Two-photon autofluorescence and second-harmonic imaging of adult stem cells

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Abstract. Human and animal stem cells (rat and human adult pancreatic stem cells, salivary gland stem cells, and human dental pulp stem cells) are investigated by femtosecond laser 5-D two-photon microscopy. Autofluorescence and second-harmonic generation (SHG) are imaged with submicron spatial resolution, 270 ps temporal resolution, and 10 nm spectral resolution. In particular, the reduced coenzyme nicotinamide adenine (phosphorylated) dinucleotide [NAD(P)H] and flavoprotein fluorescence is detected in stem cell monolayers and stem cell spheroids. Major emission peaks at 460 and 530 nm with typical long fluorescence lifetimes (τ_2) of 1.8 and 2.0 ns, respectively, are measured using spectral imaging and time-correlated single photon counting. Differentiated stem cells produce the extra cellular matrix (ECM) protein collagen, detected by SHG signals at 435 nm. Multiphoton microscopes may become novel noninvasive tools for marker-free optical stem cell characterization and for on-line monitoring of differentiation within a 3-D microenvironment.
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Keywords: stem cell; 5-D two-photon imaging; autofluorescence; second-harmonic generation.

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1 INTRODUCTION

In stem cell research, there is a high demand on techniques for the noninvasive, marker-free observation of growth, proliferation, differentiation, and stability of living stem cells under physiological conditions. The use of exogenous markers may alter the metabolic balance, influences the cell life, and avoid further therapeutic application. Noninvasive multiphoton microscopes with near-infrared (NIR) femtosecond laser sources[1–4] have been applied to image living single cells and different tissues with a high spatial resolution without any staining. Two-photon autofluorescence is obtained due to intrinsic fluorophores such as nicotinamide adenine (phosphorylated) dinucleotide [NAD(P)H], flavins, porphyrins, elastin, and melanin. In addition, second-harmonic generation (SHG) is induced by certain biomolecule structures such as collagen.[5–9]

In addition to the measurement of the fluorescence/SHG intensity by optical sectioning (3-D imaging), fluorescence lifetime imaging (FLIM, 4-D) and spectral imaging (5-D) can be performed. In particular, the arrival times of the fluorescence photons with respect to the excitation of the molecule and the particular location (pixel) can be determined by time-correlated single photon counting (TCSPC) and the use of photomultipliers with as short rise time. When using a photomultiplier tube (PMT) array in combination with a polychromator, the "color" of the emitted photon per pixel can be also determined (spectral imaging).

Multiphoton imaging is very suitable for long term analysis of living cells due to the absence of out of focus photostress. The excitation volume is limited to a subfemtoliter focal volume. NIR femtosecond lasers enable deep light penetration into tissues and provide the possibility of optical sectioning of 3-D biological objects. Nondestructive imaging of living specimens is restricted to a certain optical window determined by the spectral range and the light intensities.[10] Intrinsic cellular fluorophores deliver information on the cell structure as well as on cell activities. Authors could distinguish certain cancer cells from normal cells,[11,12] even malignant from benign tumors,[13] by optical properties of native fluorescent molecules. Many studies on the intrinsic metabolic state show that innate cellular fluorescence has the potential to discriminate proliferating and nonproliferation cell population,[14] and also metabolic changes of self-renewing and differentiating cells.[15,16] The differentiation of human mesenchymal stem cells into an adipogenic pathway (formation of lipid droplets, change of morphology and autofluorescence) has been investigated recently by two-photon microscopy.[17]

A unique feature of stem cells is the extended self-renewal of the cell population and their differentiation potency. The

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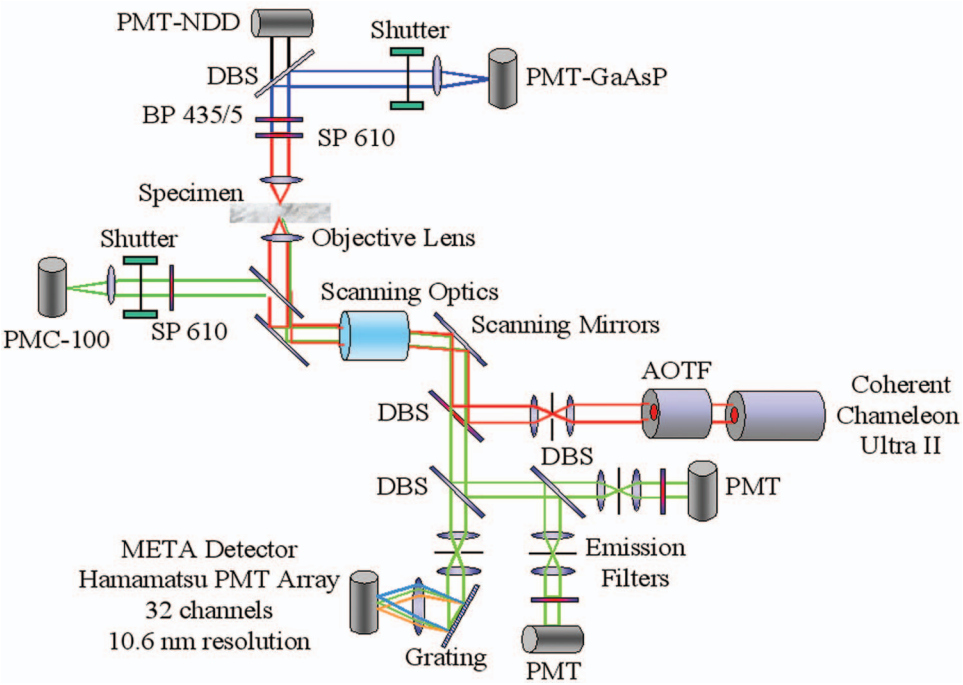

Fig. 1 Experimental setup. PMT is the photomultiplier tube, NDD is the nondescanned detector, DBS is the dichroic beam splitter, BP is the bandpass filter, SP is the short pass filter, AOTF is the acousto optical tunable filter, and GaAsP is gallium arsenide phosphide.

aim of the present study was to investigate the two-photon excited autofluorescence of human stem cells and the onset of collagen production of differentiated cells by the detection of SHG signals. An interesting question arises as to whether it is possible to select differentiated cells from undifferentiated cells without interfering with the native environment and without the use of external markers.

Here we report on the application of a 5-D two-photon microscope for imaging different human and animal stem cell lines by autofluorescence and SHG in monolayers and spheroids.

2 Material and Methods

2.1 *Stem Cell Lines*

The stem cell lines human salivary gland stem cells (hSGSC), human pancreatic stem cells (hPSC), rat pancreatic stem cells (rPSC), and human dental pulp stem cells (hDPSC) were used[18–21] for two-photon autofluorescence imaging. FLIM measurements and spectral imaging were performed on hSGSC stem cells. The first three cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Germany) containing 4.5 g/L glucose, L-glutamine, and supplemented with 15% fetal calf serum Gold (FCS-Gold) (PAA Laboratories GmbH, Germany), 100 U/ml penicillin, and 100 mg/ml streptomycin. hDPSC were cultured in α-MEM with 15% FCS, 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

For imaging, cells were transferred (confluence 80 to 90%) into sterile miniaturized cell chambers with two 0.17 mm thick glass windows with etched grids (MiniCeM-grid, JenLab GmbH, Jena) and additionally incubated for another day until they were attached to the glass surface.

2.2 *Pellet Culture and Chondrogenic Differentiation*

For chondrogenic differentiation, a pellet culture was used. Approximately 200,000 cells were placed in a 15 ml polypropylene tube and centrifuged to a pellet for 5 min (1000× /min). The flattened pellet at the bottom of the tube was cultured at 37 °C with 5% CO_2 in the culture medium for 5 to 6 days until a spherical form (spheroid) was formed. For the differentiation, the spheroids were cultured in 1 ml of the chondrogenic medium DMEM (GIBCO, Germany) supplemented with 10 mg/ml transforming growth factor (TGF)-β3, 100 nM dexamethasone, 50 mg/ml ascorbic acid-phosphate, 4 mg/ml L-proline, 100 mg/ml natrium piruvate, 15% FCS-Gold, 100 U/ml penicillin, and 100 mg/ml streptomycin. The medium was replaced every 3 to 4 days for a period of 4 to 5 weeks. For microscopy, the pellets were transferred to dishes with 0.17 mm thick glass coverslips.

2.3 *Instrumentation and Measurements*

A laser scanning microscope (LSM 510 Meta NLO, Zeiss, Germany) equipped with a tunable 80 MHz Ti:sapphire laser source (Chameleon, Coherent Inc., Santa Clara, California) was used to study autofluorescence and SHG of stem cells. For autofluorescence imaging, laser beams at 750, 850, and 900 nm (140 fs laser output) at 5 mW mean power were used. The beamsplitter HFT KP 650 and the short pass filter KP 685 were employed. The cells were scanned in a 512×512 pixel field at 25.6 μs pixel dwell time. Fluores-

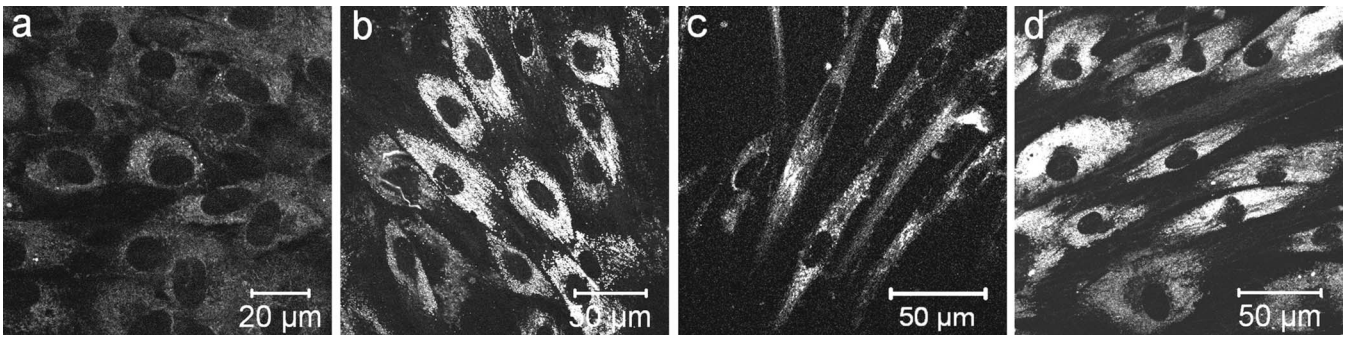

Fig. 2 Autofluorescence images of (a) rat pancreatic stem cells (rPSC), (b) human pancreatic stem cells (hPSC), (c) dental pulpa stem cells (DPSC), and (d) salivary gland stem cells (hSGSC). Excitation wavelength was at 750 nm. Fluorescence emission was recorded through a Plan-Neofluar 40×/NA 1.3 oil objective.

cence emission was recorded through a Plan-Neofluar 40×/NA 1.3 oil objective. The experimental parameters such as scanning time and the settings for contrast and brightness were identical for all autofluorescence images obtained from different cell lines for comparison purposes.

For SHG detection, the external PMT-GaAsP (H7422P-40, Hamamatsu, Japan) was mounted to the microscope in a forward direction. A SP 610 filter was installed in front of the detector to prevent scattered laser light onto the detector. SHG signals were collected through a 435 nm bandpass filter at an excitation wavelength of 870 nm.

3-D two-photon autofluorescence and SHG images were obtained by optical sectioning with z-intervals of 5 to 10 μm. Fluorescence lifetime imaging (FLIM) was performed by single-photon counting (TCSPS) using the board SPC-830 (Becker and Hickl GmbH, Germany) and the fast detector PMC-100 with an SP 610 filter.

The instrumental function (IRF) of the optical system was measured using the second-harmonic generated signal from collagen II (Sigma-Aldrich, Germany). SPCImage software was used to analyze the fluorescence lifetime decay curves as well as the calculation of histograms (Becker and Hickl GmbH, Germany). Spectral imaging was performed with the 32 channel PMT array (META, Hamamatsu). The grating in the polychromator provided a resolution of 10 nm per channel. Typically, the spectral range of 382 to 714 nm was imaged. Figure 1 shows the experimental setup.

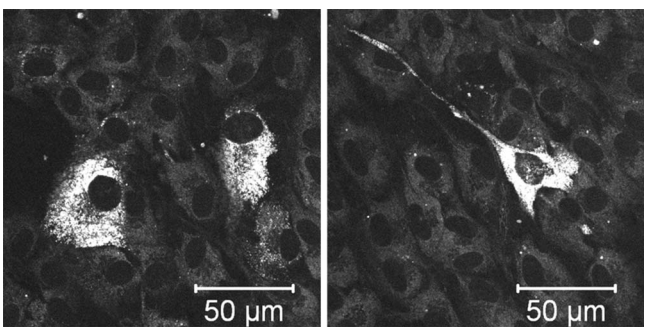

Fig. 3 Autofluorescence image of rPSC cells. Note that some cells (<5%) exhibited a very intense fluorescence.

3 RESULTS

3.1 *Two-Photon Autoflourescence Imaging and Spectral Analysis*

The two-photon measurements showed that different stem cell lines have an autofluorescence with different intensity. hDPSC, hPSC, and hSGSC human stem cells exhibited an intense autofluorescence compared to rPSC rat cells. Figure 2 demonstrates the differences in the fluorescence intensity between human and rat pancreatic cells. Also, we noted significant fluorescence intensity variations between cells of the same line (Fig. 3). Intrinsic fluorescence from living cells was detected at different NIR excitation wavelengths (750, 850, and 900 nm) to separate different native fluorescence molecules. When using 750 nm, NAD(P)H as well flavins/flavoproteins can be excited by a two-photon process. However, when changing to 850 or even 900 nm, flavins/flavoproteins (e.g., FAD) only and not NAD(P)H can be efficiently excited.[22] Spectral measurements (Fig. 4) showed that the detected fluorescence light was intense in the blue/green spectral region. The maximum of the fluorescence was found to be at 460 to 470 nm when excited with 750 nm

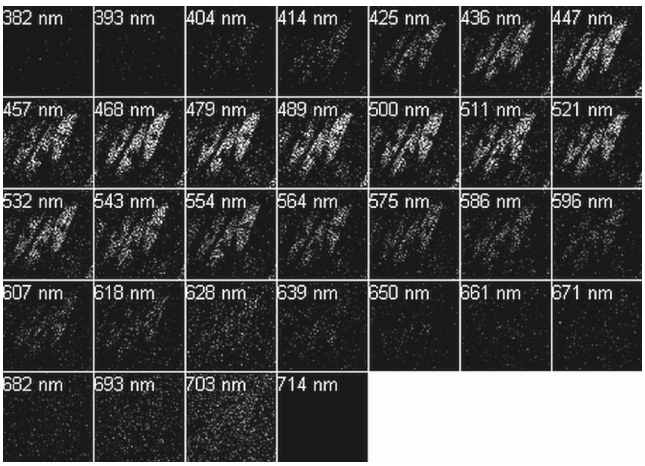

Fig. 4 Spectral analysis of hSGSC stem cells obtained from 32 images in the spectral range of 382 to 714 nm. Excitation wavelength was at 750 nm.

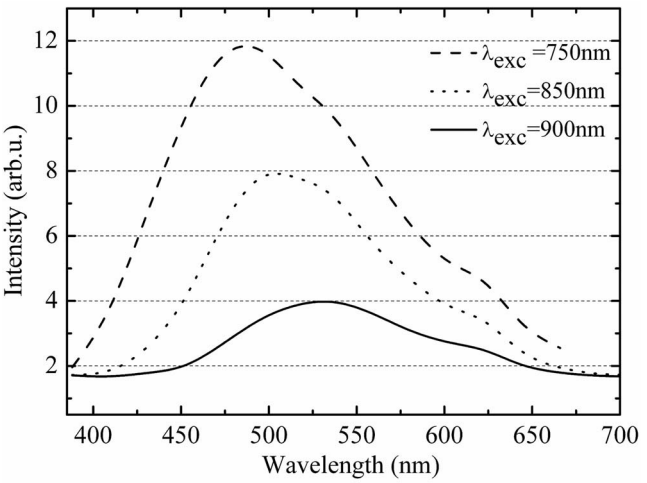

Fig. 5 Spectral measurement of hSGSC stem cells.

light, which is consistent with the emission behavior free and protein bound NADH. The maximum shifted to 530 to 535 nm when using 900 nm light, which is consistent with flavin emission (Fig. 5). We have proven that the presence of the culture medium did not add significant background to the autofluorescence signals from the cells.

3.2 *Fluorescence Lifetime Imaging Measurement and Analysis*

Fluorescence decay curves have been obtained by exciting at 750 and 900 nm. Figure 6 presents a typical 750 nm excited FLIM image, a histogram, and a fluorescence decay curve of hSGSC stem cells. The decay curve reflects the autofluorescence of one mitochondrium in a specific cell. 6740 photons were detected for this curve. The biexponential fit with the fitting parameter $\chi^2=1.00$ reveals two components. A fast one with a short lifetime (τ_1) of 0.17 ns and an amplitude of $a_1=72\%$, and a second one with $\tau_2=1.8$ ns and the amplitude $a_2=28\%$.

To get a statistical information on the variance of intracellular decay curves, we analyzed the curves of ten different perinuclear fluorescence regions of the bright cell on the right side within Fig. 6. The data are depicted in Table 1. Interestingly, only one major short component with an average value of 0.202 ns [standard deviation (SD) 0.019, amplitude 70.5%] was found in this particular cell. The long component had an average value of 2.014 ns (SD 0.077).

When calculating the mean fluorescence lifetime τ_2 of the long-lived component within this frame of about ten cells in Fig. 6, we obtained a major value of 1.87 ns, as depicted in the histogram. When analyzing ten frames of about 100 cells in total of the same cell line, we obtained a mean τ_2 value of

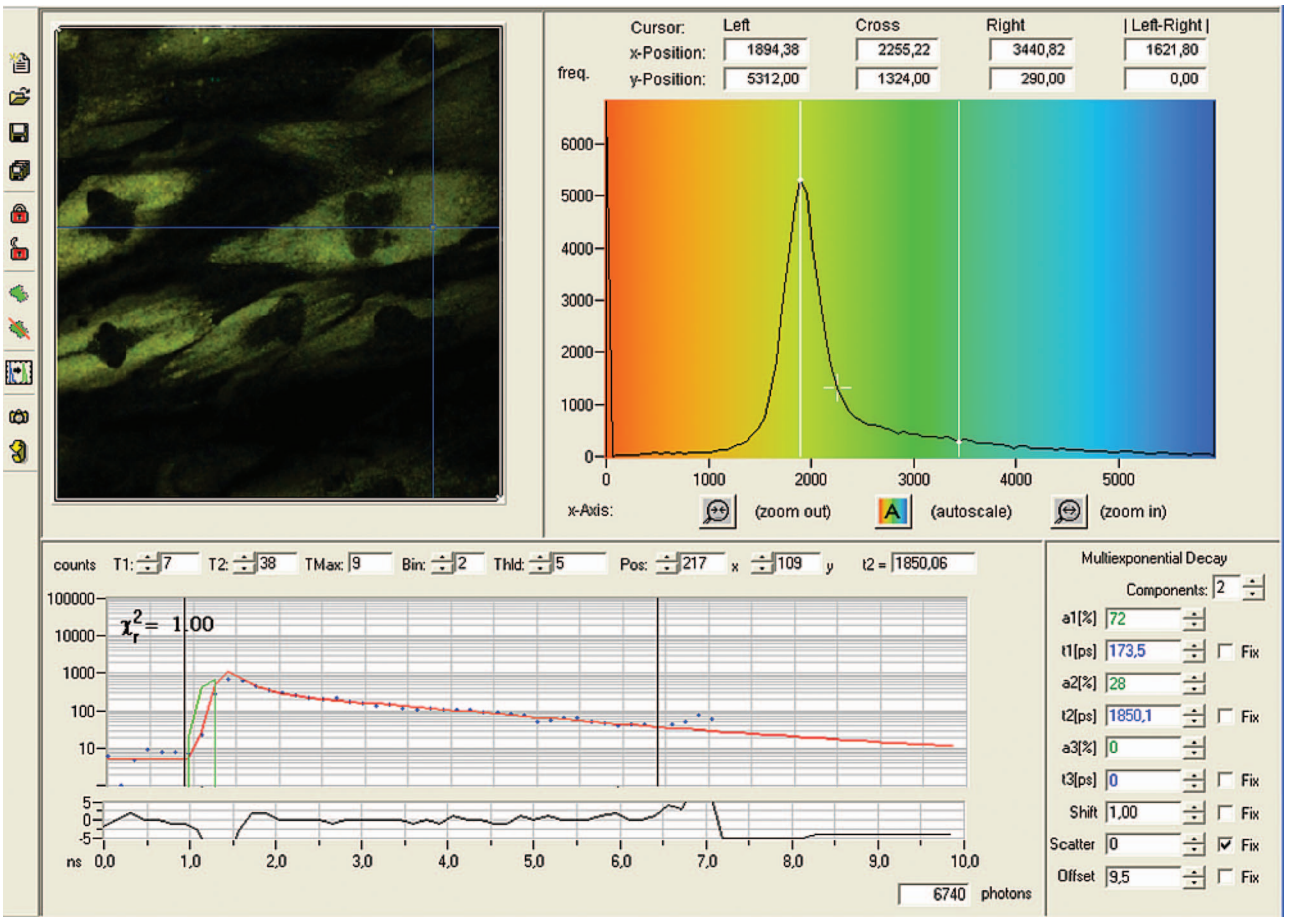

Fig. 6 Data analysis window of the SPCImage software.

Table 1 Intracellular FLIM data obtained from ten decay curves within the cytoplasm of the bright cell on the right in Fig. 6.

	τ_1 (ns)	τ_2 (ns)	τ_m (ns)	a_1 (%)	χ^2
1	0.193	1.892	0.729	68.5	1.01
2	0.244	2.004	0.778	69.7	1.14
3	0.202	2.089	0.722	72.4	1.44
4	0.208	2.087	0.736	71.9	1.40
5	0.197	2.060	0.721	71.9	1.22
6	0.169	1.933	0.731	68.1	1.48
7	0.203	1.979	0.717	70.5	1.08
8	0.211	2.128	0.776	70.5	1.59
9	0.214	2.025	0.729	71.6	1.65
10	0.186	1.945	0.722	69.6	1.56
Average	0.202	2.014	0.736	70.5	1.36
SD	0.019	0.077	0.022	1.5	0.2

(1.82 ± 0.02) ns. In contrast, when changing the excitation wavelength to 900 nm, an average value τ_2 of 100 cells of 2.00 ± 0.03 ns was obtained.

When calculating the mean lifetime $\tau_m = (a_1\tau_1 + a_2\tau_2)/(a_1 + a_2)$, per pixel we obtained an average value of 0.7 ns for the image in Fig. 6. Considering 100 cells within ten frames, the mean value was found (0.680 ± 0.04) ns. The employment of the 900 nm excitation wavelength resulted in a longer value of (0.820 ± 0.05) ns.

Interestingly, when analyzing the short fluorescent components within these FLIM images (frames), we obtained up to three maxima in the τ_1-histogram (Fig. 7). The first one at

Table 2 Average FLIM data calculated from ten regions of interest covering one single cell in each region. "x" means no component was detected.

cell	τ_{1a} (ns)	τ_{1b} (ns)	τ_2 (ns)	τ_m (ns)	a_{1a} (%)	a_{1b} (%)	ratio a_{1a}:a_{2b}	χ^2
1	0.208	x	2.059	0.795	72.1	x	x	1.02
2	0.215	x	2.118	0.781	72.9	x	x	1.08
3	0.216	0.687	2.103	0.798	71.9	79.7	2.216	1.13
4	0.201	0.673	2.118	0.785	71.9	79.9	1.792	1.11
5	0.241	0.695	2.133	0.781	72.3	79.9	1.367	1.10
6	0.283	0.708	1.952	0.785	73.3	79.9	1.815	1.13
7	0.230	x	1.977	0.708	72.9	x	x	1.13
8	0.181	0.696	1.941	0.661	73.9	79.9	1.244	1.13
9	0.197	0.764	2.046	0.701	72.9	79.7	1.219	1.17
10	0.226	0.730	2.097	0.768	71.1	79.3	5.834	1.31
Average	0.220	0.708	2.054	0.756	72.5	79.8	2.212	1.13
SD	0.028	0.031	0.073	0.048	0.8	0.2	1.638	0.07

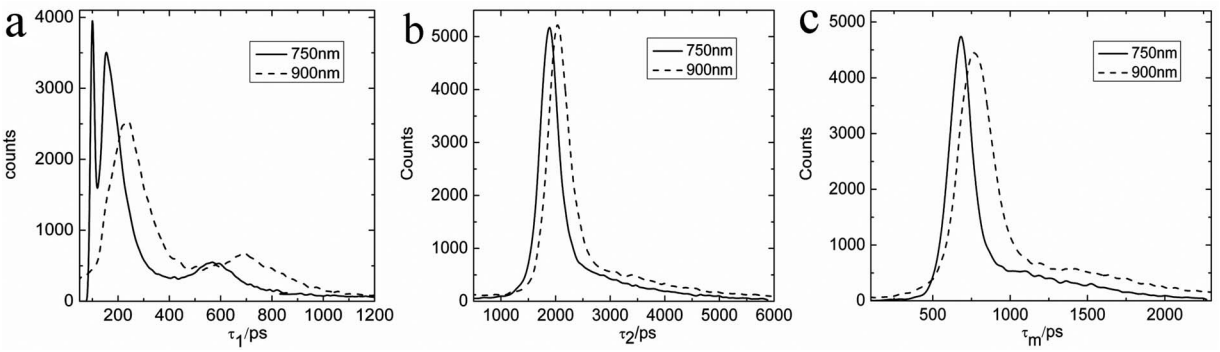

Fig. 7 Histograms for the distribution of the fluorescent components (a) τ_1, (b) τ_2, and (c) τ_m at 750 and 900 nm of hSGSC stem cells.

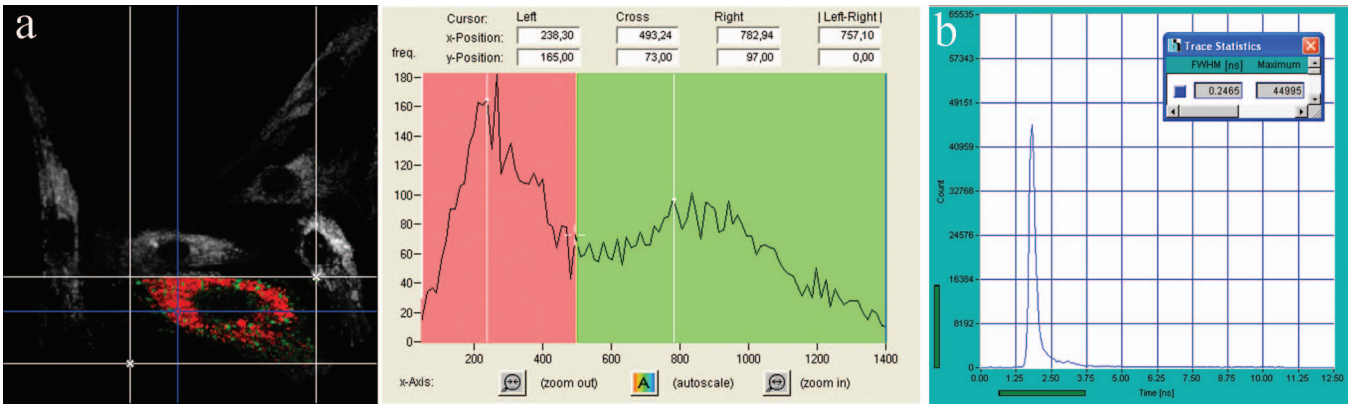

Fig. 8 (a) Average FLIM data of a particular cell were obtained from a region of interest (white rectangle).The histogram reveals two short-lived components with two fluorescence lifetime maxima at 0.238 and 0.783 ns, respectively. The fluorophores with a lifetime in the range of 0 to 0.5 ns are depicted red, and the fluorophores with a lifetime of 0.5 to 1.4 ns are shown green in the false color t_1 image. (b) The measured instrumental response function with a full width half maximum of 0.247 ns was obtained as SHG signal from collagen II.

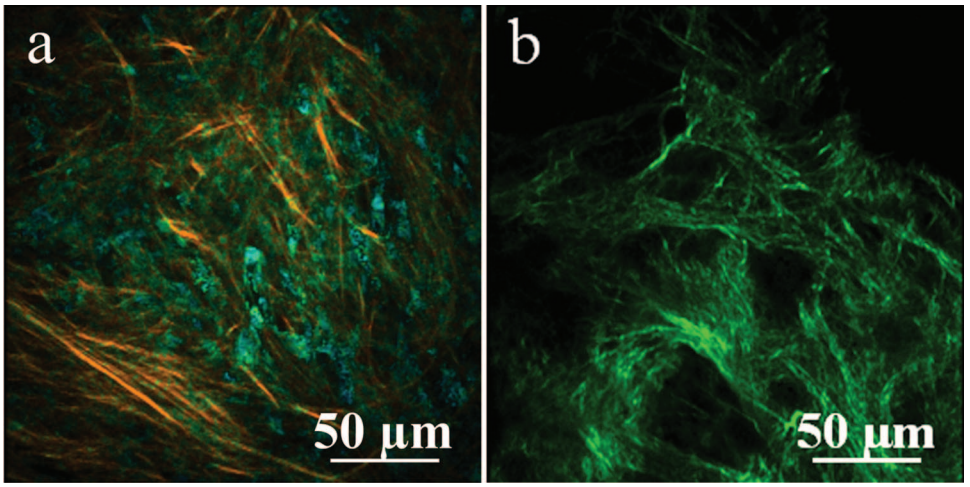

Fig. 9 Multiphoton autofluorescence and SHG images of hSGSC stem cells after chondrogenic differentiation. (a) Image taken with the short-pass filter SP 610 autofluorescence (green) and SHG (red-yellow fibrils), and (b) imaging with the bandpass filter BP 435/5 (mainly SHG signals).

around 0.07 ns reflects the influence of backscattered laser light, which was able to transmit through the beamsplitter and the short pass filter (SP 610). When adding a second short pass filter, this peak nearly disappeared. The other two peaks occurred around (0.170 ± 0.015) ns and (0.660 ± 0.05) ns at 750 nm excitation wavelength and shifted to (0.200 ± 0.015) ns and (0.800 ± 0.05) ns, respectively, at 900 nm. Control measurements were done in culture medium as well as after washing the cells with phosphate buffer saline (PBS). The bands were therefore not background signals from the system such as autofluorescence of optics, etc., but of biological origin. We analyzed these two short components in more detail. For that purpose, we performed a statistical analysis of the histograms (Table 2) on ten bright fluorescent cells out of Fig. 6 and 8. Rectangular regions of interest (ROI) were created as depicted in Fig. 8. Each ROI covered one cell. According to the table, just three cells exhibited only one short fluorescence lifetime of about 0.2 ns, whereas seven out of ten cells possessed two short components with lifetimes of 0.2 and 0.7 ns, respectively.

3.3 *Second-Harmonic Generation Detection*

We monitored the biosynthesis of collagen in 3-D salivary gland and pancreatic stem/progenitor cell cultures by monitoring the occurrence of SHG signals for a long time period up to 5 weeks. The spheroids had dimensions of 0.2 to 2 mm in diameter. The two-photon autofluorescence and SHG images of spheroids were obtained by optical sectioning in z-steps of 5 to 10 μm. The occurrence of the first SHG signal was detected after eight days after the introduction of the stimulating agent TGF-β3 [Figs. 9(a) and 9(b)].

4 Conclusion

Detected two-photon autofluorescence with emission maxima at 460 to 470 nm and 530 to 535 nm, and the long fluorescence lifetimes of 1.8 and 2.0 ns, respectively, prove the presence of the biomolecules NAD(P)H and flavins/flavoproteins such as FAD in stem cells and stem cell clusters. The occurrence of some extremely bright cells shows the heterogeneity within a cell population of the same culture dish.

Interestingly, the analysis of the short-lived component of human salivary gland stem cells was found to be even more complex. Some cells exhibited only one short-lived fluorophore with a fluorescence lifetime of about 0.2 ns only (excellent biexponential fit with χ^2 of 1.00, Fig. 6), whereas the majority of cells revealed an additional short-lived component with 0.7 ns fluorescence lifetime.

The interpretation of these two short fluorophores is complicated. Free NAD(P)H has a short lifetime around 0.200 to 0.300 ns, whereas a variety of flavoproteins have short picosecond lifetimes. The free flavin mononucleotide (FMN) has a typical lifetime of 4.7 to 5.2 ns,[23,24] while flavin adenine dinucleotide (FAD) has a lifetime of 2.3 to 2.8 ns.[24–26] When binding to a variety of proteins, the fluorescence lifetime of NAD(P)H in a variety of cells and solutions shifts to higher values of about 2 ns.[23] Chia et al.[27] reported on the presence of three NAD(P)H fluorophores in rat brain tissue. A very short one of 0.48 ns for the free (nonbound) coenzyme, a second short one of 0.77 ns and a long lifetime of 3 to 6 ns for bound NAD(P)H.

Future work needs to be done for the interpretation of the cellular FLIM data and spectra with regard to the specific cellular metabolism and differentiation value. For that purpose, the particular imaged cell of interest should also be characterized with selective biochemical means, such as antibody staining of the cell as well as the surrounding cells.

Very interestingly, SHG can be used to detect the biosynthesis of collagen as a result of the differentiation process.[28,29] We performed long-term studies of up to 35 days on 3-D stem cell spheroids and were able to monitor the expression of the extracellular matrix protein. The possibility of nondestructive marker-free imaging allows the study of the organization and development of the ECM structure, and of feedback mechanisms. Recently it was shown that collagen influences the differentiation of stem cells.[30]

Femtosecond laser microscopes with their capability of nondestructive two- and three-photon excited autofluorescence and SHG/THG imaging may become novel noninvasive multidimensional tools for marker-free optical stem cell characterization and for on-line monitoring of differentiation of living cells in a 3-D environment, including ECM components.

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