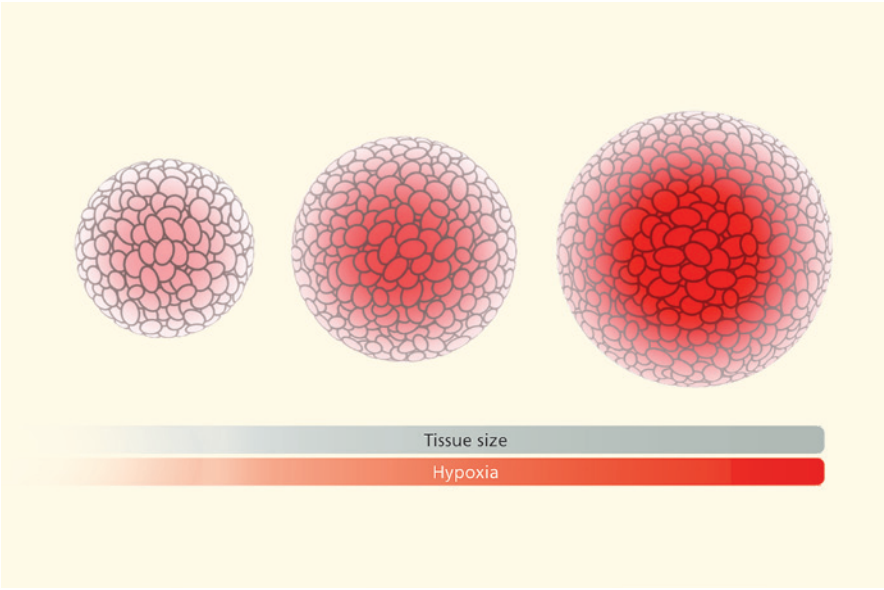

APPLICATION NOTE

Cellular Imaging and Analysis

Key Features

- High Content Analysis of InSphero® microtissues using the Operetta system
- 3D cancer microtissues as a tumor model for drug efficacy testing
- Quantification of cancer biomarker expression with in vivo NIR agents

Quantitative Analysis of 3D Microtissue Growth and Biomarker Intensity

Background

In vivo cells form three-dimensional (3D) microenvironments through cell-cell and cell-matrix interactions to create complex tissues and perform higher level functions. Biochemical signals and nutrient gradients are essential for tissue functioning and therefore also physiological cell behavior. Conventional two-dimensional cell culture does not provide such a complex microenvironment and consequently cells can lose some of their physiological properties (Pamploni *et al.*, 2007). 3D cell culture methods are now widely accepted as being more physiologically relevant and are believed to improve prediction of drug candidates at an earlier stage of the development process (Fayad *et al.*, 2011, Hirschaeuser *et al.*, 2010).

There are several methods for culturing cells in 3D that involve scaffolds and matrices. One scaffold-free procedure is the hanging-drop method. In a hanging drop, cells of either one cell type or of several cell types spontaneously aggregate to form spheroids. InSphero® AG has developed a 96-well plate that allows the automation of this 3D cell culture technology to make 3D microtissues available for high throughput screening applications (Drewitz *et al.*, 2011).

PerkinElmer®
For the Better

PerkinElmer in vivo near infrared (NIR) agents are designed to monitor and quantify biological events such as cancer or inflammatory diseases in small animals. The NIR agents are composed of two parts. The biological part mediates the interaction with the target molecule or gets cleaved by a target protein. The second part, a near-infrared dye with an emission wavelength of 700 nm, visualizes the biological event. As light absorption and scattering in biological tissue reaches a minimum at wavelengths between 600-900 nm, these dyes are especially well suited to imaging non-surface targets *in vivo* and are also very useful for imaging tissue samples such as microtissues. An ever growing collection of in vivo agents allows the imaging of disease biomarkers, e.g. cancer biomarkers such as cathepsins (Mohamed & Sloane, 2006, Conus & Simon, 2010), matrix metalloproteases (MMP) (Nelson *et al.,* 2000) and hypoxic conditions (Harris, 2002).

Here, we present a high content screening application to analyze different characteristics of 3D microtissues with the Operetta® High Content Imaging System. We analyzed the sensitivity of tumor microtissues to treatment with cytotoxic drugs and visualized and quantified several cancer-associated biomarkers by staining the microtissues with different in vivo NIR agents.

Drug Sensitivity Tests Using 3D Microtissues

Assay-ready, live 9 day-old 3D tumor microtissues consisting of HT-29 colorectal adenocarcinoma cells (hCo-CCL02, Catalog No.: MT-01-004-01) were provided by InSphero® and the transport medium was replaced by serum supplemented medium. The microtissues came in a 96-well GravityTRAP™ plate that allows easy localization of the tissues in the wells. Microtissues were incubated for 3 days at 37 °C and 5% CO_2 prior to use in a drug sensitivity assay. For this assay, live 3D microtissues were treated with different concentrations of the reference compounds 5-Fluorouracil (5-FU, Sigma-Aldrich®, F6627) and Staurosporine (Sigma-Aldrich®, S5921). Compound dilutions were added in a mixture with 1.6 µM Hoechst 33342 in serum supplemented medium. Following treatment, microtissues were imaged every 24 hrs over 5 days in treatment cocktail on the Operetta system. Between measurements, the microtissues were incubated at 37 °C and 5% CO_2. Brightfield and Hoechst images were acquired in widefield mode at a focus height of 20 µm with the 10X high NA objective.

To analyze the sensitivity of live microtissues to the reference compounds, the microtissue growth was chosen as a sensitive parameter. Quantitative image analysis of microtissue size was performed using the Hoechst images.

The microtissues were detected using the *Find Nuclei* building block of the Harmony® High Content Imaging and Analysis Software followed by calculation of the tissue area with the *Calculate Morphology Properties* building block.

Treatment with both compounds led to a dose-dependent inhibition of microtissue growth, with Staurosporine having a stronger inhibitory effect on the tissue area than 5-FU (Figure 1).

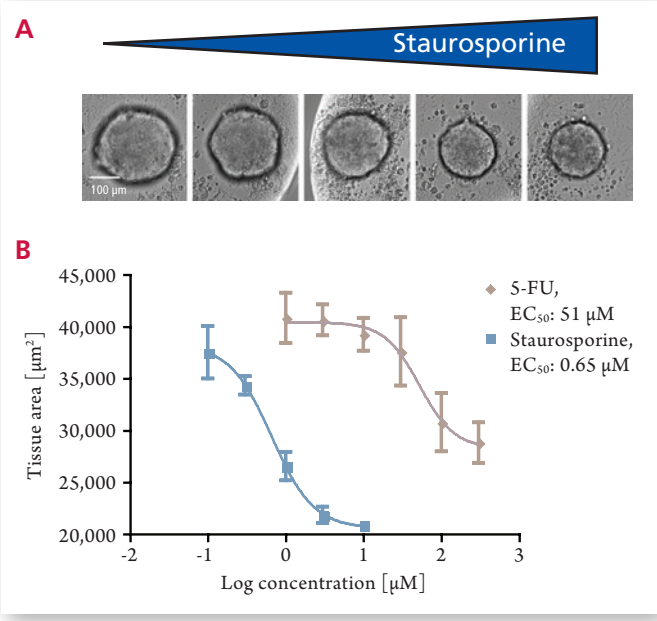

Figure 1. **Inhibition of growth of 3D tumor microtissues after 72 hrs of incubation with 5-FU or Staurosporine.** A) Brightfield images illustrating the growth-inhibiting effect of Staurosporine on microtissues. B) Dose-response curves of microtissues treated with 5-FU and Staurosporine resulting in an EC_{50} of 51 µM for 5-FU and 0.65 µM for Staurosporine.

Quantification of Cancer Biomarker Expression in 3D Microtissues with in vivo NIR Agents

3D microtissues were stained with 100 nM ProSense® 680 Fluorescent Imaging Agent (PerkinElmer®, NEV10003), MMPSense® 680 Fluorescent Imaging Agent (PerkinElmer, NEV10126) and HypoxiSense® 680 Fluorescent Imaging Agent (PerkinElmer, NEV11070) to analyze the cathepsin and MMP activity and to visualize hypoxic areas in the microtissues. ProSense 680 and MMPSense 680 agents are cathepsin and matrix metalloprotease activatable agents and HypoxiSense 680 agent specifically binds to the hypoxia marker carbonic anhydrase IX. In addition, the staining cocktails contained 1.6 µM Hoechst 33342. Five GravityTRAP™ plates with HT-29 tumor microtissues, each representing one timepoint, were incubated with the staining cocktails until imaging. Prior to imaging, the staining cocktail was replaced with PBS. To determine the timepoint with maximal dye activation or binding, images were acquired every 24 hrs over 5 days at a focus height of 20 µm in confocal mode with the 10X high NA objective.

72 hrs after staining the NIR fluorescent agents show maximum activation, and different staining patterns can be observed for the 3 dyes. ProSense 680 agent homogeneously stains the microtissue and isolated cells that are present in the well. MMPSense 680 agent is mainly activated by isolated cells, while hypoxic conditions inside the microtissue core are indicated by a strong HypoxiSense 680 fluorescence signal in the center of the microtissue (Figure 2).

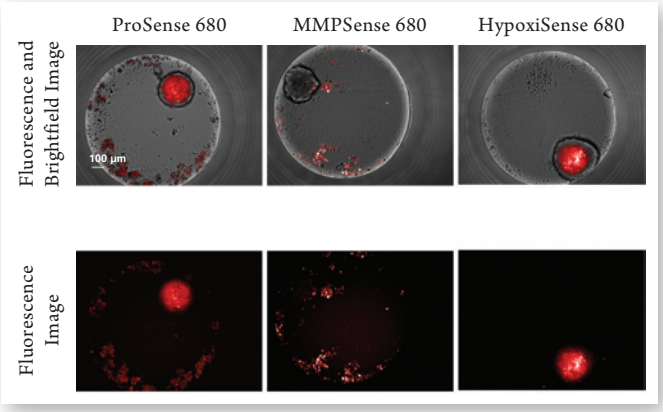

Figure 2. **Staining of microtissues with ProSense 680, MMPSense 680 and HypoxiSense 680 results in characteristic staining patterns.** Microtissues were incubated with 100 nM of the NIR agents for 72 hrs. The top row shows an overlay of brightfield and fluorescence images, while the lower row shows only the fluorescence images. ProSense 680 (left) shows a homogeneous staining of the whole microtissue and the isolated cells in the well. MMPSense 680 (middle) is strongly activated by cells outside of the microtissue and shows a weak fluorescence signal within the microtissues. HypoxiSense 680 (right) stains the microtissue with fluorescence maxima in the core region.

To quantify the different labeling phenotypes, microtissues were segmented using the Harmony software's *Find Nuclei* building block, based on the Hoechst channel. To quantify regional agent intensities, the segmented microtissues were subdivided into a core region, a surrounding core region and a border region by using the *Select Region* building block of the Harmony software (Figures 3A and B). The regional agent intensities were calculated using the *Calculate Intensity Properties* building block and compared to the intensity of the whole microtissue region (Figure 3C).

ProSense 680 agent is homogeneously distributed in the different regions of the tissues, which corresponds to observations *in vivo* where ProSense agent stains all cells in the tumor homogeneously. No significant variations in the MMPSense 680 signal could be observed in the defined tissue regions. MMPs are known to play a crucial role for cancer cell migration and metastasis (Dai *et al.*, 2011). The activation of MMPSense agent in isolated cells suggests the contribution of MMP activity to degradation of extracellular matrix and escape of these cells from the tissue. The high HypoxiSense 680 agent intensities in the core of the microtissue (Figure 3C) clearly indicate the presence of an oxygen gradient towards the core region.

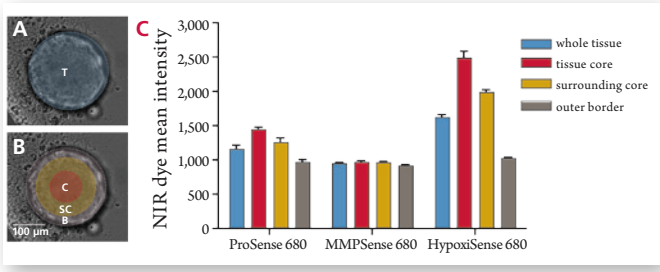

Figure 3. **Quantitative analysis of regional intensities of the in vivo NIR agents in microtissues.** Microtissues were subdivided into different regions (whole tissue, tissue core, surrounding core and outer border) to quantify the different staining patterns. A) Whole tissue area (T, blue). B) Microtissue with indicated outer border (B, gray), surrounding core (SC, yellow) and tissue core region (C, red). C) Mean fluorescence intensities of ProSense 680, MMPSense 680 and HypoxiSense 680 agents in the different regions (N=3 per dye).

To determine if larger microtissues show increased hypoxia, we analyzed the HypoxiSense 680 intensities in the core region of microtissues with different sizes. The mean intensity in the core region of microtissues only slightly increases with size, resulting in a slope of 0.01 for the regression line. However, in larger microtissues the maximum intensity in the core region shows a strongly elevated fluorescence signal with a much higher slope (0.04). This indicates that the degree of hypoxia increases locally with tissue diameter in small hypoxic centers (Figure 4).

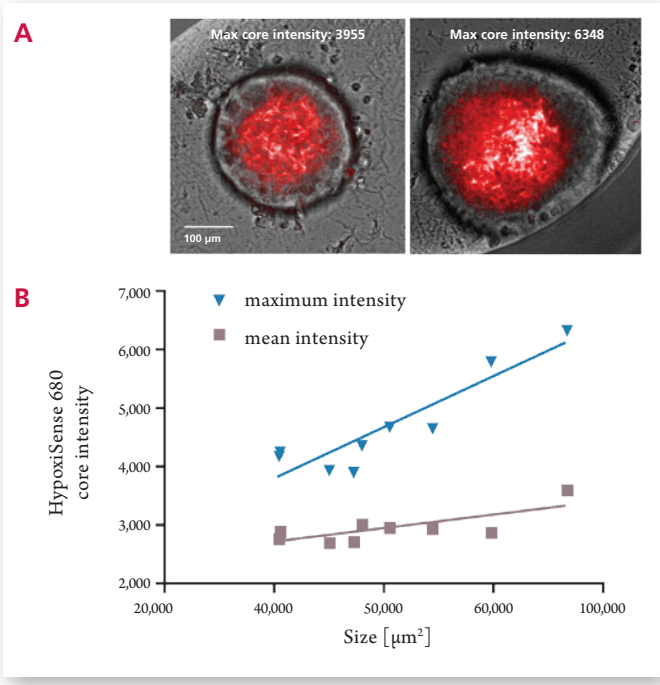

Figure 4. **HypoxiSense 680 staining of microtissues is size-dependant.** A) Overlay of brightfield and fluorescence images of two representative microtissues. The bigger microtissue on the right (area = 93,197 µm²) shows a strongly increased HypoxiSense maximum intensity compared to the smaller tissue on the left (area = 49,844 µm²). B) With increasing size the microtissue cores show a slightly higher mean intensity (slope 0.01) and a strongly increasing maximum intensity (slope 0.04). This suggests the presence of small hypoxic centers with strong HypoxiSense 680 intensity.

Conclusion

In this high content imaging study we used cancer microtissues as a 3D tumor model to analyze drug efficacy and we quantified microtissue cancer biomarker expression using in vivo NIR agents on the Operetta system. The analyzed microtissues were sensitive to anti-cancer drugs and showed a clear inhibition of tissue growth. The analysis of microtissues with in vivo NIR agents resulted in a successful quantification of disease-associated cancer biomarkers in microtissues and confirms them to be a physiological cell model that resembles solid tumors. Due to their long excitation (680 nm) and emission (700 nm) wavelength, the NIR agents are an ideal tool to study microtissues, as light absorption and scattering in tissue is very low at these wavelengths. The possibility of using in vivo NIR agents as translational tools in biochemical, cellular and in vivo experiments enables comparable biologic interactions, increasing the reliability of experiments throughout the drug discovery process.

The Operetta system and the easy-to-use Harmony software provide an ideal platform to study InSphero® 3D microtissues.

In summary, the availability of InSphero® 3D microtissues suitable for high-throughput allows a completely new generation of screening applications that can be run on the Operetta High Content Imaging System.

The applicability of *in vivo* NIR dyes to this new model system will help to increase the predictive power of preclinical experiments and to reduce costs during the drug development process.

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010294A_01 July 2012 Printed in USA