Label-free Raman Micro-Spectral Imaging of the Microenvironment in Three Dimensional Human Tumor Spheroids

Tatyana Chernenko[1], Ruchi Shah[1], Evgenia Zuser[2], Milos Miljkovic[2], Max Diem[2], and Mansoor Amiji[1]
[1]Department of Pharmaceutical Sciences and [2]Department of Chemistry and Chemical Biology, Northeastern University, Boston, MA 02115, USA

ABSTRACT

Sensitivity of tumors to chemotherapy greatly relies on capability of the administered drug to traverse the tumor via its leaky vasculature and eradicate cancer cells. Unfortunately, in order for the drug to exhibit a toxic effect it must traverse the tumor interstitium which, due to its abnormal physiology, poses a barrier to efficacy of the drug. As the tumor micro-environment may influence its susceptibility to chemotherapy, a detailed understanding is needed in order to improve efficiency of various therapeutics. Three dimension spheroids have thus been used as models for tumors, to research their interstitium *in vitro*. We have been utilizing these tumor models in order to characterize their architecture and development using label-free Raman micro-spectral imaging technique.

Keywords: Spheroids, tumor-microenvironment, label-free Raman imaging

1. INTRODUCTION

Progress in cancer chemotherapy involves the ability of the drug to traverse and penetrate the tumor tissue, via its poorly-structured vasculature [1-2]. However, an *in vivo* distribution of the cytotoxic agents is highly variable within the tumor mass. Thus cancer cells will be eradicated based on the gradient of the drug penetration within the tumor. Apart from an irregular vasculature attributed to the tumor, its micro-environment also poses another obstacle to an effective chemotherapeutic agent, compromising further efficacy of the drug [3-4].

The micro-environment of a growing tumor is characterized by a proliferating outer shell and various regions of hypoxic and necrotic centers. Tumor mass is known to possess features attributed to multi-cellular resistance, which is characterized by an actively dividing outer shell, a quiescent intermediate layer, as well as a hypoxic core. Chemotherapeutics, which do reach the tumor site, unfortunately, may only eliminate cells in the outer-most layer, proximal to the blood vessel, while the drug penetration into the core is vastly reduced. Thus, understanding the biochemical organization of the spheroid will render a much better understanding of tumor growth and development, elucidating drug penetration patterns, subsequently offering insight into its suppression.

In order to accurately research the tumor-microenvironments and characterize tumor growth and development patterns various models have been utilized in the field of cancer therapy. One such system includes *in vitro* growth of spheroids as tumor models, which are 3-dimensional aggregates of adherent cancer cells [1, 5]. They possess many properties pertaining to tumors, including the development of the extra-cellular matrix as well as a gradient of nutrient and O_2-deprived hypoxic core.

Three dimensional tumor spheroids thus provide a glimpse into the early tumor architecture and development. Susceptibility of the cancer cells to various chemotherapeutics can thus be easily studied with the use of tumor-models, which subsequently portray the kinetics and dynamics of administered chemotherapeutics. Hence, we have utilized spheroids in order to conduct research on dynamics of this tumor-model using a label-free non-perturbing Raman micro-spectroscopy, as an imaging modality [6-7]. Without extraneous labels or dyes, we have thus been able to verify the various micro-environments within the spheroid.

2. EXPERIMENTAL METHODS

2.1 2D and 3D Cell Culture

Human pancreatic adenocarcinoma (Panc-1) and human ovarian adenocarcinoma (SKOV-3) cell lines, purchased from ATCC, were cultured in Dulbecco's Modified Eagle's Medium (DMEM, CellGro) supplemented with 10% fetal bovine serum (FBS, Gemini), and 5% penicillin/streptomycin (Gibco). Panc-1 spheroids were formed using hanging drop method. Aggregates of cells of day 1, day 2, day 3 and day 5 were fixed, washed with 1x PBS and analyzed. The fixative used was 10% formaldehyde (Sigma-Aldrich, St. Louis, MO).

2.2 Live/Dead Assay

Fluorescence assays were conducted with Live/dead Viability/Cytoxicity Assay kit. Two-day Panc-1 spheroids were collected and placed into solution containing 0.8μL of calcein AM and 2.5 μL of ethidium homodimer-1 in 1 ml of phosphate-buffered saline (PBS).

Then spheroieds were incubated for 45 min at 37°C. Images were acquired using a Zeiss LSM 700 Confocal Microscope, 10X.

2.3 Raman Data Acquisition

Raman spectra and spectral images were acquired using a WITec, Inc. (Ulm, Germany) Model CRM 200 Confocal Raman Microscope. Excitation (ca. 40 mW at 488 nm) is provided by an air-cooled solid state laser (Spectra Physics, Cyan). A Nikon Fluor (60x/1.00 NA, WD = 2.0 mm) water immersion objective was used in the studies presented here. The sample is located on a piezo-electrically driven microscope scanning stage with an x,y resolution of ca. 3 nm and a repeatability of ± 5 nm, and z resolution of ca. 0.3 nm and ± 2 nm repeatability. Spectra are collected at a 0.5 µm grid, with a dwell time of 0.25-0.4 seconds.

2.4 Multivariate Analysis

The mathematical methods of analysis applied to the acquired data set were Hierarchical Cluster Analysis (HCA, based on spectral similarity), and Vertex Component Analysis (VCA, a hyperspectral unmixing method). In HCA the spectra are merged into a few clusters to which color codes are assigned, and pseudocolor map may be constructed based strictly on spectral similarities. In spectral unmixing, the spectra from individual pixels are decomposed into several constituent endmembers, the fractions of which indicate to what extent each constituent spectrum contributes to the signal in each pixel. VCA is a method that expresses all spectra in a data set as linear combinations of the most dissimilar spectra.

3. RESULTS AND DISCUSSION

Raman micro-spectroscopy, due to its high spatial resolution and its ability to probe samples under *in vivo* and *in vitro* conditions, enables rapid and non-destructive analysis of living biological systems. We have thus applied it to analyze two types of spheroids cultured from two cell lines, namely the SKOV-3 cell line as well as the Panc-1.

A confocal setup of the instrumentation utilized allows for biochemical analysis of the spheroid micro-environment without superfluous dyes. Applications of various statistical methods of analysis on the hyperspectral datasets obtained allows for a reconstruction of a biochemical functional map of the specimen. We have utilized Vertex Component Analysis (VCA) as a discriminatory method for the acquired spectral datasets. VCA is based on the principle that the biological samples are comprised of "pure" chemical components and thus the entire spectral dataset is clustered according to degree of abundance of each of the components therein. Typically, three monochrome intensity images may be obtained and pseudo-colored red, green and blue. An overlay of these RGB maps renders an intensity profile of each of the three clusters assigned by user. An example of the VCA-reconstructed biochemical map of Panc-1 cells in culture is shown in Figure 1, where three major regions of interest are observed, namely the nucleus, membrane-rich organelles, as well as inherent phospholipid inclusions.

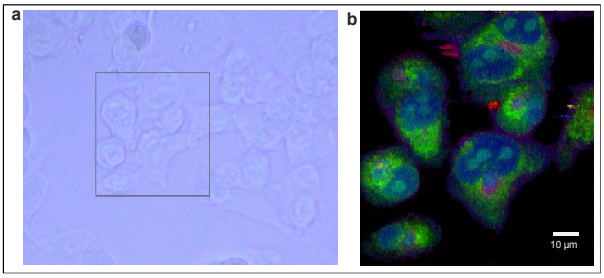

Figure 1. Visual image (A) of 2-dimensional cell culture of PANC-1 spheroids grown on CaF_2 slides. Image acquired at 20x. VCA-analyzed image (B) of the cells shown in inset in (A), acquired at 60x. Blue regions represent nucleus/cell body, red regions denote phospholipid-rich intra-cellular inclusions, while green regions correlate with membrane-rich organelles.

In order to assess the tumor micro-environment, a 3-dimensional tumor-model has been analyzed in lieu of conventional cell culture. Lateral sections of the spheroids have been obtained and analyzed, from the top of the spheroid to a depth of 100 µm, shown in Figure 2. As the focus at the top of the spheroid was determined by visual inspection, individual cells are easily distinguished. Statistical method of analysis employed on the dataset reveals distinct biochemical environments within the spheroid without application of stains. They are pseudo-colored blue – for nucleus and cell body, red – for phospholipid-rich inclusions inherent to the cell lines, while gradations of green and yellow delineate intra-cellular and extra-cellular matrices.

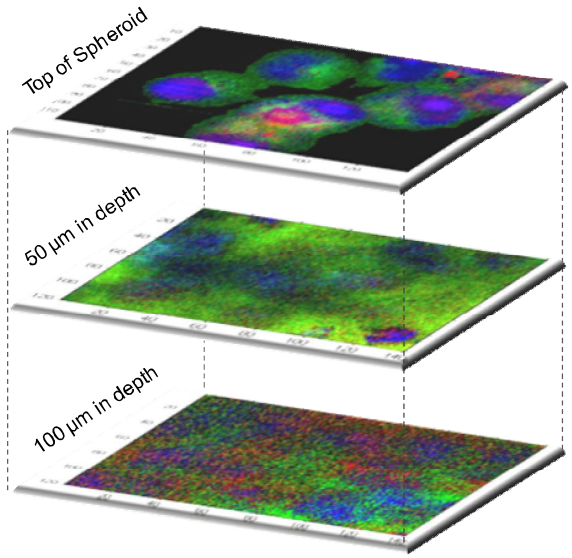

Figure 2. VCA-analyzed depth lateral scans of a PANC-1 spheroid. Blue regions indicate nucleus/cell body, red regions represent inherent phospholipid inclusions, while the green/yellow regions represent intra- and extra-cellular matrices.

Hence, mathematical methods of analysis applied to Raman hyperspectral datasets reveal various intracellular

compartments, such as the nucleus as well as various inclusions within the cytoplasm, in the outer-most layer of the spheroid, as is seen in Figures 2 and 3.

The spectra adjacent to the depth scan in Figure 3, further portray the prominent cellular features with corresponding spectral signatures in the fingerprint region (750 – 1800 cm^{-1}) as well as in the C-H stretching region (2800 – 3100 cm^{-1}). The peaks characteristic of peptide are seen at 1660 cm^{-1} and 1260 cm^{-1}, DNA/RNA specific peaks are seen below 1000 cm^{-1}, while a peak specific to phenylalanine is seen at 1005 cm^{-1}. As we probe deeper into the core of the tissue by taking an axial scan, as is denoted in Figure 3, a general scheme of the tumor environment is observed. While individual cells are still apparent, the extra-cellular matrix is identified as a distinct entity via VCA. Discrete spectral signatures of the extracellular matrix are also observed with the increasing depth of the spheroid, and are pseudo-colored yellow in Figure 3. The spectral signatures from the yellow cluster tend to correlate with the increase in pH within the inner region of the tumor-model, underscoring existence of a necrotic core. This is corroborated with performing a Live/Dead Assay on spheroids of the same cell line (Figure 4), where a trend is visible of an accumulation of necrotic cells (red) within the core of the spheroids, with live cells (green) lining its periphery.

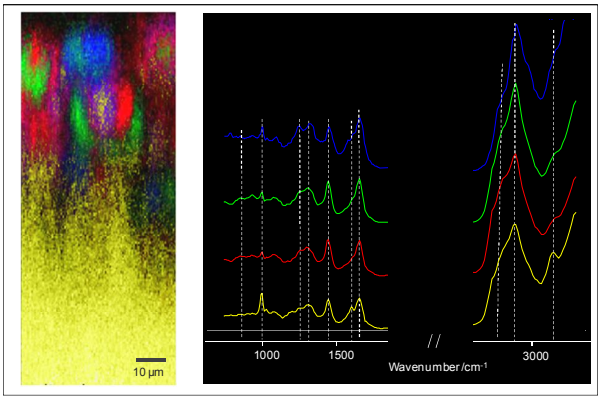

Figure 3. (A) Raman image of depth profile of 24 hr spheroid (100 µm deep), reconstructed via VCA. (B) Stacked abundance spectra from the corresponding VCA intensity map. Peaks of interest are identified within fingerprint region (750 – 1800 cm^{-1}) and C-H stretching region (2800 – 3100 cm^{-1}).

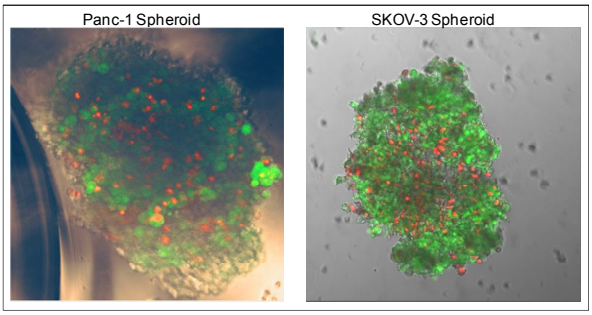

Figure 4. DIC visual images of 3-day grown Panc-1 human pancreatic adenocarcinoma(A) and SKOV-3 human ovarian adenocarcinoma (B) spheroids overlaid with Live/Dead assay fluorescence stain. Green fluorescence corresponds to live cells, while red fluorescence correspond to dead cells. Objective utilized is10x.

4. CONCLUSIONS

Raman micro-spectral imaging modality offers a biochemical snapshot of the spheroid interstitium. Utilizing this technique in synergy with unsupervised mathematical algorithms, such as the Vertex Component Analysis (VCA), allows us to view a biochemical reconstruction of any biological sample.

5. ACKNOWLEDGEMENTS

This study was supported by the National Cancer Institute's (NCI) Alliance in Nanotechnology for Cancer through the Center for Cancer Nanotechnology Excellence (CCNE) grant U54- CA151881 as well another NCI grant CA090346.

6. REFERENCES

1. Minchinton, A.I. and I.F. Tannock, *Drug penetration in solid tumours.* Nat Rev Cancer, 2006. 6(8): p. 583-592.
2. Brown, J.M. and A.J. Giaccia, *The Unique Physiology of Solid Tumors: Opportunities (and Problems) for Cancer Therapy.* Cancer Research, 1998. 58(7): p. 1408-1416.
3. Tannock, I.F., *Tumor Physiology and Drug Resistance.* Cancer and Metastasis Reviews, 2001. 20(1): p. 123-132.
4. Vaupel, P., *Tumor microenvironmental physiology and its implications for radiation oncology.* Seminars in Radiation Oncology, 2004. 14(3): p. 198-206.
5. Sutherland, R., *Cell and environment interactions in tumor microregions: the multicell spheroid model.* Science, 1988. 240(4849): p. 177-184.
6. Chernenko, T., Matthaeus, C., Milane, L., Quintero, L., Amiji, M., Diem, M., *Label-Free Raman Spectral Imaging of Intracellular Delivery and Degradation of Polymeric Nanoparticle Systems.* ACS Nano, 2009. 3(11): p. 3552-3559.
7. Zuser, E., Chernenko, T., Newmark, J., Miljkovic, M., Diem, M. *Confocal Raman microspectral imaging (CRMI) of murine stem cell colonies.* Analyst, 2010. 135(12): p. 3030-3033.