Pushing the Boundaries of 3D Microtissue Analysis using High Content Imaging

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1 Introduction

3D cell culture methods are widely accepted as being more physiologically relevant than conventional 2D cell culture methods, and are believed to improve the prediction of drug candidates at an early stage in the drug development process. The visualization of 3D structures is challenging, for example when imaging microtissues there is light scattering and absorption which prevents imaging deep into the center of the microtissue. Here, we show how the boundaries of microtissue imaging can be pushed further by using the Opera® High Content Screening System (equipped with a water objective lens) in combination with a microtissue pretreatment. The aqueous reagent (Sca*l*e) renders the biological sample optically more transparent and allows a greater imaging depth. We also describe the analysis of a spherical colon cancer microtissue model using *in vivo* near infrared (NIR) agents. These agents allowed visualization and quantification of cancer-associated biomarker intensities and their distribution in microtissues. The expression of these biomarkers and the observed oxygen gradients correlate with the development of solid tumors *in vivo*, confirming microtissues to be a physiological cell model suitable for cell-based drug screening.

2 Enabling a greater imaging depth into 3D microtissues using the Opera High Content Screening System

HCT116 microtissues (human colon carcinoma cell line stably expressing GFP) and NIH3T3 microtissues (mouse embryonic fibroblast cell line stably expressing RFP) were produced using the GravityPLUS™ system from InSphero AG. This system allows for automation of the hanging drop method and provides an optimal tool for making microtissues amenable to cell-based drug screening (1). Microtissues were fixed, stained with Hoechst (16 µM, 1h), treated with Sca*l*e reagent (2) for 1 day and then imaged with the Opera system using a 20x water objective. A 200 µm z-stack with a plane distance of 2 µm was acquired. To obtain an objective measure of how far into the microtissue core structures were visible, SER Edge texture properties were analyzed using Acapella® High Content Imaging and Analysis Software (Fig. 1). The detection depth was determined for each microtissue type and condition (Fig. 2).

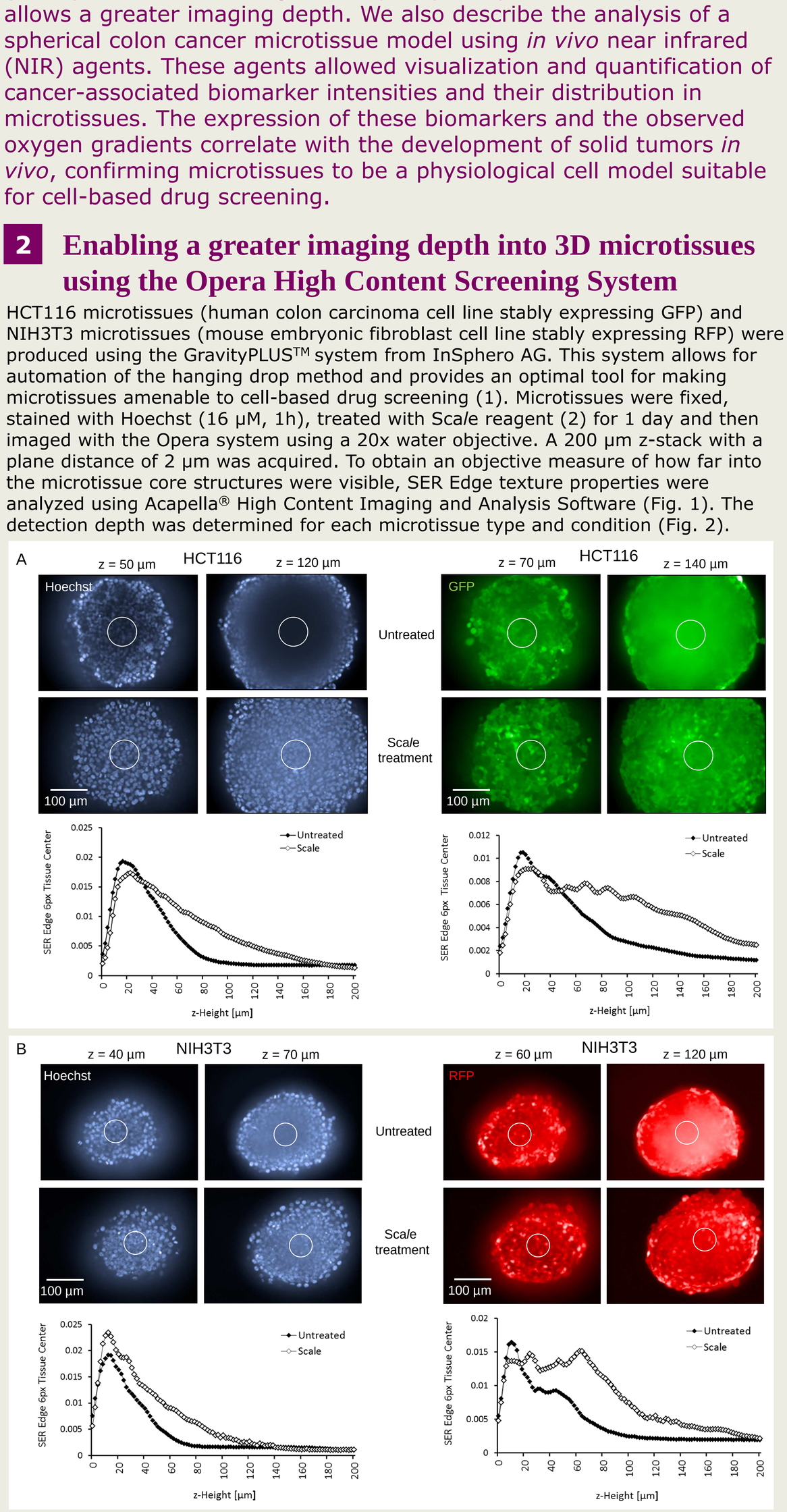

Figure 1: Comparison of texture properties in untreated and Sca*l*e treated microtissues. Results from HCT116 microtissues (A) and NIH3T3 microtissues (B) are shown. Images show selected planes of the Hoechst Channels (left) and GFP or RFP channels (right). Using the Acapella software, the texture (SER Edge, 6px) of the microtissue center (white circle) was analyzed for each plane. The calculated SER Edge values in Sca*l*e treated microtissues are higher than in untreated microtissues. Treatment with Sca*l*e therefore allows detection of structures at higher planes.

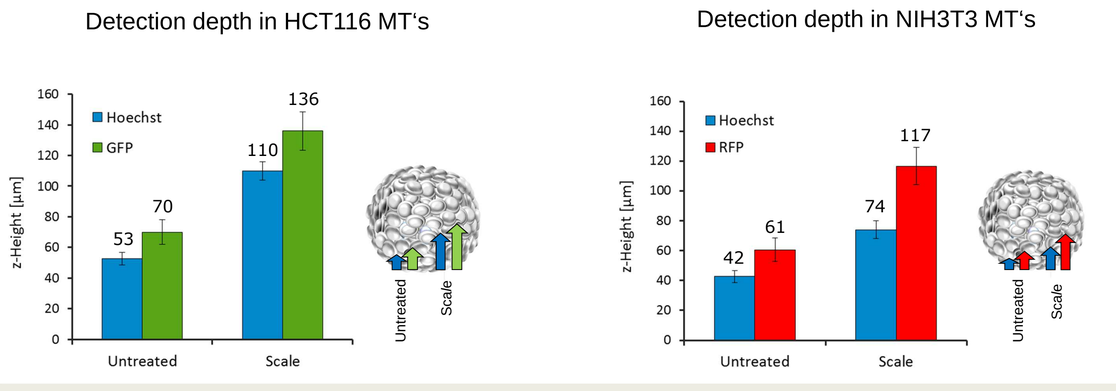

Figure 2: Detection depth in HCT116 (left) and NIH3T3 (right) microtissues. The calculated detection depth is the z-Height [µm] of the highest image plane suitable for single cell or nuclei segmentation. Using the 20xW objective of the Opera system, untreated HCT116 microtissues could be imaged up to 53 µm (Hoechst) and 70 µm (GFP). The Sca*l*e treatment doubled the detection depth in HCT116 microtissues (110 µm for Hoechst and 136 µm for GFP). In NIH3T3 microtissues with a more compact structure, Sca*l*e treatment increased the detection depth from 42 µm to 74 µm (Hoechst) and from 61 µm to 117 µm (RFP). n = 3 microtissues.

3 Quantification of cancer biomarker expression and distribution in 3D microtissues with *in vivo* NIR agents using the Operetta High Content Imaging System

To analyze the activity of the cancer-associated biomarkers cathepsin and MMP, and to visualize hypoxic areas, microtissues were stained with 100 nM of the ProSense® 680 (NEV10003), MMPSense® 680 (NEV10126) and HypoxiSense® 680 (NEV11070) NIR agents, respectively (Fig. 3). Microtissues were incubated for 72 h and then imaged on the Operetta® High Content Imaging System with a 10x objective. To study hypoxic conditions, microtissues of varying sizes were produced by seeding different cell numbers during spheroid formation. Microtissues were stained with HypoxiSense 680 agent, imaged on the Operetta system, and the maximum intensity in the spheroid core region was analyzed using Harmony® High Content Analysis Software (Fig. 4).

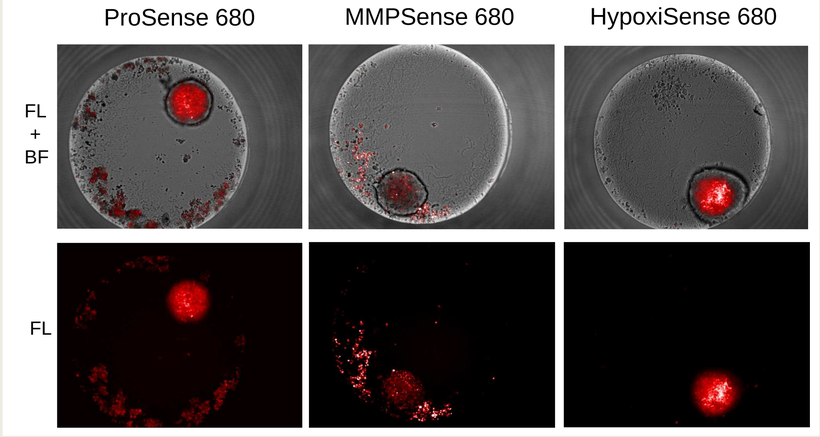

Figure 3: Staining of microtissues with *in vivo* NIR agents results in characteristic staining patterns. Fluorescence (FL) and Brightfield (BF) images are shown. ProSense 680 (left) shows a homogeneous staining of the whole microtissue. MMPSense 680 (middle) is strongly activated in isolated cells and shows a weak fluorescence signal within the microtissue. HypoxiSense 680 (right) stains the microtissue with fluorescence maxima in the core region.

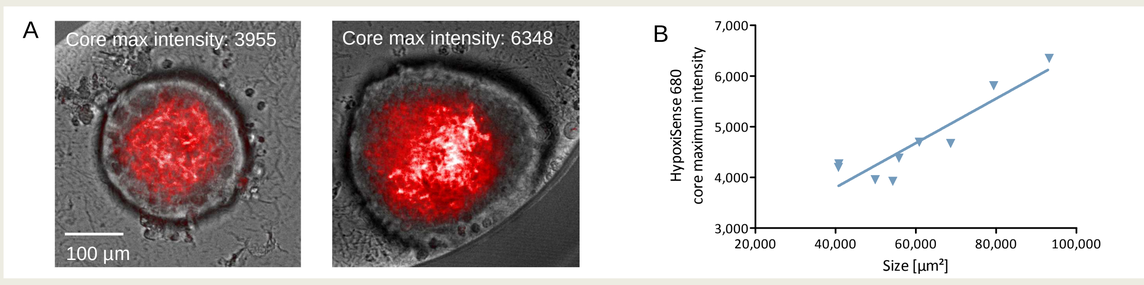

Figure 4: HypoxiSense 680 staining intensity of microtissues is size-dependent. A) Overlay of brightfield and fluorescence images of two representative microtissues. The larger microtissue on the right (area = 93,197 µm²) shows a strongly increased HypoxiSense maximum signal intensity compared to the smaller tissue on the left (area = 49,844 µm²). B) With increasing size the microtissue cores show a strong increase in maximum signal intensity. This suggests the presence of small hypoxic centers. The hypoxia gradient towards the core can be attributed to a limited penetration of oxygen, and potentially other nutrients, into the tissue, as has been described for avascular tumors *in vivo* (3).

4 Summary

In this study we evaluated 3D microtissue analysis on the Opera and Operetta systems. To push the boundaries of imaging limitations in 3D microtissues, we used the Opera system equipped with a 20x water objective, and pre-treatment of microtissues with Sca*l*e reagent. Sca*l*e treatment doubled the imaging depth in all channels, allowing more information to be obtained from deeper inside the 3D tissues. Moreover, we used cancer microtissues as a 3D tumor model to quantify microtissue cancer biomarker expression using *in vivo* NIR agents on the Operetta system. The characteristic labeling patterns of the NIR agents is observed in a similar way *in vivo* and confirms that microtissues are a physiologically relevant cell model that resemble solid tumors.

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3. Lin RZ *et al* (2008): Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *J. Biotechnol.*, 3(9-10):1172-1184.

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