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In Vivo Non Linear Optical (NLO) Imaging in Live Rabbit Eyes Using the Heidelberg Two-Photon Laser Ophthalmoscope

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Abstract

Imaging of non-linear optical (NLO) signals generated from the eye using ultrafast pulsed lasers has been limited to the study of ex vivo tissues because of the use of conventional microscopes with slow scan speeds. The purpose of this study was to evaluate the ability of a novel, high scan rate ophthalmoscope to generate NLO signals using an attached femtosecond laser. NLO signals were generated and imaged in live, anesthetized albino rabbits using a newly designed Heidelberg Two-Photon Laser Ophthalmoscope with attached 25 mW femtosecond laser having a central wavelength of 780 nm, pulsewidth of 75 fs, and a repetition rate of 50 MHz. To assess two-photon excited fluorescent (TPEF) signal generation, cultured rabbit corneal fibroblasts (RCF) were first labeled by Blue-green fluorescent FluoSpheres (1 μm diameter) and then cells were micro-injected into the central cornea. Clumps of RCF cells could be detected by both reflectance and TPEF imaging at 6 hours after injection. By 6 days, RCF containing fluorescent microspheres confirmed by TPEF showed a more spread morphology and had migrated from the original injection site. Overall, this study demonstrates the potential of using NLO microscopy to sequentially detect TPEF signals from live, intact corneas. We conclude that further refinement of the Two-photon laser Ophthalmoscope should lead to the development of an important, new clinical instrument capable of detecting NLO signals from patient corneas.

Keywords

Two-Photon Excited Fluorescence; Microspheres; Rabbit Cornea; Fibroblast; In Vivo Confocal

Introduction

Recently, imaging of non-linear optical (NLO) signals has been used to probe the cellular and extracellular organization of the cornea. These NLO signals are generated by fast-pulsed, femtosecond lasers with very short pulse durations, <100 fs, that are capable of achieving extremely high photon densities when the light is focused within very small tissue volumes (femtoliters: $1\mu m^3$). These pulsed, high photon densities lead to near simultaneous,

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multiple photon absorption by biological molecules without consequent tissue damage associated with longer pulsed or continuous lasers of the same power. Using longer wavelength, near-infrared coherent light (700–900 nm), multiple photon interactions can generate visible, NLO signals by either two-photon excited fluorescence (TPEF) or second harmonic generation (SHG) (Denk, et al., 1990; Guo, et al., 1996). TPEF signals generated by either endogenous or exogenously applied fluorophores have an emission spectra similar to single photon excitation that varies in intensity depending on the excitation wavelength. However, SHG signals are generated by non-centrosymmetric molecules, such as collagen, that interact with two excitation photons and emit a single emission photon that is always half the excitation wavelength. These differences between TPEF and SHG emission spectra make it possible for a single excitation wavelength to generate both TPEF and SHG signals that can be separately identified. Additionally, imaging of NLO signals using near-infrared coherent light provides greater depth of penetration into tissues, less phototoxicity, higher contrast and spatial resolution than conventional single photon approaches.

Piston, Masters and Webb were the first to report the detection of endogenous corneal TPEF signals from rabbit corneal epithelial cells in situ that were shown to be generated from NAD(P)H (Piston, et al. 1995). Later studies have reported on the imaging of both NAD(P)H and oxidized flavin adenine dinucleotide (FAD) autofluorescence detected in corneal epithelial cells, keratocytes and endothelial cells of mouse corneas (Lyubovitsky, et al., 2006), as well as SHG signals from corneal collagen in rabbit corneas (Yeh, et al., 2002; Zoumi, et al., 2002). Since the NAD(P)H/FAD emission spectra extends from 420–600 nm, the SHG signal generated by excitation wavelengths from 800 nm and shorter can be separated for simultaneous detection of cells and extracellular collagen.

Using NLO imaging, the cellular and extracellular organization of the cornea in various species including pig, mouse, cow and human has been evaluated without tissue processing, sectioning or staining (Chen, et al., 2009; Lyubovitsky, et al., 2006; Morishige, et al., 2006; Teng, et al., 2006; Wang, et al., 2008). SHG imaging of corneal collagen has been used to evaluate the effects of stromal edema and intraocular pressure (Hsueh, et al., 2009; Wu, et al., 2008), as well as to identify structural changes associated with disease, such as keratoconus (Morishige, et al., 2007; Tan, et al., 2006), wound healing (Farid, et al., 2008; Han, et al., 2004; Morishige, et al., 2008; Teng, et al., 2007; Wang, et al., 2007) and infectious keratitis (Tan, et al., 2007). While these reports have predominantly been conducted on excised tissue using laboratory based NLO laser scanning microscopes, as noted by Masters NLO imaging of live, intact corneas may have important clinical applications as this novel imaging paradigm is further developed, integrated and correlated with our current knowledge of corneal structure and function (Masters, 2009).

Towards the development of a clinical based NLO imaging system, we have evaluated the ability of a modified Heidelberg Two-Photon Ophthalmoscope with attached 25 mW, 780 nm femtosecond laser to detect TPEF signals within live rabbit corneas. In this study, Blue-green FluoSpheres-labeled rabbit corneal fibroblasts were injected into the corneal stroma of live rabbits. The spread of injected labeled fibroblasts could be imaged, sequentially in the same cornea by both reflectance confocal microscopy and NLO-TPEF imaging. Further development of this new, non-invasive microscope may facilitate the future study of corneal physiology and function in vivo, and over time in patients.

Materials and Methods

Heidelberg Two-Photon Laser Ophthalmoscope

The optical setup for Two-Photon Laser Ophthalmoscope is shown in Figure 1 (Heidelberg Engineering GmbH, Heidelberg, Germany). A compact femtosecond laser (central

wavelength 780 nm, pulsewidth 75 fs, repetition rate 50 MHz, average output power 25 mW, Femtolite Ultra, IMRA Inc., MI, USA) was focused using a 40×/0.8 objective (Obj., Olympus LUMPlanFL, PA, USA) onto the cornea. The laser beam was deflected in x- and y-direction via two scanning mirrors to trace out a square raster of 300×300 μm^2, 200×200 μm^2, or 150×150μm^2 at a frame rate up to 16 Hz. The back-reflected light was focused onto a confocal pinhole (PH) and detected by an avalanche photodiode (CD (Refl.)). The TPEF blue/green light was collected by the same objective lens and separated from the reflection light by a dichroic mirror (DiM). Backscattered laser light is further rejected by an IR-cut filter (SP, 750 nm cut-off wavelength, OD > 6). As TPEF light originates from the focal plane only, the signal is projected directly onto a non-descanned detector (NDD (TPEF)). The laser intensity was controlled using a rotating half-wave-plate (L/2) / polarizer (PBS) combination.

To examine TPEF in the live rabbits, animals were first anesthetized using ketamine (30 mg/Kg) and xylazine (3 mg/Kg) with topical proparacaine anesthesia. The rabbit was then scanned using the TPEF Laser Ophthalmoscope by applanating the 40× (N.A.=0.8) water-immersion objective lens to the rabbit cornea using a polymethylmethacrylate cap (Tomocap, Heidelberg Engineering GmbH, Heidelberg, Germany) to focus and collect signals. Contact gel (Comfort Gel, Bausch & Lomb, Berlin, Germany) was used as a coupling agent between the Tomocap and the cornea. By adjusting the controller and focus knob, the TPEF signals were excited by a femtosecond pulsed turnkey fiber laser source with 780 nm excitation wavelength. The acquisition of the image was achieved in high resolution as 1536×1536 in a 300 μm transversal field-of-view. Images were acquired and stored by using the Heidelberg Eye Explorer (Heidelberg Engineering GmbH, Heidelberg, Germany)

Rabbit Corneal Fibroblast Cultures

Corneal keratocytes were initially isolated from rabbits corneas obtained from Pel-Freez (Rogers, AR, USA) according to previously published methods (Gatlin, et al., 2003; Jester, et al., 1994). Briefly, eyeballs were washed with RPMI (31800–022 Gibco, Invitrogen Co., Carlsbad, CA, USA) supplemented with sodium bicarbonate (S-5761, Sigma-Aldrich Co., St. Louis, MO, USA) and antibiotics (penicillin/streptomycin/fungicide, 17–745E Biowhittaker, Lonza Group Ltd., Basel, Switzerland). Epithelium was scraped off of the cornea, the corneas cut off the eye and endothelium removed using a cotton-tipped applicator. Corneas were placed into the centrifuge tubes (15–20 corneas per tube) containing 50 mg hyaluronidase (LS002592 Worthington Biochemical Co., Lakewood, NJ, USA), 200 mg collagenase (17018-037 Gibco, Invitrogen Co., Carlsbad, CA, USA) and antibiotics in DMEM medium (31600-034 Gibco, Invitrogen Co., Carlsbad, CA, USA). Corneas were then incubate at 37°C in a 5% CO_2, water jacketed, cell culture incubator overnight.

Cells were then spun down at 1500 rpm for 5 minutes (Allegra Centrifuge, Beckman Coulter Inc., Fullerton, CA, USA), cell viability checked and cells counted using an automated cell analyzer (Vi-cell, Beckman Coulter Inc., Fullerton, CA, USA). Rabbit keratocytes were then seeded onto polystyrene treated 75 cm^2 cell culture flasks (CLS430720 Corning Inc., NY, USA) and grown in DMEM Serum positive medium containing 10% fetal bovine serum (FBS) (F-2442 Sigma, St Louis, MO, USA), and antibiotics. Cells were then passed in culture and used as rabbit corneal fibroblasts (RCF).

Cell Labeling

To label RCF, cells were first grown to confluence and then cultured in the presence of Blue-green FluoSpheres beads (430/465) (F-13080, Molecular Probes Inc., Eugene, OR,

USA) for 24 hours at a ratio of 1:10 to 1:100 cells to beads. Cultures were then rinsed three times with sterile PBS to remove the non-internalized beads. Labeled cells were then trypsinized for 5 minutes, and the cells collected by centrifugation at 1500 rpm for 5 minutes and washed 3 times in sterile PBS. Labeled cells were then counted and the concentration adjusted to 1.5×10^6 cells/ml before injection into the rabbit corneal stroma. The final suspension was transferred to a Hamilton syringe under sterile conditions. At the same time, 200 μl of labeled cells were seeded on 35 mm collagen coated tissue culture dishes (P35G-0-14-C MatTek Co., Ashland, MA, USA) for in vitro observation.

The Microspheres uptake and TPEF signals were confirmed using a laser confocal microscope system (LSM 510 META; Zeiss, Jena, Germany) with 40× (N.A.=1.3) oil-immersion objective lens, equipped with a mode locked titanium/sapphire laser (Chameleon 820 nm; Coherent, Santa Clara, CA). Samples were scanned using a 0.5 μm z-axis step size. Images were processed using the LSM Image Examiner (Zeiss, Jena, Germany). In vitro RCF cells were also observed by using inverted epifluorescent microscope (Leica DMIRB, Wetzlar, Germany) equipped with bandpass FITC excitation and emission filters with 20× (N.A.=0.4) and 40× (N.A.=0.55) dry objective lens . Fluorescence was detected using a CCD Camera (Hamamatsu ORCA II, Meyer Ins., Houston, TX, USA). Images were captured and overlaid using the MetaMorph Imaging System (Universal Imaging Co. Dowington, PA, USA).

Cell Injection

The rabbit was first anesthetized with 30mg/kg ketamine (57319-542-02 Ketaject, Phoenix, St Joseph, MO, USA) and 5mg/kg xylazine (139–236 AnaSed, Akorn Inc., Decatur, IL, USA) by intramuscular injection using a 23-gauge needle and the eye was topically anesthetized using one drop of 0.5% proparacaine (Alcon, Forth Worth, TX, USA). Using a stereofluorescent microscope (Leica L5 Fl, Leica Microsystems, Wetzlar, Germany), 50 μl of cells (~75,000 cells) were injected into the central cornea of the left eye using a 30 gauge needle. The eye was then treated with 0.3% gentamicin antibiotic ophthalmic drops (Bausch & Lomb, Tampa, FL) and taped closed.

Results

Microspheres endocytosis

After 12 hours co-incubation with FluoSpheres at a concentration of 1:10 (cells to beads), cultured RCF cells were 3-dimensionally scanned using a z-axis step size 0.5 μm in both reflectance to detect cells and TPEF to detect micrcospheres at 780 nm excitation. Labeled cells seen in reflectance show cell out line and focal adhesions (Figure 2, black and white). TPEF image (yellow) shows microspheres inside RCF in both XY and XZ planes.

In vitro labeled RCF observation

Before injection of labeled RCF, cells were seeded on to a glass cover-slip bottom culture dishes and then observed using an inverted epifluorescent microscope (Figure 3). RCF after trypsinization showed a spherical shape and contained numerous, phagocytosed microspheres (A. Green). Note that no free, unphagocytosed microspheres were detected. After 6 hours incubation in culture, attached and spread RCF continued to retain microspheres, which appeared to accumulate in the perinuclear region of the cells (Figure 3B).

Two Photon Excited FluoSpheres Tracking in Living Rabbit Cornea

Images acquired from normal, non-injected live rabbits showed no TPEF or SHG signals, indicating that the laser power from the Two Photon Laser Ophthalmoscope was not

sufficient to generate endogenous NLO signals from the cornea. However, the Laser Ophthalmoscope could detect fluorescent microspheres within injected RCF (Figure 4). Six hours after injection of labeled cells, reflected light signals from RCF cells showed clumps of cells at the injection site (A and C). TPEF images of the same regions showed that small fluorescent microspheres were contained only within the cells seen by reflectance imaging (B and D). Six days after RCF injection cells showed spreading and migration from the injection site as detected by reflectance imaging (Figure 5, A and C). Injected RCF (arrows) also showed brighter reflectance than adjacent, host corneal cells (arrowheads) due to the presence of phagocytosed microspheres. TPEF imaging also showed that only injected RCF cells retained fluorescent microspheres (Figure 5, B and D). These findings suggest that labeled RCF cells can still be easily track and followed by both reflection and TPEF mode. More importantly, no adverse effects of imaging using the Heidelberg Two-Photon Ophthalmoscope could be detected. Adjacent keratocytes appeared to remain quiescent and the corneal epithelium overlying the area of injection remained normal.

Discussion

Confocal microscopic examination of living tissue has been used to obtain live cellular images from selected tissues including kidney, liver, thyroid, muscle and connective tissue of rabbit and rats (Jester, et al., 1991; Petroll, et al., 1994; Petroll, et al., 1996), and has been used as a powerful methodology for examining the cornea (Li, et al., 1997) and evaluating corneal wound healing following excimer laser photorefractive keratectomy (Cavanagh, et al., 1993; Chang, et al., 1999; Maurer, et al., 1999). Here we report the first application of cornea-specific in vivo Two-Photon Laser Ophthalmoscope that provides high resolution TPEF images of labeled RCF injected into living rabbit corneal stroma. The major advantage of Two-photon Microscopy is that, compared with confocal microscopy, emission pinholes are not necessary (Centonze, et al., 1998; Gauderon, et al., 1999) to achieve axial depth discrimination because of the localized excitation volume. This specific feature also results in reduced sample photobleaching without out-of-focus photodamage. The wide spectral separation between emission and excitation of TPEF signals also offers improvement in the signal-to-noise ratio. Owing to the lack of efficient endogenous absorbers and the low scattering pattern from the long-wavelength photons employed in NLO microscopy, much deeper penetration is possible than with single photon or conventional fluorescent approaches (Helmchen, et al., 2005).

Unfortunately, the currently configured Two-Photon Ophthalmoscope with a 780 nm femtosecond laser could not detect any intrinsic TPEF signal that might have been generated by NAD(P)H/FAD from cells or SHG from collagen. The low power of the current laser (25 mW) is presumably below that required to generate such autofluorescence from keratocytes, corneal epithelial cells or stromal collagen. As currently configured, the 25 mW femtosecond laser with 50 MHz repetition rate and a scan speed of 16 Hz over a 300 μm^2 field of view provides a spot laser power that is well below the ANSI standards for retinal toxicity. Although we do not know what power levels will be required to generate endogenous TPEF and SHG signals in the Heidelberg Two Photon Ophthalmoscope, studies using a Zeiss 410 LSM and Chameleon femtosecond laser indicate that both NAD(P)H and SHG signals can be repeatedly obtained from intact corneas of live rabbits without tissue damage or photobleaching (Wang and Halbhuber, 2006). Additionally, comparison of the threshold values of laser power for performing nanosurgery using femtosecond laser induced optical breakdown and TPEF indicate that the power required for imaging is substantially below that required for cutting (Wang, et al. 2007). Furthermore, as long as the femtosecond laser light remains focused within the corneal volume, laser energy reaching back to the retina would be substantially less than that achieved by current femtosecond lasers used in corneal and lens surgery. Together these findings suggest that the currently designed system

with increased laser power may provide for NLO imaging of live corneas. This ability may have important implication for assessing overall NAD(P)H levels within the corneas of patients as well as identifying structural difference in stromal collagen of patients prior to refractive surgery.

In the present study, we have assessed the TPEF signal generated by FluoSpheres that were phagocytosed by corneal fibroblasts. The corneal fibroblasts that were investigated have been shown to have a pronounced capability to uptake a wide variety of foreign particles (Klintworth, 1969). Transmission electron microscopy has revealed that phagocytosed particles were membrane bound within the cytoplasm of keratocytes (Lande, et al., 1981), which matched the results obtained from LSM in our experiment. Most fluorescent tracers diffuse widely from their injection sites or remain in closely neighboring structures. However, previous reports that cells labeled with microbeads remain confined to the injection sites, make it possible to study the cell organization of many systems (Katz, et al., 1990). Furthermore, polystyrene latex beads have been shown to persist in corneal fibroblast for at least 6 months after injection. (Fujita, et al., 1987). Cells in our experiments appeared to show a similar retention of label for the 6 days of observation.

It should be noted that few if any beads were released from cells under both in vitro and in vivo conditions in our experiment. These data suggest that using this labeling approach one might be able to perform long-term and time-related 4D tracking and follow-up of labeled cells. This might prove useful in labeling cells where transduction with fluorescent reporter genes may be difficult, as in the case of studying the transplantation of corneal keratocytes. Recently, several laboratories have reported on corneal cell transplantation using umbilical cord mesenchymal or keratocyte stem cells to treated corneal disease (Liu et al. 2010; Du et al, 2009). Since keratocytes are difficult to transfect without losing their phenotypic characteristics, labeling with nanospheres maybe an alternative to more conventional transfection strategies using fluorescent gene transfer.

The disadvantages of using microinjection of labeled corneal cells, however, are the invasive nature and limited ability to evenly distribute cells throughout the cornea. Although the green fluorescent latex microspheres have been in use for several years as neuronal tracers in vivo (Thanos, et al., 1990), the biohazard potential of labeling in the cornea needs to be evaluated in future studies (Sayes, et al., 2009). Clearly, additional work is necessary to optimize the cell injections as well as identify other fluorescent marker that can be detected using TPEF.

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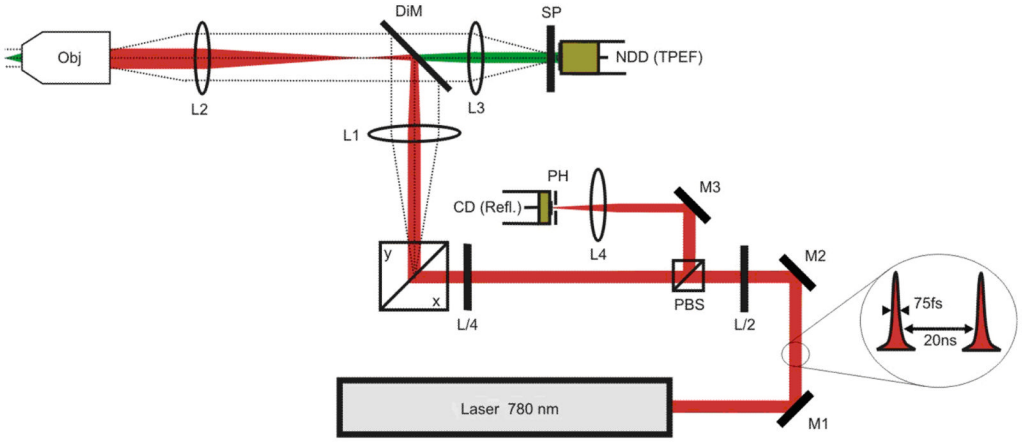

Figure 1.
Optical setup of the Heidelberg Engineering Two-Photon Excited Fluorescence (TPEF) Laser Ophthalmoscope.

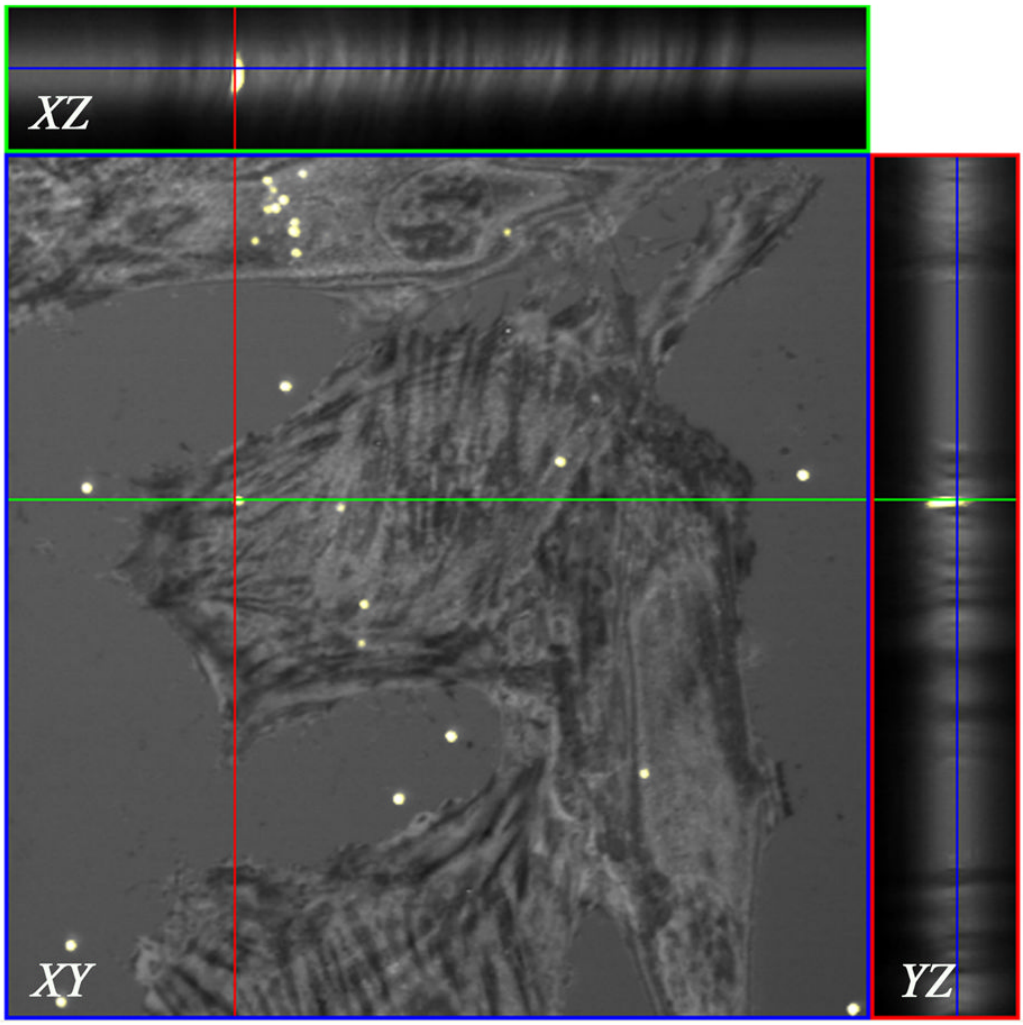

Figure 2.
Twelve hours co-incubate of RCF with FluoSpheres at a concentration of 1: 10 (cells to beads). . *XY*, *XZand YZ* planes (Z depth = 7.5 μm) are presented respectively for 3-dimensional .data sets combing reflectance (Gray) and TPEF (Yellow) signals. Single MircoSpheres can be detected inside a cell from all 3 planes.

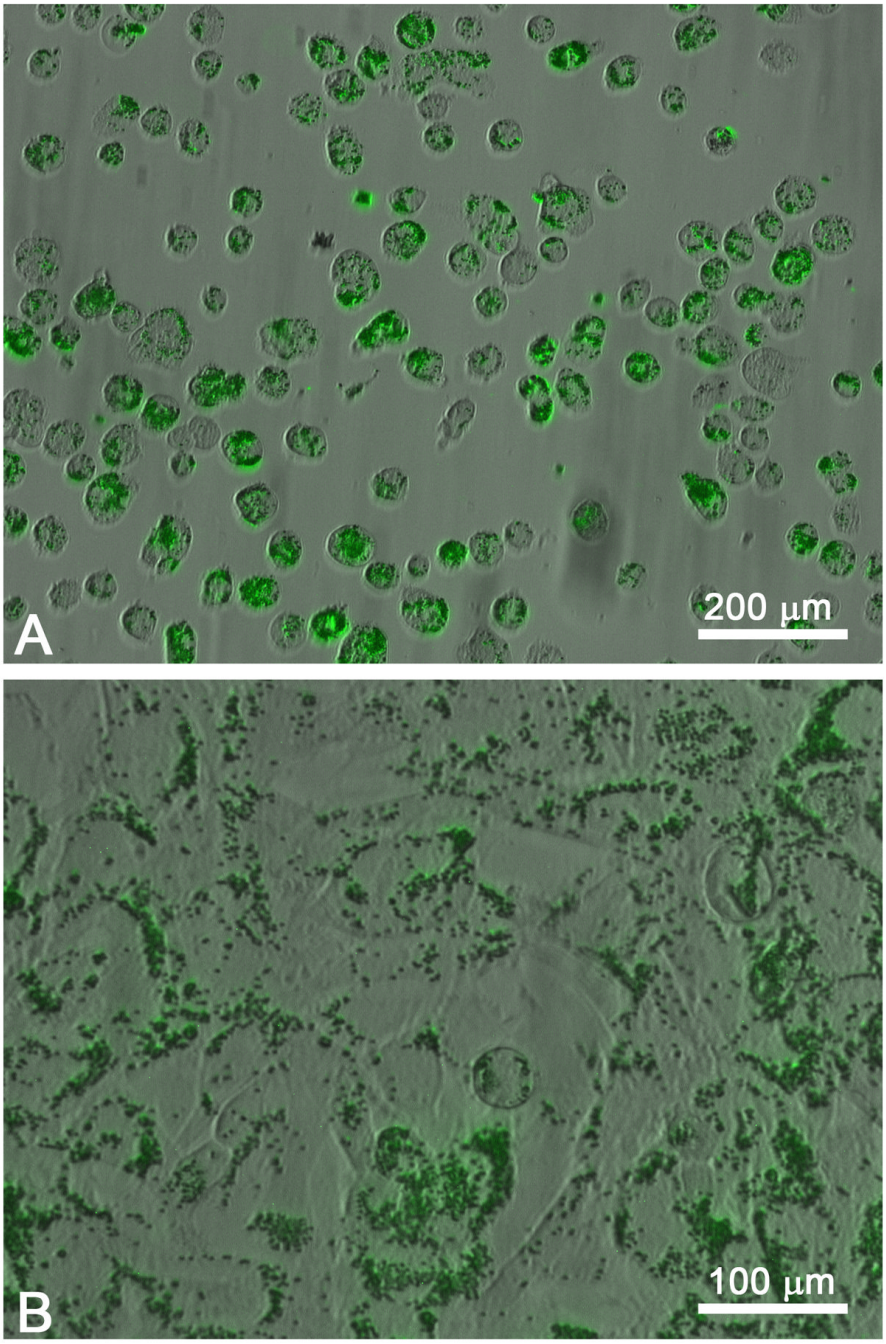

Figure 3.
In vitro labeled RCF cells were observed by using inverted Epifluorescent microscope. **A.** RCF labeled cells after trypsinization and before injection. Note that no fluorescence was detected outside of cells. B. Labeled RCF cells 6 hours after being placed in culture. Note cell spreading and retention of MicroSpheres.

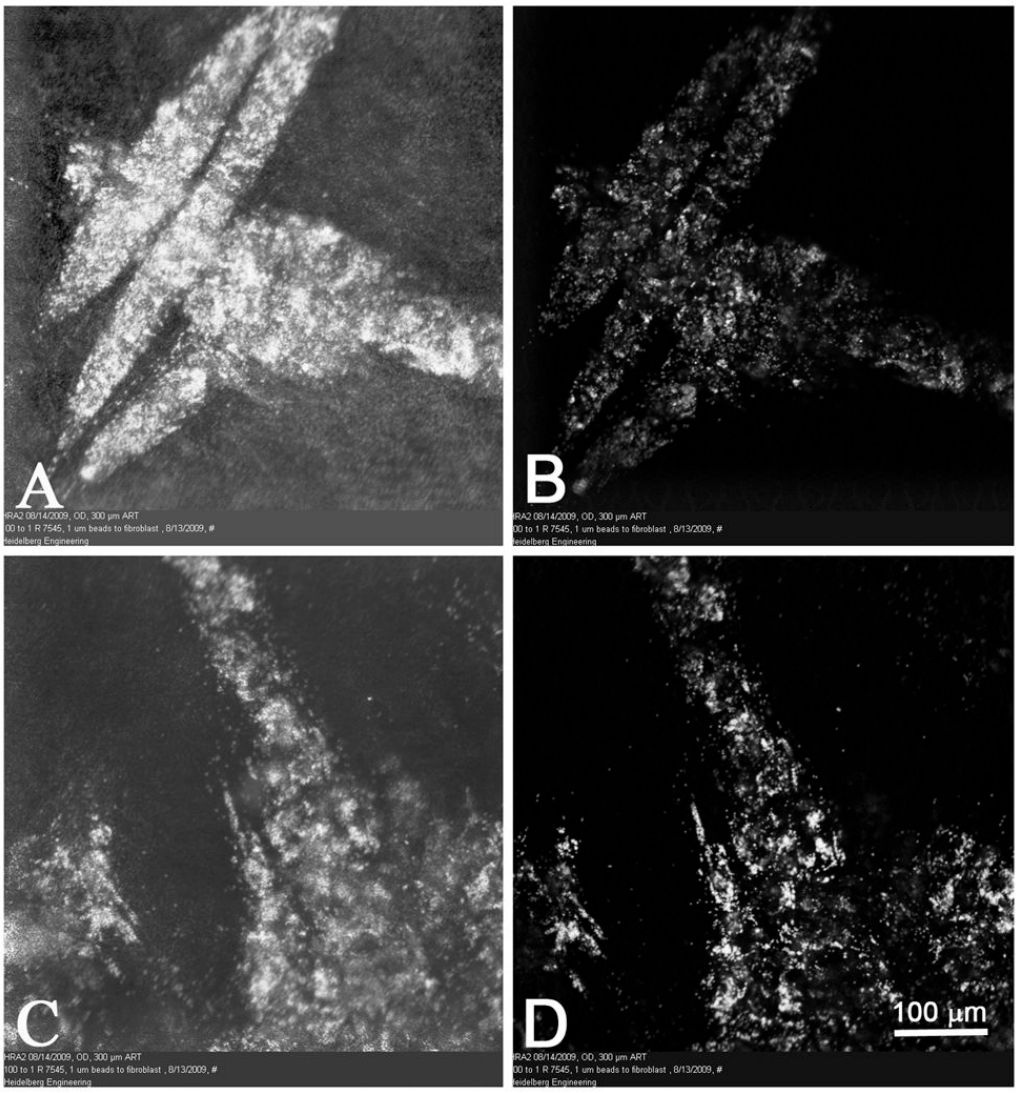

Figure 4.

Two-photon Laser Ophthalmoscope images in living rabbit eye 6 hours after injection. **A,C** Injected RCF cells and corneal stroma viewed in reflection mode. **B, D** High speed mode image of TPEF signal from RCF. Only injected RCF appeared to contain fluorescent MicroSpheres. (All images were viewed in 300 μm transversal field of view.)

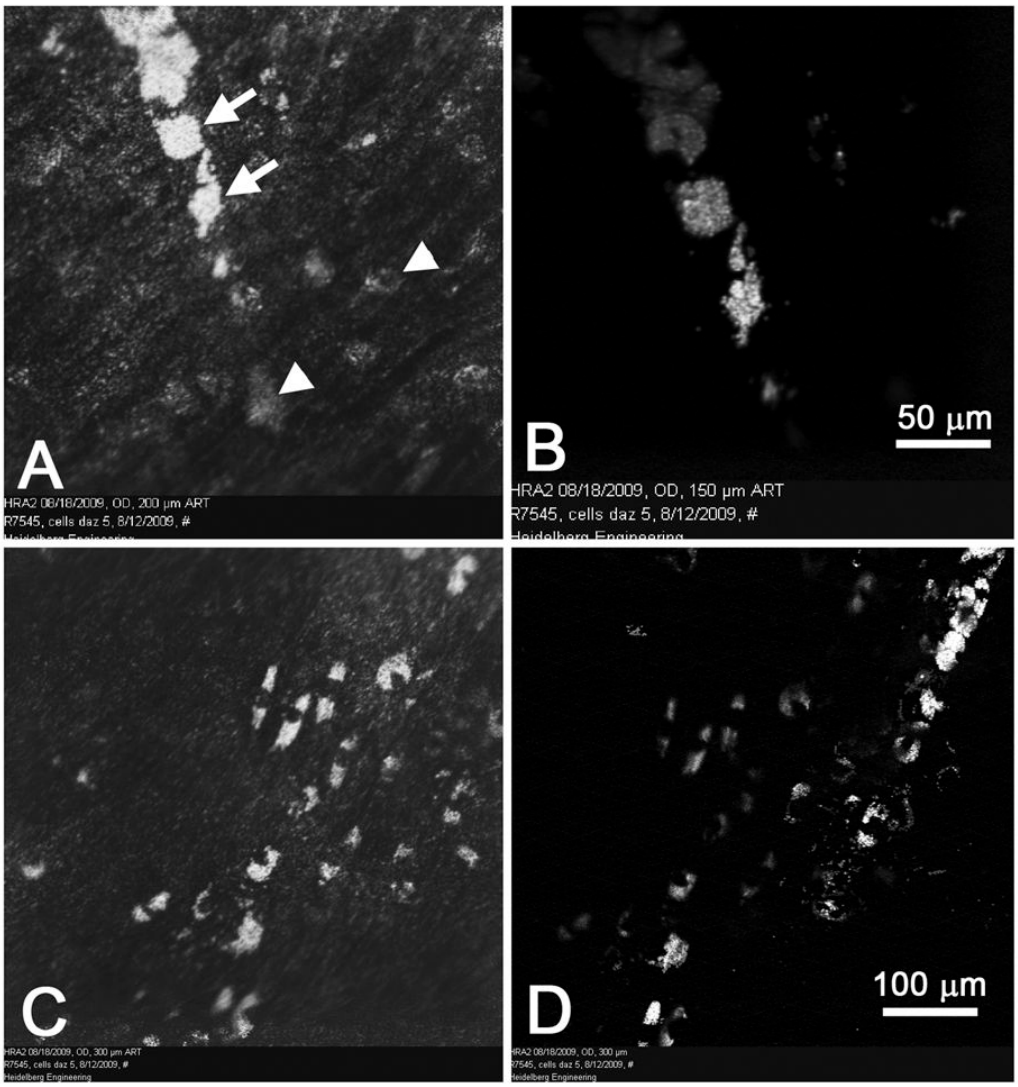

Figure 5.

Two-photon Laser Ophthalmoscope images of living rabbit eye 6 days after injection: **A,C** reflection mode; **B, D** TPEF mode. **A**, **B** were observed in 150 µm transversal field of view, **C, D** in 300 µm transversal field of view. Only labeled cells can be viewed in TPEF mode (A, arrows) while surrounding stromal cells showed no labeling (A, arrowheads).