Coregistered Spectral Optical Coherence Tomography and Two-Photon Microscopy for Multimodal Near-Instantaneous Deep-Tissue Imaging

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Abstract

Two-photon microscopy (2PM) has brought unique insight into the mechanisms underlying immune system dynamics and function since it enables monitoring of cellular motility and communication in complex systems within their genuine environment—the living organism. However, use of 2PM in clinical settings is limited. In contrast, optical coherence tomography (OCT), a noninvasive label-free diagnostic imaging method, which allows monitoring morphologic changes of large tissue regions in vivo, has found broad application in the clinic. Here we developed a combined multimodal technology to achieve near-instantaneous coregistered OCT, 2PM, and second harmonic generation (SHG) imaging over large volumes (up to $1,000 \times 1,000 \times 300 \mu m^3$) of tendons and other tissue compartments in mouse paws, as well as in mouse lymph nodes, spleens, and femurs. Using our multimodal imaging approach, we found differences in macrophage cell shape and motility behavior depending on whether they are located in tendons or in the surrounding tissue compartments of the mouse paw. The cellular shape of tissue-resident macrophages, indicative for their role in tissue, correlated with the supramolecular organization of collagen as revealed by SHG and OCT. Hence, the here-presented approach of coregistered OCT, 2PM, and second harmonic generation has the potential to link specific cellular phenotypes and functions (as revealed by 2PM) to tissue morphology (as highlighted by OCT) and thus, to build a bridge between basic research knowledge and clinical observations. © 2020 The Authors. Cytometry Part A published by Wiley Periodicals, Inc. on behalf of International Society for Advancement of Cytometry.

Key terms

two-photon microscopy; in vivo imaging; spectral domain optical coherence tomography; second harmonics generation

In the last three decades, two-photon microscopy (2PM) has proven to be the method of choice to perform deep-tissue imaging in tissue slices, explanted organs and in living animals (1,2). Especially in immunology, 2PM has brought unique insights into cellular motility patterns, cellular communication, and tissue functions, in health and disease, and helped to decipher key pathophysiologic phenomena involving the immune system (3–7). In order to acquire information on immune cell subsets from 2PM, fluorescent labeling of cells within organisms, either using viable dyes or using fluorescent reporter constructs, is needed. The endogenous tissue fluorescence ( autofluorescence), while providing valuable information about the tissue state (5,6,8,9), is not specific enough to reveal the orchestration of phenomena governing organ and tissue functions on a cellular level. Additionally, 2PM requires complex devices such as femtosecond-pulsed lasers and dedicated optomechanics.
and optoelectronics. Hence, this technology is broadly used in research, but not in the clinic.

Optical coherence tomography (OCT), also developed three decades ago, is a true tomographic high-resolution imaging modality based on the interference of a reference beam and a sample beam that has been reflected by various layers within the tissue (10–15). The technology provides image contrast by relying on changes in refractive index within the sample, that is, on the various scattering properties of the tissue. Its depth resolution, that is, axial resolution, is independent of the numerical aperture (NA) and is determined only by the spectral properties of the illumination source. Whereas time-domain OCT proved to be rather slow, with the advent of frequency-domain OCT, especially of spectral-domain OCT, acquisition speed has increased and the technology now finds broad application both in clinical diagnosis and in research (15,16). Spectral domain OCT measures the interference depth profile in each pixel (A-scan). When the reference beam is scanned along a line over the sample, an interference depth profile of the line is acquired (B-scan). A series of B-scans results in a three-dimensional (3D) volumetric image of the sample. OCT was mostly employed at low NA values (~0.1) and, thus, low lateral resolutions (~5 μm) (11). Due to a low signal-to-noise ratio, OCT cannot typically resolve cellular structures in tissue but can only distinguish between various tissue layers and compartments. Prototype OCT devices with higher NA (0.5), that is, optical coherence microscopy, have been designed; however, due to its limited field of view, this technology has not yet found broad application in either the biosciences or biomedicine, nor in the clinic (13). Taken together, the main limitation of current OCT technology is the fact that it retrieves information on organ morphology at the level of tissue compartments, but not at the cellular level. Moreover, OCT also cannot provide information on tissue or cellular function with the specificity of other methods such as 2PM.

In general, the radiation used by optical methods always presents a danger for the sample. For this reason, imaging of organs and organisms requires a precise control of sample illumination to minimize phototoxicity, that is, to avoid cellular and tissue damage. Due to the nonlinear excitation of femtosecond pulsed lasers, the illumination in 2PM affects only a thin layer of tissue located at the focal plane. By controlling the laser power in the range of few tens of milliwatts, photodamage is typically reduced to a negligible level for mammalian tissues (17–19). Based on elastic reflection and interference, OCT typically employs low coherence light sources at extremely low powers (in the range of microwatts) (14). Thus, OCT causes practically no damage to the sample and is particularly well suited for in vivo imaging, even of photosensitive tissues such as the retina.

Thus, both 2PM and optical coherence technologies have particularly complementary strengths: 2PM provides information with high specificity on morphology and tissue function at the cellular and subcellular level, but is difficult to utilize in the clinic on a daily basis. On the other hand, while OCT (with its lower resolution than that achieved by 2PM) provides information solely on tissue morphology, it has already been successfully implemented both in research setups and in the clinic. Hence, a coregistered, simultaneous combination of OCT and 2PM would allow translation of the inherent molecular specificity (and thus functional information) of 2PM to clinical studies by establishing a link between functional and morphologic information in animals. Based upon the fact that OCT enables imaging of tissue compartmentalization over larger volumes than 2PM does, we expect that, in the research context, the combination of these technologies will add comprehensive and valuable information on tissue organization to the specific information on cellular and tissue functions.

Various prototype setups combining 2PM and OCT (20–25), and these combined with second harmonic generation (SHG) (26–28) or with fluorescence lifetime imaging (29,30), have previously been proposed. Combinations of OCT and 2PM based on microendoscopy have also expanded the range of possible applications (31–35), including cancer research. However, near-instantaneous coregistered OCT, 2PM, and SHG imaging (i.e., fast switch between the imaging modes, in the range of seconds to milliseconds) of one and the same tissue region, in the range of hundreds to thousands of micrometers, has not been developed nor applied yet to live tissues.

In the clinic, OCT already found broad application in ophthalmology and dermatology, and more recently, in neurology (36) and cancer research (37). Due to an emerging interest in using OCT for the clinical diagnosis of tissue degeneration in rheumatology and immunology (38,39), the application of such a multimodal imaging technology in joints, secondary lymphoid organs or the marrow of long bones is of central relevance.
In this work, we integrated a spectral domain OCT module into a customized two-photon microscope allowing for dynamic and functional high-resolution multimodal imaging of large tissue volumes (up to $1,000 \times 1,000 \times 300 \, \mu m^3$), through the same objective lens, both intravitally and in explanted organs. The main advantages of our approach follow from the below mentioned facts: (1) imaging of exactly the same large volume in the sample is performed by both 2PM and OCT; (2) switching between OCT and 2PM modes takes place fast, within milliseconds, resulting in near-instantaneous multimodal imaging; (3) with both OCT and 2PM, cellular or even subcellular resolution is achieved; (4) both OCT and 2PM acquisition rates of large tissue volumes ($1,000 \times 1,000 \times 62 \, \mu m^3$) are sufficient to track motile immune cells in tissues.

We applied our near-instantaneous coregistered OCT and 2PM imaging approach to study the dynamics of CX3CR1$^+$ cells, which includes tissue-resident macrophages, in the tendons and other surrounding tissue compartments in the paws of CX3CR1:eGFP mice. Exploiting this unique, multimodal imaging setup in vivo, we found a strong interdependency between macrophage morphology and motility patterns, on one side, and the tissue architecture, on the other side. Tissue-resident macrophages play a crucial role in the pathogenesis of rheumatic arthritis and other chronic inflammatory diseases of the joints (40). Based on the ability of OCT to highlight modifications in tissue architecture in patients, we expect that the correlative findings provided by our coregistered imaging approach will provide the basis for a better interpretation of clinical diagnosis, to identify tissue degeneration or to evaluate responses to therapies.

**RESULTS**

**Combined Two-Photon Laser Scanning Microscopy and OCT for Near-Instantaneous Coregistered Multimodal Imaging**

We extended our customized 2PM setup with a spectral domain OCT module to perform coregistered multimodal two-photon excitation fluorescence, second harmonic, and OCT imaging (Fig. 1A). Our 2PM setup allows for multiplexed optical nonlinear imaging, that is, up to four spectrally resolved fluorescence signals and SHG. The spectral domain OCT module is based on a system (Ganymede II, Thorlabs, Dachau, Germany) that uses a super-luminescence light source (SLD, continuous wave laser diode, 930 nm). The coherence length of the light source, which corresponds to the supported axial resolution, is $2.1 \, \mu m$ in water or tissue and $3 \, \mu m$ in air. The OCT beam is split into two by a 50/50 polarizer beam splitter (BS). One beam is directed to the microscope, where it is deflected over the sample using the same galvonometric scan mirrors (GS) as those used for 2PM. Also focusing into the sample is performed using the same multi-immersion objective lens (Apochromat, 10x, NA 0.6, Olympus, Hamburg, Germany) used for 2PM (Fig. 1A). The back aperture of the objective lens is not completely illuminated to avoid the formation of side-lobes in the point spread function and to increase the depth range of optimal signal. This affects the lateral but not the axial resolution of OCT, since the axial resolution depends only on the coherence length of the light source. The second OCT beam is directed into the reference arm. This beam arm is reflected back to the BS by a corner cube (a retroreflector prism) which is computer-controlled to move back and forth around an optical length nearly equal to the sample arm. Sample and reference beams are brought to interfere, spectrally dispersed by a holographic volume lattice and detected by a CMOS line-scan imager with a line length of 2,408 pixel and an acquisition rate of 110 kHz (Piranha 4, Teledyne Dalsa, Waterloo, Canada). The switch between the OCT and 2PM modes occurs automatically and within 30 ms by changing the galvonometric mirror M between two fixed positions (Fig. 1A). This allows near-instantaneous, sequential OCT, and 2PM acquisition of the same volume within the sample.

The two-photon microscope uses either a femtosecond-pulsed TiSa laser (Chameleon Ultra II, Coherent, Glasgow, UK), or alternatively, an optical parametric oscillator (OPO Compact, APE, Berlin, Germany) for excitation. The TiSa and OPO laser beams are scanned and focused into the sample by the same galvonometric mirrors (GS) and objective lens as the OCT sample beam (Fig. 1A). The fluorescence and SHG signal emitted by the sample are spectrally separated by a system of dichroic mirrors and interference filters and detected by photomultiplier tubes (PMTs).

We typically use OCT laser powers of 0.8 mW at 930 nm. For two-photon imaging, we did not exceed 10 mW average TiSa laser power, corresponding to a photon flux of $4.65 \times 10^{30} \text{photons}/(\text{cm}^2 \text{s})$ at 930 nm, to avoid sample photodamage.

Dispersion effects in OCT are corrected by a compensation glass block in the beam path of the reference arm (Fig. 1A). Hence, we could retrieve the expected thickness of several stacked $140 \, \mu m$ thick coverslips. From B-scans of the coverslip stack (Fig. 1B), we determined an average coverslip thickness of $139.5 \, \mu m$ down to an imaging depth of $440 \, \mu m$, with an axial resolution of $2.7 \, \mu m$. In order to acquire maximum information from the OCT data, we repeatedly acquired B-scans at different focal depths and chose the focal depth for which the OCT signal was detectable deepest in tissue for further data acquisition.

The spatial resolution in 2PM is governed by diffraction, being deteriorated by Rayleigh scattering and wave-front distortions in tissue. In our case, the 2PM resolution amounts to $680 \, \mu m$ laterally and $3.9 \, \mu m$ axially, as measured on $200 \, \mu m$ fluorescent beads ($605 \, \mu m$ emission wavelength) excited at 930 nm (19,41). The lateral resolution of OCT is slightly lower than that of 2PM due to the underfilled back aperture of the objective lens and is deteriorated by scattering and wave-front distortions in tissue as well. Axially we measured a resolution of $2.7 \, \mu m$ by OCT, which is in good agreement with the detected bandwidth of the interference spectra.

Using our setup, we performed multimodal 2PM and OCT imaging in the paw of CX3CR1: eGFP mice, in which myeloid cells expressing the fractalkine receptor CX3CR1 are genetically marked by green fluorescent protein (GFP). In xy-images acquired in $220 \, \mu m$ tissue depth, we detected structures as...
small as 5 μm by both 2PM (based on GFP fluorescence) and OCT (Fig. 1C and insets [i] and [ii] in Fig. 1C). In axial direction, that is, along the optical axis of the microscope, we measured structures as small as 9 μm by 2PM and 4 μm by OCT (Fig. 1D and insets [i] and [ii] in Fig. 1D). Thus, we confirmed that the spatial resolution in both 2PM and OCT mode reaches at least a cellular level, sufficient for the analysis of immune cells, since typically, a hematopoietic cell is ≥10 μm in diameter.

As both OCT and two-photon laser beams are scanned over the sample by the same galvanometer scanner and are

Figure 1. Legend on next page.
focused into the sample by the same objective lens, we are able to colocalize the resulting 3D images of both modes over the entire imaging volume, that is, up to $1,000 \times 1,000 \times 300 \, \mu m^3$ ($1,010 \times 1,010 \times 150 \, \text{voxel}$), with voxel accuracy. However, due to the different beam paths of OCT and Ti:Sa laser, respectively, the OCT and 2PM images are slightly shifted. We determined a shift in $x$ direction of 90 $\mu m$ and in $y$ direction of 14 $\mu m$ (Fig. 1E). In $z$ direction, the shift is smaller than the axial resolution in 2PM and, thus, cannot be measured.

The acquisition rate in the 2PM mode is determined by the scanning rate of the galvanometric mirrors, that is, 944 ms for a $1,000 \times 1,000 \, \mu m^2$ ($1,010 \times 1,010 \, \text{pixel}$) image. Volumes of $1,000 \times 1,000 \times 62 \, \mu m^3$ ($505 \times 505 \times 31 \, \text{voxel}$) are recorded within 14.6 s by additionally moving the objective lens along the microscope axis, thus, changing the position of the focal plane within the sample. As described above, 3D OCT images are directly recorded when scanning the light beam over the sample. Hence, there is no need to additionally move the objective lens as in 2PM. In order to improve the signal-to-noise ratio in OCT mode, we typically performed multiple scans (5x) resulting in an acquisition time of 9.3 s/$1,000 \times 1,000 \times 500 \, \mu m^3$ ($1,010 \times 1,010 \times 250 \, \text{voxel}$) volume.

Taken together, the main advantages of the coregistered OCT and 2PM multimodal imaging approach are: (1) near-instantaneous OCT and 2PM acquisition due to fast switch between imaging modes within milliseconds, (2) coregistration of the OCT and 2PM imaging volumes as large as $1,000 \times 1,000 \times 300 \, \mu m^3$ ($1,010 \times 1,010 \times 150 \, \text{voxel}$) with voxel accuracy, (3) cellular or even subcellular lateral and axial resolution in both 2PM and OCT mode, and (4) time-lapse 2PM imaging of $1,000 \times 1,000 \times 62 \, \mu m^3$ ($505 \times 505 \times 31 \, \text{voxel}$) at an acquisition rate of 14.6 s/imaging volume, sufficient to track motile immune cells in tissue with typical mean velocities of 5–10 $\mu m$/min (3).

**Multimodal Coregistration of 3D Volumes and Time-Lapse Imaging of Tissue-Resident Macrophages in the Murine Paw**

We correlatively imaged by OCT and 2PM limb paws of CX3CR1/eGFP mice ($n = 7$ animals) at various locations of up to $1,000 \times 1,000 \times 300 \, \mu m^3$ volumes, within the metatarsal area. We detected by OCT and SHG various tissue compartments, identified by GFP fluorescence CX3CR1+ cells and by Rhodamine dextran fluorescence perfused blood vessels and phagocytes (Fig. 2A,B).

Tendons and bones are visible both in the OCT and in the SHG channel based on their specific supramolecular organization of collagen fibers and on differences in their refractive index (Fig. 2A). As revealed by the SHG channel, collagen fibers are parallel to each other in tendons, whereas in cortical bone they are more irregularly organized. Apart of the longitudinal orientation of the collagen fibers in tendons, OCT reveals transversally aligned fiber bundles. Presumably, they allow tendon stretching and compression also in transversal direction (Fig. 2B). In tendons, we found CX3CR1+ cells but no perfused blood vessels. OCT additionally reveals several tissue layers of the paw situated under the tendons as indicated by arrow heads in Figure 2C. In these surrounding tissues, we found perfused blood vessels and also CX3CR1+ cells.

The bone marrow is characterized by a different aspect of the CX3CR1+ network and by abundant vascularization as compared to tendons (Fig. 2A). In OCT channel, the cellular structure of the bone marrow is also visible (Fig. 2A).

By analyzing the morphology of CX3CR1+ cells in the tendons as compared to surrounding tissue compartments, we found that in tendons, these cells adapt their shape to the orientation of collagen fiber bundles and are mostly elongated, with a sphericity factor of $0.38 \pm 0.07$ (experiments were performed in seven animals, 4–4 different volumes in the metatarsal area per animal). In other tissue compartments (highlighted by OCT), the CX3CR1+ cells adopt rather round shapes with a sphericity factor of $0.53 \pm 0.08$ determined in the same experiments as mentioned above (Fig. 2D,E). Furthermore, the volume of CX3CR1+ cells in tendons is slightly larger than in surrounding tissue, that is, 4,942 ± 195 $\mu m^3$ as compared to 4,531 ± 270 $\mu m^3$. All volume values correspond to the expected volume of macrophages with an average diameter of 20 $\mu m$, that is, 4,200 $\mu m^3$ calculated by spherical approximation of the cellular shape. Hence, our morphological analysis indicates that CX3CR1+ cells in tendons are...
Figure 2. 3D coregistered OCT and 2PM imaging in the paw of CX3CR1:eGFP mice reveals the link between shape and dimensions of CX3CR1* cells and tissue architecture as shown by OCT and SHG. (A) 3D reconstructed images of an 1,000 × 1,000 × 200 μm³ (1,010 × 1,010 × 101 voxel) volume in the metatarsal region of a CX3CR1:eGFP mouse paw: label-free SHG signal of collagen in tendons and cortical bone (blue), GFP fluorescence of CX3CR1* cells—among them tissue resident macrophages (green), rhodamine dextran fluorescence in blood vessels and phagocytes (red), and OCT signal (magenta). The individual 2PM, SHG, and OCT channels are shown in gray. The upper left image represents the merged 2PM (green and red) and SHG (blue) reconstruction highlighting a tendon, the bone and bone marrow of a metatarsal long bone and densely vascularized surrounding tissues. In the lower left image the OCT signal (magenta) highlighting both the tendon, the large blood vessel, cortical bone, and the cellular structure of the bone marrow is superimposed on the 2PM and SHG signals. Scale bar = 200 μm. (B) 3D reconstructed images of a 1,000 × 1,000 × 294 μm³ volume showing the properties of surrounding tissue and a tendon in detail.
Figure 3. Intravital time-lapse imaging in paws of CX3CR1:eGFP mice reveals different motility patterns of CX3CR1+ cells in tendons as compared to surrounding tissue areas. (A) Left images: 3D reconstructed zoom-in images from a 1,000 × 1,000 × 62 μm³ (505 × 505 × 31 voxel) volume in the metatarsal region of a CX3CR1:eGFP mouse paw: label-free SHG signal of collagen in tendons (blue), GFP in CX3CR1+ cells—among them tissue resident macrophages (green), and rhodamine dextran in blood vessels and phagocytes (red) in the metatarsal area of paws. Right images: Corresponding tracks of CX3CR1+ cells in the surrounding tissue compartments (upper images) and in a tendon (lower images). Representative images for four different areas, all corrected for xy and z drift. Corresponding OCT signal superimposed on the 2PM and SHG signals for one of these areas is shown in Supporting Information Figure S1. Time-lapse imaging is depicted in Supporting Information Movies S1 and S2. Scale bar (grid unit) = 10 μm. (B, C) Quantification of mean velocity and displacement rate of CX3CR1+ cells (from three different areas) in tendons as compared to surrounding tissue compartments in metatarsal areas of paws. Statistical analysis was performed using the Mann–Whitney test (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).
Figure 4. 3D coregistered instantaneous 2PM and OCT imaging of the lymph nodes, spleens, and retinas of CX3CR1:eGFP mice and of the femoral epiphysis of a Cdh5:tdTomato-Histone:GFP mouse. (A) Lymph node imaging: 3D reconstructed images of a 1,000 × 1,000 × 300 μm³ (1,010 × 1,010 × 151 voxel) volume in the popliteal lymph node of a CX3CR1:eGFP mouse: label-free SHG signal of collagen in the lymph node capsule (blue), GFP fluorescence of CX3CR1⁺ cells (green), and OCT signal (magenta). Corresponding 3D images of the single GFP fluorescence, SHG, and OCT channels are shown in gray. The images reveal an area abundant in CX3CR1⁺ cells (area 1) and an area with a sparse distribution of these cells (area 2). The OCT signal reveals cellular structures in area 2, presumably a lymphocyte rich region of the lymph node. The lower image in (A) represents the same volume as the upper images, from an xz-perspective. This view confirms the cellular structures in the OCT signal (magenta) as well as the capsule in the OCT and SHG channels (magenta and blue). Scale bar = 200 μm. Representative data for n = 3 mice. (B) Lymph node imaging: 3D reconstructed image of a 1,000 × 1,000 × 62 μm³ (505 × 505 × 31 voxel) volume in the popliteal lymph node of a CX3CR1:eGFP mouse: OCT signal (magenta) in the
mostly tissue resident macrophages, which adapt their shape to the tissue architecture, whereas in the surrounding compartments the CX3CR1+ macrophage population is more heterogeneous and may comprise both tissue resident macrophages and other phagocytic cells.

Furthermore, we monitored the dynamics of CX3CR1+ macrophages in tendons and surrounding tissue compartments of paws in living mice, over time (Supporting Information Movies S1 and S2, Fig. 3A,B). Similar to the surveying CX3CR1+ microglia in the brain (42) and to tissue-resident CX3CR1+ macrophages in the small intestine (5), CX3CR1+ macrophages in the tendons are mostly sessile (mean velocity = 1.94 ± 0.30 μm/min; displacement rate = 1.65 ± 0.38 μm/min) and probably fulfill different functions in tissue. The subset of sessile CX3CR1+ cells in surrounding tissue compartments are presumably also tissue-resident macrophages responsible for the maintenance of tissue homeostasis. In the surrounding tissue compartments, most CX3CR1+ cells are motile (Fig. 3B,C; mean velocity = 3.63 ± 0.56 μm/min; displacement rate = 2.06 ± 0.60 μm/min), and probably fulfill different functions in tissue. The term mean velocity is the average of all instantaneous velocities determined for each cell in the 3D stack. The instantaneous velocity of a cell is defined as the distance the cell covers between consecutive imaging events of the 3D stack divided by the time span between these events—in our case 30 s. The mean velocity is a measure for cellular motility, regardless of whether the motion is directed or not. The displacement of each cell in the 3D stack is defined as the straight segment connecting the positions of the cell at the first and the last time point the cell was detected during the time-lapse imaging, respectively. The displacement rate is calculated as the displacement of the cell divided by the time span the cell was visible within the imaging volume (3D stack). We excluded cells dwelling less than 5 min, that is, 10 time points, in the imaging volume. In contrast to the mean velocity, the displacement rate is a measure of directed cellular motion.

The different shape and motility behavior of CX3CR1+ macrophages strongly correlate with the tissue compartment where they reside, that is, tendons as compared to surrounding tissue compartments. Thus, our approach retains the unique potential to establish a link between tissue architecture accessible by OCT and the phenotype and related function of tissue-resident macrophages indicative for health or pathology, for example, during chronic inflammation of joints.

Multimodal Coregistered Imaging of 3D Volumes of Lymph Node, Spleen, Femoral Diaphysis, and Retina

We were able to correlate image OCT, 2PM fluorescence and SHG, and the extracellular and cellular structures of lymph nodes in CX3CR1:eGFP mice (n = 3 mice, Fig. 4A, B). Both 2PM and SHG imaging depth at an excitation wavelength of 900 nm and OCT imaging depth at an illumination wavelength of 930 nm amounted to 220 μm and were limited by Rayleigh scattering characteristic for lymph node tissue.

The lymph node capsule is visualized in both OCT and SHG by signal coming from collagen fibers. OCT further resolves cellular structures in tissue compartments with a low density of CX3CR1+ cells (area 2 in Fig. 4A), as highlighted by their GFP fluorescence. We assume that the cellular structures with a high OCT signal are lymphocytes due to their round shape and their dimensions comparable to GFP+ cells. The medullary cords and the T cell zone show a high density of CX3CR1+ cells, whereas the medullary cords are also rich in collagen, that is, they are characterized by a high SHG signal (Fig. 4B). The subcapsular sinus (Fig. 4B), localized directly under the capsule, is characterized by a high abundance of phagocytes labeled by Rhodamine dextran. In this area, also round, motile CX3CR1+ cells are present (mean velocity = 7.78 ± 1.34 μm/min; displacement rate = 2.87 ± 1.15 μm/min). In the other lymph node compartments, that is, B follicles, T cell zone and medullary cords, both round and CX3CR1+ cells and CX3CR1+ cells having cellular

whole lymph node, SHG signal (blue) of collagen in the lymph node capsule, GFP fluorescence of CX3CR1+ cells (green), and rhodamine dextran signal in blood vessels in the whole lymph node and phagocytes, especially in the subcapsular sinus (red). Scale bar = 200 μm. In this image, we could identify the main lymph node compartments: the capsule (SHG and OCT channels), the subcapsular sinus with a high abundance of phagocytes containing rhodamine dextran (red) and of CX3CR1+ monocytes (green), B cell follicles with a sparse distribution of CX3CR1+ cells as well as the neighboring T cell zones and the medullary cords with a high abundance of CX3CR1+ cells. The motility patterns of CX3CR1+ cells in the subcapsular sinus, B cell follicles, and medullary cords are analyzed by tracking these cells in time-lapse images acquired at the region as shown in the merged image in (B) (Supporting Information Movie S3). Mean velocity and displacement rate of the CX3CR1+ cells are shown in the graphs in (B). Statistical analysis was performed using the one-way ANOVA test with Bonferroni post hoc multicolumn test (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). (C) Spleen imaging: 3D reconstructed images from xy- and xz-perspective of a 1,000 × 1,000 × 200 μm3 (505 × 505 × 101 voxel) volume in the spleen of a CX3CR1+ eGFP mouse (representative images for n = 3 mice): label-free SHG signal of collagen in the spleen capsule (blue), GFP fluorescence of CX3CR1+ cells (green), Qd065 fluorescence in phagocytes (red), and OCT signal (magenta) contrasting in a label-free manner the spleen capsule and cellular structures underneath. Scale bar = 200 μm. (D) Retinal flat mount imaging: yz perspective of a coregistered 2PM and OCT image of a 500 × 500 × 100 μm3 (505 × 505 × 51 voxel) volume in the flat mount of the retina of a CX3CR1+ eGFP mouse: GFP fluorescence of resident CX3CR1+ cells, mainly microglia (green) and OCT signal (magenta) contrasting in a label-free manner the retinal neuronal fibers layer (RNFL) together with the ganglion neuronal cell layer (GCL), the inner photoreceptor layer (IPL), the outer photoreceptor layer (OPL), the outer nuclear layer (ONL) and the external limiting membrane (ELM). The white vertical line shows the position of the optic nerve head (ON head). Scale bar = 100 μm. (E) Femoral imaging: 3D reconstructed image of a 1,000 × 1,000 × 150 μm3 (1,010 × 1,010 × 75 voxel) volume in the longitudinally sectioned femur of a CdGt:tdTomato x Histone:GFP mouse: label-free SHG signal of collagen cortical and trabecular bone (CB and TB, blue), GFP in the nuclei of endothelial cells forming the blood vessels (green) within the bone marrow regions (BM)—right panel. Coregistered 3D image of cellular structures and tissue zones highlighted by OCT contrast in a label-free manner (magenta) including the trabecular and cortical bone as well as bone marrow—left panel. Scale bars = 100 μm.
processes are present. These cells are also motile, that is, in the T and B cell zones, mean velocity = 4.04 ± 1.32 μm/min, displacement rate = 1.44 ± 0.70 μm/min and in the medullary cords, mean velocity = 4.56 ± 1.07 μm/min, displacement rate = 1.72 ± 0.53 μm/min. However, they tend to move slower and their motion is less directed than that of the cells in the subcapsular sinus. We assume that the rather sessile CX3CR1+ cells in these lymph node areas are tissue-resident cells, among them also tissue-resident macrophages (Fig. 4B).

Similar to the imaging experiments in lymph nodes, we performed coregistered 2PM, SHG, and OCT imaging of spleens of CX3CR1:eGFP mice (n = 3 mice) after i.v. injection of Qdots655 (Fig. 4C). The imaging depth for all signals, that is, 2PM fluorescence, SHG, and OCT, was at most 200 μm and was limited by the Rayleigh scattering of spleen tissue. The collagen-rich capsule is highlighted by both SHG and OCT signals. The GFP fluorescence in 2PM additionally highlights CX3CR1+ cells, whereas Qdots655 fluorescence makes phagocytes visible. In spleen, OCT is able to detect cellular structures underneath the capsule; however, it does not allow any conclusions regarding the phenotype or function of these cells (Fig. 4C).

Fresh flat mounts of murine retinae of CX3CR1:eGFP mice (n = 2 mice) imaged by using the coregistered OCT and 2PM technology (Fig. 4D), confirmed the performance of the approach. Whereas the OCT signal highlighted several retinal layers, 2PM allowed us to retrieve the localization of CX3CR1+ cells, presumably tissue-resident microglia based on their GFP fluorescence. These cells play a crucial role in the disease development in the chronically inflamed central nervous system (6,43), in general, and chronically inflamed retina (18,44), in particular, as shown by us and by others.

In Cdhs5:tdTomato x Histone:GFP mice, the membrane of endothelial cells express tdTomato and their nuclei express GFP. Hence, after excitation at 900 nm by 2PM, nuclear GFP fluorescence (but no tdTomato fluorescence) can be detected. In explanted femurs of Cdhs5:tdTomato x Histone:GFP mice sectioned longitudinally (Fig. 4E), various organ regions could be distinguished based only on the contrast of the OCT channel. Hence, bone cortex and trabecular bone were identified based on their distinct, lower signal in the OCT channel. Their correct annotation was confirmed by the SHG signal of collagen in the cortical and trabecular bone (delimited by dashed lines in Fig. 4E). Furthermore, bone marrow regions containing hematopoietic cells were identified based on their high OCT signal intensity. These bone marrow regions were superimposed with densely vascularized areas, as revealed by GFP fluorescence in the nuclei of endothelial cells (indicated by stars in Fig. 4E).

**DISCUSSION**

Multimodal optical imaging technologies have evolved, both in the research environment as well as in the clinic, due to a tremendous need to obtain comprehensive spatiotemporal information on the pathophysiology of diverse organs and tissues. In this respect, various optical methods have been proposed and applied for visualizing biological samples including explanted tissues and organs or living organisms.

As far as OCT and intravitral 2PM are concerned, each of them separately has found broad application in the research context, for example, in neurosciences (42), immunology (3), and cancer research (45). We and others have demonstrated the optical performance and advantages of the individual techniques for intravitral imaging, that is, imaging in living animals (31,46,47), but also their inherent, specific limitations. 2PM provides information on tissue morphology and function at the cellular and even subcellular level and have helped to elucidate key pathophysiologic mechanisms in various organs and tissues (2). However, 2PM requires special sample labeling, complex experimental devices, and cannot access deep tissue layers in humans, all properties that make the technique difficult to apply in the clinic. The only exception to this has been the use of 2PM to visualize epidermal tissue layers in skin (DermalInspect, Jena, Germany). In contrast, OCT is an imaging technology based only on the detection of differences in refractive index between tissue structures, that is, the signal is endogenously generated, and, as such, does not require any labeling. Moreover, typical OCT setups are rather simple and, thus, very robust and can easily be employed in the clinic. However, OCT can only provide information on tissue morphology, without phenotypic specificity. Whereas the axial resolution of OCT is typically higher than that of microscopy technologies including 2PM, its poor lateral resolution and poor contrast (due to the use of low NA lenses) are only able to determine information about organ structure at the tissue, and not at the single cell, level. In order to partially counteract this deficit, special experimental OCT setups have been developed and employed to retrieve skin architecture (25,29), the thickness of retinal layers (43), and to develop sensor-free adaptive optics for in vivo imaging in the mouse retina (44,48). Still, OCT remains a technology characterized by poor lateral resolution.

Considering the advantages and drawbacks of the two technologies, it is obvious that 2PM and OCT may complement each other and that their combination would be beneficial for relating information on cellular phenotypes and functions to tissue and organ morphology and, thus, for translating this information to the clinic. Combinations of 2PM and OCT has been previously proposed but only recently demonstrated on biological samples (14), for example, to correlated skin morphology with lipid-rich cellular structures (29). Moreover, previous approaches have been used on different spatial scales and their ability to monitor dynamic changes in tissues at the cellular level has not been demonstrated. While OCT typically covers a large volume in the tissue, the subsequently acquired 2PM represents only a small fraction of the OCT imaging volume. In this way, the information connecting specific phenotypes and functions (as provided by 2PM) and morphology (as provided by OCT) is limited to small regions of only a few tens of micrometers. An extrapolation to the whole organ will be rather difficult due to tissue heterogeneity.
The main benefits of the multimodal imaging approach presented in this work are (1) the consecutive acquisition of the 2PM and OCT signals within a few milliseconds of each other, (2) imaging of both 2PM and OCT within large volumes in tissue, that is, up to \(1,000 \times 1,000 \times 300 \ \mu m^3\) depending on the imaged sample, (3) coregistered 2PM and OCT acquisition with 2PM voxel level accuracy, and (4) fast time-lapse data acquisition of \(1,000 \times 1,000 \times 62 \ \mu m^3\) volumes in tissue allowing to track motile immune cells by 2PM. The acquisition rate of spectral domain OCT reaches values in Hz-range (13) and is comparable to that of 2PM. Hence, our coregistered technology correlates functional and dynamic information provided by 2PM with tissue architecture as highlighted by OCT, thereby enabling conclusions at a single cell level, with high temporal resolution. Thus, it retains the potential to translate mechanistic information on pathophysiology to clinical settings where only OCT measurements can be performed on a regular basis.

Currently, OCT finds broad applicability in ophthalmology and dermatology, and more recently, in neurology (49). With our present study, we account for the increasing interest in rheumatology to employ OCT in diagnosis and drug monitoring by repeatedly visualizing cartilage degeneration. The imaging technologies typically used to survey cartilage degeneration during rheumatic diseases are magnetic resonance imaging (MRI) and ultrasonography (US) (50). In order to make the interpretation of these data accessible to rheumatologists without extensive expertise in clinical imaging, standardization protocols of image acquisition have been proposed to account for anatomic diversity (51) and automated analysis tools based on deep-convolutional algorithms have been developed and applied (52). Still, the spatial resolution in both MRI and US is much lower than OCT, making the detection of subtle tissue changes in the early phase of cartilage degeneration practically impossible. In contrast, arthroscopy (a technology broadly used in rheumatology (53) that is able to detect tissue changes at higher spatial resolution) requires surgical intervention and has a rather small field of view. This limits the techniques accessibility to reliable anatomical landmarks and thus makes the detection of pathology-related tissue changes during the disease’s course complicated. As mentioned previously, OCT is able to access large tissue volumes, at higher spatial resolution and in a noninvasive manner. Counteracting the techniques limited tissue imaging depth will be just a matter of technological development. As OCT requires only moderate-cost devices, we expect the technique to find broad application in rheumatology.

We specifically focused in our work on target organs of chronic inflammation, especially relevant in the musculoskeletal system, that is, tendons and surrounding tissues in mouse paws, and on organs of the immune system: lymph nodes, spleens, bones, and bone marrow. As for the causes of tissue degeneration in tendons and joints, cells of mesenchymal origin such as fibroblasts, various mesenchymal stroma subtypes, endothelial cells, or tissue-resident macrophages in the synovial membrane and in tendons are known to induce and maintain inflammation using diverse molecular and cellular mechanisms (54). In particular, tissue-resident macrophages have a highly diverse range of biological functions. While being appropriately positioned and transcriptionally primed to respond to local environmental challenges, they maintain tissue homeostasis, mediate immune surveillance, and may induce inflammation. At the same time, subpopulations of tissue-resident macrophages are also needed to promote the subsequent resolution of inflammation (40). Given the biological complexity of these functions, tissue-resident macrophage subsets within a single tissue type are highly diverse phenotypically and functionally, as was recently demonstrated for CX3CR1+ tissue resident macrophages in joints (55). Understanding the dynamic changes in phenotype and function within the subsets of tissue-resident macrophages, their interactions with other cells present in tissue and their impact on tissue integrity, from the tissue level down to single cells, is mandatory to elucidate the mechanisms underlying organ and tissue (dys)functions during rheumatic diseases. Still imaging technologies linking cell phenotypes and functions with tissue architecture, at high temporal and spatial resolution (i.e., cellular resolution in living organisms) are not yet available.

Using our coregistered, near-instantaneous OCT and 2PM technology in paws of CX3CR1:eGFP mice, we were able to correlate shape and dynamics of tissue resident macrophages, as acquired by 2PM, with tissue compartment architecture, as highlighted by OCT. Following the parallel organization of collagen fibers in tendons, tissue resident macrophages take on elongated shapes and are mostly sessile. In surrounding tissues, with less organized tissue architecture, CX3CR1+ cells adopt a more irregular shape (rather round), and most are highly dynamic; comparable to CX3CR1+ cells in the subcapsular sinus of lymph nodes.

As we expect OCT to find even more use in the clinic in the future, our approach offers the power to build a unique bridge between new cellular (and even molecular) aspects of the pathogenesis of chronic inflammatory diseases and daily clinical diagnosis.

**MATERIALS AND METHODS**

**Two-Photon Microscope Setup**

Two-photon fluorescence imaging experiments were performed as previously described, using a specialized laser-scanning microscope based on a commercial scan head (TriMScope II, LaVision BioTec, Bielefeld, Germany) (47). A near-infrared laser (Ti:Sa, Chameleon Ultra II, Coherent, Glasgow, UK) and an infrared laser (OPO, APE, Berlin, Germany) were used as excitation sources. The Ti:Sa and OPO beams, both linearly polarized, were combined in the scan head using a dichroic mirror. A multi-immersion objective lens (10x, NA 0.6, Apochromat, Olympus, Hamburg, Germany) was used to focus both 2PM and OCT laser beams into the sample. The laser power was controlled by combinations of /2 wave-plates and polarizers. The ultrashort pulses of Ti:Sa were compressed using external compressors.
Fluorescence and SHG were collected in the backward epi-direction using dichroic mirror (775, Chroma) and directed to four PMTs (H7422, Hamamatsu, Hamamatsu City, Japan). All PMTs were assembled in a detection system with different optical channels, where every channel was determined by individual fluorescence filter and a set of dichroic mirrors: 466 ± 20, 525 ± 25, 593 ± 20, and 655 ± 20 nm. The acquisition time for an image with a field-of-view of 1,000 × 1,000 μm² and a digital resolution of 1,010 × 1,010 pixel was 944 ms. We acquired 60 μm z-stacks (z-step 2 μm) every 30 s over a total time course of typically 30 min.

Mouse Strains and Handling
All mice used in the imaging experiments were 8–10 weeks old and had a C57Bl/6 background. We used CXCR1+eGFP mice (56) and Cdhl5 tdTomato-HistoneGFP mice. All intravital imaging experiments were performed in accordance with the regulations and guidelines and approved by the responsible authorities.

Specimen Preparation for Imaging

Intravital imaging of limb paws
We injected i.v. in CXCR1+/-GFP mice Rhodamine dextran for vasculature labeling. The dextran also labels phagocytes. The mice were anesthetized using inhalation anesthesia (isoflurane). After removing the hair, we carefully made an incision and removed the skin above in the metatarsal area, in order to optically access the tendons and underlying tissue compartments. The mouse was placed on a heating pad (34–36°C). The paw was fixed with tape on a pad and rinsed with isotonic NaCl solution (0.9%). A cup of low melting agarose was carefully formed on top of the paw, directly above the incision to keep the tissue hydrated throughout the entire imaging session. Coregistered OCT and 2PM imaging was immediately performed.

Ex vivo imaging of limb paws
CXCR1+/-GFP mice were sacrificed by cervical dislocation. After removing the hair, we carefully made an incision and removed the skin above the metatarsal area in order to optically access the tendons and underlying tissue compartments. The mouse was placed on a heating pad. The paw was fixed with tape on the pad and rinsed with isotonic NaCl solution (0.9%). A cup of low melting agarose was carefully placed on top of the paw, directly above the incision to keep the tissue hydrated throughout the entire imaging session. Coregistered OCT and 2PM imaging was immediately performed.

Ex vivo imaging of spleens or lymph nodes
CXCR1+/-GFP mice were injected i.v. either with Rhodamine dextran or with QDots655 5 to 10 min prior to imaging and sacrificed by cervical dislocation. The spleens and lymph nodes were removed and placed in isotonic NaCl solution at 37°C. Coregistered OCT and 2PM imaging was immediately performed.

Femoral bone
Cdhl5 tdTomato × HistoneGFP mice were sacrificed by cervical dislocation. The femurs were removed, longitudinally sectioned and placed in isotonic NaCl solution at 37°C. Imaging was performed immediately.

Retinal flat mount
CXCR1+/-GFP mice were sacrificed by cervical dislocation. Their eyes were explanted, eye lens was carefully removed, retinae were placed onto a microscope slide in isotonic NaCl solution at 37°C. Imaging was performed immediately.

Data Analysis
Image reconstruction was performed either using Fiji/ImageJ or Imaris (Bitplane, UK). MorphoLibj in ImageJ was used to segment and quantify the sparcity and volume of CXCR1+ cells. The xy and z drift in the time-lapse imaging data was corrected using 3DCorrectDrift Fiji plugin implemented in Imaris. Imaris (Bitplane) was used to track these cells and determine their mean velocity and displacement rate. Statistical analysis was performed using GraphPad.

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