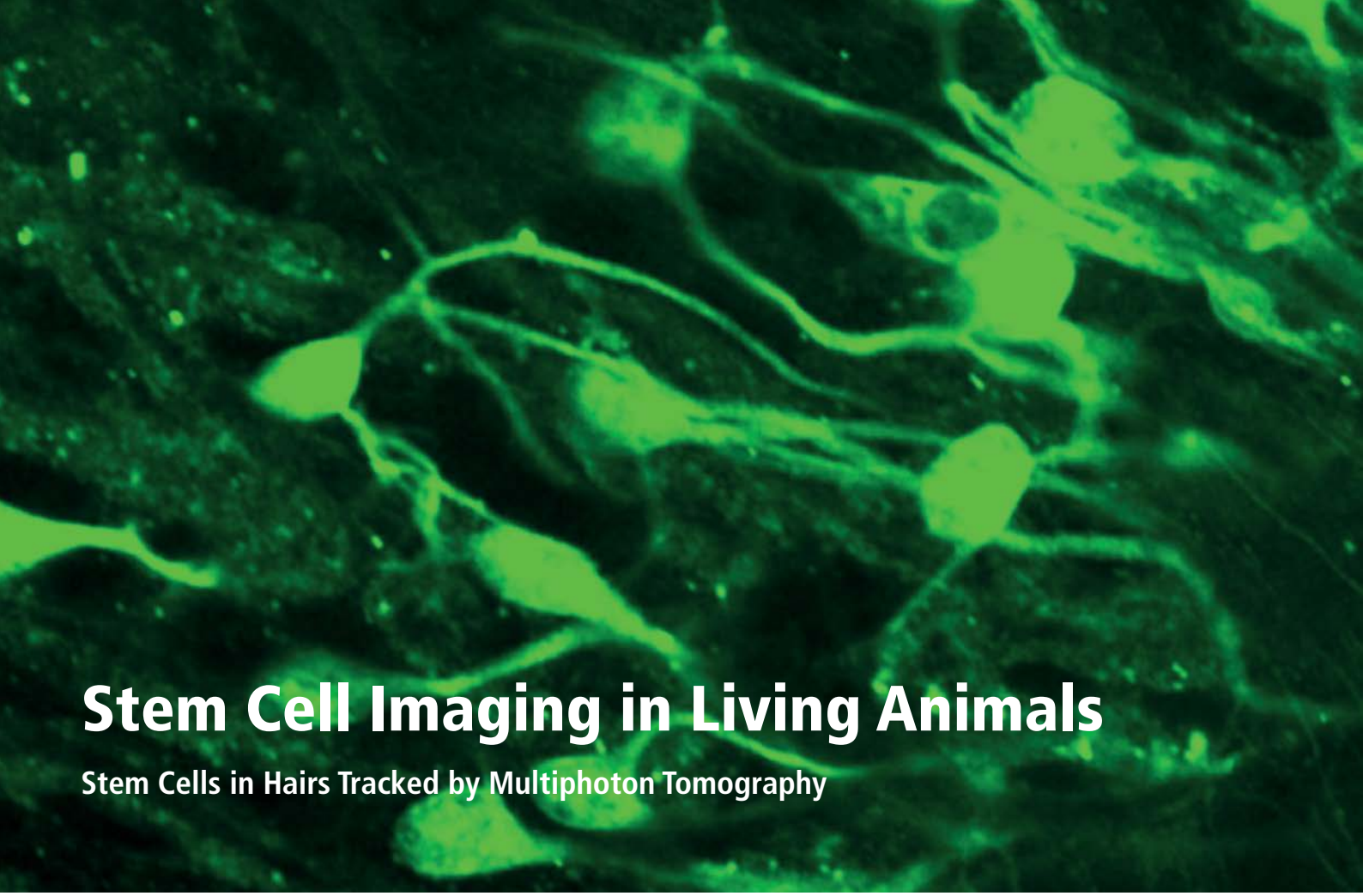

Stem Cell Imaging in Living Animals

Stem Cells in Hairs Tracked by Multiphoton Tomography

Monitoring stem cells in their natural physiological environment is crucial to understanding stem cell differentiation and the generation of tissue. We applied high-resolution 3D multiphoton tomography in order to non-invasively visualize the stem cells of hair follicles. Single nestin GFP-expressing stem cells were tracked for up to five hours in living transgenic mice. The microenvironment was monitored by two-photon autofluorescence and second harmonic generation.

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Background

Hair follicle stem cells that express nestin located in the bulge area and dermal papilla have therapeutic potential due to their easy accessibility and capacity to develop various tissues such as nerves, blood vessels, and smooth muscles [1–4]. Harvesting nestin-expressing stem cells from the hair follicle is less invasive compared to other tissue-specific stem cell sources. Hair follicle stem cells can effect nerve and spinal cord injury repair in mice upon transplantation [5-6]. However, the molecular mechanisms underlying stem cell differentiation and tissue regeneration is not yet fully understood.

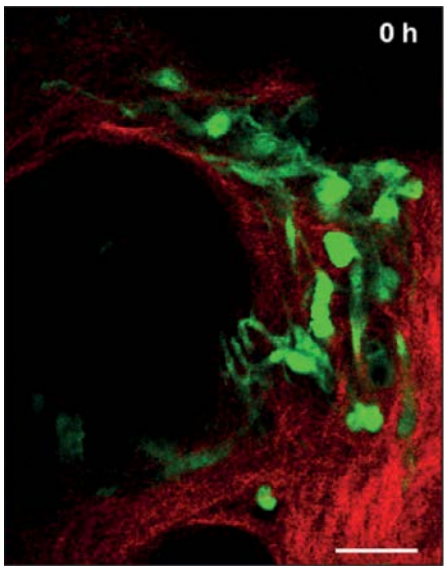
Label-free observation of proliferation, and differentiation of stem cells as well as migration behavior under natural conditions is of high interest. The use of exogenous labels may alter the physiological balance as well as the differentiation and reproduction capability of stem cells. Multiphoton imaging can overcome this problem because it is based on two-photon excitation of intrinsic fluorophores such as NAD(P)H, flavins, porphyrins, elastin, and melanin [7]. In addition, second harmonic generation (SHG) images can be obtained from certain biomolecule structures such as collagen and myosin [8–10].

High-resolution, label–free multiphoton tomographs with articulated flexible arms have become promising clinical tools to detect a variety of skin deceases at a single cell level such as melanoma and various other forms of skin cancer. They are employed in cosmetic research and skin aging measurements as well as *in situ* drug monitoring, and tissue engineering. This novel form of tomography is extremely sensitive due to single photon counting (SPC) technology and has the highest resolution of all *in vivo* tissue imaging methods [11-12].

We review here tracking of nestin-expressing stem cells in their natural physiological environment within the bulge of the hair follicle of living mice by using high-resolution *in vivo* multiphoton tomography. This method is completely non–invasive and in particular, suitable for *in vivo* long-term tracking of intratissue stem cells without any significant effect on cell metabolism, reproduction, and viability.

Keywords

Multiphoton Tomography, Stem Cells, Hair, Skin, Transgenic Mice



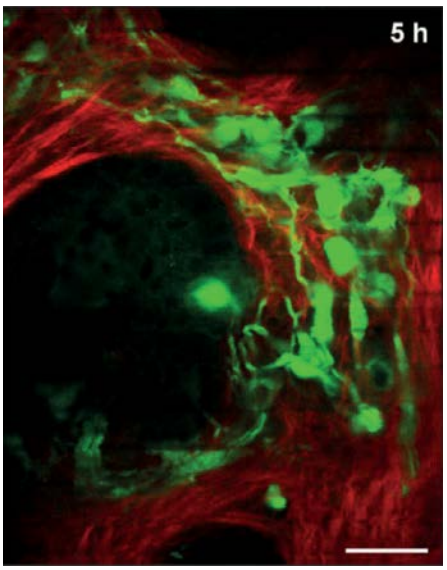


Fig. 1: Non-invasive tracking of migrating hair stem cells in a live mouse by multiphoton tomography. Time-lapsed multiphoton optical sections through intact skin. Dynamics of green fluorescent stem cells are seen initially and after 5 hours. SHG-active collagen bundles are depicted in red. Bar: 30 µm.

Imaging Tools and Animals

The multiphoton tomograph MPTflex (JenLab Jena, Germany), equipped with a sealed turn-key tunable 80 MHz titanium: sapphire femtosecond laser (710–920 nm) was used for stem cell tracking in living animals. The optical unit consists of an active optical power attenuator to regulate the *in situ* power of the laser corresponding to tissue depth, an active beam stabilization device, a safety unit and a flexible articulated mirror-arm with a compact scan head. The scan head consists of a fast galvo-scanning device to generate 2D (XY and XZ) scans, a piezodriven z-scanner, high NA focusing optics (NA 1.3) and a dual-photon detector unit for the measurement of fluorescence and SHG. The *in situ* laser pulse width was determined to be 250 fs. Optical sections can be generated as deep as 300 µm. The overall field-of-view of the optical system covers 350 x 350 µm^2. The acquisition time for one optical section is typically two seconds. Multiphoton imaging is performed at low picojoule pulse energy.

Nestin-GFP transgenic C57/B6 athymic nu/nu nude and C57/B1 mice, with green fluorescent protein (GFP) expression, driven by the nestin regulatory element (nestin-driven GFP [ND-GFP]) (AntiCancer, Inc., San Diego, CA, USA), were used for *in vivo* imaging. Mice were anesthetized by 30 µl ketamin solution 2-5 minutes before imaging and positioned under the flexible scan head of the multiphoton tomography.

Results and Discussion

Hair follicle stem cells express the neural stem-cell marker nestin [1–6, 13, 14].

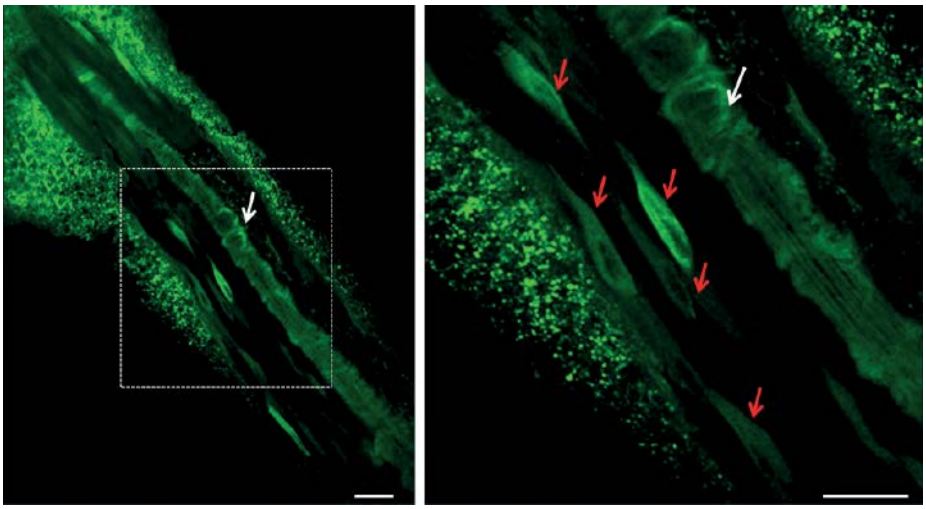


Fig. 2: Imaging of migrating stem cells (red arrows) along a hair shaft (white arrow) within an extracted whisker follicle by 3D sectioning and multiphoton imaging. Bar: 15 µm.

Therefore, nestin-driven GFP-expressing transgenic mice were used to detect stem cells in the hair follicles in their native 3D microenvironment. High-resolution multiphoton tomography was used to visualize the stem cells in their native niche [14]. The microenvironment consists of non-labeled autofluorescent cells and the extracellular matrix (ECM) components elastin and collagen as well as GFP-expressing stem cells. The cellular autofluorescence based on NAD(P)H as well as flavins/flavoproteins were detected at 750 nm. Autofluorescence and SHG can be detected simultaneously at 790 nm. Nestin-GFP was excited at 930 nm. 3D optical sectioning provided information on the cell morphology, cell size, and stem cell distribution in the tissue. Long-term imaging allowed detection of stem cell dynamics (fig. 1). Optical sections were obtained in typical z-steps of 1–2 µm down to a total depth of about 200–300 µm. Nestin-expressing stem cells were found to have a different morphology than the main skin cell population in their physiological natural micro-environment. The typical size of stem cells was found to be about 7 µm, whereas the surrounding cells had a typical size of 15 µm [14]. Often they occurred in clusters comprising of varying numbers of cells per bulge.

Stem cells migrating from the bulge have been detected inside the skin during optical deep tissue sectioning. Stem cells migrating along the hair shaft were also visualized (fig. 2).

Using multiphoton tomography, we showed the *in vivo* natural environment of stem cells. Our investigation demonstrated that hair follicle stem cells also may participate in wound healing and regeneration processes of the skin [15-16].

Currently, there are no label-free tools available to distinguish stem cells from

tissue specific functional cells. Multiphoton imaging systems equipped with precise single photon counting units and spectral modules can discriminate stem cells from their progenitors and locate them in their native niche. It was shown that fluorescent fingerprints of stem cells are naturally distinct from those differentiated ones, e.g. the ratio of NADH/flavins etc. [17-18]. Fluorescence properties such as spectrum and fluorescence lifetime alter during differentiation processes due to metabolic changes [17, 20]. This specific autofluorescence behavior of stem cells can be utilized as a biomarker.

Summary and Outlook

Multiphoton imaging provides non-invasive assessment of distribution and differentiation of stem cells with sub-cellular resolutions in their natural tissue microenvironment as it was demonstrated in hair follicles [14]. Non-destructive multiphoton sectioning can visualize long-term behavior of single stem cells and allows repeated tracking of single stem cells and cell populations over a period of hours [14]. Non-labeled stem cells and extracellular matrixes can be imaged simultaneously and without labeling due to autofluorescence and SHG signals [18]. This *in vivo* method enables the study of stem cell dynamics, interaction of stem cells within their microenvironment, and also the therapeutic benefits of stem cells. In addition to multiphoton imaging tools, CARS imaging techniques can be applied as further label-free techniques using chemical fingerprint information [21]. Near-infrared as lasers, such as for multiphoton imaging (but at higher intensities) can be employed to manipulate cells/cellular organelles with higher precision [22]. Laser-induced ablation of mesenchymal dermal papilla cells and simultaneous long-term observations have shown the importance of mesenchymal dermal papilla cells for stem cell activation and regeneration during hair growth [23]. Nestin-expression stem cells participate in nerve growth of the hair follicle and other nerve growth and are another target of visualization by multiphoton tomography [24].

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