Technical Advance

In Situ Characterization of Genetically Targeted (Green Fluorescent) Single Cells and Their Microenvironment in an Adoptive Host

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Stable expression of transgene-encoded enhanced green fluorescence protein (eGFP) was used as a sensitive and specific marker to detect *in situ* donor cells engrafted into different tissues of adoptive hosts. eGFP⁺ lymphoid or myeloid cells (eg, CD4⁺ T cells or bone marrow-derived dendritic cells) from eGFP-transgenic C57BL/6 donor mice were injected into congenic, immunodeficient RAG1^{-/-} C57/BL6 hosts. eGFP⁺ cells were detected in the adoptive host from 2 days to 4 weeks after transfer using an optimized method of fixed cryopreservation to process the tissue. This allowed the simple, sensitive, and specific detection of eGFP⁺ donor cells in histological sections of transplanted hosts. We further demonstrate that this technique can be combined with other established labeling methods such as 1) immunofluorescent labeling to characterize the host cells interacting with engrafted cells and to determine the phenotype of the engrafted cells *in situ*; 2) terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining to detect apoptotic death of engrafted and autochthonous cell populations; and 3) fluorescent antibody labeling of incorporated bromodeoxyuridine to measure the fraction of proliferating cells in the graft. (Am J Pathol 2001, 158:1975–1983)

Adoptive transfer systems are extensively used to study *in vivo* differentiation and homing of cell populations and to test their ability to induce or suppress diseases.^{1,2} *In situ* techniques can generate critical information on the distribution of cells in the host and their interaction with the target tissues of interest. Techniques to identify donor cells rely mainly on immunohistology. These techniques of labeling transferred and engrafted cells *in situ* with antibodies have limitations. They are time consuming,

have low sensitivity (often caused by high background reactivity of the tissue of interest), and may have limited specificity because of cross-reactivities with host cells that are difficult to control. The availability of monoclonal antibodies (mAbs) suitable for immunohistology in the murine system is limited. Hence, simple and specific techniques to identify *in situ* engrafted cells and to simultaneously determine their differentiation, activation, and viability status would greatly facilitate many studies *in vivo*.

Green fluorescent protein (GFP) is a bioluminescent protein from the jellyfish *Aequorea victoria*.^{3,4} Mutant variants of this protein have been developed that show enhanced fluorescence. A commonly used variant with enhanced fluorescence is GFPmut1 (designated eGFP in this article).⁵ eGFP has been used as a reporter gene in prokaryotic and eukaryotic cells *in vitro*. A transgenic C57BL/6 mouse line has been constructed that expresses eGFP under the chicken β -actin promoter control.^{6–10} These mice have been used to study hemopoietic and lymphoid development,^{11–13} spermatogenesis,¹⁴ and maternal cell transmission to offspring through placenta during pregnancy and by breast-feeding after birth.¹⁵ These studies required the isolation of viable cells from the host and their subsequent flow cytometric identification and characterization. Because of the lack of optimized techniques to detect eGFP-expressing cells *in situ*, an analysis of cell migration, proliferation, and differentiation by immunohistological techniques was difficult to perform in this attractive model.

We transferred eGFP⁺ cells of well-defined lymphoid or myeloid subsets, ie, splenic CD4⁺ T cells or bone marrow-derived dendritic cells (DCs), from eGFP-transgenic (eGFP-tg) C57/BL6 (B6) donor mice into congenic, immunodeficient RAG1^{-/-} B6 hosts. We used the stable expression of eGFP as a sensitive and highly specific

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marker for the *in situ* detection of donor cells in repopulated tissues of the host. We describe a method for processing tissue that permits the simple, sensitive, and specific detection of eGFP⁺ donor cells in histological sections of transplanted hosts. In addition, we demonstrate that this technique can be combined with three other labeling techniques to analyze *in situ* the fate of the engrafted eGFP⁺ cells. It can be used with 1) fluorescent antibody staining to determine *in situ* the heterogeneity and distribution of marker profile expression in engrafted and autochthonous cell populations indicative of their differentiation and/or activation; 2) terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining to detect apoptotic death of engrafted and resident cells; and 3) immunolabeling of incorporated bromodeoxyuridine (BrdU) to measure the fraction of proliferating graft and host cells.

Materials and Methods

Mice

eGFP-tg C57BL/6J mice and C57BL/6J-*Rag1*^{tm1Mom} (*RAG1*^{-/-}) mice¹⁶ (stock no. 2216; Jackson Laboratories, Bar Harbor, ME) were used. Dr. Masaru Okabe, Osaka University Research Institute for Microbial Diseases (Osaka, Japan) kindly provided eGFP-tg B6 mice.^{6,11} These mice carry copies of the eGFP-transgene cloned into a pCAGGS expression vector. Breeding colonies of *RAG1*^{-/-} mice were established under specific pathogen-free conditions in the animal colony at the University of Ulm.

Isolation and Adoptive Transfer of eGFP^{bright} CD4⁺ Cells and DCs

Spleen cells were obtained from eGFP-tg B6 mice. CD8⁺ T cells were depleted from these spleen cells by treatment with anti-CD8 antibody and low toxicity rabbit complement (catalog no. CL3051; Cedarlane, Hombly, Canada) following the manufacturer's instructions. CD4⁺ T cells were enriched to >98% purity by positive selection using the magnetic-activated cell separation (MACS) system (Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, 10⁷ cells were incubated with CD4(L3T4) Micro Beads (1:10 dilution, catalog no. 130-049-201; Miltenyi Biotec) in 100 μ l of MACS buffer [phosphate-buffered saline (PBS) supplemented with 2 mmol/L ethylenediaminetetraacetic acid (EDTA) and 0.5% bovine serum albumin] for 30 minutes at 8°C. Cells were washed twice in MACS buffer, resuspended in 500 μ l of MACS buffer, and pipetted onto a prerinsed LS separation column (catalog no. 130-042-401, Miltenyi Biotec) attached to a Midi-MACS separation unit (catalog no. 130-042-302, Miltenyi Biotec). After washing three times with 3 ml of MACS buffer, columns were removed from the separation unit. To elute bound cells, 6 ml of MACS buffer were passed through the columns using a plunger supplied with the columns. Cells from eGFP⁺-tg B6 donors were always selected for high eGFP expression (eGFP^{bright} cells) by fluo-

rescence-activated cell sorting before transfer into the eGFP⁻ host. The purity of the positively separated CD4⁺ population was always >98%. We injected intraperitoneally into *RAG1*^{-/-} B6 hosts 3 $\times$ 10⁵ CD4⁺ eGFP^{bright} T cells per mouse.

The *in vitro* generation of DCs from murine bone marrow has been described.¹⁷ Briefly, bone marrow cells prepared from femurs were depleted of CD4⁺ and CD8⁺ T cells, B220⁺ B cells, and MHC-class-II⁺ maturing myeloid cells (antibody catalog no. 492-01, 494-01, 495-01, 524-01; Miltenyi Biotec) by MACS sorting. These progenitor-enriched marrow cells were cultured at a density of 10⁶ cells/ml in serum-free UltraCulture medium (BioWhittaker, Verviers, Belgium) supplemented with 5 ng/ml GM-CSF and 10 ng/ml Flt₃ ligand (catalog nos. 315-03 and 300-19; PeproTech, Rocky Hill, NJ), 2 mmol/L glutamine, and antibiotics. Cultures were incubated at 37°C in humidified air with 5% CO₂. Cells were fed by medium exchange on day 3 and day 5 of culture. DCs were harvested at day 8 of culture. We injected intraperitoneally into *RAG1*^{-/-} B6 hosts 1 $\times$ 10⁷ DCs per mouse.

Only 30 to 45% of the homozygous transgenic B6 mice used in this study express the transgene. The expression pattern of the eGFP-encoding transgene was heterogeneous. In some organs, eg, liver and kidney, strongly eGFP⁺ epithelial cells were found beside cells without detectable eGFP expression. Tissues from eGFP⁺-tg B6 mice were used to study tissue processing for the presence of eGFP fluorescence.

Tissue Processing

Tissue specimens obtained from eGFP-tg B6 mice or transplanted *RAG1*^{-/-} mice included the mesenteric and peripancreatic lymph nodes, spleen, thymus, liver, kidney, small and large intestine, femur, and vertebral column. The tissues were either snap-frozen in liquid nitrogen and stored at -70°C, or treated with a 4% phosphate-buffered paraformaldehyde solution (pH 7.2) for 16 hours at 8°C. For bone tissue, a prolonged fixation in paraformaldehyde for 2 days was chosen followed by decalcification in an EDTA solution (200 g/L, pH 7.2) at 37°C for 1 week.

Tissue samples submitted to chemical fixation and paraffin embedding were further processed in a Tissue-Tek VIP automate (Sakura, Torrance, CA) set to the following program: formalin-fixation at 35°C (1 $\times$ 30 minutes), dehydration in an ascending ethanol row at 40°C (1 $\times$ 75%, 1 $\times$ 80%, 2 $\times$ 96%, 2 $\times$ 100%; 30 minutes per each step), equilibration in xylene at 40°C (1 $\times$ 30 minutes plus 1 $\times$ 1 hour) and soaking in paraffin at 56°C (3 $\times$ 30 minutes plus 1 $\times$ 1 hour). After embedding in paraffin, tissue was cut into 2- μ m sections that were subsequently deparaffinized in xylene, rehydrated by decreasing ethanol concentrations, and embedded in Vectashield mounting medium (catalog no. H1000; Vector, Burlingame, CA). As an alternative to paraffin embedding, tissue samples were incubated for 48 hours at 8°C in 4% phosphate-buffered paraformaldehyde solution (pH 7.2) containing sucrose at various concentrations (0 to 60%),

snap-frozen in liquid nitrogen and processed as described below.

Paraformaldehyde fixed or untreated cryopreserved tissue was embedded in OCT compound (catalog no. 4583; Miles Inc., Naperville, IL). Two- μm cryosections were cut with a cryostat (Leica, Wetzlar, Germany). The sections were mounted on poly-L-lysine-coated microscopic slides. The mounted sections were air-dried for 12 hours at room temperature. They were then left untreated, or were immersed for 30 minutes at room temperature in either paraformaldehyde solution, or acetone, or 70% ethanol. Fixed sections were subsequently rinsed in PBS buffer for 5 minutes at room temperature. They were thereafter incubated for 1 minute with 0.3 $\mu\text{g}/\text{ml}$ 4,6-diamidino-2-phenylindole (catalog no. D9542; Sigma-Aldrich, Deisenhofen, Germany) for fluorescence nuclear counterstaining and embedded in Vectashield medium. In addition, hematoxylin and eosin (H&E) staining was performed.

In Situ Detection of eGFP⁺ Cells that Have Bound Fluorochrome-Conjugated Antibodies

For immunohistology, 2- μm cryosections of paraformaldehyde-fixed and subsequently snap-frozen tissue were mounted on poly-L-lysine-coated microscopic slides and air-dried for 12 hours at room temperature. These cryosections were first incubated with a biotin-conjugated anti-CD45R/B220 clone RA3-6132 (dilution 1:100, catalog no. 01122D; Pharmingen, Hamburg, Germany) or anti-CD45 clone 30-F11 (dilution 1:30, catalog no. 01112D; Pharmingen) mAb for 1 hour, washed twice in PBS buffer, and then incubated with Cy3-conjugated streptavidin (catalog no. 016-160-084; Dianova, Hamburg, Germany) for 30 minutes at a dilution of 1:1000 to detect bound mAb. All incubation steps were performed at room temperature in PBS. To test if eGFP fluorescence can withstand demasking of formalin-resistant epitopes, sections were immersed in 200 ml of citrate-buffer (0.01 mol/L, pH 6.0) and heat-treated in a microwave oven at 800 W for 20 minutes.

In Situ Detection of in Vivo Proliferating eGFP⁺ Cells by BrdU Labeling

Proliferating cells labeled with BrdU *in vivo* were detected *in situ*. Animals were intraperitoneally injected 1 hour before sacrifice with 1 mg/mouse BrdU (catalog no. 85002, Sigma-Aldrich) dissolved in 0.2 ml of PBS. Cryosections of paraformaldehyde-fixed intestinal tissue were denatured in various concentrations of hydrochloric acid (0.002 to 2.0 mol/L) and neutralized in borate buffer (0.1 mol/L, pH 8.0) as described.¹⁸ BrdU-positive nuclei were detected by the biotin-conjugated anti-BrdU mAb clone BR-3 (catalog no. MD5215; Caltag, Burlingame, CA) and Cy3-labeled streptavidin. Immediately after staining and embedding in Vectashield mounting medium, sections were evaluated by fluorescent microscopy.

In Situ Detection of Apoptotic eGFP⁺ Cells by TUNEL Staining

Cells undergoing apoptosis were detected *in situ* by labeling DNA strand breaks using TUNEL.¹⁹ Briefly, cryosections of paraformaldehyde-fixed mesenteric lymph nodes were digested with proteinase K (20 $\mu\text{g}/\text{ml}$, Sigma-Aldrich) for 15 minutes at 37°C. The labeling reaction was performed using 10 U of TdT (catalog no. M1871; Promega, Mannheim, Germany) and 20 $\mu\text{mol}/\text{L}$ of biotin-16-dUTP (catalog no. 1093070, Boehringer-Mannheim) in 50 μl of TdT buffer (0.5 mol/L cacodylic acid, sodium salt, pH 6.8; 1 mmol/L CoCl_2 , 0.5 mmol/L dithiothreitol, 0.05% w/v bovine serum albumin, 0.15 mol/L NaCl). Labeled cells were detected using Cy3-conjugated streptavidin (catalog no. 016-160-084, Dianova) diluted 1:5000.

Fluorescence Microscopy

Slides were examined under an Axioskop fluorescence microscope (Zeiss, Jena, Germany) using a mercury-vapor light source and an appropriate filter set. Images were recorded by a video camera and processed on a computer using the ISIS3-software (version 3.02; Meta-systems, Heidelberg, Germany).

Results

Modification of Protocols for Tissue Processing to Optimize Detection of eGFP⁺ Cells

We obtained tissues from either eGFP-tg B6 mice (selected for eGFP expression), or RAG1^{-/-} B6 mice transplanted with purified eGFP⁺ cells. We tested different protocols for tissue processing to optimize detection of eGFP⁺ cells *in situ*.

eGFP fluorescence was almost completely eliminated in paraformaldehyde-fixed, paraffin-embedded tissue. Only minimal eGFP fluorescence could be observed in organs with high levels of eGFP expression, such as liver and kidney (data not shown).

We tested if eGFP⁺ cells can be detected in snap-frozen, cryopreserved tissue. eGFP fluorescence was virtually undetectable on cryosections from liver and kidney that were counterstained and embedded in Vectashield mounting medium. This effect is certainly because of the high solubility of eGFP in aqueous media that has been described before.⁹ We therefore treated air-dried cryosections with fixative agents for 30 minutes at room temperature. Among these, acetone had no effect whereas 4% paraformaldehyde solution and 70% ethanol rescued eGFP fluorescence only incompletely which moreover was faint and ill circumscribed. The low effectiveness of ethanol treatment can be ascribed to its limited ability to inhibit diffusion of eGFP, whereas cross-linking by paraformaldehyde fixation was probably too slow to counteract the diffusion process.

We fixed cryosections from tissues in paraformaldehyde solution before snap-freezing in liquid nitrogen. This

Figure 1. eGFP⁺ cells detected on paraformaldehyde-fixed cryosections of tissues from eGFP-tg B6 mice. eGFP-expressing hepatocytes (**A**) and renal epithelium of the cortical and papillary region (**B**) display an intense green fluorescence. The histomorphology of paraformaldehyde-fixed cryosections of kidney tissue (**C**) is improved when tissue was treated with a 50% sucrose solution (**D**). **C** and **D**: H&E stainings. Original magnifications: $\times 54$ (**A**), $\times 27$ (**B**), $\times 216$ (**C** and **D**).

procedure maintained bright eGFP fluorescence readily detectable by fluorescent microscopy. Individual eGFP⁺ cells or eGFP⁺ cell groups could be clearly identified in different tissues (Figure 1, A and B). The high intensity of eGFP fluorescence permitted sensitive detection of positive cells even in organs displaying a high autofluorescent background, such as liver tissue (Figure 1A).²⁰ The eGFP signal was remarkably stable as it was detectable without significant loss of signal strength in sections after

storage for several weeks at room temperature. When H&E stainings from cryosections of paraformaldehyde-fixed tissue were examined, the histomorphology was found to be impaired by intercellular retraction artifacts and cytoplasmic clumping (Figure 1C). This was overcome by adding sucrose to the paraformaldehyde solution. Of the different sucrose concentrations tested, 50% seemed to be optimum and resulted in improved histomorphology (Figure 1D).

Figure 2. eGFP⁺ cells detectable in decalcified tissue. eGFP⁺-positive cells are easily identified on cryosections of bone tissue that was fixed in paraformaldehyde and decalcified with EDTA. **A**: At low magnification, brightly eGFP-positive chondrocytes of articular cartilage are conspicuous. **B**: Cells of the bone marrow show a moderate expression of eGFP that becomes detectable at higher magnification. Original magnifications: $\times 54$ (**A**), $\times 106$ (**B**).

Figure 3. Immunohistology, BrdU labeling, and TUNEL staining of tissues harboring eGFP⁺ cells. *In situ* labeling techniques are performed on tissue from eGFP-tg B6 mice (**left**) and RAG1^{-/-} mice adoptively transplanted with eGFP⁺ T cells (**right**). **A:** Mantle-zone B cells in a mesenteric lymph node of an eGFP-tg animal stain red with anti-CD45R/B220 mAb. eGFP⁺ B220⁻ cells are visible in the same follicle. **B:** Leukocytes of the colonic submucosa in eGFP⁺ T-cell-transplanted RAG1^{-/-} mice are labeled with anti-CD45, some of which can be identified as donor T cells by their eGFP fluorescence. At the base of intestinal crypts, proliferating epithelial cells with BrdU-incorporating nuclei labeled red are found adjacent to eGFP⁺ enterocytes in eGFP-tg B6 mice (**C**) and in the vicinity of eGFP⁺ T cells in the mucosal lamina propria of transplanted RAG1^{-/-} mice (**D**). TUNEL staining reveals several apoptotic bodies (red) in the mesenteric lymph node of an eGFP-tg mouse (**E**) and a RAG1^{-/-} mouse adoptively transplanted with eGFP⁺ T cells (**F**). Individual apoptotic cells display residual eGFP, resulting in a yellow color (**arrows**). Original magnifications: $\times 106$ (**A**, **E**, and **F**), $\times 340$ (**B** and **C**), $\times 216$ (**D**).

Detection of eGFP⁺ Cells in Calcified Tissues

To detect eGFP⁺ cells in bone marrow, paraformaldehyde-fixed femur and vertebrae were decalcified in the presence of EDTA and subsequently snap-frozen in liquid nitrogen. This protocol allowed us to readily detect eGFP⁺ cells on frozen sections containing bone tissue that were cut with a conventional cryostat (Figure 2, A and B). The prolonged treatment with EDTA had no detrimental influence on eGFP

fluorescence, as can be demonstrated by the brightly fluorescent chondrocytes in Figure 2A.

In Situ Labeling of eGFP⁺ Cells and Host Cells with Fluorochrome-Conjugated Antibodies

Because visualization of eGFP in unfixed cryosections was virtually impossible because of quick diffusion in

Figure 4. eGFP⁺ DCs from eGFP-tg B6 donor mice engrafted in RAG1^{-/-} hosts. Four days after transfer, few engrafted eGFP⁺ DCs are found in the thymus (**A**) whereas in the mesenteric lymph nodes the number of engrafted DCs is significantly higher (**B**). Original magnifications: $\times 340$ (**A**), $\times 216$ (**B**).

aqueous media, immunohistology had to be performed on paraformaldehyde-fixed tissue and was therefore restricted to the use of antibodies against formalin-resistant epitopes. We used the anti-CD45R/B220 (clone 16A) and anti-CD45 (clone 30-F11) mAbs that are able to recognize formalin-fixed antigen (BD Pharmingen, technical information). With CD45R/B220, it was possible to label B lymphocytes in lymph node tissue while simultaneously detecting eGFP⁺ cells (Figure 3A). CD45 displayed a moderately strong staining of leukocytes, including eGFP⁺ adoptively transferred T cells (Figure 3B). Recognition of antigens in tissue fixed by cross-linking agents is often improved by target retrieval using heat.²¹ Following established protocols, we tried boiling the sections while immersed in citrate buffer in a microwave oven. This procedure led to a nearly complete loss of eGFP fluorescence that can probably be ascribed to the applied heat (the T_m of wild-type GFP is 70°C) but not to the mildly acidic buffer (pH 6.0) because eGFP is reported to retain 50% of its fluorescence at this pH.²²

In Situ Detection of in Vivo Proliferating eGFP⁺ Cells and Host Cells

For the detection of proliferating cells, mice were injected with BrdU 1 hour before sacrifice. Cryosections of paraformaldehyde-fixed tissue were treated with hydrochloric acid to denature the double-stranded DNA thus exposing incorporated BrdU to the mAb. As shown in Figure 3, C and D, nuclei of proliferating cells at the base of intestinal crypts were specifically labeled. At the same time, eGFP fluorescence was preserved to an extent that readily permitted unambiguous microscopic detection of GFP⁺ cells in eGFP-tg B6 mice (Figure 3C) and RAG1^{-/-} hosts transplanted with eGFP⁺ T cells (Figure 3D). Titration of hydrochloric acid revealed that a concentration of 0.2 mol/L was optimum to sufficiently retain both eGFP⁺ fluorescence and BrdU staining. These data demonstrate that a low pH of 0.7 does not completely (or not irreversibly) abolish the fluorescence activity of eGFP. It was crucial to evaluate the BrdU stainings immediately after their

processing and labeling because the intensity of the fluorescent signals markedly deteriorated within a few hours.

In Situ Detection of Apoptotic eGFP⁺ Cells and Host Cells

Apoptotic cell death was detected by TUNEL staining of DNA strand breaks performed at cryosections of paraformaldehyde-fixed tissue. Numerous apoptotic cells could be found in the mesenteric lymph nodes of eGFP-tg B6 mice (Figure 3E) and RAG1^{-/-} hosts transplanted with eGFP⁺ T cells (Figure 3F). Some of the apoptotic bodies proved to be remnants of eGFP⁺ cells, as demonstrated by their yellow color resulting from the addition of green and red fluorescence. Staining was specific as no apoptosis was visible in negative control reactions on the omission of TdT. eGFP fluorescence was not significantly impaired in TUNEL reactions. Note that proteolytic digestion with proteinase K, a step in the TUNEL-staining protocol, had no obvious influence on eGFP fluorescence.

Two Instructive Examples to Demonstrate the Power of the System and the Protocols

Transfer of eGFP⁺ DCs into Congenic RAG1^{-/-} hosts

In vitro cultured bone marrow-derived DCs from eGFP-tg B6 donor mice were transferred into congenic RAG1^{-/-} hosts. We found low numbers of eGFP⁺ DCs in the thymus of the transplanted host 2 to 5 days after transfer (Figure 4A). Higher numbers of eGFP⁺ DCs were found in the mesenteric and peripancreatic lymph nodes of the adoptive host (Figure 4B). The level of eGFP fluorescence of DCs was similar to the high fluorescence intensity of engrafted CD4⁺ T cells and permitted the sensitive detection of the *in situ* distribution of these potent antigen-presenting cells.

Figure 5. Detection of eGFP⁺ CD4⁺ T cells from eGFP-tg B6 donor mice engrafted in RAG1^{-/-} hosts. eGFP^{bright} CD4⁺ T cells adoptively transferred into RAG1^{-/-} hosts are readily detected in the adoptive host 15 days after transfer. **A:** Transplanted T cells aggregate into follicle-like structures in the spleen. **B:** In the liver eGFP⁺ T cells are found in the periportal fields and the lobular parenchyma. Large numbers of eGFP⁺ T cells accumulate in the colonic lamina propria (**C**) and in the mesenteric lymph nodes (**D**), whereas only a few eGFP-expressing cells are found in the mucosa of the small intestine (**E**). **F:** In the bone marrow, engrafted T cells are few yet easily detected (**arrow**). Original magnifications: $\times 54$ (**A**, **B**, and **D**), $\times 108$ (**C** and **F**), $\times 27$ (**E**).

Transfer of eGFP⁺ CD4⁺ T Cells into Congenic RAG1^{-/-} Hosts

We transferred purified eGFP⁺ CD4⁺ T cells from eGFP-tg B6 donor mice into congenic, immunodeficient RAG1^{-/-} hosts. CD4⁺ T cells were selected for the eGFP^{bright} phenotype on the fluorescence-activated cell sorting because eGFP is heterogeneously expressed from the transgene in the CD4⁺ T cell population of tg mice. Transferred eGFP⁺ cells were easily

detected on cryosections of paraformaldehyde-fixed tissue from transplanted mice. Repopulation of the host with eGFP⁺ donor T cells could be readily monitored in any target organ of interest, such as liver, mucosal tissues, bone marrow, spleen, or lymph nodes (Figure 5, A–E) even if only low numbers of eGFP⁺ cells were present (Figure 5; B, E, and F). All CD4⁺ lymphoid cells found in transplanted RAG1^{-/-} host 3 to 5 weeks after transfer were eGFP⁺ (Figure 6). Thus, within the adoptive host, eGFP expression by T cells was stable.

Figure 6. eGFP is a specific and stable marker of adoptively transferred cells. Lymphoid cells were isolated from the spleen, liver, and colon of RAG1^{-/-} mice transplanted with purified splenic eGFP^{bright} CD4⁺ T cells from eGFP-tg B6 donor mice. Fluorescence-activated cell sorting analyses shows that all engrafted eGFP^{bright} cells are CD4⁺ (and CD3⁺; data not shown).

Discussion

We describe a method to trace *in situ* different cell subsets using eGFP as a marker. In fluorescence microscopy, wild-type GFP is detected with a higher sensitivity as compared to staining with fluorescent-labeled antibodies.²³ Based on spectral analysis, fluorescence of eGFP is 35-fold higher than that of wild-type GFP.^{5,24,25} This makes eGFP an attractive marker for identifying adoptively transferred cells *in situ*. We show in this article that the repopulation of an adoptive host with GFP⁺ cells from eGFP-tg B6 donor mice can be readily followed. The method we describe is sensitive and specific. It can generate critical information on the *in situ* homing, differentiation, proliferation, and apoptosis of cells in adoptive transfer experiments and thus might become a powerful tool in many fields such as cellular immunology and stem cell research.

The *in situ* detection of donor eGFP⁺ cells is readily achieved on histological sections processed according to the simple technical guidelines summarized in Table 1. Unlike paraformaldehyde-fixed frozen tissue, paraffin-embedded tissue showed a nearly complete loss of eGFP-fluorescence. In contrast to published data and the results shown in this article, Walter and colleagues²⁶ found only a minimal reduction of eGFP fluorescence after a paraffin-embedding procedure when they examined tissue from mice that express transgene-encoded eGFP under CMV promoter control. The difference in

these findings is difficult to explain. Loss of eGFP fluorescence may be because of the high background fluorescence present in paraffin-embedded material.⁹ Alternatively, eGFP fluorescence may be destroyed during dehydration, paraffin embedding, and rehydration. The method we describe also offers the advantage to be suitable for direct and technically simple identification of donor-type cells in EDTA-treated bone marrow, thereby circumventing cumbersome procedures required for producing cryosections of bone tissue that has not been previously decalcified.²⁷ This is of considerable practical significance because *in situ* evaluation of the bone marrow is likely to play a pivotal role in many adoptive transfer experiments.

We show that eGFP⁺ cells can be further characterized *in situ* by established methods of immunohistology. Their surface marker expression can be determined by staining sections with fluorochrome-labeled antibodies. Immunostaining can yield information on the *in situ* differentiation and activation of engrafted eGFP⁺ cells or alternatively provide crucial data to characterize their cellular microenvironment. After BrdU labeling *in vivo*, the fraction and distribution of proliferating eGFP⁺ donor cells as well as resident cells can be quantitatively evaluated *in situ*. We show that by slightly modifying published protocols for labeling incorporated BrdU, eGFP⁺ fluorescence can be detected simultaneously. The sub-

Table 1. Protocol for *in Situ* Detection of eGFP-Positive Cells

1. Fix thin tissue slices (less than 3-mm thick) in 4% phosphate-buffered paraformaldehyde solution (pH 7.2) for 16 hours at 8°C
2. Optional for improved histomorphology, incubate tissue in paraformaldehyde solution (see no. 1) containing 50% sucrose for 48 hours at 8°C and briefly rinse sample in PBS before proceeding with No. 3
3. Freeze sample in liquid nitrogen
4. Store at -70°C
5. Embed frozen tissue in OCT compound. Cut 2-μm sections on a cryostat set at ~-20°C, mount sections on poly-L-lysine-coated slides
6. Optional perform nuclear counterstaining with DAPI (0.3 μg/ml) in PBS for 1 minute and rinse 2× with PBS
7. Embed sections in suitable mounting medium (for example Vectashield) and cover with coverslip
8. Sections can be stored in the dark at room temperature for several weeks without significant loss of eGFP fluorescence

set of eGFP⁺ cells that undergo apoptosis can be followed by combining the described tissue processing technique with the labeling of fragmented DNA by the established TUNEL-staining method. Even during the late stage of apoptosis characterized by DNA fragmentation²⁸ apoptotic bodies contained amounts of intact eGFP that were sufficient for microscopic identification of donor cells in TUNEL stainings. Confirming published data,^{29,30} in our system eGFP seemed to be relatively stable in the course of programmed cell death. This can be regarded as a potential advantage compared to identifying donor cells by antigens that might be subject to proteolytic digestion starting during the early phase of apoptosis. In fact, GFP or its variants have been repeatedly used for *in vivo* studies of apoptosis relying on their resistance against proteolytic caspase activity.^{31,32}

Taken together, the usage of eGFP⁺ donor cells for adoptive transfer experiments combined with the novel histological tissue processing we describe facilitates many studies on the *in situ* development of differentiating cell systems, eg, in lymphoid or myeloid cell lineages. In addition, eGFP⁺ cells can be recovered at different time points after transfer from the adoptive host and assayed *in vitro* for expression of cell surface markers or for functional reactivities.

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