Analyzing cell type-specific dynamics of metabolism on kidney

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Method Article

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Abstract

Conventional single-cell metabolomics approaches such as matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) generate biochemical snapshots, neglecting the inherent dynamic nature of metabolism. Here we describe a platform based on isotope tracing and MALDI-MSI that allows *in-situ* dynamic measurements of cell type-specific metabolism at single-cell resolution, and thus unravel cell metabolism within its tissue architecture. We applied different ^{13}C-isotope-labeled nutrients on vibratome slices of fresh mouse kidney. MALDI-MSI at single-cell resolution (i.e. pixel size of 5 × 5 μm^2) was then applied to detect metabolites and lipids from the harvested tissues. Following MALDI-MSI analysis, post-MSI-analyzed sections were stained and subsequently imaged using multiplexed immunofluorescence (IF) microscopy for cell-type identification. This platform allows to achieve single-cell resolution *in situ* and hence interpret cell type-specific metabolic dynamics in the context of structure and metabolism of neighboring cells.

Introduction

Single-cell omics tools are becoming increasingly important to provide new insights into cellular heterogeneity, and the way cells interact and behave in their respective microenvironments. The aim to analyse the entity of cellular metabolites within a single cell provides a challenging task due to the enormous structural diversity, rapid turnover, and low analyte abundances in a limited sample volume. Mass spectrometry-based single-cell metabolomics methods have been proposed previously, including those applying MALDI-MSI [1, 2]. To date, MALDI-MSI-based single-cell metabolomics studies have, however, focussed on analysing single time point metabolic 'snapshots' providing valuable information on *in situ* metabolic heterogeneity, but crucially lack insight into the dynamic component of cellular metabolism [3-5]. However, the need exists for approaches that provide a truly comprehensive understanding of the interplay between biochemical alterations and cellular functions, metabolic fluxes and dynamic interpretations at the single-cell level. To achieve this, isotope tracing has been used to model dynamic processes of metabolism using bulk metabolomics [6], in which complete tissues are homogenized and reduced to a single metabolic profile disregarding tissue and metabolic heterogeneity. The combination of isotope tracing and subsequent analysis by MALDI-MSI could be considered as an approach to assess metabolic dynamics *in situ* [7-9]. Here we describe a platform based on high spatial resolution MALDI-MSI (5 × 5 μm^2 pixel-size) that utilizes isotope tracing to allow *in situ* cell type-specific dynamic metabolism measurements to unravel cell metabolism within its tissue architecture. Since the average diameter of most cells in kidney tissue is around 10 μm, thus single measurements will be at subcellular level and therefore each cell will be measured by approximately 4 pixels using MALDI-MSI. All pixels, importantly not segmented into individual cells, will be used for following cell type-specific metabolomics analysis.

This method was designed to use different ^{13}C-isotope-labeled nutrients that allowed tracing of spatio-temporal incorporation of the ^{13}C-isotopes into the main intermediates of glycolysis and the TCA cycle (Figure 1A). Using various labeled nutrients not only allowed visualization of the dynamic changes over time, but also allowed us to resolve their contributions to specific metabolic pathways. To accomplish this, we applied a tissue culture system, in which 350 µm-thick vibratome slices of fresh mouse kidney [10] (Figure 1B) were incubated for up to 2 hours. During incubation, parallel introduction of different ^{13}C-isotope-labeled nutrients to the tissue culture medium at selected time points allowed efficient and biochemically meaningful labeling of metabolically active cells[11] to measure the metabolic changes in a non-steady state. Subsequently, tissue slices were quenched with liquid nitrogen and sectioned further into 10 µm-thick tissue sections, thaw-mounted on conductive glass slides and coated with a chemical matrix for MALDI-MSI measurements. Negative ion-mode MALDI-MSI at 5 × 5 µm^2 pixel size was applied to detect metabolites and (phospho)lipids[12] from the harvested tissues. Following MALDI-MSI measurements, post-MSI-analysed sections were stained and subsequently imaged using multiplexed immunofluorescence (IF) microscopy for cell type identification (Figure 1C). By combining IF and spatial segmentation of the MALDI-MSI data (based on uniform manifold approximation and projection (UMAP) of the lipid signals in particular), cell type-specific signatures were established using the following assumptions: i) MALDI-MSI lipid profiles are cell type-specific and ii) major phospholipid species, important for cell typing, are mainly cell membrane components[13] and thus stable during the 2 hour isotopic labeling. MALDI-MSI measurements were performed on sections from different time points and using different added nutrients. To show all the dynamic metabolic changes within one pixel without batch effects, a two-step process was followed as introduced by Stuart *et al.* in the *Seurat* package[14]: i) single-pixel lipid profiles were used to identify anchors between two datasets. ii) ^{13}C-labeled metabolite production was imputed onto the control dataset by transferring the abundance of all measured metabolites from the labeling datasets using k-nearest neighbours (KNN) analysis. (Figure 1D) An imputed dataset containing the complete ^{13}C-labeling information, representing the predicted values of mass isotopomers for each tracing experiment at a single pixel level with a similar lipid profile, from different timepoints and nutrients could thus be established. Dynamic metabolic calculations and ^{13}C enrichment of isotopologues were performed on single pixel data including metabolic changes and pathway convergence. Finally, the *in situ* heterogeneity of the metabolic dynamics was visualized in pseudo-images generated from the calculated values (Figure 1E).

Reagents

Hanks' Balanced Salt Solution (HBSS, Gibco, 2458830)

Glucose (Gibco, 15023-21)

Penicillin/streptomycin (Gibco, 15070-063)

Agarose (Roche, 11388991001)

Seahorse XF DMEM assay medium (Agilent, 103681)

Fetal Bovine Serum (Gibco, 10270-106)

Linoleic acid (Sigma, L1376)

Bovine serum albumin (Sigma, A7030)

Glutamine (Sigma, G8540)

Sodium acetate (Sigma, S5636)

Sodium citrate (Sigma, S1804)

U-$^{13}C_6$-glucose (99%, Sigma, 389374)

U-$^{13}C_6$-glutamine (99%, Cambridge Isotope Laboratories, Inc., CLM-1822-H)

U-$^{13}C_{18}$- linoleic acid (98%, Cambridge Isotope Laboratories, Inc., CLM-6855-0)

Gelatin (Merck, 9000-70-8)

Indium-tin-oxide (ITO)-coated glass slides (VisionTek Systems Ltd., Chester, UK)

N-(1-naphthyl) ethylenediamine dihydrochloride (NEDC) (Sigma-Aldrich, N9125)

Methanol (ACTU-All chemicals, UN1230)

Acetonitrile (ACTU-All chemicals, UN1648)

Phosphorus, red (Sigma, 04004)

Ethanol (Merck, 64-17-5)

4% paraformaldehyde (Sigma, 158127)

Antigen retrieval buffer, Citrate pH 6.1 (Dako, Agilent Technologies, S1699)

Normal donkey serum (Sigma, D9663)

Triton-X100 (Merck, 108603)

PBS (Fresenius Kabi, the Netherlands, 16RA3360)

ProlongTM gold antifade mountant with DAPI (Thermo Fisher Scientific, P36931)

Equipment

Equipment:

Vibratome VT1200 (Leica Microsystems, Germany)

Cryostar NX70 cryostat (Thermo Fisher Scientific, MA, USA)

Vacuum freeze-dryer

SunCollect sprayer (SunChrom GmbH, Friedrichsdorf, Germany)

RapifleX MALDI-TOF/TOF system (Bruker Daltonics GmbH, Bremen, Germany)

12 T solariX FTICR mass spectrometer (Bruker Daltonics)

3D Histech Pannoramic MIDI Scanner (Sysmex, Etten-Leur, the Netherlands)

Software:

FlexControl (Version 4.0, Bruker Daltonics)

FlexImaging 5.0 (Bruker Daltonics)

FtmsControl (Version 2.1.0, Bruker Daltonics)

mMass

SCiLS Lab 2016b (SCiLS, Bruker Daltonics)

R

RStudio

CaseViewer (3DHISTECH Ltd, Hungary)

Matlab R2019a

Adobe photoshop 2021

Adobe Illustrator 2021

R packages:

IsoCorrectoR

Seurat 3.0

Monocle3

pheatmap

plotly

Code:

The codes used in this article are available in Github (https://github.com/Gangqiwang/scDYMO).

Procedure

Vibratome sectioning and tissue slice incubation

1. After sacrifice, collect mouse kidneys and keep them in ice-cold sterile HBSS buffer with 5 mM glucose and 1% penicillin/streptomycin until vibratome sectioning.

2. Embed kidney in 4% low temperature-melting agarose gel.

3. Obtain 350 µm thick tissue slices from fresh tissue under ice-cold HBSS with 5mM glucose and 1% penicillin/streptomycin using a Vibratome VT1200. Slicing speed is 0.1 mm/s, and vibration amplitude is 2 mm.

4. Place tissue slices into culture plates (24 well) and incubate them in a 0.6 mL well-defined medium (nutrients-free Seahorse XF DMEM assay medium, supplemented with 2% FCS, 3 mM linoleic acid (dissolved with addition of 1% BSA), 5 mM glucose, 500 µM glutamine, 100 µM sodium acetate, 50 µM sodium citrate and penicillin/streptomycin (pH adjusted to 7.4) for up to 2 hours at 37°C and 5% CO_2.

5. During incubation, change medium to media containing the various ^{13}C-labeled nutrients at different time points. For ^{13}C-metabolite labeling incubation, equal amounts of either U-$^{13}C_6$-glucose, U-$^{13}C_6$-glutamine or U-$^{13}C_{18}$- linoleic acid are used to replace un-labeled nutrients in each medium.

6. At the end of the experiment, wash tissue slices with milli Q water shortly and quench them with liquid N_2. Store samples at -80 °C.

Tissue Preparation and Matrix Deposition

1. Embed frozen tissue samples in 10% gelatin on dry ice.

2. Cryosection into 10 µm thick tissue sections using a Cryostar NX70 cryostat at -20 °C.

3. Thaw-mount the sections onto indium-tin-oxide (ITO)-coated glass slides by putting a finger on the opposite side of the slide at -20 °C. Store the slides at -80 °C until MALDI-MSI measurement.

4. Place the mounted sections in a vacuum freeze-dryer for 15 minutes prior to matrix application.

5. After drying, apply a 7 mg/mL NEDC MALDI-matrix, dissolved in a methanol/acetonitrile/deionized water (70, 25, 5 %v/v/v) solution, on the sections using a SunCollect sprayer. A total of 21 matrix layers are applied with the following flow rates: layer 1-3 at 5 µL/min, layer 4-6 at 10 µL/min, layer 7-9 at 15 µL/min and 10-21 at 20 µL/min (speed *x*, medium 1; speed *y*, medium 1; *z* position, 35mm).

MALDI-MSI measurement

1. Perform MALDI-TOF/TOF-MSI measurement on the matrix coated sections using a RapifleX MALDI-TOF/TOF system. A matrix alone area is measured as control. Negative ion-mode mass spectra are acquired at a pixel size of 5 × 5 μm^2 over a mass range from *m/z* 80-1000. Prior to analysis the instrument is externally calibrated using red phosphorus. Spectra are acquired with 15 laser shots per pixel at a laser repetition rate of 10 kHz and a laser intensity just above the ionization threshold. Data acquisition is performed using flexControl and flexImaging 5.0.

2. Follow up with a MALDI-FTICR-MSI measurement on a 12T solariX MALDI-FTICR mass spectrometer (Bruker Daltonics) in negative-ion mode, using 30 laser shots and 50 µm pixel size. Prior to analysis the instrument is calibrated using red phosphorus. The spectra are recorded over a *m/z* range of 100-1000 with a 2M data point transient and transient length of 0.5592 seconds, providing an estimated resolution of 130,000 at *m/z* 400. Data acquisition is performed using ftmsControl, and flexImaging 5.0.

Post-MALDI-MSI Immunofluorescence staining

1. After the MALDI-MSI data acquisition, remove excess matrix from the MSI-analyzed-tissue-sections by washing in 100% ethanol (2 × 5 min), 75% ethanol (1 × 5 min) and 50% ethanol (1 × 5 min).

2. Fix tissue sections using 4% paraformaldehyde in PBS for 15 min. In case antigen retrieval will be performed in the next step, tissue sections can be fixed in 4% paraformaldehyde for 30 min at room temperature or overnight at 4 °C.

3. Perform antigen retrieval using antigen retrieval buffer (Citrate pH 6.1) in an autoclave. (optional for optimized usage of some antibodies)

4. Block tissue sections with 3% normal donkey serum, 2% BSA and 0.01% Triton-X100 in PBS for 1 hour at room temperature.

5. Incubate tissue sections with primary antibodies overnight at 4°C.

6. Wash tissue sections with PBS for 3 x 5 min.

7. Incubate tissue sections with fluorescent-labeled secondary antibodies for 1 hour at room temperature.

8. Wash tissue sections with PBS for 3 x 5 min.

9. Embed tissue sections in ProlongTM gold antifade mountant with DAPI.

10. Record the fluorescent images of the tissue sections using a 3D Histech Pannoramic MIDI Scanner.

MSI data pre-processing and exporting

1. Import MSI data into SCiLS Lab 2016b with baseline correction using convolution algorithm.

2. Import the average spectrum into mMass. Re-calibrate the spectrum in mMass. Export the *m/z* feature list with peaks that have a signal-to-noise-ratio > 3. Exclude the matrix peaks from the *m/z* feature list (obtained from the matrix control area), and use the remaining peaks to import as *m/z* feature list in SCiLS Lab 2016b with an interval width of ±30 mDa.

3. The *m/z* features present in both MALDI-FTICR-MSI and MALDI-TOF-MSI datasets, with similar tissue distribution are further used for identity assignment of lipid species. The *m/z* values from MALDI-FTICR-MSI are imported into the Human Metabolome Database (https://hmdb.ca/) and annotated for lipid species with an error < ±5 ppm. For small molecules detected only by MALDI-TOF-MSI, the *m/z* values are imported into the Human Metabolome Database and annotated for metabolites with an error < ±20 ppm. The ^{13}C-labeled peaks are selected by comparing the spectrum of control and ^{13}C-labeling experiments, and annotated based on the presence of un-labeled metabolites and their theoretical *m/z* values.

4. Normalize the MALDI-TOF-MSI data to the total ion count in SCiLS Lab 2016b. Export peak intensities of the selected features for all the measured pixels to a CSV file from SCiLS Lab 2016b. Several values will be present for each feature in the CSV file, due to interval width. Select the maximum values for each feature, represented by the maximum peak intensity from the measured pixels. Export the pixel coordinate information from SCiLS Lab 2016b.

5. Perform natural isotope abundance correction for metabolites used for fraction enrichment calculation with R package IsoCorrectoR.

Single cell clustering and cluster identification

1. Transform the MSI lipidome dataset into a count matrix by multiplying the TIC-normalized intensities by 100 and taking the integer.

2. This count data matrix is normalized and scaled using SCTransform to generate a 2-dimensional UMAP map using Seurat 3.0 in R.

3. Export the cluster information of all the pixels from Seurat 3.0 in R. Combine cluster information and coordinate information to generate images that show the distribution of the pixels from different clusters on tissues using pheatmap package in R.

4. In CaseViewer, annotate the MALDI-MSI measured area (using the saturated fluorescence signal to define the edge between measured and non-measured area) and export as a high resolution IF tiff-file with known (pixel) dimensions of the MALDI-MSI measured area. This allows to align accurately to the measured size of the MALDI-MSI information..

5. Co-register images of the pixel distribution from each cluster with the high-resolution IF tiff image section. It is important to maintain the width and height proportions of the images during co-registration. Annotate the cluster identity based on both staining and kidney morphology.

6. (optional) Change the pixel resolution of the IF staining image to the same resolution as the MSI image in Matlab R2019a, resulting in an IF image with 5×5 μm^2 pixel size. Export the IF staining values of each pixel and combine those with MALDI-MSI data for the targeted cell-type analysis.

7. Annotated Seurat projects are further integrated into the same UMAP to compare their clusters derived from lipid profiles by using FindIntegrationAnchors and IntegrateData functions in R.

Data integration and imputation

1. Remove the metabolite *m/z* features from ‘control’, so only lipid *m/z* features are left, which are used as query.

2. Use the MALDI-MSI data from the ^{13}C-labeling experiments as a reference to transfer metabolite production into the query using FindTransferAnchors and TransferData function from the Seurat 3.0 package in R. Both the query and reference are normalized and scaled using SCTransform.

3. Combine all the imputed metabolite productions into one query dataset, which contained the ^{13}C-labeling information over time as well as from different nutrients.

4. Calculate the fraction enrichment of isotopologues based on the ratio of ^{13}C-labeled metabolites and the sum of same metabolites on each pixel.

5. Generate pseudo-images using the calculated fraction enrichment of isotopologues together with pixel coordinate information exported from SCiLS Lab 2016b. Hotspot removal (high quantile 99%) are applied to all the pseudo-images generated from calculated values. The average fraction enrichment values of identified clusters are used for generating graphs and statistical analysis.

Molecular histology generated from 3D UMAP analysis

1. Integrate different Seurat projects using FindIntegrationAnchors and IntegrateData functions from Seurat 3.0 in R. (optional)

2. Generate a 3-dimensional UMAP map using package plotly in R.

3. Export the pixel embedding information of the 3-dimensional UMAP, and transfer UMAP1, UMAP2 and UMAP3 values into RGB color coding by varying red, green and blue intensities on the 3 independent axes.

4. Combine the RGB color coding with pixel coordinate information exported from SCiLS Lab 2016b to generate a MxNx3 data matrix in R. Use the MxNx3 data matrix to generate molecular histology images in Matlab R2019a.

Trajectory analysis using MALDI-MSI data

1. Integrate different Seurat projects using FindIntegrationAnchors and IntegrateData functions from Seurat 3.0 in R. (optional)

2. Generate a data form for Monocle3 from a Seurat project in R.

3. Run trajectory analysis using Monocle3 in R.

4. Export the embedding information of UMAP and pseudotime values calculated by Monocle3.

5. Transfer UMAP1, UMAP2 and pseudotime values into RGB color coding by varying red, green and blue intensities on the 3 independent axes.

6. Combine the RGB color coding with pixel coordinate information exported from SCiLS Lab 2016b to generate a MxNx3 data matrix in R. Use the MxNx3 data matrix to generate spatial trajectory images in Matlab R2019a. A 3D trajectory image is generated using colorcloud function in Matlab R2019a.

Troubleshooting

Time Taken

Anticipated Results

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Figures

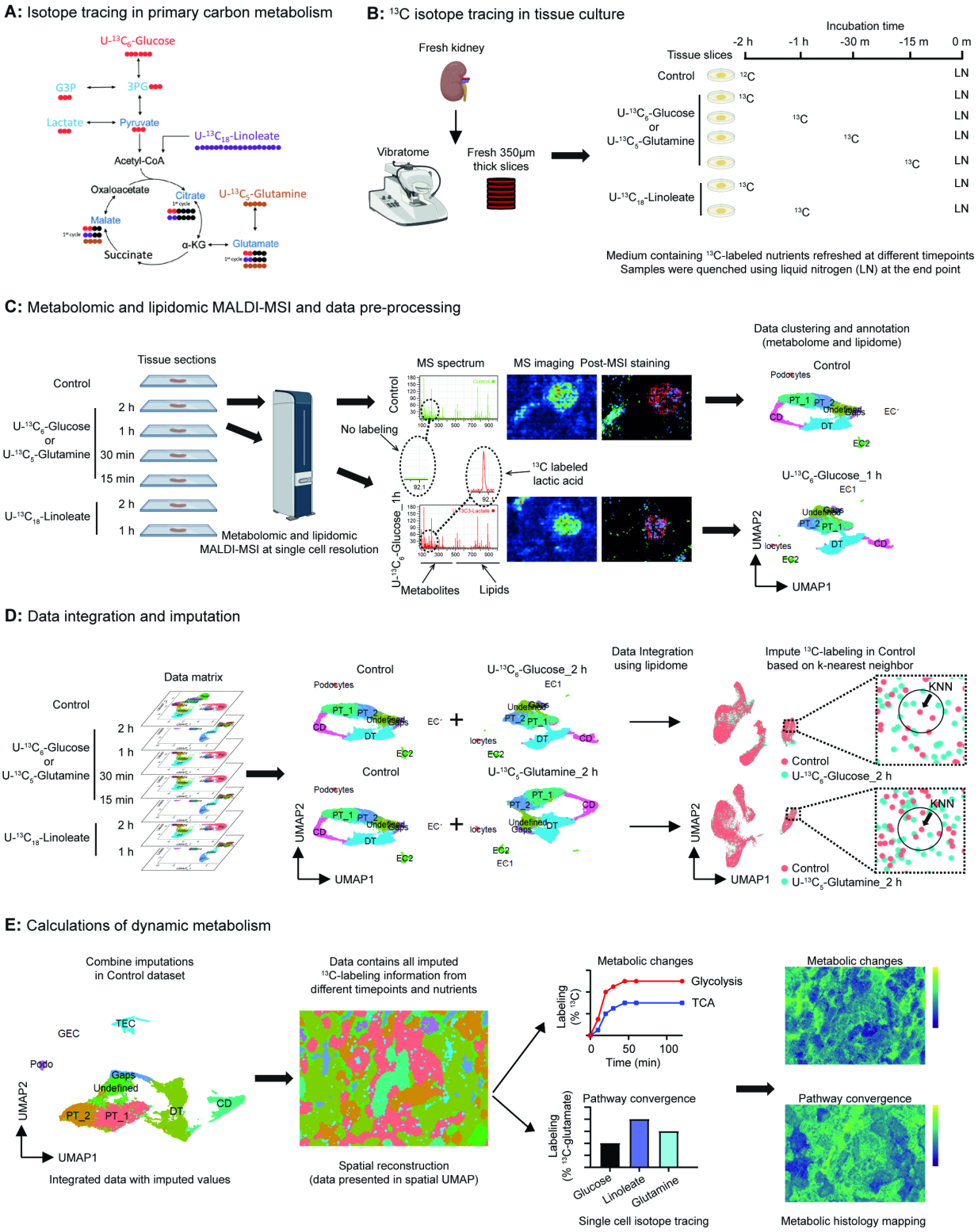

Figure 1

Workflow of cell type-specific dynamic metabolic measurements and analysis. A, Overview of the traced isotopes in the primary carbon metabolism. Different U-^{13}C-labeled nutrients (U-$^{13}C_6$-glucose, U-$^{13}C_{18}$-linoleate and U-$^{13}C_5$-glutamine) were used to trace their contribution to glycolytic- and TCA intermediates (light blue). **B,** Fresh mouse kidney tissue was cut into 350 µm-thick slices using a vibratome. ^{13}C isotope

tracing in tissue culture was performed by addition of different ^{13}C-labeled nutrients into a well-defined medium at different timepoints. Samples were quenched using liquid nitrogen (LN). **C,** The metabolome and lipidome were measured in all samples using MALDI-MSI at high spatial resolution (5 x 5 µm^2 pixel-size). MALDI-MSI data was pre-processed and transferred into a data matrix. Cell types were identified based on lipid profiles and post-MALDI-MSI immunofluorescence staining. Images from B and C were created using Biorender. **D,** Cell-type specific (phospho)lipid data was used to characterize various cell types. The lipid data was used for anchor-based data integration of the "control" data matrix with data matrices of sections with ^{13}C-labeled metabolome. Using k-nearest neighbor (KNN) analysis, the molecular information contained in the ^{13}C-labeling time course data matrices were imputed in the control data matrix. **E,** Establishment of the imputed dataset, in which each pixel contains all the added ^{13}C-labeling information from each timepoint and ^{13}C-nutrients. Dynamic metabolic calculations were performed on single-pixel, including metabolic rates and pathway convergence. To visualize the heterogeneity in tissue metabolic dynamics, a series of pseudo-images, which were generated from calculated values, were generated by tracing pixel coordinates back to the original spatial information from the MALDI-MSI analysis. Abbreviations: *3PG, 3-phosphoglycerate; G3P, glycerol-3-phosphate; a-KG, alpha-ketoglutaric acid; KNN, k-nearest neighbor*.