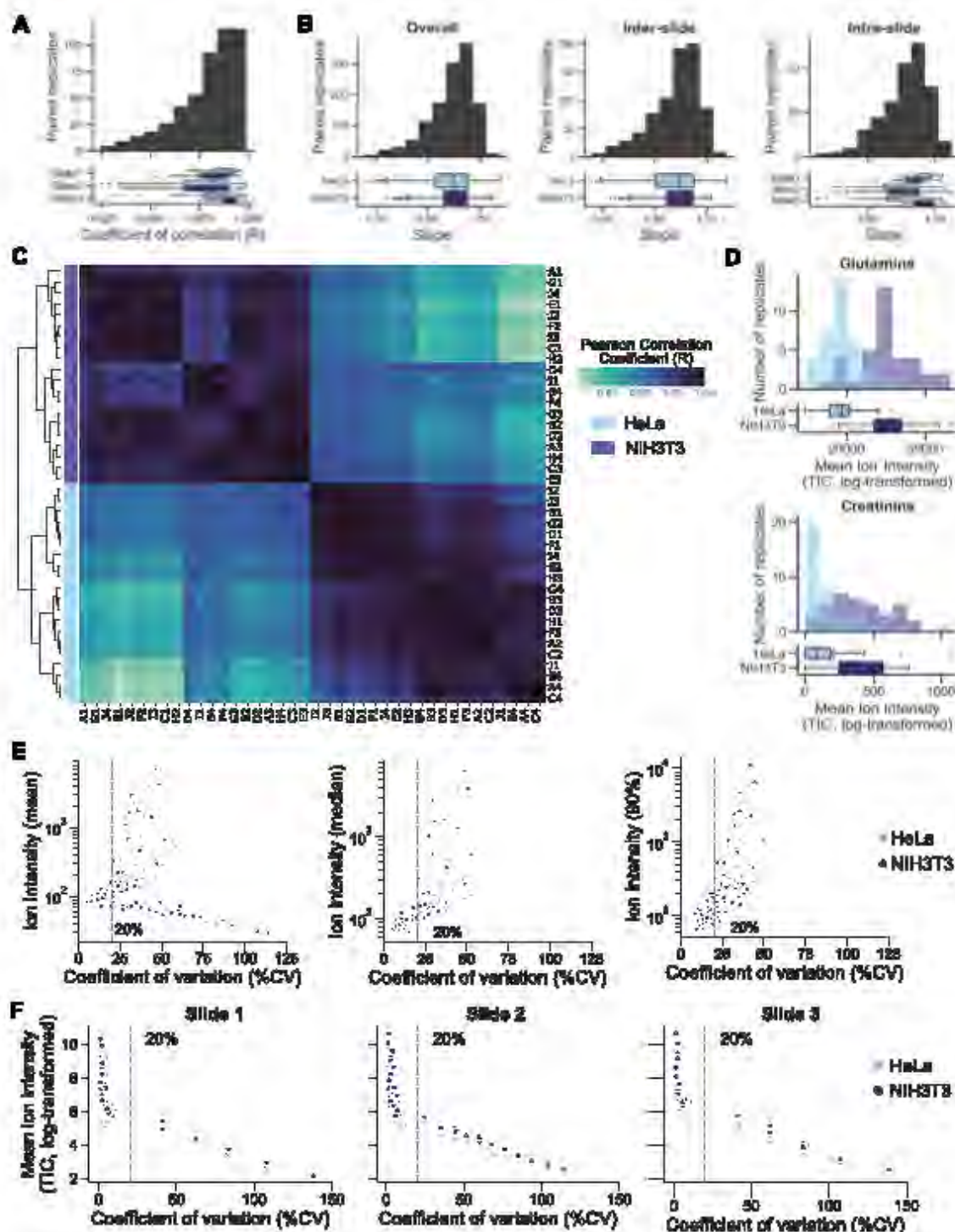


Figure S2. Evaluation of data reproducibility across wells and slides and metabolite variability, related to Figure 3

- (A) Pearson R for intra-slide replicates, considering slides and lines separately.
 (B) Slope distribution for overall, inter-slide, and intra-slide replicates.
 (C) Pearson R among wells of slide 2.
 (D) Normalized mean ion intensity distribution (glutamine and creatinine) per condition.
 (E) Mean, median, and 90% quartile ion intensity over %CV for HeLa and NIH3T3.
 (F) Normalized mean ion intensity over %CV for HeLa and NIH3T3, per slide.

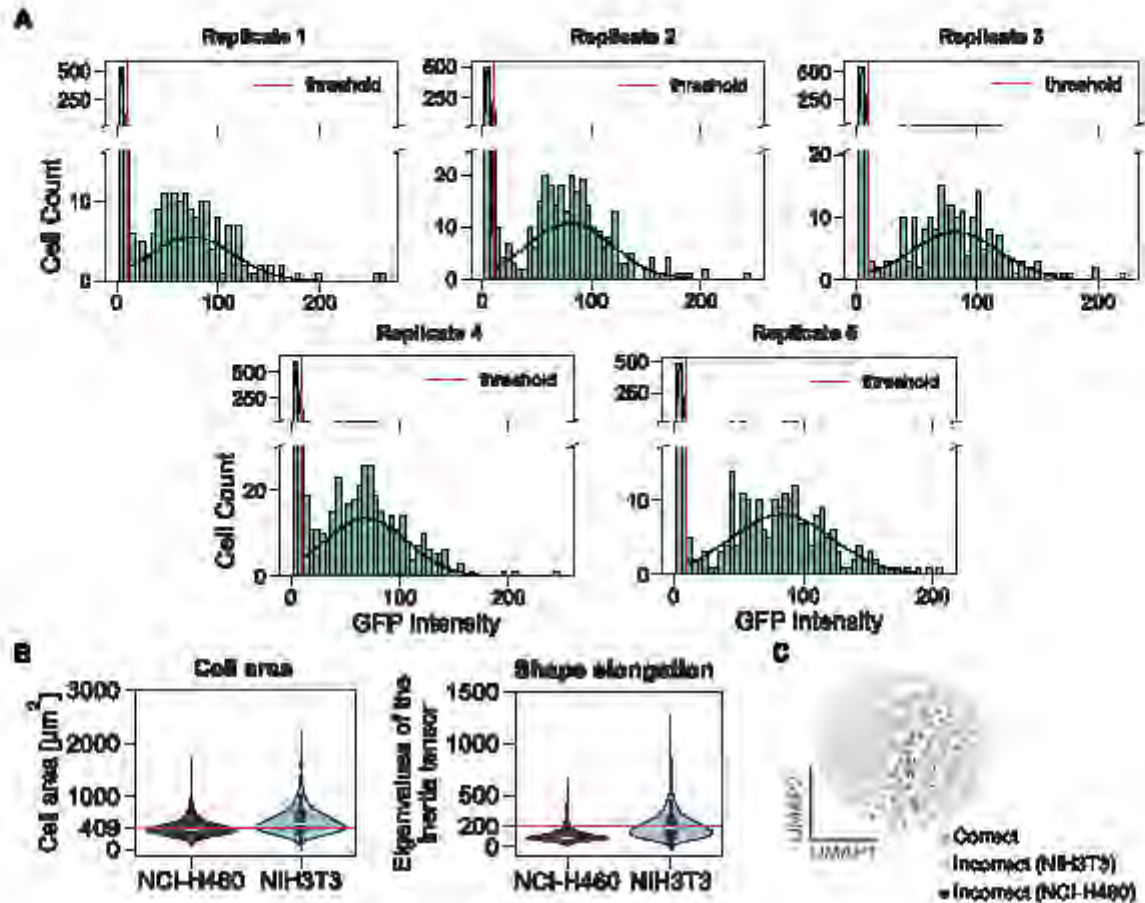


Figure S3. Co-cultured cell lines classification based on GFP intensity and morphology, related to Figure 4

(A) GFP bimodal distribution per well-replicate with threshold = 10 for cell-type assignment.

(B) Comparison of cell sizes and shape elongation between NCI-H460 and NIH3T3.

(C) UMAP highlighting cells correctly classified into NCI-H460 and NIH3T3 cell types (gray) and the mislabeled cells (incorrect NCI-H460 in dark blue and incorrect NIH3T3 in light blue).

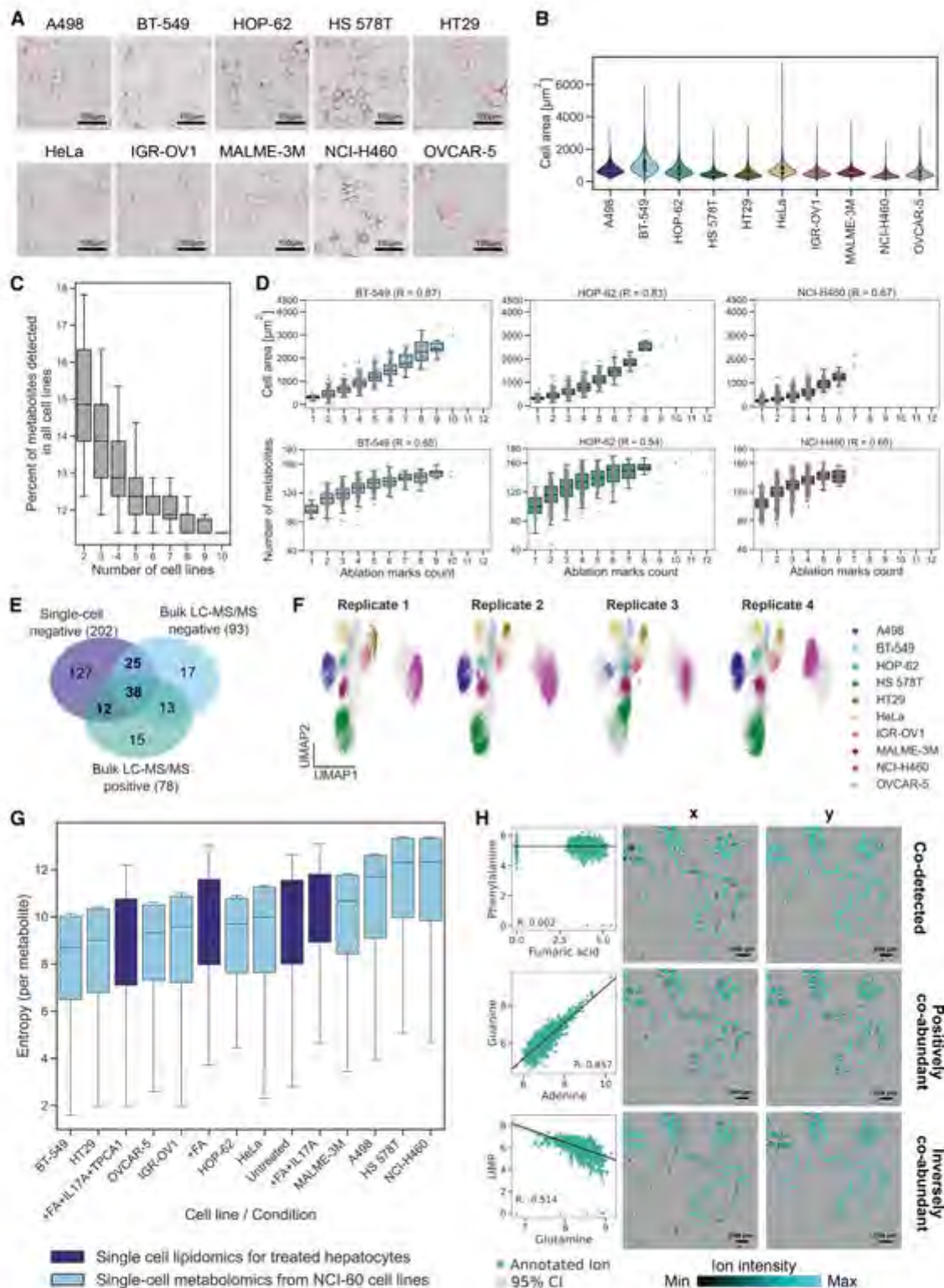


Figure S4. Assessing metabolite variability and well reproducibility in the NCI-60 panel and HeLa, related to Figures 5 and 6

(A) Composite image (bright field and Hoechst, in red) of the NCI60 cell lines, alongside HeLa cells. Scale bar at 100 μm .

(B) Cell size distribution per cell line.

(legend continued on next page)

(C) Distribution of shared metabolites (in percent, %) detected across cell lines within each combination group. Presence of metabolites is considered when intensities are >0 in 99% of the sampled cells.

(D) Distribution of number of metabolites or cell area (μm^2) per ablation marks count demonstrating positive correlation.

(E) Venn diagram comparing the number of ions detected by HT SpaceM (negative ion mode) and bulk LC-MS/MS (negative and positive ion modes).

(F) UMAP of the single-cell data colored by cell line for each replicate.

(G) Metabolite-level (or lipid-level from SpaceM paper²⁰) Shannon's entropy across cell lines and conditions. Groups are ordered by median entropy and colored by dataset.

(H) Examples of co-detected, positively co-abundant, and inversely co-abundant metabolites. Scatterplot of single-cell TIC-normalized ion intensities ($\log_2 p$) between two metabolites. Spatial distribution highlighting the metabolite intensity per cell (x and y axes of the scatterplot).

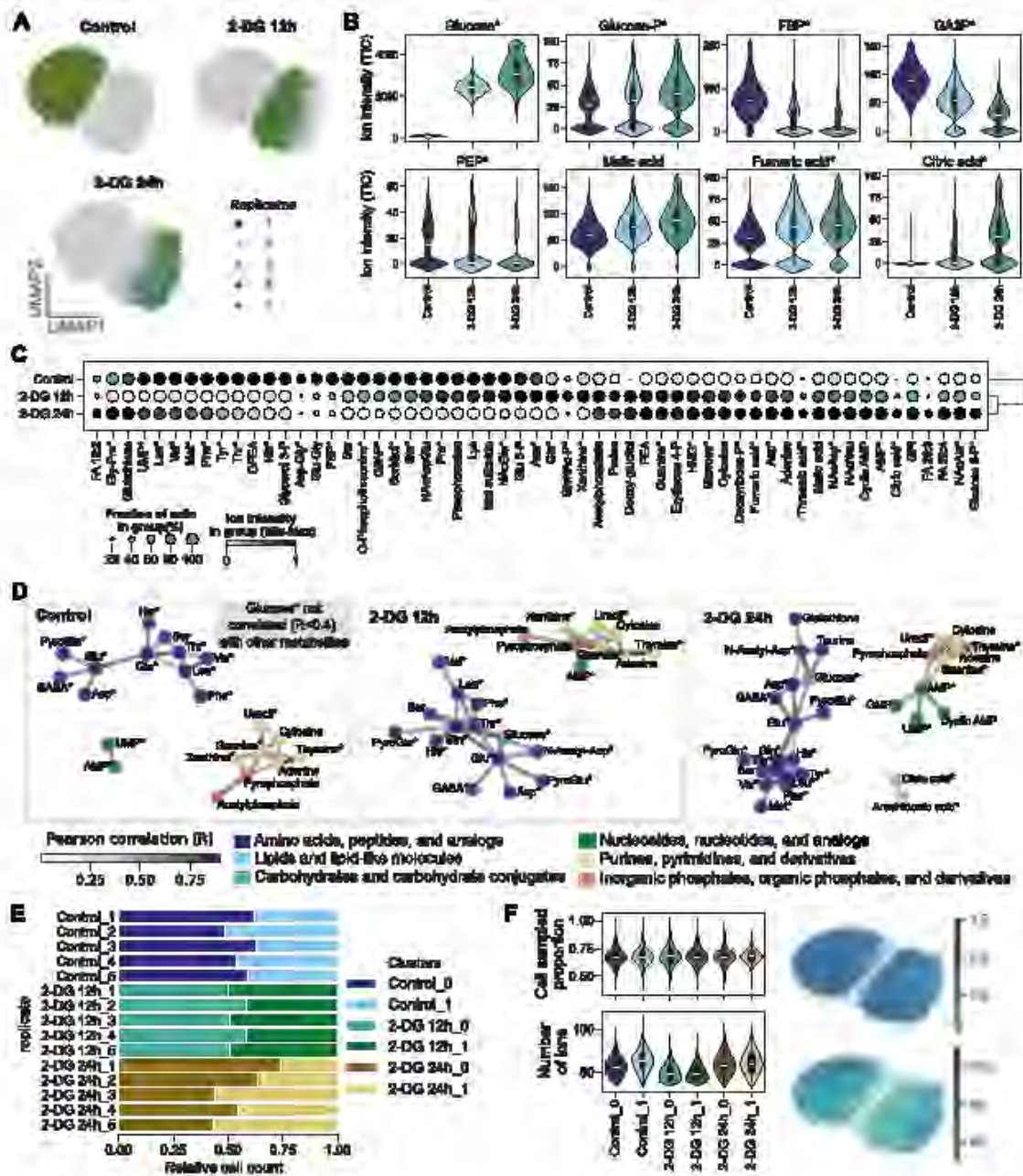


Figure S5. Metabolic alterations induced by 2-DG on HeLa cells, related to Figure 7

(A) UMAP of the single-cell data colored by replicate for each condition.

(B) Distribution of glycolysis-TCA cycle metabolite intensities among conditions (control, 2-DG 12 h, and 2-DG 24 h).

(C) Dot plot of conditions' markers showing average metabolite intensity feature-scaled and the fraction of cells expressing the metabolite.

(D) Single-cell metabolite co-abundance networks for each condition. Node color represents metabolite class, and edge represents the Pearson R, and only edges with $R \geq 0.4$ are shown.

(E) Bar plot of the relative cell count present in each cluster per replicate.

(F) Distribution of cell sampled proportion and total number of annotated ions. (*) denotes the presence of isomers.