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Imaging and Tracking of Bone Marrow-Derived Immune and Stem Cells

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Abstract

Bone marrow (BM)-derived stem and immune cells play critical roles in maintaining the health, regeneration, and repair of many tissues. Given their important functions in tissue regeneration and therapy, tracking the dynamic behaviors of BM-derived cells has been a long-standing research goal of both biologists and engineers. Because of the complex cellular-level processes involved, real-time imaging technologies that have sufficient spatial and temporal resolution to visualize them are needed. In addition, in order to track cellular dynamics, special attention is needed to account for changes in the microenvironment where the cells reside, for example, tissue contraction, stretching, development, etc. In this chapter, we introduce methods for real-time imaging and longitudinal tracking of BM-derived immune and stem cells in in vivo three-dimensional (3-D) tissue environments with an integrated optical microscope. The integrated microscope combines multiple imaging functions derived from optical coherence tomography (OCT) and multiphoton microscopy (MPM), including optical coherence microscopy (OCM), micro-vasculature imaging, two-photon excited fluorescence (TPEF), and second harmonic generation (SHG) microscopy. Short- and long-term tracking of the dynamic behavior of BM-derived cells involved in cutaneous wound healing and skin grafting in green fluorescent protein (GFP) BM-transplanted mice is demonstrated. Methods and algorithms for nonrigid registration of time-lapse images are introduced, which allows for long-term tracking of cell dynamics over several months.

Keywords

In vivo microscopy; Multimodal; Skin regeneration; Bone marrow cells

1 Introduction

Bone marrow (BM)-derived stem cells have captured increasing interest from scientists because of their important functions in tissue regeneration and repair (1, 2). In addition to the adult stem cells, such as mesenchymal (MSC) and hematopoietic (HSC) stem cells, a large variety of immune cells derived from BM have crucial roles and functions in regulating the immune system during a peripheral infection or inflammation (3, 4). Especially in skin biology, BM-derived cells not only contribute to aid skin regeneration by differentiating into keratinocytes but also are actively involved in the immune response in

the form of surveillance and inflammation (5–7). A better understanding of the functions and dynamics of BM-derived cells will necessarily foster advances in regenerative medicine and the clinical application of BM transplant-related therapy for a variety of diseases. Considering the complicated behaviors of BM-derived cells, which involve fast cellular dynamics, including differentiation, migration, and interactions with other cells and the microenvironment of natural tissue, a high-resolution real-time 3-D in vitro and in vivo imaging tool is needed to track these cellular dynamics in order to study their functions, roles, and contributions in health and disease.

Histology has been the gold standard for cell biology research. Due to its destructive nature, however, histology can only study cell structure, or potentially cell function, at a fixed time point. This end-point method is thus not suited for real-time imaging and longitudinal studies. By virtue of its high spatial resolution and noninvasive nature, optical microscopy has been conventionally used for in vitro cell tracking. Because the conventional optical microscope does not offer sufficient depth resolution, it only works well for thin samples, and cell tracking with a conventional optical microscope is primarily performed on the basis of a 2-D culturing environment, for example, in cell culture plates. Confocal microscopy is a 3-D imaging technique that can be used for imaging relatively thick living tissue, and can be operated in both fluorescence and reflectance mode (8). Due to the short wavelength of the excitation beam (normally blue or UV light), confocal microscopy can be problematic due to photodamage and is limited by a relatively short penetration depth. Fortunately, these problems can be overcome with multiphoton microscopy (MPM) which utilizes longer wavelength excitation (normally in the near-infrared) and achieves high 3-D resolution based on nonlinear optical effects, such as two-photon excited fluorescence (TPEF) (9) and second harmonic generation (SHG) (10). High penetration depths of over 1 mm have been achieved with 1.3 μm excitation light in brain tissue (11). Presently, intravital cellular imaging with TPEF is routinely performed. Optical coherence tomography (OCT) is another promising noninvasive imaging technology that is capable of 3-D visualization (12). OCT constructs depth-resolved images by using interferometric techniques to measure the time-of-flight of scattered photons (13). OCT generates images based on the light-scattering properties of samples, making it a noninvasive technique. However, OCT lacks molecular contrast which can be helpful for assessing the functional states of cells and tissues.

As any single imaging technology has specific advantages and limitations, multimodal imaging methods that provide complementary information are desirable. Various multimodal methods have been developed for real-time imaging, such as integrating MPM and OCT (14), coherent anti-Stokes Raman scattering (CARS) and OCT (15), CARS and MPM (16), and reflective confocal and MPM (17, 18), among others. In particular, the combination of optical coherence microscopy (OCM (19, 20), the high-resolution variant of OCT) and MPM has drawn considerable attention as this combination can provide co-registered structural and molecular information of the sample, and can be based on a single laser source (21–24). In this configuration, OCM provides structural information and MPM provides molecular or functional information about the sample. The two modalities are thus complementary, and enable a new method to visualize the dynamic behavior of cells with high 3-D resolution in complicated microenvironments.

This chapter will focus on imaging and tracking BM-derived cells in in vivo mouse skin with a combined MPM/OCT microscope. We show the experiments and results on short- and long-term tracking of GFP-labeled cells derived from transplanted BM, and their responses and functions in the processes of wound healing and skin grafting. The technologies described in the chapter include imaging the collagen structure with SHG, morphological changes and migration of GFP-labeled immune cells with TPEF, and structural information and microvasculature regeneration with OCT/OCM. Experimental procedures and protocols associated with the use of GFP-labeled BM transplants and skin grafts are detailed. An algorithm for image registration with non-rigid samples (such as soft tissues, including skin) is included as well, which enables long-term tracking of cellular dynamics in dynamic tissue microenvironments. The technical and experimental information detailed in this chapter will provide guidance to researchers interested in this topic and in performing related research.

1.1 Multiphoton Microscopy

MPM is a high-resolution imaging technique that is based on nonlinear optical processes, such as two-photon excitation fluorescence (TPEF) imaging (9) and second harmonic generation (SHG) imaging (10). TPEF imaging is based on the absorption of two near-infrared photons and the subsequent emission of a single photon in the visible wavelength range. TPEF can work both for exogenous and endogenous fluorophores. SHG imaging is based on the process of frequency doubling by which two near-infrared photons are converted into a single photon with exactly twice as much energy as the input photons. SHG signals arise from symmetry properties of molecules, which in biological imaging, is most often present in collagen (25).

Due to the nonlinear processes involved in MPM, a higher density of photons is required, and thus, an ultrashort-pulse laser such as a femtosecond titanium-sapphire laser is typically used in combination with a high numerical aperture (NA) objective lens. The high intensity requirement of MPM also reduces photobleaching that is often common in confocal microscopy (CM), and restricts both TPEF and SHG signals from being generated away from the focal volume. This eliminates the need for spatial filtering that is essential for CM. As well, the use of near-infrared excitation wavelengths results in increased penetration depth due to the low absorption and scattering from tissue in this wavelength range. As a result of these properties, MPM presents many advantages over many traditional optical imaging techniques for the acquisition of real-time cellular level images from biological samples (26).

1.2 Optical Coherence Tomography (OCT)

Optical Coherence Tomography (OCT) is a noninvasive scattering-based imaging technique used to obtain high-resolution cross-sectional images of tissue samples (12). OCT operates on the principle of optical ranging by which time-of-flight information from scattered photons is obtained to determine the depth from which they were scattered. This is very similar to ultrasound imaging except that OCT imaging uses optical radiation as opposed to acoustic waves. As the speed of light is much faster than can be accurately detected by direct measurement, low coherence interferometry, requiring the use of a broad bandwidth light

source, is employed to acquire the time-of-flight information from the sample. In this scheme, light is split into a sample and reference arm and light scattered from the sample is recombined with the reference light at the detector to form an interference pattern, from which the depth information in the sample is encoded. The beam in the sample arm is scanned in both transverse directions in order to obtain a full 3-D OCT volume. In OCT, the axial resolution is determined by the bandwidth of the source and is often on the order of a few microns, while the transverse resolution is determined by the size of the focused beam and is typically on the order of 10 μm .

Optical coherence microscopy (OCM) is a variant of OCT that utilizes a high NA lens to achieve higher transverse resolution (19, 20). This, however, comes at the expense of a shorter depth-of-field due to the confocal gating of the high NA lens, allowing *en face* planes to be obtained instead of a depth-resolved cross-sectional image as in OCT. The confocal gating provided by the high NA lens combined with the coherence gating provided by the low coherence interferometry used in OCM provides a higher rejection of out-of-focus photons when compared with reflectance confocal microscopy, thus allowing for significantly deeper imaging depths in highly scattering tissues (20).

In addition to measuring scattering signals in tissue, OCT can also be used to visualize the microvasculature in living tissue. This is typically performed by measuring the time-dependent changes in the scattering signal from the sample (27–29). The signal inside blood vessels will be changing over time, and at a much higher rate than outside of the vessels where the scatterers are relatively static. One such method used to visualize the microvasculature in live tissue is known as phase-variance OCT (30, 31). Phase-variance methods are based on making several measurements at a given location and computing the changes in the signal over time. In areas of greater change, a higher signal will be generated. Phase-variance methods are of particular use in assessing the state of skin as it undergoes repair, allowing the microenvironment with which BM-derived cells interact to be better visualized and understood, particularly in skin grafting and wound healing studies.

1.3 Time-Lapse Imaging of Nonrigid Tissue

For long-term time-lapse imaging in skin, registration of consecutive images is critical for tracking bone marrow-derived cells. A challenge is presented when the sample to be imaged undergoes significant mechanical deformations. This can occur in normal daily variations of skin position, but most problematically in situations such as wounding or grafting of the skin, where these deformations cannot be accounted for by traditional linear registration techniques. Instead, nonrigid image registration methods may be employed to better account for these deformations (32). Nonrigid registration algorithms utilize physical landmarks that present themselves with unique spatial relationships that are preserved between imaging sessions. Using these spatial relationships, a transformation grid can be applied to align images at different time points by nonrigid warping of the image. In skin, this method can easily be implemented by using the SHG signal from collagen. By exploiting the spatial relationships between adjacent hair follicles, improved image registration can be readily obtained through nonrigid transformation in wound healing and skin grafting studies.

1.4 GFP Bone Marrow-Derived Cells and Their Function in Regeneration of Skin

BM contains stem and immune cells which are known to play key roles in regeneration and repair of many tissue sites (1, 2). BM stem and progenitor cells consist of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs have the potential to differentiate into all blood cell lineages and reconstitute the entire hematopoietic and immune systems following transplantation in vivo. MSCs can differentiate into osteocytes, adipocytes, chondrocytes, hepatocytes, myocytes, cardiomyocytes, neurons, and keratinocytes as well. In addition to these predominant stem cells, BM-derived cells have been found to be actively involved in immune processes (3, 4, 33). In skin regeneration, BM-derived cells contribute not only by differentiating into keratinocytes but also by immune surveillance and inflammation, functions which are commonly found in skin wound healing and following skin grafting (5–7).

Since the most efficient and widely used intravital microscopy techniques are based on fluorescence, we used BM from GFP-expressing mice. GFP is a very important in vivo biomarker for cellular imaging that fluoresces at the peak of 509 nm when excited with blue or UV light (34, 35). GFP has a large two-photon absorption coefficient, which makes it well suited for cellular imaging with MPM. GFP can be readily transfected into a variety of cell types, and transgenic mice can express GFP constitutively in all of its cells. Therefore, a wild-type host mouse with transplanted BM from a GFP-transfected mouse is a good model for tracking the dynamics of different types of cells originating from the transplanted BM and expressing GFP, all within a non-GFP-expressing tissue environment of a wild-type mouse.

2 Materials

2.1 Wild Type Mice (Recipients)

6–10-week-old female wild-type C57BL/6 mice.

2.2 GFP Transfected Mice (Donor)

Male transgenic mice with global GFP expression (C57B/6-Tg (CAG-EGFP) 10sb/J).

2.3 Materials and Tools for BM Transplant and Skin Wounding

1. Isoflurane anesthesia gas source.
2. Oxygen source.
3. 70 % ethanol.
4. Sterile phosphate buffered saline (PBS).
5. ACK Lysis buffer.
6. DMEM solution.
7. 100 × 20 mm plastic petri dishes.
8. Mouse nose cone.

9. 10 % povidone/iodine.
10. Sterile gauze pads.
11. 3/4 in. sheer Band-Aid-type sheer bandage (CVS, Curad).
12. Microdissecting forceps (4 in., half-curved, serrated; 5 in., straight, serrated or non-serrated).
13. Microdissecting scissors (4½ in., curved, with very sharp points; 4 in., straight).
14. Watchmaker's or jeweler's forceps (4½ in., 0.17 mm wide, 0.10 mm thick; Dumont no. 7).
15. Scalpel blades (Bard Parker no. 10).
16. 10 ml syringes (Luer Lock).
17. 263/8 G needles.
18. 40 µm filter paper.
19. Stereo microscope.

2.4 Integrated Microscope

The schematic of the integrated OCM/MPM microscope is shown in Fig. 1. This system combines OCT (including the variant functions of OCM and phase variance detection for microvasculature imaging) and MPM (including SHG and TPEF). The multiple imaging modalities are integrated into a single microscope frame, and are thus able to generate spatially co-registered multimodal images.

1. The source of the integrated microscope is based on a single laser but with a specially designed dual-spectrum configuration (36). This design is advantageous in that the single laser enables a more robust and reliable system, while the separately controlled dual-band configuration allows for optimal performance of both modalities (OCM/MPM). The laser is a high-power, widely tunable titanium:sapphire laser (Mai-Tai HP, Spectra-Physics) which outputs 100 fs pulses with a bandwidth of 10 nm at a center wavelength tunable within the range of 730–1000 nm. The linearly polarized output (with maximum average power of 3 W) from this laser is divided by a 90/10 beam-splitter into two beams. The higher power beam is coupled by a 0.4 NA aspheric lens into a 0.5 m long photonic crystal fiber with a NA of 0.1 and a mode field diameter of 7 µm (LMA-8, Crystal Fiber A/S) to generate super-continuum (SC). This spectrally broadened beam with a bandwidth up to 150 nm is collimated by an objective, and used as the OCT/OCM source. The other low energy beam which has a narrow bandwidth but large wavelength tuning range functions as the excitation light for MPM. The power of the MPM and OCM sources can be independently controlled by a set of neutral density filters.
2. For OCM, the beam is split by a 50/50 beam splitter into the reference and sample arms of a Michelson interferometer. Linearly polarized light in the OCM sample arm is rotated 90° by an achromatic half-wave plate and then recombined with the

narrowband MPM excitation beam at the polarizing beam splitter. The two collinearly aligned beams are expanded by a telescope and focused by a microscope objective (20x, 0.95 NA, water immersion, Olympus, Inc.) onto the sample. The objective can also be changed to a low NA objective or lens for OCT imaging with a larger field-of-view. The sample is positioned on a motorized stage which can translate the sample in three directions (x,y,z). A pair of galvanometers (Micromax 671, Cambridge Technology) positioned before the telescope scan the beam across the sample.

3. For OCT/OCM, the reference arm light is reflected by a plane mirror mounted on a translation stage. The spectral interference pattern of the reference and sample arm beams is detected for OCM acquisition by a spectrometer which is based on a diffraction grating and CCD line camera (P2-22-02k40, Dalsa). The frame rate for the line camera depends on the speed and mode of image acquisition (galvanometer vs. stage) and can be up to 35 kHz. OCT/OCM images are generated after several processing steps including compensating for unbalanced dispersion in the sample arm and for nonuniform distribution of the spectrum on the CCD due to nonlinearity of the diffraction grating. The epi-collected MPM fluorescence signal is diverted by a long-pass dichroic mirror and band-pass filtered. This filter is easily interchanged to detect various fluorescence or second-harmonic generation signals. The fluorescence signals of different spectral band are detected separately by two photomultiplier tubes (H7421, Hamamatsu). Control of the integrated system, as well as image formation and display, is managed through a Labview interface. For large field-of-view OCM/MPM images, mosaic image acquisition is performed by translation of the motorized stage between single images.

3 Methods

3.1 Bone Marrow Transplant

Bone marrow harvested from donor male mice with global GFP expression (C57BL/6-Tg (CAG-EGFP) 10sb/J) (37) is transplanted to female wild-type recipient mice (C57B/6). These GFP BM-transplanted mice are informative animal models for investigating the dynamic behavior of BM-derived cells, which express GFP, and can be clearly visualized and differentiated with MPM. Radiate 6–10 week female wild-type recipient mice (C57B/6) with up to 2 doses of 6 GY (administered 4 h apart).

1. Sacrifice donor male mice with global GFP expression (C57BL/6-Tg (CAG-EGFP) 10sb/J) via CO₂ inhalation.
2. Dissect and clean hind limb bones, and place them in phosphate buffered saline (PBS).
3. Crush dissected bones with mortar and pestle.
4. Strain the crushed solution with 40 μ m filter paper and lyse red blood cells with an ACK lysing buffer.
5. Count and dilute cell concentration to approximately 7×10^6 cells/ml. Keep the solution on ice prior to transplant.

6. Transplant the prepared bone marrow cells (150 μ l, 10^6 cells) into irradiated wild-type mice by tail vein injection.

3.2 In Vivo Multimodal Skin Imaging of GFP BM-Transplanted Mice

In vivo skin imaging with the integrated multimodal microscope is performed in GFP bone marrow transplanted mice.

1. A 6–10-week-old wild-type mouse is anesthetized with isoflurane gas and placed on a heating pad.
2. Shave the area of skin to be imaged with an electric clipper. Remove any residual hair using surgical scissors and a stereo microscope. Try to remove as much hair as possible without injuring the skin (Note 1).
3. Position the anesthetized mouse on the motorized stage of the multimodal microscope and hold it in place by gently clamping the skin. The skin site to be imaged will be pressed against a coverslip.
4. Apply drops of glycerol to the skin as an index-matching agent.
5. MPM and OCT imaging of the skin is performed through the coverslip. Multimodal images of wounded mouse ear skin are shown in Fig. 2.
6. OCT images with a large field-of-view are obtained by using a low NA objective (Fig. 2a). OCT images show the structural morphology of skin tissue with a larger penetration depth, since OCT is based on the light-scattering properties of the sample.
7. TPEF images (Fig. 2b) will show the locations of BM-derived GFP cells.
8. SHG images (Fig. 2c) will show the collagen distribution in the dermis.
9. Images from different modalities can be overlaid (Fig. 2d), which shows the spatial co-registration of all the images. Complementary information is present from the overlaid image. A cross-sectional image extracted from the overlaid volumetric dataset shows the depth-dependent information and the sample properties obtained from the different modalities.
10. High-resolution OCM images can be obtained using a high NA objective (Fig. 3a).
11. Zoomed-in views of the TPEF images (Fig. 3b) show the detailed morphology of BM-derived GFP cells.
12. Overlaid OCM and TPEF images (Fig. 3c) show the positions of the GFP cells in 2-D.
13. 3-D orthoslices of the volumetric OCM-TPEF (Fig. 3d) overlays reveal the locations of GFP cells in the 3-D background of the skin structure.

¹The two-photon-generated autofluorescence background from hair shafts is subtracted from volumetric TPEF data using a segmentation procedure. This was achieved by applying a threshold to the volume, identifying isolated features, and assessing them based on their size, aspect ratio, and length. The inverse of the resulting binary mask was multiplied with the original image volume to remove the hair shafts. Although this method does not account for weaker autofluorescent features, it enables the removal of the largest features which can affect the interpretation of the images.

3.3 Skin Wounding

A skin wound is made on a GFP BM-transplanted mouse for real-time imaging and tracking of the dynamics and responses of BM-derived cells in wound healing processes.

1. Anesthetize the BM-transplanted mouse with isoflurane gas.
2. Shave the skin (either dorsal or ear) with electric clippers.
3. Carefully remove remaining hair from the region to be imaged using surgical scissors and a stereo microscope.
4. Wound the skin site to be imaged by taking either a 1 mm diameter punch biopsy or making a 1 mm long linear incision manually with a fine-tipped scalpel.
5. Wounding and imaging experiments preferably should be performed at least 2 months after the BM transplant, when complete marrow engraftment and bone marrow cell production has resumed.

3.4 Nonrigid Co-registration Algorithm

Natural skin is a highly flexible organ which experiences significant mechanical distortion during daily activities as well as contraction during wound healing. In this case, a nonrigid image registration process must be introduced to account for the nonrigid changes during wound healing in order to track the same skin site over several months. This nonrigid registration process also enables one to separate the small-scale movements and dynamics of single cells from the large-scale changes due to tissue deformation. The nonrigid registration algorithm in this method is based on using the locations of hair follicle as landmarks. The registration procedure consists of detecting hair follicle positions, initializing a follicle matching algorithm, performing a grid transformation, and iteratively optimizing the solution. The different steps of the algorithm are illustrated in Fig. 4.

1. Choose two *en face* images from two volumetric skin image datasets that were acquired from two time points, and are to be registered (Fig. 5a and b). The images can be either SHG or OCM structural images that have good contrast between hair follicles and dermis (Note 2).
2. Filter the images with a difference-of-Gaussian (DOG) kernel at different scales. Local maxima in the DOG image are detected following the application of a threshold. These maximum positions are considered as the center locations of the follicles (Note 3).
3. Evaluate the match quality of each hair follicle in one image to each follicle in the other image to be co-registered. This is done by selecting a fixed number of neighboring follicles (typically 10–15) and calculating the matching quality

²While the registration is performed in two dimensions, it is possible to correct the axial dimension as well by computationally shifting the SHG volumes along the axial dimensions to make the surface of the dermis flat.

³Local minima in the scale-space are selected as possible feature points. The initial set of possible hair follicle points that is found is very high and contains many incorrect matches. Points are eliminated based on thresholding the DOG images and an additional metric that evaluates the radial symmetry of the local image gradients, resulting in a more accurate estimate of the follicle positions. This process is performed on SHG images from multiple depths, and matches of the same follicle at different depths are merged to give the final estimate of the hair follicle locations.

between the two sets of neighboring points after local rotation and scaling operations. This method assumes that the local deformation within the range of the neighboring points can be approximated by a rigid transformation.

4. Determine matched follicles that have a higher match quality than a given threshold. Use the positions of those matched follicles to define an initial transformation between the two images.
5. Match remaining follicles by an iterative procedure that adds new follicle matches to the transformation model each time a match is determined.
6. Interpolate the transformation function to determine a non-rigid warping transformation matrix and 2-D warping grid (Fig. 5d).
7. Warp the original image based on the warping grid to obtain a registered image (Fig. 5c).

3.5 Time-Lapse Imaging and Tracking of BM Cells in Wound Healing

The combination of multiple imaging modalities allows in vivo visualization of BM-derived cell dynamics and their function in wound healing processes. In addition to the short-term cell migration dynamics, the nonrigid image registration enables fundamental dynamics of BM-derived cells to be tracked over a period of several months.

1. A full-thickness excisional wound is made in the dorsal skin of a GFP BM-transplanted mouse, following the protocols of Section 3.3.
2. Following the wound, MPM images are acquired at several time points over 45 minutes on the first day of wounding (Fig. 6) to reveal the short-term dynamic behavior of BM-derived cells.
3. SHG images provide the structural information of the skin environment around the wound site as well as provide guidance for image registration to track cell dynamics.
4. Time-lapse images reveal dynamics of a cluster of Langerhans cells in the vicinity of wound site within 24 h (Fig. 6). These cells are identified on the basis of their unique morphology and location within the epidermis of the skin (Note 4) (38).
5. High-magnification TPEF images (Fig. 6) show the detailed morphology of the GFP BM-derived cells. The cells transform their morphology from one having many extensions for sensing the extracellular environment for antigens, to one of an amorphous shape suitable for migration. A magnified view of the cell cluster

⁴Langerhans cells (LC) are a class of dendritic cells whose primary function is to sense the extracellular environment for the presence of antigens. These cells reside in the epidermis of the skin and are typically immobile under steady-state conditions. Upon detection and uptake of antigens, these cells migrate to the locoregional lymph nodes to present antigens to other cells in the immune system as part of the adaptive immune response. Under steady-state conditions, LCs have a very distinct morphology which consists of dendrites that probe the local micro-environment for antigens. Upon activation, these dendritic processes are retracted, resulting in an amorphous morphology that enables migration of the LCs to the lymph nodes. Understanding the functional role of LCs is an active area of research. While these cells were initially thought to play a role in initiating immune responses in reaction to foreign antigens, recent studies have suggested their primary function may be to support immune tolerance (41). The time-lapse imaging shows that a large number of these cells undergo a reorganization of their morphology over a 45 minutes time period and migrate from their initial locations. This is, to our knowledge, the first in vivo time-lapse imaging of the activation of individual Langerhans cells.

shows the morphological change and the eventual disappearance of many Langerhans cells in response to the wound. This behavior is consistent with the known function of Langerhans cells and suggests that many of the cells have migrated from the skin wound site to the locoregional lymph nodes.

6. Following the short-term (24 h) imaging sequence, OCM/MPM images of the wound site are acquired every 24 h for 2 weeks (Fig. 7).
7. In the GFP/SHG overlays (Fig. 7a–e), the GFP fluorescence channel shows the dynamic changes in the BM-derived cells. The most notable effect is the large increase in the number of GFP cells around the wound site on day 2 (Fig. 7b). Based on the proximity of these large cell clusters around the edge of the wound and the timing of their arrival and disappearance, it is likely that these cells are involved in the natural inflammatory and immune responses.
8. Based on the SHG and OCM signals, the wound site is initially visible as a round hole and the wound gradually heals over 2 weeks (Fig. 7j), as shown by the OCM images. However, the SHG signal in the center of the wound fails to reappear by the end of the 2 week period (Fig. 7e), and the scattering signal (shown by the OCM images) from the wound remains different than the surrounding areas. These results suggest that while the wounded skin has healed, the wound is still lacking the organized collagen found in normal skin.

3.6 Skin Graft

Skin grafts from wild-type donors to GFP BM-transplanted recipient mice are performed following a similar protocol described in ref. (39).

1. Sacrifice a wild-type C57BL/6 donor mouse by CO₂ inhalation.
2. Drench the entire mouse with 70 % ethanol.
3. Dissect and clean ears, and place them in PBS.
4. Surgically remove a 7 × 7 mm² area of donor ventral ear skin and place it in cold PBS. The harvested skin will consist of both the epidermis and dermis of the skin, but not the underlying cartilage.
5. Anesthetize the GFP BM-transplanted recipient mouse with isoflurane gas. Skin grafting should be performed at least 8 weeks after BM transplant.
6. Shave the host skin site for the graft bed (either back or ear) with electric clippers.
7. Surgically remove a 7 × 7 mm² piece of ventral skin from the prepared site on the host using forceps and dissecting scissors (Note 5).

⁵Using watchmaker's forceps, pinch the skin where the topmost horizontal cut will be made, which is at the top of the scapula, just lateral to the spine. Rest the blades of the dissecting scissors flat on top of the pinched skin and cut skin as superficially as possible. Take care not to angle the scissors downward when cutting or the panniculus carnosus will be cut. If the panniculus is accidentally removed, the skin will not engraft. If the cut is not sufficiently deep to reach the panniculus, repinch the skin and carefully cut to the level of the panniculus. There should be no white or pigmented dermal tissue present in the cuts, as this will interfere with removal of the intact skin. Complete a square with four cuts. Using watchmaker's forceps, grasp a corner of the square skin graft and gently pull it off the skin bed.

8. Immediately place the skin graft from the donor onto the prepared graft bed of the host.
9. Cover the graft site with petrolatum gauze and wrap the mouse with a strip of elastic bandage.
10. Monitor the integrity of the bandage at least daily, and remove the bandage following 7 days of recovery.

3.7 Time-Lapse Imaging of BM Cells After Skin Grafting

Multimodal images of the skin graft are acquired at different time points after the removal of the bandage. Because the skin graft is from a wild-type mouse, any GFP cells present within the graft area are from host BM-derived cells and their dynamic behavior will be visualized by the TPEF images (40).

1. SHG images are acquired from the graft site, which provide the structural information of the skin environment of the graft as well as provide guidance for image registration to track the cell dynamics (Fig. 8a).
2. TPEF images are acquired, which show the morphology and dynamics of BM-derived GFP cells (Fig. 8b).
3. SHG and TPEF images are overlaid to show the position and dynamics of the BM-derived GFP cells. The presence of clusters of GFP cells in the epidermis of the grafted skin is observed (Fig. 8c). The isolated nature of this cluster suggests that a local precursor cell was derived from the BM and then proliferated to form this cluster of cells. Based on the morphology of these cells, it is likely that they are Langerhans cells.

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Fig. 1. Schematic of the integrated optical microscope. Abbreviations: *BS* beam splitter; *C* collimator, *CCD* charge-coupled device line-scan camera, *CL* coupling lens, *DC* dichroic mirror, *DG* diffraction grating, *F* filter, *GS* galvanometer scanner, *HWP* half-wave plate, *L* lens, *O* objective, *M* high reflection mirror, *PCF* photonic crystal fiber, *PBS* polarizing beam splitter, *PH* pinhole, *PMT* photo-multiplier tube, *RM* reference mirror, *S* sample, *TL* telescope lens (24)

Fig. 2.

Multimodal imaging of ear skin in a GFP BM-transplanted mouse 30 days following an excisional wound. (a) *En face* structural OCT section showing individual hair follicles. (b) Wide-area TPEF mosaic of the GFP expressing BM-derived cells in the skin. (c) Wide-area SHG mosaic of collagen. The central dark region represents the wound while the hair follicles appear as smaller dark regions. (d) *En face* image of the three modalities overlaid. Scale bar is 500 μm

Fig. 3.

(a) OCM and (b) TPEF images of skin from a GFP BM-transplanted mouse. A hair follicle is visible in the OCM image while TPEF visualizes individual GFP cells in addition to autofluorescence from hairs. (c) The overlay of the OCM and TPEF images allows the tissue microenvironment of the GFP cells to be seen. (d) 3-D orthoslices of the volumetric OCM-TPEF overlays

Fig. 4.
Block diagram of the nonrigid image registration algorithm

Fig. 5. SHG images of mouse skin on (a) day 1 and (b) day 2 after wounding. (c) Warped image after the nonrigid image registration process. (d) Warping grid of image shown in (c)

Fig. 6.

Time-lapse imaging of dendritic cells in vivo, showing their activation over 45 minutes following a cutaneous wound. Magnified view of the cell cluster indicated by the *red box* in the wide-area SHG and TPEF images, showing the morphological change and migration of a dendritic cell in response to the wound. This behavior is consistent with the known function of Langerhans cells and suggests that cells may be in the process of migrating from the skin to the lymph nodes. Scale bars are approximately 100 μm

Fig. 7. Three-dimensional (a–e) MPM fluorescence images overlaid on SHG and (f–j) OCM renderings of the wound site at multiple time points during healing. In (a–e), the GFP is represented by the *green channel* and the SHG data is represented in *grey scale*

Fig. 8.

Axial projections of in vivo volumetric SHG and TPEF images of grafted skin in GFP bone marrow transplanted mice. **(a)** The SHG projection visualizes the collagen network while **(b)** the TPEF projection visualizes the bone marrow-derived cells present throughout the depth of the skin. **(c)** An overlaid projection with high SHG opacity allows the GFP cells in the epidermis to be visualized while masking the GFP cells in the dermis. The presence of a focal cluster of epidermal GFP cells is apparent in the skin graft **(c)**. Scale bar is 300 μm .

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