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Mapping immune processes in intact tissues at cellular resolution

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Understanding the spatiotemporal changes of cellular and molecular events within an organism is crucial to elucidate the complex immune processes involved in infections, autoimmune disorders, transplantation, and neoplastic transformation and metastasis. Here we introduce a novel multicolor light sheet fluorescence microscopy (LSFM) approach for deciphering immune processes in large tissue specimens on a single-cell level in 3 dimensions. We combined and optimized antibody penetration, tissue clearing, and triple-color illumination to create a method for analyzing intact mouse and human tissues. This approach allowed us to successfully quantify changes in expression patterns of mucosal vascular addressin cell adhesion molecule–1 (MAdCAM-1) and T cell responses in Peyer’s patches following stimulation of the immune system. In addition, we employed LSFM to map individual T cell subsets after hematopoietic cell transplantation and detected rare cellular events. Thus, we present a versatile imaging technology that should be highly beneficial in biomedical research.

Introduction

A variety of different imaging strategies have been utilized to study immune cell activity and migration in animal models of human disease. Imaging techniques such as MRI, PET, and single-photon emission tomography (SPECT) are particularly attractive, since they can be readily translated from preclinical animal models to clinical application in humans (1). However, these techniques generally do not provide sufficient resolution to visualize individual cells and in many cases lack sensitivity to detect rare biological events in different areas throughout the body. In contrast, high-resolution imaging techniques such as confocal microscopy and multiphoton laser scanning microscopy (MPM) have limited penetration depth due to specimen-induced aberrations and, therefore, do not allow study of the area of interest within a large tissue volume.

So far, enormous efforts have been undertaken to visualize single immune cells throughout the body in murine models of human disease, particularly by high-precision cryosectioning of entire organs, and subsequent compilation of consecutive images (2, 3). This laborious approach provided valuable insights; however, it was prone to tissue distortions and artifacts. Recently, several techniques for morphological and functional imaging have been developed to visualize mesoscopic specimens employing 3D microscopy strategies including optical projection tomography (OPT) (4) and optical coherence tomography (OCT) (5), as well as light sheet–based techniques such as selective plane illumination microscopy (SPIM) (6) and ultramicroscopy (7). So far, light sheet fluorescence microscopy (LSFM) has been employed only in a very few intact adult mouse organs such as the brain (8), spinal cord (9), and the middle ear (10), using single-color illumination. Other microscopy techniques such as OPT and OCT do not achieve cellular resolution (11) or do not allow a multicolor application (12).

In light-sheet microscopy, a tissue-clearing procedure (replacement of water by a more highly refractive index substance) is typically used to make the specimen transparent. Here, we combined deep tissue staining protocols with optimized clearing procedures and advanced LSFM for the first time to be used as a quantitative triple-color technique investigating intact murine and human samples. Complex immune processes, as for instance in hematopoietic cell transplantation or in antitumor responses, can now be analyzed on a single-cell level in large tissue specimens or even entire organs.

Results

A virtual journey through intact tissues by multicolor LSFM. Using LSFM, we imaged Peyer’s patches (PPs), which are initiation sites for adaptive immune responses as they occur in infections and autoimmune diseases. We visualized intact PPs of adult mice after specific deep tissue antibody staining and clearing of the tissue specimens (Figure 1, A and B).

Three spectrally distinct light sheets were created for a one-sided illumination, and images were detected sequentially (Figure 1C). Intrinsic autofluorescence in the green color channel provided microanatomical details of the intestinal mucosa and submucosa. Even subepithelial dome regions and B cell follicles of PPs could be visualized and clearly differentiated from adjacent crypts and villi. To highlight T lymphocytes, we stained with red-labeled anti-CD4–Alexa Fluor 647. High endothelial venules (HEVs), which play an important role in lymphocyte entry into PPs, were labeled with
mucosal addressin cell adhesion molecule–1 (MAdCAM-1–Alexa Fluor 546, measured in the yellow color channel). High-precision optical z-stacks provided 1,800 optical sections (×5 objective) with an increment of 5 μm and allowed for scanning of whole PPs and lymph nodes (size, >18 mm³) in 3 color channels within minutes. The resulting z-stack required no further image processing and could be directly used for 3D tissue reconstruction (Figure 1, D and E), volume measurements, and quantification of protein expression. This precise 3D tissue reconstruction additionally allowed a virtual journey through the small intestine, which offered unique insights into the whole PP as well as adjacent areas (Supplemental Videos 1 and 2; supplemental material available online with this article; doi:10.1172/JCI65100DS1). Using a ×20 objective enabled us to visualize single CD4+ cells (Figure 1F) and even subcellular components, such as individual nuclei (Figure 1G), deep within the tissue. In our hands, LSFM has proven to have a major acquisition speed advantage over MPM in imaging whole transparent PPs (LSFM <12 minutes, MPM approximately 6 hours; Supplemental Figure 1) and reached a higher penetration depth (LSFM >2 mm).

Application of LSFM in multiple tissues of mice and humans. Labeling of intact tissues with multiple fluorescent antibodies for 3D imaging opens a broad range of biomedical applications, because any molecule of interest can be stained. For optimal analysis of whole organs, a homogeneous and specific antibody staining deep into the tissue is required. To this end, two strategies turned out to be efficient. We injected fluorochrome-labeled antibodies i.v. and shortly thereafter euthanized animals for microscopic analysis. We confirmed the specificity of antibody binding and distribution with immunofluorescence microscopy and CD4+ T cells (red) as single and color-merged single optical sections (objective, ×5; scale bar: 100 μm) and (D) after computational 3D reconstruction. (E) 3D reconstruction of the whole organ reveals co-localization of MAdCAM-1 expression and CD4+ T cells in a PP. Using a ×20 objective allowed a higher magnification of (F) single CD4+ T cells (z-projection scale bar: 100 μm) and (G) subcellular precision imaging single-cell nuclei (stained with DAPI) within lymph nodes (z-projection scale bar: 100 μm).
Technical advance

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Brightening by peroxidase treatment before sample clearing. In addition to performing studies in the mouse, we applied LSFM to analyze human specimens such as tonsils and colon cancer biopsy samples. Here, we successfully stained and displayed CD8⁺ T cells in relation to CD31⁺ endothelial cells in tonsils (Figure 2D) and tumor-infiltrating CD4⁺ and CD8⁺ T cells in colon cancer (Figure 2E and Supplemental Figure 4). Of note, the desmoplastic stroma reaction that is often present in cancer samples required modification of the processing procedure to allow antibody access into these very dense tissues.

Quantification of immune processes in entire organs. As LSFM proved valuable for mapping individual immune cells in murine and human tissues, we applied this technique to investigate evolving immune processes by repeated sampling. We used a model of acute GVHD to study T cell interactions with HEVs in whole PPs after allogeneic hematopoietic cell transplantation (allo-HCT) (13). We induced acute GVHD in myeloablatively conditioned BALB/c mice by transplanting bone marrow cells from C57BL/6 (CD90.2⁺, CD45.2⁺) donors and luciferase transgenic C57BL/6 CD45.1⁺ or CD90.1⁺ T cells. We followed the in vivo progression of GVHD on a macroscopic level by non-invasive bioluminescence imaging (BLI), which pinpointed organs of donor T cell infiltration (Figure 3). At the transition from GVHD initiation to effector phase (day +3 to day +4) (14) and at the first peak of the effector phase (day +6), we prepared PPs (Figure 4) and other tissues (Supplemental Figure 5) of recipients for subsequent whole-organ microscopy. To identify donor T cells, we injected anti-CD45.1⁻ or anti-CD90.1⁻–specific monoclonal antibodies i.v. and stained for the adhesion molecule MadCAM-1 ex vivo on the fixed tissue. Subsequently, we mapped and quantified the expression of MadCAM-1 in relation to T cell numbers within PPs in the course of GVHD. Whereas LSFM allows clear identification of single cells from different T cell subsets, automated cell counting was impeded by dense lymphocyte clustering (Supplemental Figure 6A). As a solution, we measured the entire T cell volume in whole PPs and calculated absolute T cell numbers by

Figure 2
Multicolor LSFM of diverse murine and human tissues. (A–C) A C57BL/6 mouse was injected i.v. with fluorescently labeled antibodies CD31 (cyan) and LYVE-1 (red). Organs such as (A) heart, (B) testicle, and (C) skin were removed 1 hour after injection and prepared for LSFM. Shown are 3D reconstructions from optical sections imaged with a ×5 objective. (D and E) LSFM allowed imaging of whole human tissue biopsy samples after modification of the processing procedures. (D) z-Projection of a human tonsil biopsy (autofluorescence, green) shows the colocalization of CD31 (red) and CD8 (cyan) (scale bar: 500 μm). (E) A moderately differentiated human colon adenocarcinoma specimen with tumor-infiltrating CD8⁺ T cells (red) and CD4⁺ T cells (cyan) (scale bar: 500 μm).

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rithm that enabled us to automatically identify and count individual cells and found that TN cells (mean, 726 cells/PP) homed more efficiently than T CM cells (mean, 478 cells/PP) to PPs (Figure 5, B and C) and to mLNs (Supplemental Figure 8). Within the same experiment a comparable homing trend to secondary lymphoid organs (SLOs) was confirmed by FACS analysis of the spleen (data not shown). TEM cells migrate most efficiently to peripheral tissues as opposed to SLOs. In line with this, LSFM revealed low numbers of TEM (mean, 81/PP) homing to PPs (Figure 5, B and C). Most TEM cells located in proximity to MAdCAM-1–expressing HEVs, indicating homing to the T cell zone.

Discussion
Here, we describe a novel multicolor LSFM approach to analyze complex immune processes at the single-cell level in whole organs. Based on optical sectioning, this convenient method required combination of the protocols of optimized antibody penetration, tissue clearing, and multiple color illumination to allow an accurate computational 3D reconstruction of intact tissues. This highly versatile technique allows multicolor imaging of diverse tissue specimens from mice and humans.

LSFM fills the gap between microscopic imaging techniques such as confocal microscopy and macroscopic imaging techniques like MRI or BLI. LSFM thereby circumvents the obstacle of alternative 3D imaging methods such as OPT that are limited by the requirement of time-consuming back-projection (4). A clear advantage of LSFM is that optical sectioning allows rapid screening for rare events in mesoscopic specimens without requiring any image calculation. Therefore, areas of interest can be identified immediately during the acquisition process, which allows for...
Hence, LSFM gave us new insights into migration and dispersal of different T cell subtypes after HCT and should prove useful for optimization of cellular therapies. Advanced light sheet illumination modes (26, 27) as well as the combination with super-resolution approaches (28) even leave room for future contrast and resolution enhancements. Recent developments to optimize clearing procedures rendering organs transparent, as shown for the central nervous system (9, 29), will further assist in reducing background fluorescence and enhancing fluorescence protein stability. In cleared samples, high tissue autofluorescence, particularly in the green color channel, caused by formalin fixation (30) — allows the display of detailed microanatomical features. This proved advantageous for locating specific fluorescently labeled cell populations within their anatomical context. However, this strong autofluorescent background noise also poses certain limitations for the utilization of green fluorophores for specific labeling. Therefore, we recommend reliance on fluorophores in the red or near-infrared color spectrum, as demonstrated in this study.

In conclusion, we have shown multicolor LSFM to be a rigorous tool for effectively pinpointing and quantifying rare cellular events and cell-cell interactions within intact organs of mice and humans. Biomedical research that translates scientific progress into clinical approaches should greatly benefit from the application of this high-resolution technology. Therefore, 3D multicolor LSFM holds the promise of complementing classical histopathological diagnosis.

Figure 4
Visualizing and quantifying cellular changes after allo-HCT in 3D. Bone marrow together with CD90.1+ transgenic T cells were transplanted into irradiated allogeneic recipients to induce acute GVHD. CD90.1 or CD4 antibody was injected i.v., and MadCAM-1 was stained ex situ. (A) Representative PPs of untreated and transplant recipient mice highlight differences in MadCAM-1 expression and numbers of donor T cells. (B) After allo-HCT, MadCAM-1 is highly upregulated in the PPs, particularly in follicular areas. (C) Quantification of donor T cells in the entire PP demonstrate proliferation until day 4. By day 6 most donor T cells exit, as confirmed by BLI. Shown is 1 representative experiment of 2 with 2–4 mice per time point.
The following murine antibodies were purchased from Bio-Legend and Invitrogen: CD4 (GK1.5), CD4 (RM4-5), CD8α (clone 53-6.7), CD11c (N418), CD31 (WM59), CD35 (7E9), CD44 (IM7), CD62L (MEL-14), CD90.1 (OX-7), CD45.1 (A20), CD105 (MJ7/18), CD106 (429/MVCAM-A), MAdCAM-1 (MECA-367), and podoplanin (clone 8.1.1). Human antibodies were purchased from BioLegend: CD4 (OKT4), CD8α (HIT8a), and CD31 (WM59). FACS and standard immunofluorescence microscopy were performed using an Imager Z1M (Zeiss) and a BD FACSCanto II flow cytometer (BD Biosciences).

FACS. Magnetic bead–enriched T cells were further sorted into CD4+CD44hiCD62Llo TEM, CD4+CD44hiCD62Lhi TCM, and CD4+CD44loCD62Lhi TN using a FACSAria II cell sorter (BD Biosciences).

Preparation of mouse specimens. Organs were prepared for LSFM by modified protocols of previously described procedures (7, 31). Briefly, mice were anesthetized by intraperitoneal injection of ketamine-xylazine and transcardially perfused with 20 ml ice-cold PBS, followed by 40 ml of 4% paraformaldehyde (pH 7.4). Following the perfusion, organs were removed. The samples were placed and stored in paraformaldehyde for at least 2 hours.

Antibodies. The following murine antibodies were purchased from Bio-Legend and Invitrogen: CD4 (GK1.5), CD4 (RM4-5), CD8α (clone 53-6.7), CD11c (N418), CD31 (WM59), CD35 (7E9), CD44 (IM7), CD62L (MEL-14), CD90.1 (OX-7), CD45.1 (A20), CD105 (MJ7/18), CD106 (429/MVCAM-A), MAdCAM-1 (MECA-367), and podoplanin (clone 8.1.1). Human antibodies were purchased from BioLegend: CD4 (OKT4), CD8α (HIT8a), and CD31 (WM59). FACS and standard immunofluorescence microscopy were performed using an Imager Z1M (Zeiss) and a BD FACSCanto II flow cytometer (BD Biosciences).

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**Methods**

*Animals and hematopoietic cell transplantation.* BALB/c mice (H-2d, Thy1.2) and C57BL/6 mice (H-2b, Thy1.2) were purchased from Charles River at the age of 8–16 weeks. Transgenic mice C57BL/6.L2G85 CD90.1+ expressing luciferase (luc+) were generated by backcrossing the luc+ FVB/N-L2G85 founder line (14) with the C57BL/6 mouse for more than 12 generations. Additional transgenic luc+ mice C57BL/6.L2G85 CD45.1+ mice were bred in our colony. BALB/c mice were conditioned with a total body irradiation dose of 8 Gy and i.v. injected with $5 \times 10^6$ C57BL/6 WT sex- and age-matched bone marrow cells within 2 hours after irradiation. C57BL/6.L2G85 CD90.1+ or CD45.1+ magnetic bead– separated (Invitrogen) T cells (CD4/CD8 enrichment, approximately 80% purity) were coinjected to induce GVHD.

**BLI.** BLI was performed as previously described (13) using an IVIS Spectrum CCD imaging system (Caliper Xenogen). Mice were anesthetized with an intraperitoneally injected mixture of ketamine (50 mg/g body weight, BW) and xylazine (5 mg/g BW) in 0.1 M PBS in a total volume of 10 μl/g BW. d-Luciferin was injected at a concentration of 150 μg/g BW. Imaging data were analyzed with Living Image 4.0 (Caliper Xenogen).
at 4°C until use in further procedures. Hemoglobin-rich organs were bleached for 30 minutes in 15% hydrogen peroxide/methanol. For homogenous ex situ immunofluorescence staining of large specimens, the tissue samples were blocked for 18–24 hours with 2% FCS/PBS in 0.1% Triton-X and afterward incubated with the respective antibodies for 24 hours at 4°C on a shaker, washed in PBS, incubated with streptavidin for 24 hours, and washed again in PBS, followed by dehydration in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 96%, and in 100% for 2 hours each) at room temperature. After the samples were rinsed for 2 hours in 100% n-hexane, the n-hexane was replaced stepwise by a clearing solution consisting of 1 part benzyl alcohol in 2 parts benzyl benzoate (Sigma-Aldrich). Air exposure was strictly avoided at this step. Tissue specimens became optically transparent and suitable for the LSFM imaging after incubation in the clearing solution for at least 2 hours at room temperature.

**In vivo antibody staining.** Fluorochrome-labeled antibodies were injected i.v. 2.5 hours (300 µg CD4, CD45.1, CD90.1, or CD11c) or 0.5 hours (50 µg MAdCAM-1) before mice were euthanized. To confirm distribution and specificity of i.v. antibody staining, additional post-section staining was performed with the same antibody. To demonstrate antibody distribution and specificity of inflamed and non-inflamed tissues, CD4–Alexa Fluor 647 antibody was injected into untreated mice or into mice on day +6 after allo-HCT. Two and a half hours later, mice were euthanized, and spleens and PPs were harvested and digested according to a previously described protocol (32) with slight modifications. Briefly, organs were digested (2 mg/ml collagenase D and 0.1 mg/ml DNase I) at 37°C for 30 minutes in the presence of saturating amounts of CD4-FITC antibody (30 µg/ml) to prevent ex situ staining of remaining unbound CD4–Alexa Fluor 647. Thus, CD4-FITC single-positive cells indicate ex situ stained cells. If not specifically indicated as antibody i.v. staining, fluorescence staining was performed according to the ex situ staining protocol.

**Preparation of human tissue.** The preparation of human tissue required important modifications. Fresh biopsy tissue was fixed for 2–4 hours in PFA, washed in PBS, bleached in 15% hydrogen peroxide/methanol, and again washed in PBS. Subsequently, the sample was pretreated with 0.1% Triton-X/ PBS for 24 hours before addition of 2% FCS for another 8 hours. To allow for homogenous staining of deeper tissue layers, antibody incubation was performed for 4–6 days in 0.1% Triton-X/PBS. After washing again in PBS, the sample was dehydrated in a graded ethanol series for 4 hours each. The clearing procedure was performed exactly as for the preparation of mouse tissue.

**Multicolor LSFM.** We used an LSFM setup similar to a previously described setup (8); however, the sample was illuminated from one side by various laser sources: 405-nm diode laser (56ICS025, CVI Melles Griot), 473-nm DPSS laser (MBL-473 100 mW, CNI), and 532-nm DPSS laser (MGL-W532 500 mW, CNI), and 639-nm diode laser (Cube 640-40, Coherent). We acquired multicolor stacks, imaging each plane sequentially by each wavelength and emission filter combination. Stacks were taken in increments of either 1, 2, or 5 µm. Exposure times for image acquisition were 200 or 500 ms per frame. The excitation wavelengths, emission filter colors, data acquisition, and storage were synchronized by LabView software (National Instruments) and administered using the software package MetaMorph 7.1 (Molecular Devices). Resulting multicolor stacks were processed, if necessary (e.g., cropped or reduced), with ImageJ (NIH). Subsequently, we used the 3D image processing software Volocity (PerkinElmer) to prepare the individual images and videos.

**MPP.** Samples were embedded into a self-constructed chamber for MPM. The MPM was equipped with an optical parametric oscillator (OPA, APE) for two-photon irradiation at 1,100 nm. The excitation beam was focused with a x20 NA 0.95 water objective (Olympus). Emission was detected with HQ535/50-nm, HQ605/70-nm, and HQ710/75-nm filters. Sequential 3D stacks were obtained for up to 1-mm penetration depth at a step size of 5 µm. Single-color image stacks (overlap, 16%) were stitched together, image size was reduced from 5,566 x 3768 pixels to 756 x 512 pixels, and photomultiplier tube–induced (PMT-induced) noise outliers were removed with ImageJ (processing package Fiji, NIH) before 3D image processing with Volocity.

**Volume calculations and automated cell counting.** Multicolor stacks were processed on Volocity software (Improvision) for 3D rendering and subsequently volume quantification and cell counting. We calculated volumes of autofluorescence, MAdCAM-1, and clustered donor T cells (Volocity measurements: find objects using intensity > exclude objects touching edge of image > exclude objects larger than x standard deviation ranging from 250 to 500 to ensure automated event thresholding by LabView) by the mean of 10 individual threshold measurements for each individual sample. For automated cell counting, we used the following algorithm: Volocity measurements: find objects using intensity > clip objects to ROI) within a 3D ROI fitted to border the whole PP. The lower limit of specific threshold intensity was calculated with ImageJ by the mean of 10 individual threshold measurements for each individual sample. For automated cell counting, we used the following algorithm: Volocity measurements: find objects using intensity > exclude objects touching edge of image > exclude objects by size > filter measurements: standard deviation. The lower limit of the object size was set to 400 µm² as calculated before. We used a lower limit of the standard deviation ranging from 250 to 500 to ensure automated event identification. Mathematical analysis was performed with Excel (Microsoft) and statistical analysis with Prism 5 and InStat3 (GraphPad).

**Study approval.** All animal studies were performed according to specific animal use protocols approved by Regierung von Unterfranken, State of Bavaria, Germany. All biopsy samples were obtained for diagnostic purposes and after written informed consent was obtained from donors. The study was approved by the Ethics Committee of the Medical Faculty, University of Würzburg, Würzburg, Germany.

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