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 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 autofluorescence imaging is an important tool in clinical diagnostics and drug discovery. These methods are based on using excitation light in the near-infrared range to maximize penetration through tissue and to minimize interference with biological structures. Spectroscopic analysis 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 spheroid-like cell clusters with heterogeneous 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 can be used to mimic the transcriptional behavior of the same cell type. This scenario offers an ideal 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 this genetic lesion. 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 microimaging systems such as cells many individual fluorophores contribute to the autofluorescence that can be detected. Quantification of these distinct spectral properties produces an optical "fingerprint" of the cell. To obtain spectral information of the established isogenic glioblastoma cell model we used a quantum imaging system in combination with multiple excitation wavelengths. It consists of a tunable laser system (Chameleon Coherent) that provides sub nanosecond pulses of near infrared light (700 – 850 nm) at high repetition rate (80 MHz). The output of the laser is directed into a Ti:Saphire microscope scanning device (LaiVision BioTec, Bielefeld) that allows us to scan (illuminate the sample with up to 10 beams simultaneously). This increases fluorescence up to 54 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 over several beams, which keeps the intensity in individual beams, and thereby the damage of cells, low. The light is then reflected by a beam splitter to a Zeiss Axiovert 200 inverted microscope. A spectral range of 450 nm to 1.3 nm of emission objective onto the sample. The emitted light is collected by the same objective and passed to a InGaAs photodiode spectrometer (Pinnacle Instruments) equipped with a 150 grating grating. The dispersed light is detected by an InGaAs GE camera (PCO Lithium). The sample can be moved by a motorized x,y, z-scanning stage (Zeiss). The whole system is controlled by a computer using ImagePro 4.2 software (LaiVision BioTec, Bielefeld).

2. Microscopy and Data Analysis

Data analysis was performed using the open-source "Image" for microscopists software. Since the images of the stock display v.a. data, no cellular structures are visible. Therefore in a first step the image stack was turned in a way that the data were shown (plug/Scripts-2 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 – 800 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, cell bodies, 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 (plug/Spectral IGN background subtraction from ROI). The resulting background-corrected image was used to measure the spectra of the defined ROIs (plug/Spectral IGN Measurement). The results of the measurements were exported to a spreadsheet program (Excel, Microsoft) for display and further analysis. The spectra were normalized to the 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 475nm for cells that were excited at 752nm, which is consistent with emission of enzyme colorizer NADPH (Fig. 2b). Additionally, a distinct shoulder at a wavelength range of 500 – 550nm was observed, with higher excitation wavelengths of 850 and 890nm the peaks at 450 and 475nm decreased whereas a peak at 530nm increased. These data not only characterized the spectral pattern, with only one detectable peak at 530nm which could be associated with FAD fluorescence (Fig. 2d).

3.1 Reproducibility of spheroid size

Based on our former experience using hanging drops, agar and polyacrylamide gel conditions we could reproduce U-251MG spheroids instead of 3D control spheroids grown in U-251MG-well plates (Fig. 5). Seeding of 3000 cells was evaluated best for further analysis of spheroidal and matrigel-differentiated isogenic cell lines (fibrillar cells of the cell membrane) indicating grades of invasiveness.

Spectra derived from autofluorescence images had a very similar shape as that differences between the cell lines were not directly apparent (Fig. 3). We applied a common statistical tool, principal component analysis (PCA), to analyze normalized 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 scores in this new principle component coordinate system. The score values for the first principle component (PC1) of the spectra that were obtained by excitation at 752nm are shown in Fig. 3c. PC1 accounted for 62% of the total variation of the sample spectra. The score values of the induced and non-induced U-251MG-Control cell lines showed mostly negative values, while induced U-251MG-PTEN-harbored spheroids had positive values. Induced U251/MG-TP53-harbored spheroids produced as well as negative and positive values. These results demonstrated that PC1 revealed differences between species of U-251MG-Control cell lines and cells of species expressing TP53 or PTEN, although a few of cells of both lines were not correctly classified by PC1 score values.

In parallel to autofluorescence measurements, 251/MG-TP53 and U-251MG-PTEN cells were tested for their inducibility of transgene expression by Western blot analysis (Fig. 3d).

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. 5). This suggests that spectra generated via this strategy may to a certain degree reflect the molecular fingerprints of brain-cancer cells (Holshaus et al., 2012).

3.2 Spheroid Functional Assay – Migration / Invasion

In order to further analyze the relevance of the tumor suppressors TP53 and PTEN linked to tumor characteristics we normalized tumor spheroids generated in U-251MG-well plates into gelatin-coated flat-bottomed 96-well plates and into isoelectric gel-like matrices (Matrigel) inducing invasion-like processes contributing to cell movement and also making degradation via localized proteolysis (wandering dissemination) (Fig. 6).

Fig. 1. Multiphoton imaging and reconstruction of a glioblastoma cell line, U-251MG, which expresses a tumor suppressor gene, p53. The cell was excited at 752nm, which is consistent with emission of enzyme colorizer NADPH. A) Cell light path (53X). B) Laser scanning (53X). C) 3D reconstruction of the cell slice (53X).

Fig. 2: Window of data analysis: the main steps are shown. (A) Autofluorescence images (450 – 890nm emission) (a), c, and spectra (d). (B) Spectrum of a U251MG-PTEN spheroid cells excited at 752nm and 890nm. The Spectra of the few isolated cells are shown. (C) Spectra originating from 251/MG-TP53 and U-251MG-PTEN cells were tested for their inducibility of transgene expression by Western blot analysis (Fig. 3d). (D) 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. 5).