Study of Perifosine Penetration in 3D Cell Cultures using Peeling Analysis of MALDI MS and Immunofluorescence Images

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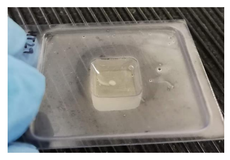
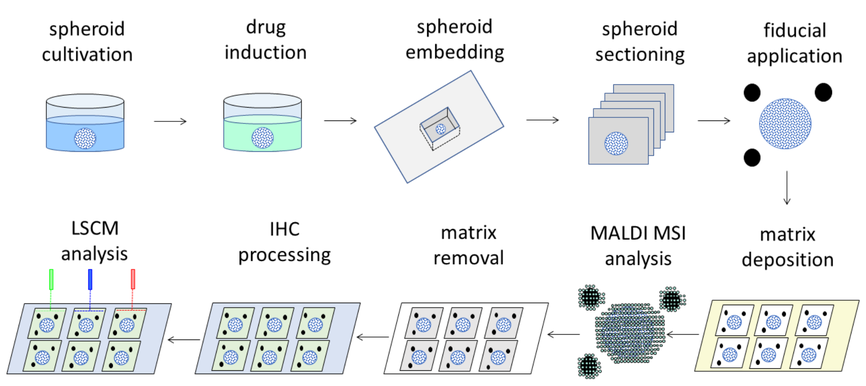
Sample preparation for subsequent MALDI MSI and immunofluorescence

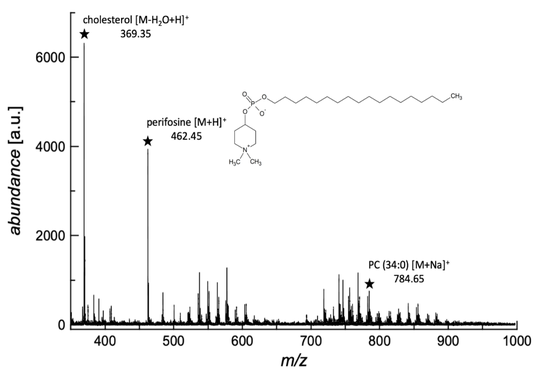


- Adenocarcinoma cell cultures HT-29 were cultivated to produce approximately 1 mm spheroids.
- Perifosine was added to the medium for a defined time interval. Spheroids were washed with PBS, transferred to gelatin and frozen.
- Samples, 12 µm thick sections on a conductive glass slide, were marked with fiducials and covered with sublimated DHB matrix. Further, sections were imaged using a MALDI TOF mass spectrometer with 50 µm pixel size and the *m/z* range 340–1000.
- After washing the matrix away with organic solvents, samples were processed by standard immunohistochemistry (IHC) protocol with selected antibodies (Ki-67, cleaved caspase 8, SNAIL/SLUG) and visualized by laser scanning confocal microscopy (LSCM) with 1.5 µm pixel size.
- Data processing software included FlexImaging, DataCube Explorer, LAS X, Image J (Fiji) and MATLAB.

MALDI MSI and immunofluorescence protocol

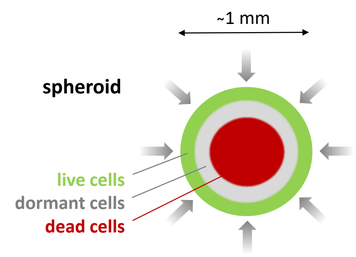


MALDI MSI settings Autoflex speed, Bruker	
ionization mode	positive
perifosine *m/z*	462.45
matrix deflection *m/z*	< 320
m/z range	340-1000
pixel size	50 µm
laser beam diameter	50 µm
number of shots/pixel	200

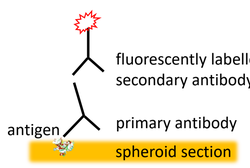


Introduction

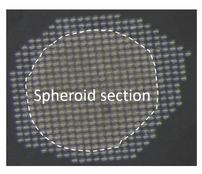
- Three-dimensional cell aggregates, or "spheroids" have gained popularity for studies of drug uptake and diffusion; they can mimic in vivo barriers and their preparation takes a few days.
- Proliferating and apoptotic cells in the spheroids can be visualized using fluorescence microscopy after immunostaining.
- Distribution of drugs, which often do not exhibit specific fluorescence, has to be revealed using another technique, such as matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI).
- To evaluate efficacy of a potential anti-cancer drug perifosine within spheroids of colorectal carcinoma, images of drug abundance from MALDI MSI and fluorescently visualized proliferating and/or apoptotic cells must be precisely colocalized and carefully compared.



IHC scheme



MALDI MSI



Spheroid peeling

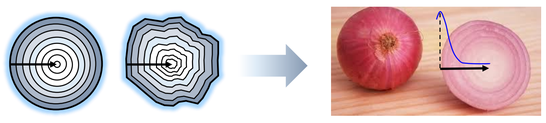
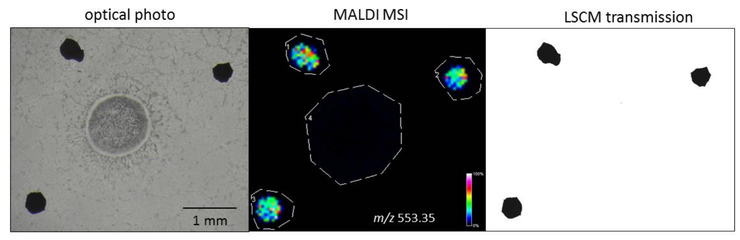


Image coregistration and spheroid peeling

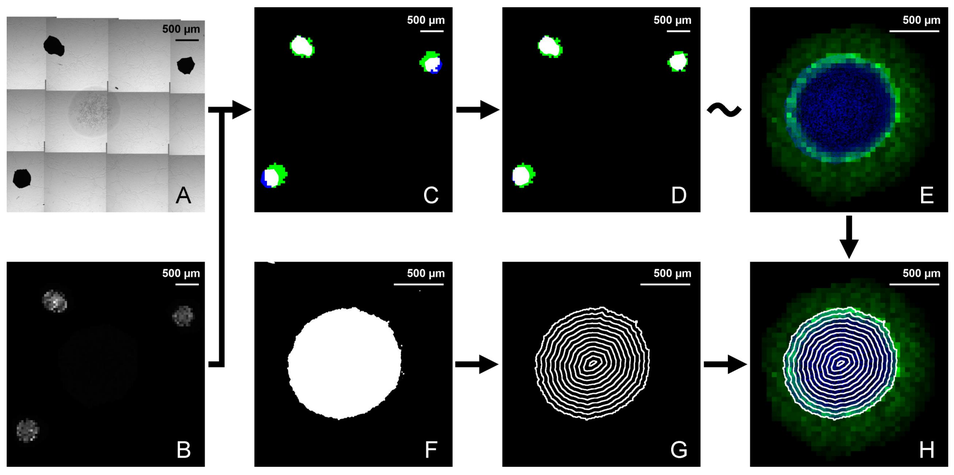
For MALDI MSI and LSCM image coregistration, four modality-specific issues had to be addressed:

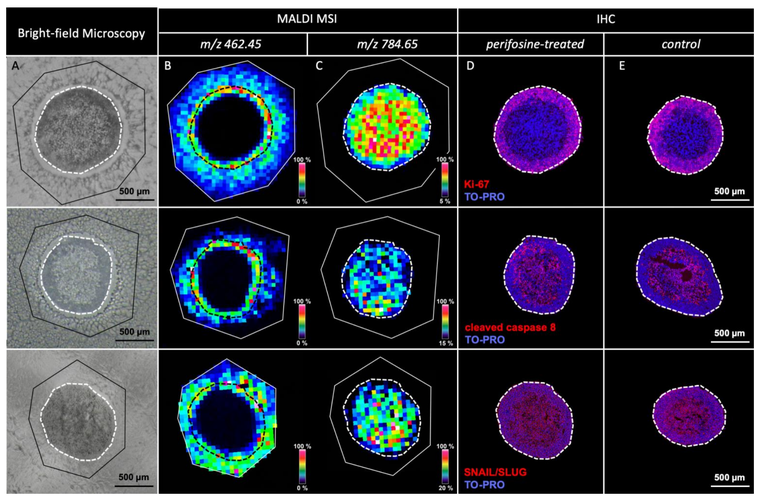
- The grayscale depth was different for the two modalities: while raw MALDI MSI data are encoded as 64-bit floating point numbers, the LSCM grayscale depth may be 8, 12 or 16 bits per channel.
- MALDI MSI spatial resolution was an order of magnitude coarser than that of LSCM. The spatial resolution of MALDI MSI in our assays was 50 µm, whereas that of the LSCM images was 1.5 µm.
- The aspect ratios of the MALDI MSI and LSCM images may not match: an assay may deliver MALDI MSI images of 65×43 pixels in size, while the corresponding LSCM mosaic to be coregistered comprises 1765×1328 pixels.
- MALDI *vs.* LSCM images can be slightly rotated with respect to each other.



The fiducials

(A) LSCM transmission mode
(B) MALDI MSI mode
(C) pre-registered by matching the binarized image centroids
(D) LSCM fiducials registered onto MALDI MSI fiducials
(E) the resulting transform applied to LSCM TO-PRO (blue) fluorescence to match the MALDI MSI perifosine (green) image
(F) fluorescence TO-PRO spheroid image segmented to obtain a binary spheroid mask
(G) peels created based on the distance transform of the binary mask
(H) an overlay combined with the registered images for analysis





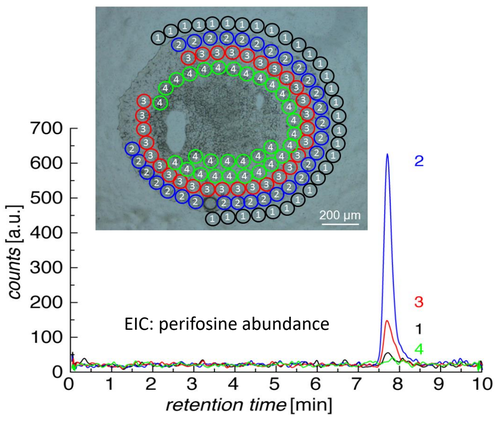
Peeling: distribution profiles

Results

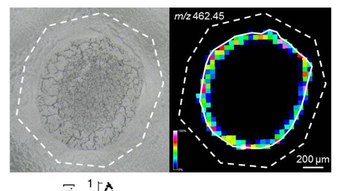
- A robust sample preparation protocol for the multimodal imaging, which includes matrix removal, was developed.
- The overlay of MALDI MS and LSCM spheroid images required fiducial-based coregistration because of lack of any morphological features in common spheroids. A commercially available white, water-based permanent paint containing TiO_2, which is not removed by washing, was selected for preparation of the suitable fiducial markers. The fiducials are detectable by MALDI MS and LSCM in the transmission mode.
- MALDI MSI and LSCM fiducial-based coregistration algorithm was developed; it addresses different depth, spatial resolution and aspect ratios as well as rotation of images taken in the two modes. Fourth, the spatial relationship between the MALDI MS and of the LSCM intensities was evaluated based on the respective intensities along equidistant layers, or "peels" of the entire spheroid, starting from the spheroid boundary.
- The study of perifosine penetration by MALDI MSI revealed that perifosine does not penetrate to the spheroid core even after 24-hour induction; its signal drops below the detection limit within 200 µm from the spheroid boundary.
- Specific characteristic of the drug, its significant diffusion from the spheroid to the gelatin medium during sample preparation, required precise coregistration and peeling data analysis.
- As expected, viability assays conducted by IHC for markers of cell proliferation and apoptosis with parallel staining of all cell nuclei revealed higher signal of the apoptotic marker in the spheroid core and the higher signal of proliferative cells on the spheroid edge.

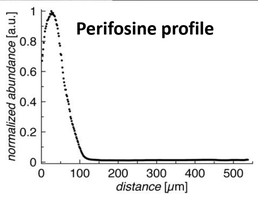
Laser capture microdissection with LC – ESI MS perifosine quantification

... confirmation of perifosine penetration profiles



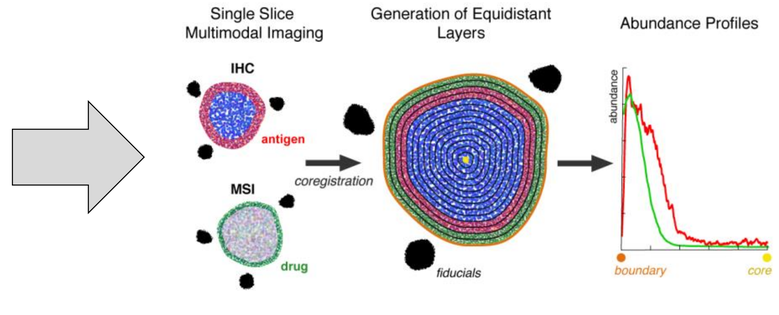
A close spheroid section: MALDI MSI





Conclusions

A robust procedure comprising sample preparation, MALDI MS and LSCM imaging and multimodal data processing was established. Fiducials were used for precise coregistration. Peeling data analysis, which used data from the entire spheroid area, was applied to construct distribution profiles of selected compounds as a function of the distance from the spheroid boundary. The accurate colocalization of MALDI MS and IHC maps showed a limited penetration of perifosine into spheroids within 24 hours and allowed differentiation between apoptosis resulting from hypoxia/nutrient deprivation and drug exposure.



Acknowledgment

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