MALDI imaging-guided microproteomics workflow for biomarker discovery of intra-tumor heterogeneity

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OVERVIEW

Purpose: To develop analytical methods for the study of intra-tumor heterogeneity.
Methods: Matrix Assisted Laser Desorption Ionization-Mass Spectrometry Imaging (MALDI MSI) of Formalin-Fixed Paraffin-Embedded (FFPE) tissues. Unsupervised grouping of MS signals leading to localization of Regions of Interest (ROI) and to the evaluation of their heterogeneity. Additional peptide identification from the comparative analysis leading to the determination of potential biomarkers in the digested tissue sections, using a cloud-based software (MultImaging) and SCiLS for large datasets.
Results: ROIs could be defined, based on the images obtained by MALDI MSI, for different types (luminal and triple negative) of breast cancer, both with good or bad prognosis. MultImaging also demonstrated its versatility and interest for further biomarker discovery, although the use of not-normalized data.
Perspectives: Laser microdissection will be used to sample the ROIs according to a recently developed and published method for processing FFPE tissue samples by LC-MS/MS to identify up to 1400 proteins[1]. The combination of these two methods will provide insight into intra-tumor heterogeneity and biomarker discovery assays.

INTRODUCTION

A single tumor sample can phenotypically have **different cell populations,** called intra-tumor heterogeneity, which can lead to differential behaviors regarding metastasis seeding and therapy resistance[2]. The presence of some cellular clones may be associated to the bad prognosis. Unfortunately, discriminating these cellular groups and predicting their implication with a prognosis is impossible with classical histological methods as the morphology remains the same within the tumor. **MALDI imaging** has proven its effectiveness for **revealing hidden molecular features** in a panel of histopathological applications[3,4]. Using adequate mathematical tools, it can give an insight into distinct cellular regions, while **proteomics** allows for depicting their **molecular framework**. Recently, Longuespée et al.[1] published a method to identify more than 1400 proteins from microdissected tissue pieces, containing only 2700 cells.

METHODOLOGY

Tissues: Breast cancer FFPE tissues (Pathology department, University of Liège, Belgium).
MALDI MSI: MALDI-TOF/TOF-MS (Bruker, Germany)
Analytical data analysis was applied to the large measured datasets using the cloud software **Multimaging** (ImaBiotech, France) and **SCiLS** (Bruker, Germany).
PERSPECTIVE METHODOLOGY:
Tissue proteomics: laser microdissection (Leica LMD 700, Germany), LC-MS/MS: UPLC Waters 2D Nanoacquity (Waters, USA) and Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap MS (Thermo Fischer Scientific, USA).
Data processing and statistical analysis: Maxquant (Label Free Quantification Algorithm) and Perseus.

RESULTS

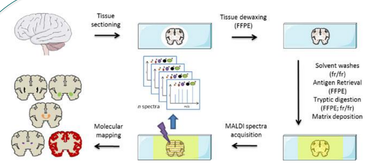


Fig. 1: MALDI imaging workflow [5]

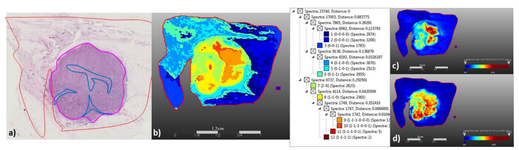


Fig. 2: Example of intra-tumor heterogeneity. a) H&E stained tissue (fibrous stroma, tumor, MALDI-TOF-MS measured area), b) segmentation image and tree (by SCiLS), *m/z* images at c) 762.5 and d) 943.6

MULTIMAGING
REVEALING TISSUE SECRETS

MultImaging cloud: All images were loaded without data reduction: 260.000 points/positions/image - up to 50Gb of compressed data can be analyzed at the same time. We used the software over the internet without loading all data into a memory through the use of secured accounts and protocols. This software integrates a biomarker discovery workflow. It allows you to overlay high definition histological images with molecular images of the compound distribution (see "Benefits of Multimaging").
Multimaging Cloud is a client software developed in C#/WPF. More details can be found at: **www.imabiotech.com**

Although the data are not (yet) normalized, the same ROIs of interest are detected compared to those obtained with SCiLS: for example for dataset [D], [F] and [J]. In some cases, we can already observe the intra-tumor heterogeneity ([F] and [J]). Also, the time required for statistical analysis is relatively short.

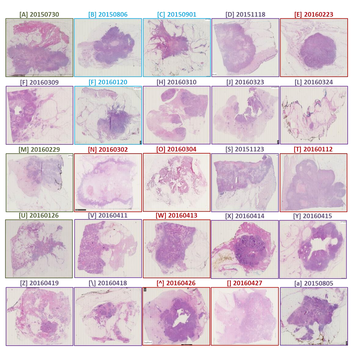


Fig. 3: 25 H&E stained breast tissue slices of different cancer types: luminal with good/bad prognosis and triple negative with good/bad prognosis.

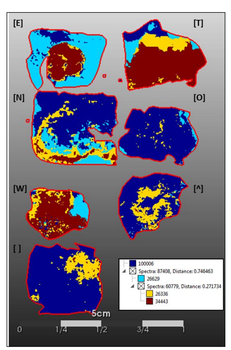


Fig. 4: Segmentation of cancer type triple negative with *bad prognosis*, intra-tumor heterogeneity ROIs: yellow and brown zones. Baseline normalization and TIC normalization by SCiLS.

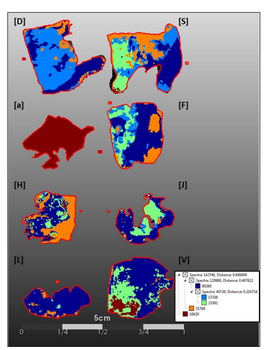


Fig. 5: Segmentation of cancer type triple negative with *good prognosis*, intra-tumor heterogeneity ROIs: light blue and green zones. Baseline normalization and TIC normalization by SCiLS.

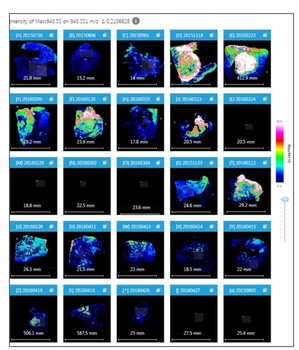


Fig. 6: Molecular distribution of *m/z*: 943.5

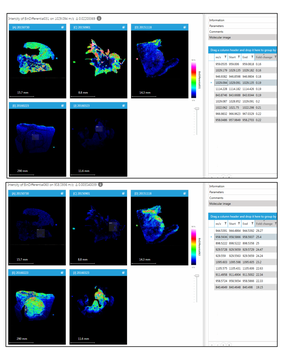
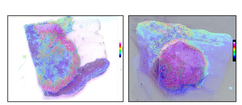


Fig. 7: Intensities of differentials on *m/z* 1029.0 and 958.6 for 5 datasets of 2 different biological classifications; Datasets [A] &[C]: luminal, Datasets [D], [E] &[J]: triple negative.

Benefits of Multimaging

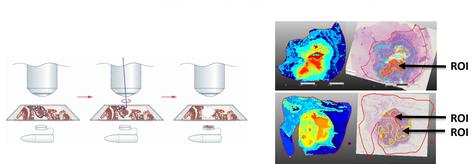
1. **Fast simultaneous visualisation of large data enriched images at the same time.**
2. **The intensity of a molecule of interest can be displayed across all datasets at the same time.**
3. **Offers the possibility to quickly detect Regions of Interest for further analysis.**
4. **Through the use of statistical analysis, it is possible to discriminate between 2 biological classifications, *i.e.* luminal and triple negative breast cancer samples. This will further lead to specific biomarkers for each classification.**



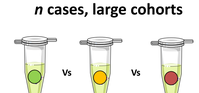
Overlap of HE image and molecular distribution of *m/z* 943.5 for dataset [D] and [E].

PERSPECTIVES

Laser microdissection of ROIs



***n* cases, large cohorts**



Statistical analysis and the combination of different visualization methods allow large scale analysis with common feature recognition to obtain small statistical ROIs across all samples (as can be seen in Figs. 4-7), which cannot be discriminated by other means.

Sample tissue pieces, using common small ROIs across all samples, by laser microdissection and perform label-free micro-proteomics .
Correlate the presence of ROIs **with a panel of biomarkers** and good or bad prognosis of the patients.

LABEL-FREE TISSUE MICRO-PROTEOMICS

This workflow is the combination of two different and complementary approaches for FFPE tissue processing: on one side mass spectrometry imaging and its data analysis, and on the other side, micro-proteomics.

The combination of the two approaches will offer a new vision on the molecular contributors of intra-tumor heterogeneity, along with the implication associated to the evolution of cancer and resistance to therapies.

REFERENCES

[1] R. Longuespee et al., *Methods* doi: 10.1016/j.ymeth.2015.12.008 (2015)
[2] F. Michor et al., *Cancer Prev. Res.* 3, 1361 (2010)
[3] A. Quaas et al., *Histopathology* 63, 455 (2013)
[4] Balluf B et al., *J. Pathol.* 235, 13 (2015)
[5] R. Longuespee et al., *Omics* 18, 539 (2014

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