Label-free microscopy: spectral imaging of multiphoton-excited cellular autofluorescence

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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.

The article focuses on methodologies of spectral imaging of endogenous autofluorescence. We describe the instrumentation, techniques and experimental procedures required for data acquisition, data handling, and analysis. Furthermore, 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.

Keywords cellular autofluorescence; spectral imaging; multiphoton microscopy; isogenic cell lines; tumor suppressor genes

1. Cellular autofluorescence

The detection of cellular endogenous autofluorescence signatures offers non-invasive, real-time diagnostic measurement, which is sensitive enough to assess both the morphology and metabolic state of live samples [1, 2, 3]. Cellular autofluorescence is the intrinsic property of cells and tissues to emit light when illuminated with an appropriate wavelength light source. Multiphoton fluorescence microscopy [4] is particularly suitable for intracellular studies of endogenous fluorescence. It allows the mapping of fluorophore distribution inside tissue and the quantification of biochemical and metabolic states, revealing high resolution optical images. Cellular autofluorescence properties can further be determined by additional approaches such as emission wavelength spectroscopy, fluorescence lifetime time and fluorescence anisotropy measurements (reviewed in [3]). The major contributors of intracellular autofluorescence are several endogenous key fluorophores, including the reduced pyridine nucleotides (NAD(P)H), oxidized flavins (FMN, FAD), aromatic amino acids like tryptophan, tyrosine and phenylalanine, lipopigments, melanin, keratin and the plant pigment chlorophyll. Endogenous extracellular fluorescence of tissues is composed mainly of fibrillar type I collagen and elastin-related fluorescence.

Each of these fluorophores exhibits its characteristic excitation and emission spectrum (Table 1). Cellular autofluorescence intensity depends on the ability of biomolecules to absorb light at the excitation wavelength (extinction coefficient) and the efficiency with which they convert absorbed light into emitted fluorescent light at the emission wavelength (quantum yield). It is further influenced by their concentration and distribution and can be affected by a number of environmental factors, including temperature, ionic strength, pH, excitation light intensity and duration, covalent coupling to other molecules, and non-covalent interactions.

However, spectra of fluorophores overlap and several fluorophores contribute to the total fluorescence measured, thus making it difficult to identify individual fluorophores in a given tissue spectrum and to selectively correlate them to certain cellular changes. Therefore, established cell lines were used to study the contribution of intracellular autofluorescence to the overall emission signature. In these in vitro cell systems the extracellular matrix emission and the influence of light absorption and scattering are eliminated, which allows an improved understanding of the biochemical changes associated with pathological processes [5, 6]. Notably, the intracellular fluorescent signatures arising from NADH and FAD can be linked to the metabolic state within a cell because these two significantly strong fluorescent coenzymes play important roles in a wide range of cellular oxidation–reduction reactions. Numerous in vitro and in vivo studies have been published on the non-invasive measurements of autofluorescence to monitor cell and tissue metabolism under different physiological and pathophysiological conditions. Furthermore, promising applications for autofluorescence spectroscopy to sensitively monitor cellular metabolism in response to drug treatment have been reported [7, 8, 9].
Table 1  Excitation and emission maxima of selected endogenous fluorophores [10].

<table>
<thead>
<tr>
<th>Endogenous fluorophore</th>
<th>Excitation maxima (nm)</th>
<th>Emission maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>280</td>
<td>350</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>275</td>
<td>300</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>260</td>
<td>280</td>
</tr>
<tr>
<td>Collagen</td>
<td>325, 360</td>
<td>400, 405</td>
</tr>
<tr>
<td>Elastin</td>
<td>290, 325</td>
<td>340, 400</td>
</tr>
<tr>
<td>FAD, Flavins</td>
<td>450</td>
<td>535</td>
</tr>
<tr>
<td>NADH</td>
<td>290, 351</td>
<td>440, 460</td>
</tr>
<tr>
<td>NADPH</td>
<td>336</td>
<td>464</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>436</td>
<td>540, 560</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>340-395</td>
<td>540, 430 - 460</td>
</tr>
<tr>
<td>Ceroid</td>
<td>340-395</td>
<td>430-460, 540</td>
</tr>
<tr>
<td>Porphyrins</td>
<td>400-500</td>
<td>630, 690</td>
</tr>
</tbody>
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Apart from the fluorescence studies of different intracellular coenzymes associated with apoptosis, redox reactions, cell morphology, glucose-sensing and mitochondrial anomalies in cancer and neurodegenerative diseases [11-15], much attention has been given to distinguishing between (pre-) malignant and benign lesions, and, consequently, to the development of minimally invasive disease diagnosis or for intraoperative delimitation of tumor-resection margins. Multiphoton microscopy applied for autofluorescence analysis of glioma tissue allowed a clear definition of the tumor-brain interface at single-cell resolution of invasive tumor cells [16]. In a combined label-free approach, distinctive glioma cell morphology and autofluorescence properties were exploited to identify potential glioma-initiating cells [17]. The molecular basis of this autofluorescence remains unknown to date, but it probably reflects higher metabolic and proliferative activity. Further elucidation of these processes will enhance our understanding of the molecular mechanisms governing tumor growth and should lead to diagnostic and therapeutic advances.

2. Spectral imaging

In multifluorophore systems such as cells many individual fluorophores contribute to the autofluorescence that can be detected. Their spectra and relative amounts define the spectrum that can be measured. Quantification of these distinct spectral signatures produces an optical “fingerprint” of the cell. To obtain spectral information the sample can be imaged with different filters covering different parts of the desired wavelength range. This multispectral imaging method produces only a few, usually less than five, image planes of spectral data. In contrast, spectral imaging methods (synonyms hyperspectral imaging, imaging spectroscopy or chemical imaging) generate data sets with higher spectral resolution that can be imagined as full spectra at every location in the image. The resulting spectral images are data cubes with spatial resolution on two axes (x,y) and spectral information on the third axis (λ). There are different spectral imaging devices that differ in resolution, speed and scanning method. The existing spectral imaging systems can be classified into staring-, whiskbroom- and pushbroom-imagers (fig. 1). Staring imagers record two-dimensional images at different wavelengths sequentially by changing filters in front of a camera. For the selection of the wavelength acusto-optical (AOTF), liquid crystal tunable filters (LCTF) or filter wheels are used. In contrast, whiskbroom imaging uses spectral detectors to scan each single point of a sample. The spatial resolution depends on the raster width that is used for sampling while the spectral resolution depends on the detector that is used. Whiskbroom imaging is typically used in confocal, IR and Raman microscopy. Another method that uses spatial scanning of the sample is pushbroom imaging. Instead of scanning the sample point by point, the spectral image is captured line by line by a spectrograph in front of a camera. The light coming from the sample is dispersed by a narrow slit, prisms or a grating on the sensor-chip of the camera. The captured images contain spatial information on one axis (x) and the spectral information on the other axis. By moving the sample relative to the detector the y-direction is scanned. The spectral resolution of the system depends on the size of the slit, the pixel-size of the camera and the grating that is used.

In our study we used a pushbroom imaging system in combination with multiphoton excitation. Multiphoton excitation has several advantages over single photon excitation. i) Multiphoton excitation provides high axial resolution almost comparable to confocal microscopy. ii) The use of near infrared light is less phototoxic to cells and iii) the rate of photobleaching is reduced. Thus cells can be observed for a longer time. iv) A single excitation wavelength can excite several molecules through 2- and 3-photon processes increasing the number of molecules that can be detected simultaneously.
Our spectral imaging system used for this study (fig. 2) consists of a tunable titanium sapphire 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. 2** 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.
3. Multiphoton-excited autofluorescence for characterisation of isogenic glioblastoma cell lines with heterologous expression of different tumor suppressor genes

3.1 Isogenic Glioblastoma Cell Lines as Model Systems for Label-Free Microscopy

Glioblastoma multiforme (GBM) is the most lethal primary brain tumor and is highly resistant to therapy. GBM tumors are characterized by high rates of mutations. Recently, the numerous identified genomic abnormalities have been catalogued allowing the classification of four subtypes of GBM and the identification of five major genetic changes, that are loss of Ink4a, Arf, p53, or phosphatase and tensin homolog deleted on chromosome (PTEN) and amplification of epidermal growth factor receptor (EGFR) [18]. The functional consequences of most aberrations conferring malignant transformation and therapeutic resistance have still to be elucidated in order to accelerate the development and approval of new targeted therapies. A suitable approach is the use of isogenic cell lines in which catalogued aberrant genes can be “knocked in” in order to dissect out the effects of oncogenic mutations. The isogenic cell lines can then be exploited to screen for compounds with selective toxicity towards the cells expressing the aberrant genes but are non-toxic to the isogenic parental cells (synthetic lethality, reviewed in [19]). 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.

We used a site-specific recombination-based strategy to construct the isogenic U-251MG cell lines. This includes a two-step procedure, in which the cells are first stably transfected with a plasmid carrying a Flp-recombinase recognition sequence that is randomly inserted into the genome. After subcloning and selection of a clone with single insertion of this plasmid, an expression plasmid with the gene of interest and a second Flp-recombinase recognition site is co-transfected along with a second plasmid coding for the Flp-recombinase. The action of the recombinase then leads to insertion of each selected gene into the predefined site, where the first vector has been inserted. Because all genes are inserted into the same location, the resulting cell lines are isogenic, only differing in the absence/presence of TP53 and PTEN, respectively. In this particular setting, we located TP53 and PTEN on expression plasmids, which have a tetracycline-inducible promoter, so that the genes can be upregulated upon addition of tetracycline or its analogue doxycycline (Tet-ON system). Insertion of an empty expression vector, i.e. without any gene, is performed in parallel, which gives rise to a control cell line (U-251MG-Control), carrying all vector elements and thus suitable to normalise against all effects that might be contributed by these. In contrast, the isogenic U-251MG-PTEN and the U-251MG-TP53 cells differ in their tumor suppressor status expressing either wild type PTEN or TP53, respectively. The use of such paired isogenic cell lines allowed us to determine the effect of the tumor suppressor genes on cellular functions and subsequently to determine the correspondingly emitted autofluorescence signatures.

3.2 Preparation of cells

Glioblastoma cells were grown in DMEM with 4.5 g/l glucose and 10% fetal calf serum. For experiments 5 x 10^5 cells create one single pixel in the recorded image. Accordingly the area for collecting photons increases and in consequence detectors, the signal-to-noise ratio can be increased by combining the information of adjacent pixels in a CCD sensor to

Spectral imaging of autofluorescence is often compromised by low signal intensities. When CCD cameras are used as detectors, the signal-to-noise ratio can be increased by combining the information of adjacent pixels in a CCD sensor to create one single pixel in the recorded image. Accordingly the area for collecting photons increases and in consequence the intensity in the recorded image increases at the cost of sampling density. Since the emission spectra of most fluorophores are broad and devoid of structure we did not need full resolution in λ-direction. We used asymmetric binning of 2x8 pixels of the camera to increase signal-to-noise ratio of the measurements, which means that signal increased 16 times while the resolution in the x-direction decreased 2x and spectral resolution decreased 8x. This setting
was suitable to reproduce cellular structure and spectra. The high sensitivity that is needed for the spectral measurements made even the light emitted by the computer monitors in an otherwise completely dark room visible. This was noticed by contaminating peaks in the red, green and blue region of the spectra. We solved the problem by placing a light-tight box on top of the microscope stage during measurement that eliminated this contaminating stray light. Optimal settings of laser power and number of beams must to be defined for the measurements in order to avoid phototoxic effects on the cells. Reducing the laser power at 750 and 800nm excitation to 50% and using 32 beams for the measurements allowed us to image the same cells at all four excitation wavelengths of 880, 850, 800 and 750nm without obvious damage to the cells.

For the measurements a line of 150µm was illuminated in the x-direction while the y-direction was scanned with the motorised x,y scanning table using its minimum step size of 250 nm. The resulting data cube had a dimension of 150µm in the x-, 75µm in the y- and 160nm (wavelength range 400 – 660nm) in the λ-direction. All images were saved in TIFF file format.

3.4 Data analysis

Data analysis was performed using the open-source “ImageJ for microscopy” software [20] which provides all the tools to analyse spectral images. 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. The ROIs can be stored using the “Multi Measure” plugin (plugins/ROI/Multi Measure/save). 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).

3.5 Results

The optical setup described allowed us to analyse living cells originated from the glioblastoma cell line U-251MG with high spectral and spatial resolution. Since we used a Tet-on regulated gene expression approach cells had to be induced 48h prior to spectral analysis to express either PTEN or TP53. Thus, three different sets of isogenic glioblastoma cells were included in the data presented here, namely the induced U-251MG-PTEN cell line, the induced U-251MG-TP53, and the induced U-251MG-Control cell line. Autofluorescence measurements were made at four different excitation wavelengths (750nm, 800 nm, 850nm, 880 nm) to ensure that as many fluorophores as possible were excited and thus contributed to the spectral profiles. Fluorescence images were acquired over a 150µm x 75µm field of view (see chapter 3.3) containing typically 10-30 cells. The emission was measured in the range of 400–660 nm and analysed with respect

![Workflow of data analysis](image-url)
to its subcellular distribution and its spectrum. Regions of interest were defined manually for analysis of individual whole cells or user-defined subcellular regions. In the work presented here, whole cells have been selectively analysed (fig. 4).

In general, regardless of the selected excitation wavelength autofluorescence images showed regions of higher fluorescence intensity within the cytoplasm whereas nuclear regions appeared far less intense (fig. 4a, c). The cytoplasmic structures had a granular, punctate pattern with perinuclear localisation.

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. 4b). 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 530nm increased (data not shown). Excitation at 880nm produced a broad spectrum, with only one detectable peak at 530nm which can be associated with FAD fluorescence (fig. 4d). Generally, signal intensities were higher when excited at 750nm compared to excitation with a longer wavelength.

Spectra derived from autofluorescence images had a very similar shape so that differences between the cell lines were not directly apparent (fig 5a). However, data could be extracted from the spectra by using multivariate analysis of measurements. 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 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 5c. 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. 5b). To confirm expression of recombinant proteins, cells were grown in the presence of 100 ng/ml doxycycline prior to lysis. Regulated induction of PTEN or TP53, respectively, was monitored by Western blotting using specific antibodies against the indicated proteins. PTEN expression was detected as early as 42 h after doxycycline treatment with maximal induction at 66 h. In contrast, the doxycycline-induced TP53 expression reached a maximum between 15 h and 42 h. In addition, we detected a basal TP53 expression even in the absence of doxycycline, due to the reactivity of the anti-p53 antibody with the endogenous mutated p53 protein in U-251MG cells. For Western blot analysis, cells were treated only once with doxycycline. Thus, the protein levels of TP53 and PTEN decreased after 42 h and 66 h, respectively, due to the limited half-life time of doxycycline (24 h) in cell culture and the degradation of recombinant proteins. Effects of induced PTEN and TP53 expression on cell proliferation and cell migration were analysed by using cell viability and scratch wound-healing.
assays, respectively. In accordance with their functions in cell cycle regulation a significant decrease of proliferation and migration rates were observed in the isogenic glioblastoma cell lines U-251MG upon induced expression of PTEN and TP53, respectively (data not shown).

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. 5c). This suggests that spectra generated by this strategy may to a certain degree reflect the molecular fingerprints of brain cancer cells.

![Fig. 5](image.png)

Fig. 5  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 750nm. 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.

3.6 Discussion

The main objective of this study was to demonstrate that multiphoton excitation combined with multi-beam scanning and spectral imaging can be used as a label-free microscopic approach to determine the autofluorescence properties of living isogenic glioblastoma cells with minimized phototoxicity and thereby best maintenance of cell viability during measurement. Furthermore, the sensitivity of this methodology was investigated to distinguish between targeted expression of cancer-relevant genes. A high sensitivity is an essential prerequisite to assess the diagnostic potential of the method and the applicability as a cellular in vitro assay to exploring the functions of somatically altered genes in cancer or other diseases.

Label-free, autofluorescence-based techniques can provide information on the native state of a living cell. The optical detection is non-invasive and can be performed in real-time, because no treatment of the sample, such as staining, is necessary. Using multiphoton-excited spectral imaging methods we were able to analyse autofluorescence of isogenic glioblastoma cell lines with high spatial and spectral resolution. Most of the observed cellular autofluorescence was located in granular structures in the perinuclear region. In cells, the highest autofluorescence can be found in mitochondria and lysosomes [21]. This is in agreement with the observed autofluorescence patterns based on size and distribution but must be proven by co-localisation studies with specific markers (e.g. Lysotracker, MitoTracker). Two major substances contributed to the measured spectra. The peaks at 450 and 470nm can be ascribed to NADH whereas the peak at 530nm results from flavins/FAD. It seems as if most, if not all, of the autofluorescence originates from these molecules, as was suggested by Aubin [22]. The measured emission spectra at 440, 460 and 535nm are slightly shifted in wavelengths compared to the reported values (tab. 1). This is caused by the applied spectrograph, mainly by the
individual polarisation properties of its grating [23]. Emission maxima were verified by spectral imaging of aqueous solutions of NADH under the same conditions.

Adjusting the optimal laser power and using a multi-beam scanning procedure was found to preserve the cell viability optimally which allowed us to image the same cells at all four excitation wavelengths (880, 850, 800 and 750nm) without obvious damage to the cells. However, it should be noted that multispectral image acquisition is a time-consuming process that includes data scanning along either the wavelength or spatial axis. Although the acquisition time per scanned image could be reduced by a factor of 32 using the multi-beam splitting in our approach, the total image acquisition time for a 150 µm x 75µm scanned field of view was still nearly 5 minutes. This relatively long scan time limits imaging rates and precludes its application to analyse fast dynamic processes. However, the described method is well-suited for steady-state measurements as they are shown in the present study. In order to acquire images on faster timescales, the multiphoton microscope could be coupled to a more sensitive electron-multiplying digital EMCCD camera which would allow to use shorter exposure times and thereby accelerate acquisition speed.

The diagnostic potential of spectrally imaging cellular autofluorescence lies in the ability to non-invasively measure molecular alterations in cells or tissues that occur during disease progression. For instance in cancer, differences in autofluorescence properties between normal, precancerous, and cancerous cells can be caused by their different cell metabolism. Since mitochondrial dysfunction has been definitively linked to a range of diseases and health problems such as neurodegenerative diseases, autoimmune diseases and cancer, the analysis of fluorescent metabolic indicators such as NADH and FAD has been gaining a lot of attention in recent years [3, 24-26]. The endogenous autofluorescence properties of cells and tissues significantly change with disease progression and thus, spectroscopic techniques monitoring the autofluorescence are utilized for in vitro, ex vivo or in vivo studies [6, 27-31]. To gain a better understanding as to how cancer-related genes influence the autofluorescence properties of cells, we applied the spectral imaging method described above to isogenic glioblastoma cell lines lacking either functional p53 or PTEN or both (control cells). Furthermore, this approach should reveal important information about the sensitivity of the applied methodology to selectively identify cells with cancer-relevant molecular changes.

In order to elucidate differences between the autofluorescence spectra obtained for the different glioblastoma cell lines the original spectral data sets were analysed using Principal Component Analysis (PCA). PCA of spectra allowed discrimination of cells expressing either TP53 or PTEN from U-251MG-Control cells with a few incorrectly classified spectra. What has to be considered is that the analysed spectra represent a relatively small data set which may impact negatively on the accuracy of statistical analysis. Furthermore, the reported preliminary results rely on the spectral analysis of whole cells. Since multiphoton microscopy combined with spectral imaging enables spectral and spatial resolution, our results can be further refined by analysing subcellular structures. Imaging of these structures is important because development of precancers and cancers is accompanied by changes in cellular and subcellular morphology [11]. Consequently, we will generate more comprehensive data sets which will allow us to validate the presented data and to use the described approach as a reliable analytical tool.

In summary, our results demonstrate that the combined approach of multiphoton excitation, multi-beam scanning and spectral imaging is a well-suited, label-free methodology to measure autofluorescence properties of living cells and thus, to assess a range of cellular processes. The use of isogenic glioblastoma cell lines allowed highly standardized measurements of autofluorescence spectra revealing spectral differences in cells expressing tumor-related genes compared to the tumor control cells. Building upon these results the contribution of other disease-related candidate genes to specific endogenous autofluorescence signatures could be investigated. Such data could potentially provide a more detailed knowledge of the optimal fluorescence-derived parameters for distinguishing between the healthy and the diseased cellular state. Furthermore, the elucidation of genotype–phenotype correlation would support further understanding of the complex processes involved in disease progression.

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