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High resolution 3D imaging of primary and secondary tumor spheroids using multicolor multi-angle Light Sheet Fluorescence Microscopy (LSFM)

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ABSTRACT

Breast cancer and Glioblastoma brain cancer are aggressive malignancies with poor prognosis. In this study primary Glioblastoma and secondary breast cancer spheroids are formed and treated with the well-known Temozolomide and Doxorubicin chemotherapeutics, respectively. A custom multi-angle Light Sheet Fluorescence Microscope is employed for high resolution imaging of both cancer cell spheroids. Such a technique is successful in realizing pre-clinical drug screening, while enables the discrimination among physiologic tumor parameters. LSFM technique, parameters and method followed are also presented.

Keywords: Light Sheet Fluorescence Microscopy (LSFM), Multicellular tumor spheroids (MCTS), drug screening and individualized therapy, Glioblastoma Brain cancer, Breast cancer

1. INTRODUCTION

Multicellular Tumour Spheroids (MCTS) are *in vitro* cancer models of increasing interest for cancer proliferation diagnostics and pharmaceutical efficiency studies, as they more closely resemble real tumours compared to monolayer cultures. However optical imaging of MCTS is technically challenging, since these are large and highly scattering samples. Light Sheet Fluorescence Microscopy (LSFM) on the other hand, has evolved into a powerful tool for three dimensional imaging of live or fixed tissues and organisms with sub-cellular resolution. It utilizes optical sectioning with various excitation lasers, along various angles for multispectral, multi-angle high resolution imaging of biological samples. LSFM exhibit major advantages over conventional microscopy, as it combines minimal phototoxic effects in the specimen, deep

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penetration of light, and high imaging speeds [1] [2]. In this study we employed LSFM to image the response of Glioblastoma and breast cancer spheroids to various chemotherapeutic drugs.

2. MATERIALS AND METHODS

2.1 LSF Microscope

Our custom built LSFM is composed of two separate light paths, one for illumination and one for detection that are established on orthogonal axes. Two continuous wave diode lasers (488nm and 635nm) are used for illumination. Each laser beam is introduced with the use of a flip mirror in the illumination path, resulting to two separate co-incident and co-aligned beams. The selected laser beam is shaped by an achromat cylindrical lens into a thin plane of light (light sheet) and focused on the corner mirror. After the mirror, the formed light sheet is imaged through a 2x telescope to the back focal plane of the illumination objective (Mitutoyo, Plan Apo, 5x/0.14, WD=34.0mm). The telescope is placed in such a way that two conjugate planes are formed on the mirror and the back focal plane of the objective, for a better and easier adjustment of the light sheet [3].

The detection path is composed of a second infinity corrected microscope objective (Mitutoyo, Plan Apo, 10x/0.28, WD=33.5mm) that is used in order to collect the emitted light and project it through an apochromatic doublet tube lens (ITL200,Thorlabs) on a thermoelectrically cooled, electron multiplying CCD camera. Right after the detection objective, an iris diaphragm is placed in order to alter the numerical aperture (NA) of the detection and thus, control the depth of field. Between the iris and the tube lens a filter wheel is placed with various emission filters (512/17, 605/70 and 650 LP) in order to record the desired part of the emitted spectrum as appropriate for each fluorescent channel of the sample.

The sample is placed and immobilized inside fluorinated ethylene propylene (FEP) tubes that contain either an index matching aqueous solution of low melting agarose (0,1-1%) (w/w) or the thermoreversible non-toxic hydrogel Cygel (Abcam). FEP tubes were chosen due to their refractive index that is similar to that of water. The tubes are then inserted inside a tank made by antireflection optical glass (Hellma Analytics), filled with a water agarose solution of the same concentration or with warmed water to enable Cygel solidification. The specimen is mounted to a sample holder with 4 degrees of freedom. Four motorized software controlled stages (Standa) allow the micrometric translation along x, y, and z-axes and rotation around the vertical y-axis.

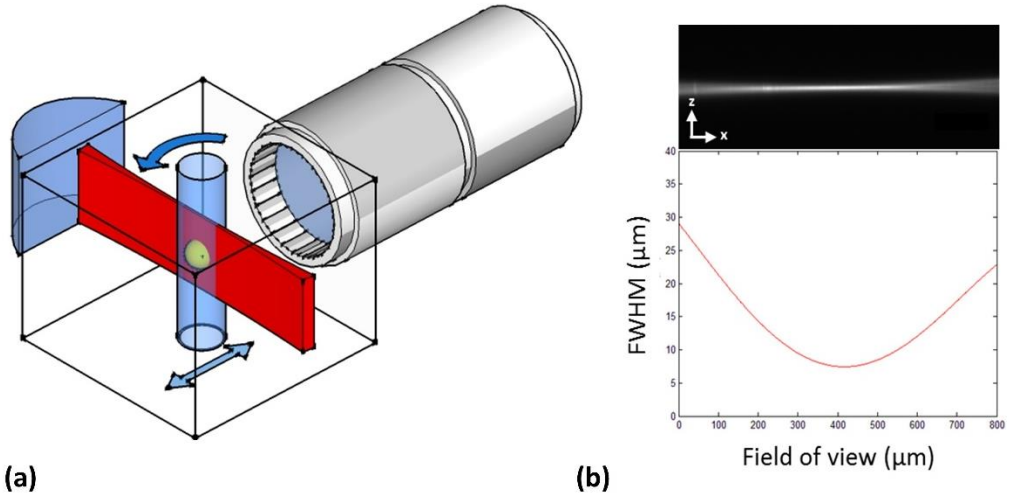

Figure 1: (a) the principle of LSFM setup (b) above: the lightsheet as seen in x-z plane, bottom: the width of the lightsheet (FWHM) plotted as a function of the x distance (field of view). Scale bar: 100μm

TABLE I. LIGHT SHEET MINIMUM THICKNESS FOR THE LASERS USED

λ[nm]	*Axial minimum thickness(z) [μm]*
488	*6.9 ±0.1*
635	*7.3 ±0.1*

2.1.1 Microscope Performance

For appropriate imaging results only a very thin region around the focal plane has to be illuminated. Moreover, the light sheet has to be established orthogonally to the detection axis with its thinnest part placed in the middle of the field of view. The resolution of LSFM is defined by the properties of the detection and the illumination axis respectively. The lateral resolution is defined by the detection objective's NA. On the other hand the axial resolution is dominated by the thickness of the light sheet. As a result the resolution is anisotropic. The combination of multiple views of the specimen along different directions can result in almost isotropic resolution similar to the lateral one [4].

2.2 Cellular spheroids

Multicellular Tumour Spheroids of two cancer cell types (primary GB, MDA-MB-231) were generated. An initial cell suspension of approximately 625 or 1250 cells was used to seed the spheroids using the hanging drop technique. Tumour spheroids are accordingly stained or treated in order to emit in different spectral regions.

An own-established primary GB cell culture was used using cells collected during the maximal safe surgical resection of a GB patient naïve from treatment (more details in [5]). The MDA-MB-231 breast cancer cells were also used and further treated to stably express GFP. The spheroids were grown for up to 23 days and treated with a range of concentrations of the anti-cancer agents DOX and TMZ from day 4 to day 7 (as in [6]), based on the IC_{50} values previously estimated in 2D. From this point onward, half of the medium was replaced with fresh every two days.

DOX penetration into the spheroids was determined by direct imaging of its inherent fluorescence. In order to assess the cell viability and death, spheroids were additionally counterstained with the far-red nuclear dye Draq7 (Biostatus) before loading them to the LSFM microscope.

2.3 Experimental procedure / processing

In our LSFM a cylindrical lens is used to form the laser beam in a plane of light and illuminate a thin region around the camera focal plane, as it is established orthogonally to the detection axis. In this way optical sectioning is performed as the sample is translated through the illuminated plane. In each measurement a stack of 50-80 optical sections is acquired.

When different laser sources are used, Multispectral imaging is achieved; different regions within an entire plane of the spheroid are selectively excited and their emission is separately acquired.

For better illumination and deeper light penetration Multiview imaging is performed. The specimen is sequentially rotated to various angles compared to the initial position and thus more projections (views) are acquired (usually four orthogonal views). Each spheroid was imaged sequentially at 4 different projections (0^o, 90^o, 180^o, and 270^o) at two fluorescent channels.

Later on, registration and fusion algorithms are applied to align and integrate the acquired projections into a final 3D image. The same procedure is repeated for all fluorescence channels, the resulting images are registered and combined to form the final volumetric multicolor isotropic image.

3. RESULTS

3.1 Breast Cancer Spheroids treated with DOX

GFP fluorescence was distributed evenly in each cell of the spheroids, which is consistent with the known diffuse expression pattern of the protein in the cytoplasm and nuclei of the cells. DOX penetration and accumulation was more pronounced in the nuclei of cells of the viable rim of the spheroid, consistent with the literature [7]. Accordingly, the fluorescence distribution pattern of Draq7 suggests that DOX was predominantly cytotoxic in the periphery of the spheroid, as the majority of dead/apoptotic cells were found in this tumor region. Finally, strong Draq7 fluorescence was detected in the center of larger spheroids (older than 7 days), consistent with the presence of a necrotic core (Fig.2).

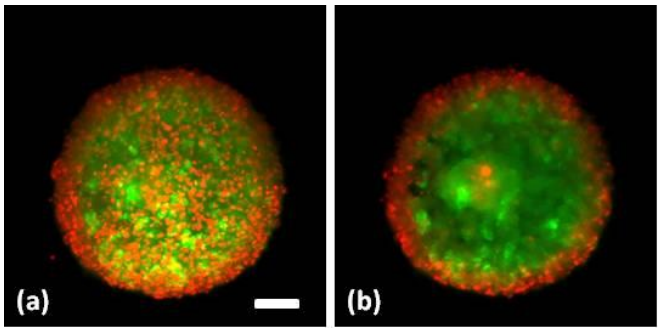

Figure 2: Drug cytotoxicity determination in a small live MDA-MB-231 breast cancer spheroid (diameter=600 μm) using LSFM. (a) Maximum intensity projection of the 52 optical sections of the spheroid. (b) The central optical section of the spheroid with the presence of the necrotic core and periphery. Scale bar: 100μm

3.2 Primary GB spheroids treated with TMZ

Primary GB spheroids were found slightly sensitive to TMZ in a dose-response relationship. LSFM further enabled discrimination between cell death and growth inhibition after treatment. As it can be seen in Fig. 3, both the control untreated GB spheroid and the TMZ-treated one appear to have the same size and same death pattern (day 7).

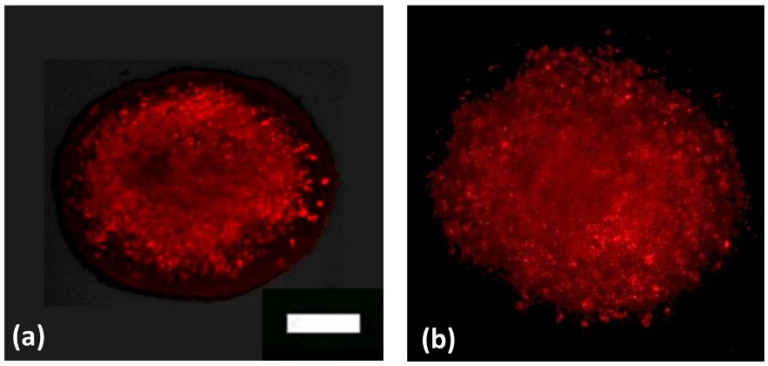

Figure 3: Cell death of the live primary GB spheroids. (a) Drug cytotoxicity determination. (b) Intrinsic cell death of an untreated control spheroid. Scale bar: 100μm.

Tumour spheroids are imaged with the LSFM along 4 orthogonal views in 2 different fluorescence channels. The acquired data are co-registered and fused in order to reconstruct the 3D distribution of the dyes and then combined to form the final isotropic multicolour high-resolution 3D image of the spheroid. The image acquisition procedure as well as the image processing steps are presented. Improvements for image acquisition and image processing are suggested and evaluated.

4. CONCLUSIONS

In this work we present the use of our custom built LSFM setup for the study of tumor spheroids, the optimization of imaging protocols and the effect of chemotherapy. LSFM is particularly well suited for fluorescence imaging of large, sensitive living specimens, such as tumor spheroids, as it provides true optical sectioning capabilities, good spatial resolution and minimal phototoxicity. To illustrate the utility of LSFM for drug screening, patient-derived GB spheroids and MDA-MB-231 breast cancer spheroids were treated with the commonly used anti-cancer agents TMZ and DOX and spheroid cell viability was estimated using the cell death nuclear stain Draq7. Our results demonstrate the potential of this technology to quantitatively assess the distribution, drug penetration and cytotoxic potency of anti-neoplastic agents in living 3-dimensional cell cultures and to serve as a useful tool in pre-clinical drug screening towards individualized therapy.

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