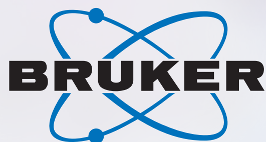

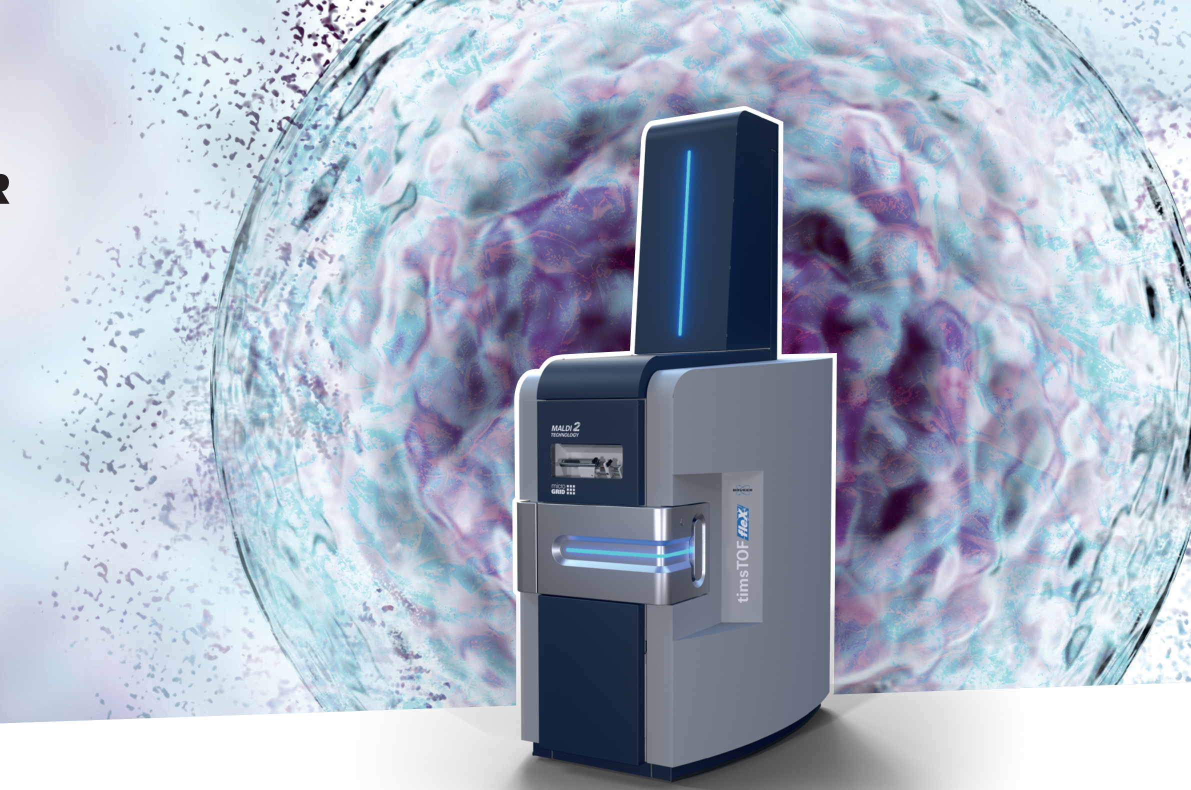


# Advancing data analysis for spatial metabolomics in 3D cell cultures

Introducing a CCS-enabled workflow for comprehensive MALDI Imaging data analysis from feature finding algorithms and molecular annotations to statistical analysis in one software.

## Introduction

Keywords:
MALDI Imaging, SCiLS Lab, 3D cell cultures, spheroids, MetaboScape

Human 3D cell cultures are widely used in the pharmaceutical industry and in the life science community for several reasons: they help reduce animal experiments, their physiology is more representative compared to 2D monolayer cell cultures, and, unlike many animal models, they express human drug targets. Because of their small size (300-3000 $\mu$m diameter) but high molecular information content, MALDI Imaging data acquisition and especially data analysis are challenging.

Spatial metabolomics data analysis workflows are often split up between several software solutions including vendor software, open-source scripts, and solutions developed by users. Such complex workflows can create interoperability or reporting challenges and may result in incomplete data analysis. Therefore, a single software framework for data processing, analysis, annotation, and reporting of results from complex biological samples like 3D cell cultures would be a major advancement in MALDI Imaging.

The latest version of SCiLS™ Lab 2024a combines the T-ReX³® feature finding algorithm for ion mobility MALDI Imaging data with powerful molecular annotation via the target list annotation tool on a MetaboScape® server. This enables ion mobility-based, i.e., collisional cross section (CCS)-enabled, annotation of hundreds of features within minutes. This tool is accessible through the Feature Table and can be easily set up within a few clicks. It automatically matches molecular information from a user-provided target list, such as LipidMaps, to SCiLS Lab CCS features, and the information output can be used for further analysis. Moreover, this advanced software offers an application programming interface (API) that permits users to stably incorporate their own python- or R-scripts into the software framework.

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## Methods

### MALDI Imaging of 3D cell cultures on a timsTOF fleX mass spectrometer

Serial cryosections of biculture spheroids, i.e., a core consisting of benign fibroblast cells surrounded by malign colon cancer cells, were obtained as described before [2,3]. Monoculture spheroids of either cell type served as references here to determine whether the molecular fingerprint of monocultures could be used to discriminate between fibroblasts and colon cancer cells in the biculture model. The samples were coated with NEDC matrix using an HTX-TM3 sprayer and measured on a timsTOF fleX with 20 µm pixel size. Data was acquired in negative ion mode using a mass range of *m/z* 300-1200 and TIMS on in the range of $1/K_0$ 0.60–1.79 $Vs/cm^2$ and a ramp time of 150 ms.

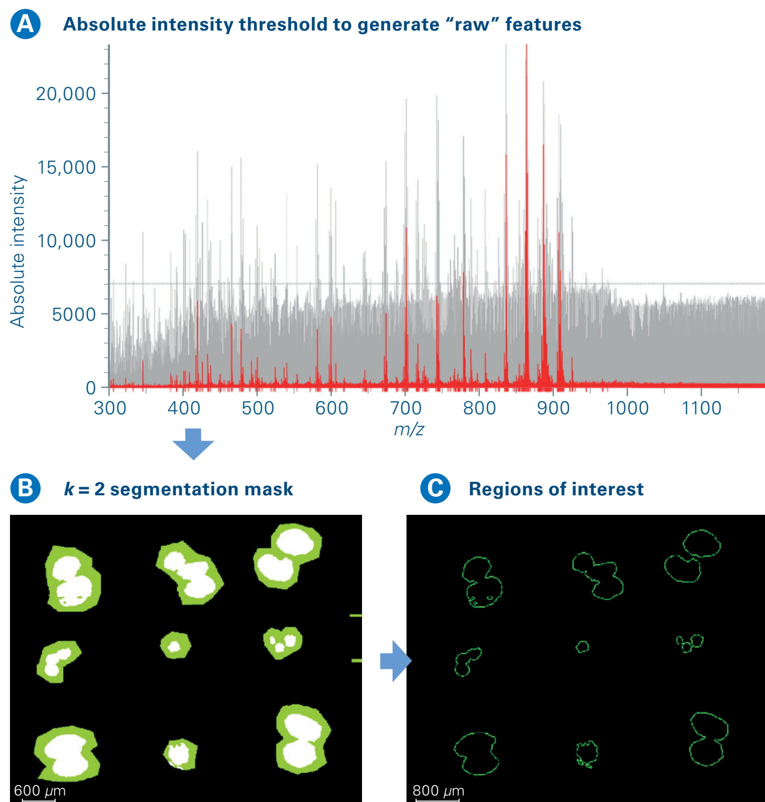


**Figure 1**
**Regions of interest (ROI) selection workflow for data analysis of spheroid 3D cell cultures.**
(A) Sliding window thresholding for selecting features for segmentation. (B) Image segmentation into background (green) and sample regions (white) using the selected features from (A). (C) Final sample ROIs selected for analysis (outlined in green).

All raw data was directly uploaded to SCiLS Lab 2024a, with resample method set to "Auto" and without baseline removal. All data shown were normalized using root mean square (RMS). Molecular annotation was done using the MetaboScape API in SCiLS Lab by first setting up an annotation method using "Annotate with Target List". For this, target lists were created from either LMSD (lipidmaps.org) or HMDB (HMDB.ca) databases following the template in MetaboScape.

Settings: the Search mode was Hierarchical, Annotation was on complete feature table, with attempting primary ion assignment enabled and with CCS-Predict Pro enabled. Tolerances and scoring were: 1 ppm narrow and 10 ppm wide for *m/z*, 100 min narrow and 200 min wide for Retention time, 25 narrow and 250 wide for mSigma, 1000 narrow and 600 wide for MS/MS score and 1% narrow and 3% wide for CCS.

### (Step 1) Selecting pixels for analysis using SCiLS Lab sofware

Background pixels were masked out by spatial segmentation (*k*-means clustering with *k*=2) of the whole dataset either by (1) using a single *m/z* feature known to be intrinsic to the sample (not shown) or by (2) using an *m/z* feature list obtained using the Sliding Window for thresholding above noise level. In Figure 1, the gray dotted line indicates the threshold level (Figure 1A), the green area showcases the background pixels eliminated through segmentation (Figure 1B), and the green outlines highlight the automatically selected ROIs of the sample (Figure 1C). Both spatial segmentation methods were able to automatically detect spheroid regions of interest for further analysis.

### (Step 2) Feature Finding

T-ReX³, Bruker's proprietary 4D feature finding algorithm, was used for extracting CCS features from "sample pixels", i.e., the spheroid ROIs. As a major advantage, T-ReX³ does deisotoping, annotates adducts (in combination with Molecular Annotation) and it unites this information into the same feature, ultimately eliminating redundancy to obtain a "clean" feature list ready for annotation and analysis. Additionally, isobaric molecules are picked as separate features due to their difference in CCS. Figure 2 illustrates T-ReX³ parameters that can be adjusted for feature finding, and how these affect the speed and number of features detected (Figure 2A). First,

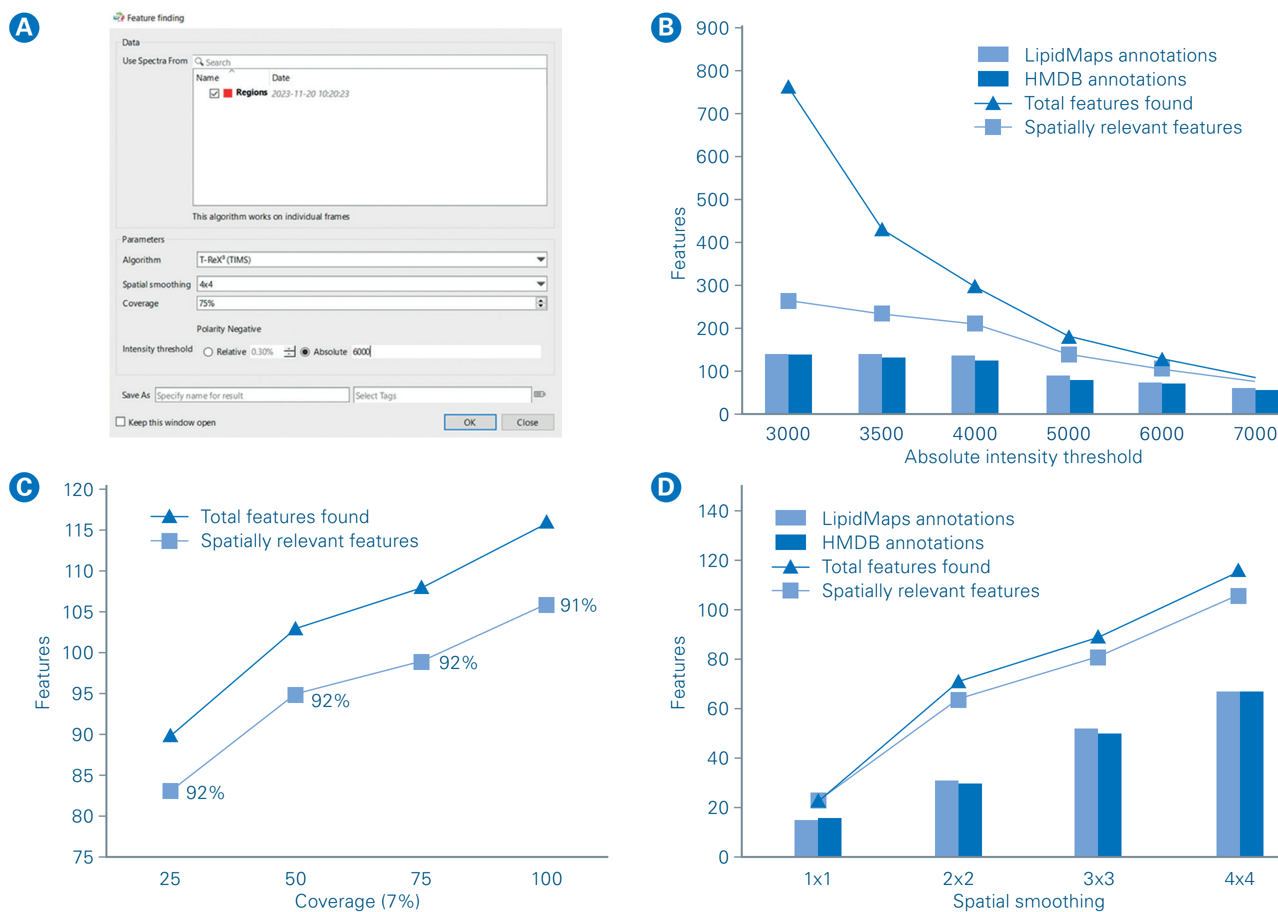


**Figure 2**

**Feature finding using the T-ReX³ algorithm (A).**

Parameters such as intensity threshold (B), coverage (C) and spatial smoothing (D) were evaluated for optimum number of features. Spatially relevant features (marked with light blue triangles) were filtered by removing the non-tissue-specific spatial distribution *m/z* features.

the optimum intensity threshold was investigated (Figure 2B), then the coverage of the sample area (Figure 2C), and spatial smoothing (Figure 2D) was evaluated. Considering the number of annotated spatial features obtained, the optimum parameters for the data were: 4 x 4 for spatial smoothing, 75% coverage, and, 6000 absolute intensity threshold.

## (Step 3) Molecular Annotation

Molecular annotation in SCiLS Lab is enabled by directly connecting to a MetaboScape server. For this to work, users establish a target list annotation method in MetaboScape once, which can then be accessed automatically from within SCiLS Lab. Here, a target list for lipidomics containing name, formula, InCHi and identifiers, based on the LipidMaps (www.lipidmaps.org) and HMDB (www.hmdb.ca) databases have been generated and the molecular annotation parameters according to instrument specifications for the timsTOF fleX have been set up. The annotation step takes ~1 minute for a list of ~300 features (both *m/z* and CCS). The number of annotated *m/z* features is dependent on the target list used. In this example dataset, results from LipidMaps and HMDB were similar (>55% annotated features) and useful for MALDI Imaging applications.

## (Step 4) Data Analysis

*k*-means clustering (segmentation) using the annotated feature list was done to differentiate between fibroblasts and cancer cells from spheroid monocultures and bicultures. A PCA confirmed the results, thus enabling further insights into metabolic behavior of 3D cell culture models (Figure 3). Results may suggest the existence of cell population subgroups, such as cancer-associated fibroblasts (CAF), in the biculture model. Thus, this 3D cell culture model may be used for studying the metabolic behavior of CAFs.

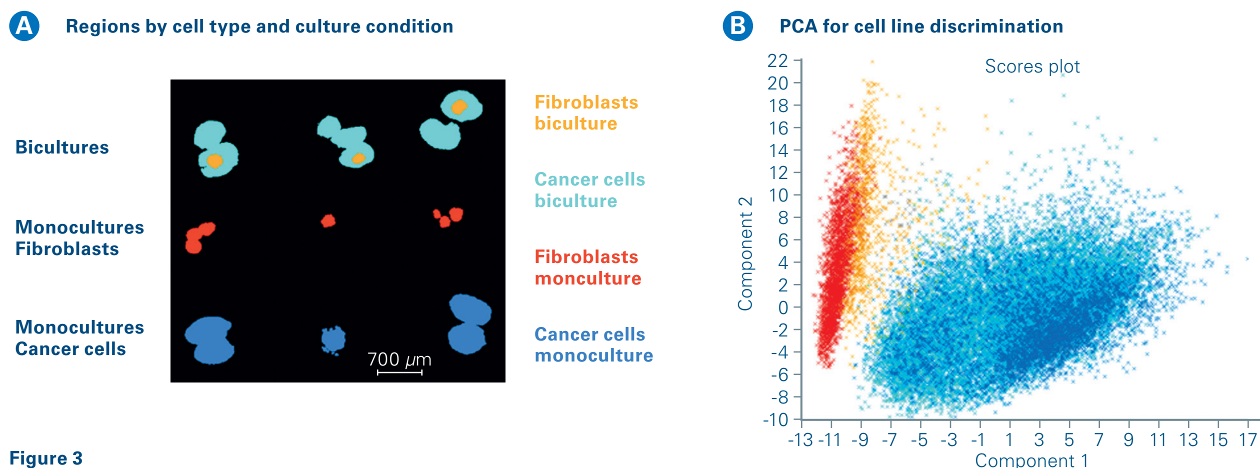


**Figure 3**

**PCA data analysis of spheroids using the annotated feature list.**

Regions and scores were colored by cell type (determined through *k*-means clustering) and culture condition in (A) and (B), respectively.

## Conclusion

From version 2024a onwards, SCiLS Lab is capable of hosting an integrated data analysis process, from importing raw data to automatic feature finding to generating and reporting analysis results. In case of spheroids, where the measured background area is comparable to that of the sample area, selecting only the tissue-relevant pixels for feature finding is crucial: it reduces the processing time considerably and aids in filtering out most of the MALDI matrix-related signals or any other background noise. Therefore, this workflow allows fast parameter optimization for feature finding, which leads to even faster annotation (even under one minute) at just a few clicks away. Overall, having these tools in the same box speeds up and simplifies the analysis process for complex biological samples such as 3D cell culture models.

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Getting a complementary view of your biological systems research by introducing workflows with metabolomics and lipidomics based LC-MS/MS using the ESI source on the timsTOF fleX. Additionally, MetaboScape offers unified workflows across ESI and MALDI Bruker platforms.


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