Zentrum für Angewandte Forschung (ZAFH)

PHOTON^{n} - PHOTONische Verfahren in neuen Dimensionen

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Spectral imaging of multiphoton-excited cellular autofluorescence using isogenic cancer cell models

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Abstract

Multiphoton-excited cellular autofluorescence is based on the emission of light by endogenous fluorescent biomolecules which are related to certain cellular functions such as structural arrangement and cellular metabolism. Because of their involvement in fundamental biological processes the analysis of endogenous fluorophores may provide information about physiological and pathological states. Consequently, non-invasive autofluorescent spectroscopy is applied in vitro and in vivo to distinguish between normal and diseased tissues, particularly in studying malignant transformation, but also for monitoring drug responses.
Here, we report the application of multiphoton-excited autofluorescence for characterization of isogenic glioblastoma cell lines with heterologous expression of different tumor suppressor genes as model system for defined, disease-relevant molecular alterations. Major advantage of isogenic cell lines is that positional and copy number effects on gene expression can be minimized and that the genetically identical, highly standardized isogenic cell lines differ only in the presence or absence of a single engineered gene of interest. The inactivation of tumor suppressor genes like TP53 and PTEN frequently determines the transition from a normal to a brain cancer cell, so that this scenario offers as model system for distinguishing between the healthy and the diseased state. To create such model systems, we started from the glioblastoma cell line U-251MG, which is deficient for PTEN and contains mutant p53. We aimed at creating U-251MG cells as isogenic cell lines that are genetically identical except for the absence/presence of TP53 and PTEN, respectively. These isogenic cell lines provide a highly standardized in vitro cell model for glioblastoma in which the genetic and epigenetic factors are held as constant as possible.

Methodology

1. Spectral Imaging

In multifluorophore systems such as cells many individual fluorophores contribute to the autofluorescence that can be detected. Quantification of these distinct spectral signatures produces an optical “fingerprint” of the cell. To obtain spectral information of the established isogenic glioblastoma cell model we used a pushbroom imaging system in combination with multiphoton excitation (fig. 1). It consists of a tunable titanium saphire laser (Chameleon, Coherent) that provides ultra short (120 fs) pulses of near infrared laser light (720 – 930 nm) at high repetition rate (80 MHz). The output of the laser is directed into a TriM Scope multibeam scanning device (LaVision BioTec, Bielefeld), that allowed us to scan / illuminate the sample with up to 64 beams simultaneously. This increases fluorescence up to 64 times depending on the number of the beams that are used, which decreases acquisition time and allows higher frame rates. In addition, the power of the laser is evenly distributed to several beams, which keeps the intensity in individual beams, and hereby the damage of cells, low. The light is then reflected by a beamsplitter into a Zeiss Axiovert 200 inverted microscope through a Zeiss EC Plan-Neofluar 40x/1.3 oil immersion objective onto the sample. The emitted light is collected by the same objective and passed to an Acton SP2150i spectrograph (Princeton Instruments,) equipped with a 150 g/mm grating. The dispersed light is detected by an Imager QE camera (PCO, Kelheim). The sample can be moved by a motorised x,y scanning table (Zeiss). The whole system is controlled by a computer using ImSpector 4.0 software (LaVision BioTec, Bielefeld).

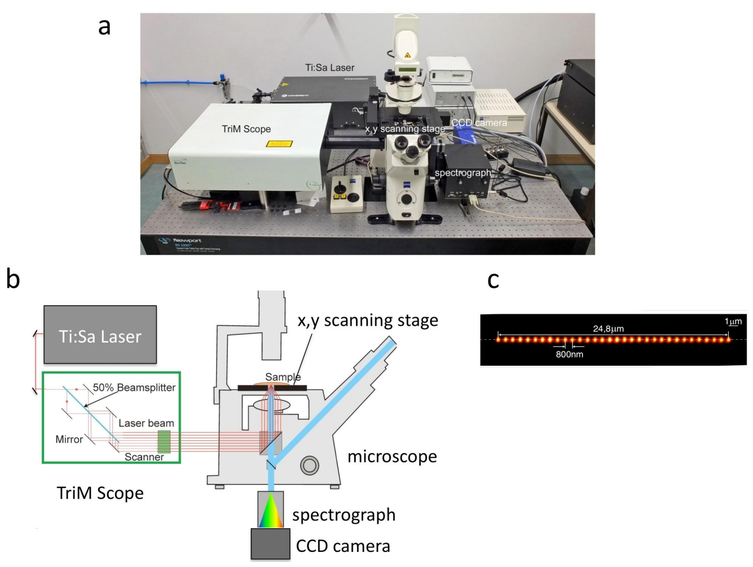

Fig. 1: Spectral imaging system with multi-beam multiphoton excitation used in this study. Photograph (a) and schematic representation of the setup (b). c) 32 beams used to scan the samples are visualised by 2-photon induced fluorescence of rhodamin-6G dissolved in water.

2. Microscopy and Data Analysis

Data analysis was performed using the open-source “ImageJ for microscopy” software. Since the images of the stack displayed x-λ data, no cellular structures are visible. Therefore in a first step the image stack was turned in a way that x,y data were shown (plugins/Stacks-Z-functions/Export coronal stack). In the next step all images of this stack were summed up, resulting in an autofluorescence image that contains all the photons captured in the observed wavelength range (400 - 660 nm). In this image the cells and subcellular structures can be easily identified. This image is therefore used to define regions of interest (ROI), for example background, cells, subcellular regions etc. After defining the background ROI the background was measured in each wavelength image of the stack and subtracted from the respective image (plugins/ROI/bg subtraction from ROI). The resulting background-corrected image stack was used to measure the spectra of the defined ROIs (plugins/ROI/Multi Measure/multi). The results of the measurements were exported to a spreadsheet program (Excel, Microsoft) for display and further analysis. The spectra were normalised to their area to make them independent of intensity and then used for principal component analysis (PCA) using the Unscrambler software package (Camo Process AS, Oslo).
Analysis of the autofluorescence revealed spectra with two peaks at 450nm and 470nm for cells that were excited at 750nm, which is consistent with emission of enzyme cofactor NAD(P)H (fig. 2b). Additionally, a distinct shoulder at a wavelength range of 500 – 550nm was observed. With higher excitation wavelengths of 800 and 850nm the peaks at 450 and 470nm decreased while a peak at 530 nm increased (data not shown). Excitation at 880 nm produced a broad spectrum, with only one detectable peak at 530nm which can be associated with FAD fluorescence (fig. 2d).

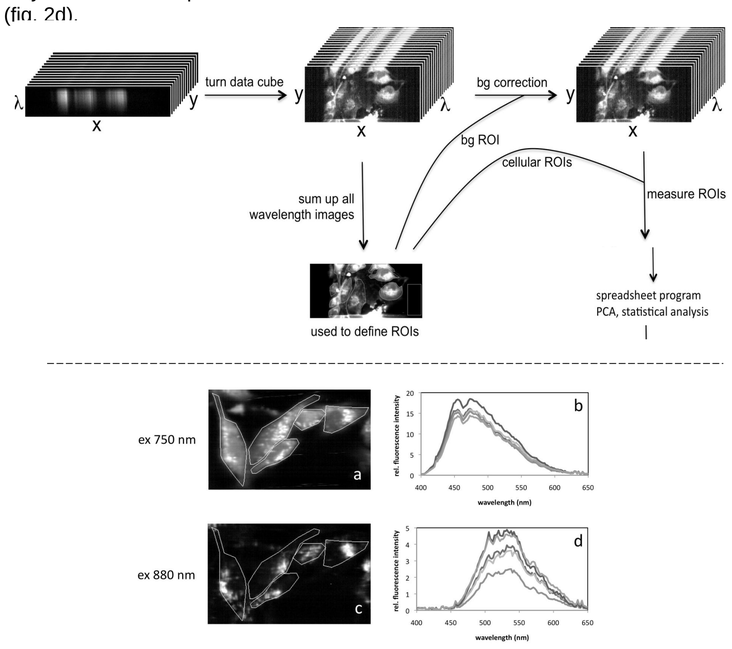

Fig. 2: *Top:* Workflow of data analysis: the main steps are shown.
Bottom: Autofluorescence images (400 – 660nm emission) (a, c) and spectra (b, d) of U-251MG-PTEN glioblastoma cells excited at 750 nm and 880 nm. The Spectra of the five indicated cells are shown.

Spectra derived from autofluorescence images had a very similar shape so that differences between the cell lines were not directly apparent (fig 3a). We applied a common statistical tool, principal component analysis (PCA), to analyse normalised autofluorescence spectra. This method reduces multi-dimensional data like fluorescence spectra into a few principle components which constitute a new, lower dimensional coordinate system for description of the data. As a result the spectra are expressed in such a way so as to highlight their similarities and differences by their score values in this new principle component coordinate system. The score values for the first principal component (PC1) of the spectra that were obtained by excitation at 750nm are shown in figure 3c. PC1 accounted for 62 % of the total variation of the sample spectra. The score values of the induced and non-induced U251MG-Control cell lines showed mainly negative values, while induced U251MG-PTEN spectra had positive values. Induced U251MG-TP53 spectra had positive as well as negative values. These results demonstrated that PC1 revealed differences between spectra of U-251MG-Control cells and spectra of cells expressing TP53 or PTEN, although a few spectra of cells were not correctly classified by PC1 score values. In parallel to autofluorescence measurements, U-251MG-TP53 and U-251MG-PTEN cells were tested for their inducibility of transgene expression by Western blot analysis (fig. 3b).
Taken together, our results show that the activity of any of the two tumor suppressors, TP53 or PTEN, results in a specific change in the spectrum, which allows discerning them from control cells (fig. 3c). This suggests that spectra generated via this strategy may to a certain degree reflect the molecular fingerprints of brain cancer cells (Holloschi et al., 2012)

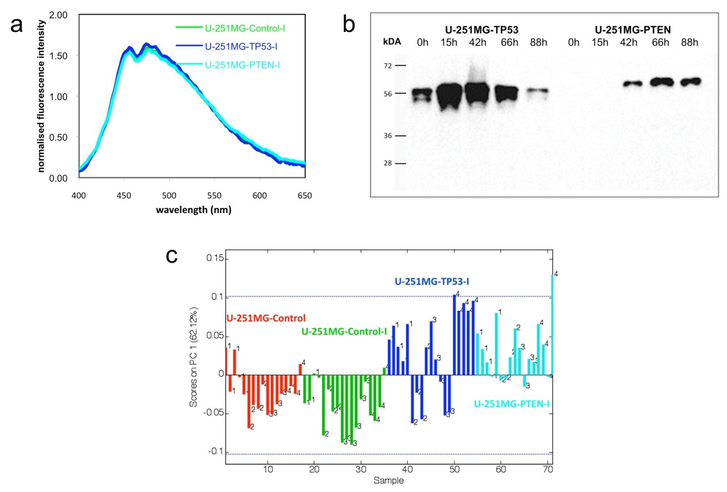

Fig. 3 Multiphoton-excited autofluorescence for characterisation of isogenic glioblastoma cell lines: a) Mean normalised spectra of induced U-251MG-Control, U-251MG-TP53 and U-251MG-PTEN cells excited at 750 nm. b) Monitoring of time-dependent expression of PTEN in U-251MG-PTEN cells (left) and TP53 in U-251MG-TP53 cells (right) by Western blotting. Prior to analysis cells were grown for the indicated time in medium containing 100 ng/ml doxycycline to induce expression of the transgene. c) Result of principal component analysis: the score values of principal component 1 which accounts for 62% of the variance between the sample spectra are shown. The data presented here included a total of 71 single-cell measurements. Exponents indicate groups of experiments.

3. Establishment of 3D-tumor spheroid models

The microenvironment of cells plays a major role in many physiological and pathological events. In this context, three dimensional (3D) tumor spheroid models are becoming important biomedical tools for cancer studies as well as for cancer drug discovery and target validation. We used a simple and standardized method (Vinci et al., 2012) for generation of homogeneous multicellular tumor spheroids using 96- round well ULA (Ultra-Low Attachment Surface) microtiter plates containing a neutral, hydrophilic hydrogel coating (fig. 4). The coating greatly reduces binding of attachment proteins which minimizes cell attachment and spreading. In addition, the well shape promotes the formation of single, centrally located spheroids of reproducible size. We applied this system to our isogenic glioblastoma cell models for measurements of tumor growth, migration and invasion in a relatively high-throughput format (fig. 5, 6).

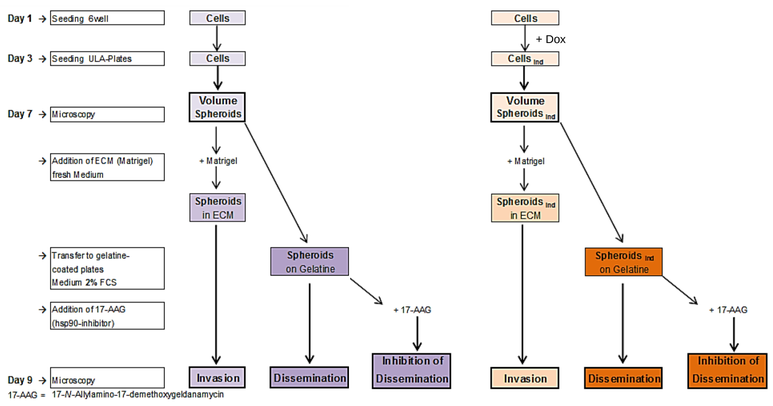

Fig. 4: Work-flow for development of tumor spheroids using the isogenic glioblastoma cell lines. We have established an optimized and standardized protocol for the isogenic cell lines applicable in a 96- round well plate format, achieving a single spheroid/well, centered for ease of optical imaging simple and simplified harvesting for further analysis. We treated U251-MG-spheroids with 17-AAG as an example for a molecularly-targeted agents (heat shock protein 90 (HSP90) inhibitor; leads to reduced invasive characteristics of glioblastoma cells).

3.1 Reproducibility of spheroid size

Based on our former experience using hanging drops, agar and polyHema growth conditions we could reproduce U251-MG spheroids most successful in ULA round-well plates (fig. 5). Seeding of 3000 cells was evaluated best for further analysis of spheroid size and development of invadopodia (invasive feet of the cell membrane) indicating grades of invasiveness.

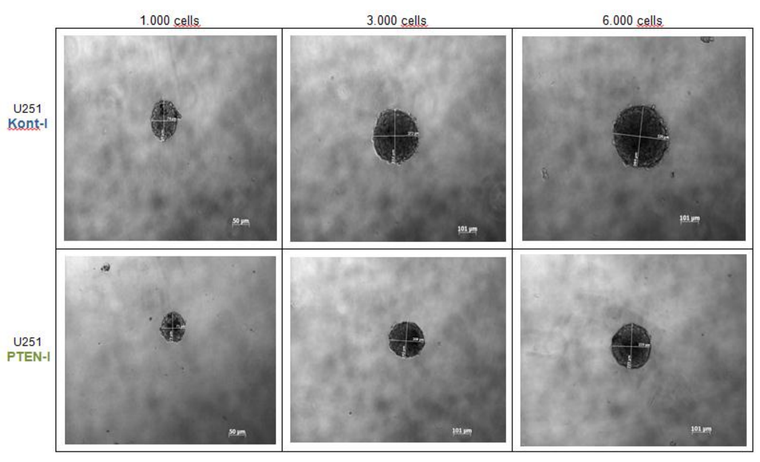

Fig. 5: Seeding different cell numbers of isogenic U251-MG cells. The resulting spheroid sizes clearly indicate the influence of the induced tumor suppressor PTEN leading to reduced spheroid sizes compared to the wild type tumor background (U251-Kont-I). Pictures were taken 96h after seeding; 10x.

3.2 Spheroid Functional Assay – Migration / Invasion

In order to further analyse the relevance of the tumor suppressors TP53 and PTEN linked to tumor characteristics we transferred tumor spheroids generated in ULA plates into gelatin-coated flat-bottomed 96-well plates and into semisolid gel-like matrix (Matrigel) inducing invasion-like processes contributing to cell movement and also matrix degradation via localized proteolysis (resembling dissemination) (fig. 6).

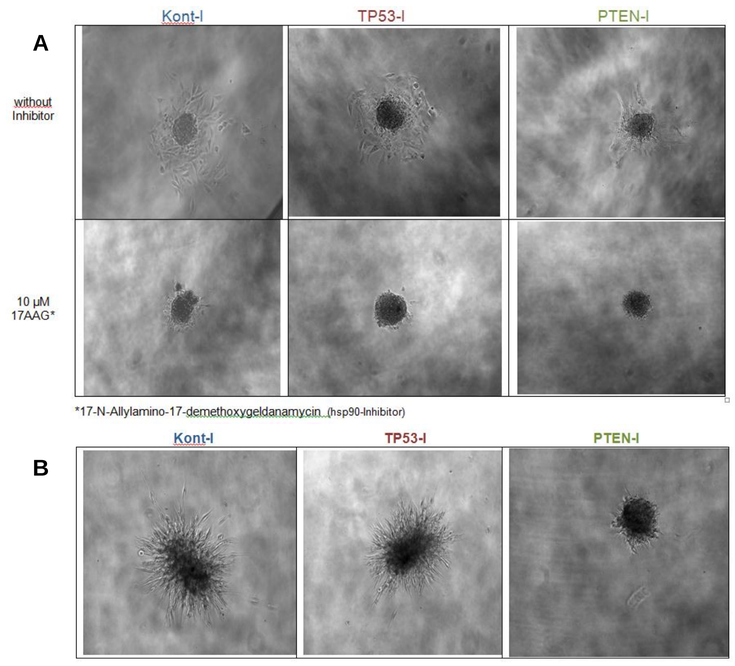

Fig. 6 A: Analysis of U251-MG tumor spheroid migration and the influence of the HSP90 inhibitor 17-AAG to cell motility. B: Analysis of U251-MG tumor spheroid invasion into Matrigel. Within a few hours, the invadopodia are apparent. Representative brightfield images were taken 72 h after cultering spheroids on gelatine (A) and 48h after transferring spheroids to Matrigel (B).