Two-Photon Imaging of the Cornea Visualized in the Living Mouse Using Vital Dyes

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PURPOSE. To acquire morphological and component information on the overall cornea in the living C57BL/6 mouse using fluorescent viability dyes and two-photon (2PH) laser microscopy.

METHODS. Corneas were scanned using a 2PH laser scanning fluorescence microscope after staining with plasma membrane stain and Hoechst 33342. Representative 2PH images of corneal cells were analyzed and restructured using Imaris software.

RESULTS. With the plasma membrane- and cell-permeant nuclear counter live cell fluorescent probe, the morphology of corneal cells and the construction of cornea were observed clearly. The general in vivo morphology of the cornea clearly showed three different cellular layers, two interfaces, and the nerve fibers. Our study detailed all of the corneal cells with clear cell nuclei and cell boundaries at the sections parallel to the surface of the cornea. Moreover, cellular stereoscopic images, the relationships of the neighbor cells, and the interfaces of different layers were also displayed distinctly with three-dimensional construction of the 2PH imaging. Our research showed for the first time that there are three or four layers of epithelial cells and seven or eight layers of keratocytes in mice.

CONCLUSIONS. Our study clearly showed the anterior limiting lamina and Descemet’s membrane in the mouse cornea and showed for the first time that there are three or four layers of cells in the epithelium and seven or eight layers of keratocytes in the stroma of mice. These results are necessary primarily to contribute important insights into the anatomy and pathology of the cornea in mice.

Keywords: corneal morphology, two-photon imaging, living mouse, vital dyes

The cornea protects the eye from foreign matter and delivers two-thirds of the eye’s refractive power. Understanding the structural and morphological information for the cornea is of key significance to physiological studies and to the diagnosis of diseases of the ocular surface. Numerous studies have focused on visualizing the normal cornea and evaluating its transformation during disease and wound healing in humans; the structures and morphologic features of individual cells of the cornea have been well studied at submicron resolution in humans. Despite these studies having significantly helped us to understand the cornea, the experimental animal cornea is not yet fully understood.

With many transgenic and knockout mice exhibiting phenotypes resembling human ocular disease, mouse models have proven to be instrumental in the investigation of human ocular physiological processes and the development of disease states, and they have recently become widely used in corneal research. An understanding of the normal corneal structure, together with its morphology, is desirable for future comparisons when using mouse models for cornea research. Up to now, several studies have provided morphometric data and the dimensions of the cornea and its components in mice. The literature provides cornea data, either based on ex vivo fixed specimens or using confocal microscopy in vivo. Tissue morphologic artifacts resulting from sample fixation, slicing, and staining are inevitable. Although the corneal morphology under in vivo confocal microscopy has maintained original aspects separate from artificial factors, the morphological and structural images of the mouse cornea are insufficiently clear. For instance, the corneal cell boundary is not distinct; the cell nucleus cannot be observed, and it is more difficult to obtain a clear picture of the three-dimensional (3D) structure of the cornea, as well as cell–cell interaction.

Real-time acquisition of structural information on the cornea, with no intervention and destruction, would be crucial for investigating and diagnosing all corneal pathologies. On the basis of recent advances in optics and laser technology, several optical section techniques, such as two-photon (2PH) microscopy, have provided alternative methods for probing the cornea without the need to section and process tissue. These minimally invasive probing methods have opened the door to in vivo investigations of the cornea. However, to the best of our knowledge, there have been no reports of high-resolution, noninvasive 2PH imaging of morphological and structural information on intact corneas in mice in vivo at the cellular level.

Previous studies have shown that 2PH microscopy with minimal invasion is an efficient method for investigating key features of the ocular surface by cellular autofluorescence. Nevertheless, autofluorescence in the cornea is not sufficiently
conspicuous to acquire distinct images at the cell level. More recently, a new generation of viability fluorescent probes has been introduced; the characteristics of live cell probes, such as CellMask Orange plasma membrane staining, in combination with the cell-permeant nuclear counterstain Hoechst 33342, have greatly improved when compared to early-generation dyes.18,19

In this study, we used fluorescent viability dyes to specifically examine the morphological information for the overall intact cornea and its components in a commonly used strain of mouse, C57BL/6, using 2PH laser microscopy in vivo.

**MATERIALS AND METHODS**

**Mice**

C57BL/6 mice, free of clinically observable ocular surface disease and all of specific pathogen-free grades, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All of the mice were used at 4 months of age. All animal care and experimental protocols were approved by the Ethical Committee of Experimental Animal Care of Henan Eye Institute and were in compliance with the National Institutes of Health guidelines, and all

**Table.** Characteristics of the Various Types of Corneal Cells

<table>
<thead>
<tr>
<th>Cell Shape</th>
<th>Nucleus Shape</th>
<th>Cell Layers</th>
<th>Permeability to Viable Dye</th>
</tr>
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<tbody>
<tr>
<td>Superficial cells</td>
<td>Flat, plate-like</td>
<td>Disc</td>
<td>1</td>
</tr>
<tr>
<td>Wing cells</td>
<td>Polyhedral</td>
<td>Less flat than the superficial cells</td>
<td>1–2</td>
</tr>
<tr>
<td>Basal cells</td>
<td>Columnar</td>
<td>Higher nuclear-to-cytoplasmic ratio</td>
<td>1</td>
</tr>
<tr>
<td>Keratocytes</td>
<td>Pyramidal or stellate shape with long, fine, unbranched cell processes</td>
<td>Oval to bean shape</td>
<td>7–8</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Polygonal</td>
<td>Soybean shape</td>
<td>1</td>
</tr>
</tbody>
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Figure 2. Representative 2PH microscopic images and 3D reconstruction of the epithelial cells in C57BL/6 mice in vivo. Columns 1 through 3 show images of superficial cells (A1–E1), wing cells (A2–E2), and basal cells (A3–E3). The first row shows images of superficial cells (A1), wing cells (A2), and basal cells (A3). Each panel shows an xy (upper) and xz (lower) slice through a 3D stack. Corneal staining with plasma membrane stain (red) and Hoechst 33342 (blue). The white arrows indicate the epithelial layers. The second row shows the 3D reconstruction of the whole cornea and the superficial cells (B1), the rendered 3D surface plots of the wing cells (B2), and the basal cells (B3). The white arrows indicate the
procedures in the study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animal Preparation and Vital Dye Stains

The mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (85 mg/kg body weight) (Sigma-Aldrich, Shanghai, China). A homemade plastic eye cup was mounted on the upward-facing eye and was sealed with Vaseline (Zhengzhou Paini Chemical Reagent Factory, Zhengzhou, China) (Fig. 1A). The corneal surface was anesthetized with 1% tetracaine hydrochloride eye drops. Under a stereo-fluorescent microscope (Zeiss SteREO Discovery V20; Zeiss Microsystems, Gottingen, Germany), 2 μL 15 μg/mL CellMask Orange plasma membrane stain (Molecular Probes/Invitrogen, New York) was intrastromally injected into the peripheral cornea of the eye using a 30-gauge needle with a bevel of 30°. After 1 hour and 30 minutes, the mice were intrastromally injected with 2 μL 250 μg/mL Hoechst 33342 (Molecular Probes/Invitrogen) at the site opposite to where the plasma membrane stain was injected. Thirty minutes later, the cornea was rinsed three times with 0.9% saline water solution for in vivo 2PH imaging.

In Vivo 3D Imaging

The mice, after being prepared for imaging, were placed with one eye up on a custom-made plastic plate; the movement of the head with breathing was restrained in the head holder (Figs. 1B, 1C), which was equipped with a nosepiece (Fig. 1B) and ear bar (Fig. 1C) (Narishige, Tokyo, Japan). The four limbs during the imaging session were limited by gentle physical restraint with adhesive tape. The eye cup was filled with 0.9% saline solution to bridge the working distance from the water-immersion objective and to minimize heartbeat- and breathing-induced eyeball movement.

Two-photon imaging was performed with a Zeiss LSM 780 NLO fluorescence microscope system (Carl Zeiss MicroImaging GmbH, Jena, Germany) using Zeiss control software (Zen 2010; Carl Zeiss MicroImaging GmbH). This system is a combination 2PH and laser scanning confocal microscope based on upright Axio Examiner microscopy. In addition to regular laser scanning confocal capabilities with 32-channel GaAsP spectral detectors, this system has a tunable, mode-locked 2PH Ti:Sapphire laser (Chameleon vision II; Coherent, Inc., Santa Clara, CA) with an excitation range of 720 nm and three reflected light non-descanned detectors.

All of the imaging was performed using a ×20 (Plan-Apochromat, Numerical aperture [NA] = 1.0) and ×63 (Plan-Apochromat, NA = 1.0, V15-IR) water-immersion objective. Two-photon imaging signals were separately detected using two reflected light Non-Descanned Detectors (NNDs) with filter windows (R1: LP555nm-BP565-610; R2: LP490-SP485). The laser power used in this study was between 24% and 28% of maximum laser power (>3.5 W). The pinhole was set to the maximal open setting for this application, and the samples were scanned using a 2-μm z-axis step size to generate 3D data sets extending from the corneal endothelium to the epithelial surface. Image sequences were recorded as 12-bit, 512 × 512-pixel images. The scanning rate used in our study was 968.14 ms per frame. Corneal 3D data were collected from the central to the paracentral region.

Ex Vivo 3D Imaging

After in vivo 3D image collection, conventional corneal whole mount was performed as described previously. Briefly, the vital dye-stained corneas captured by 2PH scanning microscopy were excised and then fixed in 10% formalin in 0.1 M phosphate buffer (PBS) at room temperature for 2 hours, then washed three times for 5 minutes each with PBS. Four radial cuts were made in the cornea so that the cornea could be flattened by a coverslip. The regions overlapping with the in vivo cornea were scanned with the same 2PH microscopy parameters.

Morphological Analysis of the Corneal Cells

All image analyses were performed by visual inspection of the individual image sections. Different corneal layers were identified by their characteristic morphometric features. Representations of 2PH images and morphological analyses of corneal cells were presented using Imaris software, version 7.3.1 X64 (Bitplane, Zurich, Switzerland). Blend or maximum-intensity projection (MIP) filters were used for 3D visualization. Imaris creates isosurface objects by filtering the original data set and overlaying mathematical models over the original
FIGURE 4. Representative 2PH images and 3D reconstruction of the keratocytes in the central stroma in C57BL/6 mice in vivo. The first row shows images of keratocytes labeled with plasma membrane stain (red) and Hoechst 33342 (blue) in the anterior (A1), middle (A2), and posterior stroma (A3). The upper panels show an xy slice through a 3D stack, and the lower panels show an xz slice through a 3D stack. The white arrows indicate the respective stromal layers. The viable keratocyte cell bodies and cell processes on the plane of focus from a random network of interconnected cells. The second row shows the 3D reconstruction of the whole cornea and the rendered 3D surface plots of the anterior (B1), middle (B2), and posterior stroma (B3).
processes. The keratocytes overlap with each other to form a mesh, and they seem to contact each other by a syncytium of long cytoplasmic processes. The quiescent or resting keratocyte nucleus appears oval or bean shaped.

The posterior stromal keratocytes (B3). The third row shows a 3D reconstruction of the MIP of the anterior (C1), middle (C2), and posterior stromal keratocytes (C3) from the side view. These images show layers of keratocytes alternating with collagen lamellae. The nuclei appear to be the dominant part of the cytoplasm when viewed in transverse section. The fourth row shows the rendered 3D surface plots of the anterior (D1), middle (D2), and posterior stromal keratocytes (D3) from the epithelial view. The keratocyte cell bodies in the anterior (D1) and middle stroma (D2) appear irregularly shaped and are often pyramidal or stellate shaped. In the posterior stroma, the cell bodies are significantly larger and more irregular in shape than the anterior and middle stromal keratocytes, with a greater number of lateral cell processes (D3). These cell bodies extend long, fine, and generally unbranched cell processes from their apices, and the processes of neighboring cells are connected with adjacent keratocyte processes. The keratocytes overlap with each other to form a mesh, and they seem to contact each other by a syncytium of long cytoplasmic processes. The third row shows a 3D reconstruction of the MIP of the anterior (E1), middle (E2), and posterior stromal keratocytes (E3) from the epithelial view. The quiescent or resting keratocyte nucleus appears oval or bean shaped.

Corneal Sections

To compare the thickness of anterior limiting lamina in vivo with the corneal section, corneal sections were prepared using traditional protocol. The normal corneas were excised and then fixed in 10% formalin. The specimens were processed and embedded in paraffin blocks. Approximately 2-μm thin slices were obtained and mounted on glass slides. The cornea sections were stained with hematoxylin and eosin (H&E) and observed with light microscopy (ECLIPSE 80i; Nikon, Yokohama, Japan).

RESULTS

With the plasma membrane– and cell-permeant nuclear counter live cell fluorescent probe, the morphology of corneal cells and construction of the cornea were presented clearly. All of the images revealed an intensely and uniformly stained network of corneal cells within every single focal plane. The general in vivo morphology of the cornea clearly showed three different cellular layers and two interfaces in the central cornea of C57BL/6 mice: the epithelium, the anterior limiting lamina, the stroma, Descemet’s membrane, and the endothelium. The characteristics of the various types of corneal cells are shown in the Table. In addition, nerve fibers were detected in the cornea.

The corneal epithelium consists of three or four layers of three different types of epithelial cells: superficial cells, wing cells, and basal cells (Fig. 2). The surface of the corneal epithelium contains one layer of superficial cells. These cells are characteristically displayed as flattened, plate-like cells with small, flat nuclei in the centers (Figs. 2A1–2E1). Beneath the superficial cells lie one or two layers of wing cells, which are polyhedral cells that are less flat than the overlying superficial cells and which have flattened nuclei. The cell membranes of the adjacent wing cells are interdigitated with cytoplasmic processes (Figs. 2A2–2E2). The basal cells are located immediately above the anterior limiting lamina. The basal layer is composed of a single layer of cuboidal to columnar cells. They are smaller and have a higher nuclear-to-cytoplasmic ratio than the other epithelial cells in the cornea, and the neighboring basal cells are interdigitated laterally (Figs. 2A3–2E3).

The anterior limiting lamina was detectable clearly at the interface between the corneal epithelium and the stroma by 2PH microscopy under water-immersion objective (Figs. 3A, 3B). However, in the H&E-stained corneal section, the anterior limiting lamina could not be distinguished (Fig. 3C).

Directly posterior to the cell-free anterior limiting lamina is the stroma. Although the cell borders were difficult to determine, with the aid of 3D image reconstruction and analysis the 3D network structure of keratocytes could be observed clearly. The keratocytes are scattered among the lamellae of the stroma (Figs. 4B1–4B3, 3C1–3C3). The quiescent or resting keratocyte nucleus appears oval to bean shaped. The nucleus appears to be the dominant part of the cytoplasm when viewed in transverse section (Figs. 4C1–4C3, 4E1–4E3). The keratocyte cell bodies in the anterior (Figs. 4A1–4E1) and middle stroma (Figs. 4A2–4E2) appear irregularly shaped and are often pyramidal or stellate shaped. These cell bodies extend long, fine, and generally unbranched cell processes from their apices, and the processes of neighboring cells are connected with adjacent keratocyte processes. The keratocytes overlap each other to form a mesh, and they seem to contact each other by a syncytium of long cytoplasmic processes. In the posterior stroma (Figs. 4A3–4E3), a morphologically distinct subpopulation of keratocytes, two or three cell layers thick, was identified immediately anterior to Descemet's membrane. The cell bodies of these posterior keratocytes are significantly larger and more irregular in shape than the anterior and middle stromal keratocytes, with a greater number of lateral cell processes. These processes were
generally shorter, and they branched more than typical stromal keratocytes, with several processes forming an irregular network connecting many of the cells.

Descemet’s membrane lies between the corneal stroma and the endothelial layer. It can be observed clearly on our collected 2PH images (Fig. 5). On oblique sections, it is thicker and can be distinguished more easily than the anterior limiting lamina (Fig. 5B).

A single layer of corneal endothelial cells covers the posterior surface of Descemet’s membrane in a well-arranged honeycomb pattern. The endothelial cells interdigitate and contain large nuclei (Fig. 6).

Corneal nerves can be observed clearly in the basal epithelium and the subbasal layer, with a well-defined parallel arrangement of linear structures (Fig. 7A). Thick nerve fibers and thinner nerve branches can be observed in the anterior stroma (Fig. 7B), as well as mainly bulky nerves distributed in the middle stroma (Fig. 7C) and posterior stroma (Fig. 7D). The nerve fibers taper from the posterior stroma to the basal epithelium.

The image sequences from cornea endothelium to epithelium and the reconstructed 3D images from in vivo and ex vivo cornea are shown in Figure 8. Compared to the in vivo corneal image, the conventional corneal whole mount showed only three different cellular layers; the two interfaces of the anterior limiting lamina and Descemet’s membrane were not detected.

The entire thickness of the cornea in whole mount was thinner than in vivo; the stroma was the leading contributor to the corneal compression. The epithelium in corneal whole mount consisted of two layers of epithelial cells, and the stroma consisted of approximately two layers of keratocyte cells. Comparisons of the morphology of corneal cells and structure in vivo and ex vivo are detailed in Figure 9. The size of the nucleus in all cells became smaller, and the density of the corneal cells increased in whole mount compared to in vivo (Figs. 9A–9G). In epithelium, three to four layers of three different types of epithelial cells were condensed to two cell layers (Figs. 9A, 9B). In the stroma, the keratocyte boundary and processes were not detected because of the crowding of keratocytes in whole mount (Figs. 9C, 9D). The phenomenon of condensation of the keratocyte cells into a single layer of endothelium in whole mount could be observed (Figs. 9E, 9G).

**DISCUSSION**

In our study, we acquired morphological and structural information for the whole cornea and its components in living C57BL/6 mice using 2PH scanning laser microscopy. With the plasma membrane- and cell-permeant nuclear counter live cell fluorescent probe, the morphology of the corneal cells and the construction of the cornea were observed clearly. The general in vivo morphology of the cornea clearly showed three

![Figure 6](https://example.com/f6.png)

**Figure 6.** Representative 2PH images (A) and 3D reconstruction of the endothelium (B–F) in C57BL/6 mice in vivo. Corneal staining with plasma membrane stain (red) and Hoechst 33342 (blue). The corneal endothelial cells (inner white circle) are in a well-arranged honeycomb pattern (upper panel of A) at the sections parallel to the surface of the cornea; the white arrows indicate the endothelial layers on an xz slice through a 3D stack (lower panel of A). Three-dimensional reconstruction of the whole cornea and the rendered endothelium (B). Rendered 3D surface plots of the endothelium are shown from the side view (C) and from the endothelial view (D). The maximum-intensity projection (MIP) of the endothelial cells and their nuclei in 3D is displayed from the side view (E) and the endothelial view (G). These images indicate that the endothelial cells contain large nuclei.

![Figure 7](https://example.com/f7.png)

**Figure 7.** Representative in vivo 2PH microscopic images of the nerve fiber in C57BL/6 mice in the subbasal (A), anterior (B), middle (C), and posterior stroma (D). Corneal staining with plasma membrane stain (red) and Hoechst 33342 (blue).
different cellular layers and two interfaces. In addition, nerve fibers were detected in the cornea.

Mice have been increasingly investigated in eye research in recent years. However, little morphological and structural detail of the cornea has been reported in most previous studies in mice. In addition, the photographs of the cornea have usually shown cross-sectional views (perpendicular to the corneal surface) of the five full corneal layers in fixed corneal samples in vitro, although images obtained with the in vivo confocal microscope generally consist of optical sections oriented parallel to the surface of the cornea. Representations of the different corneal cells without visible cell nuclei did not show the ratio of the cell nuclei to the cytoplasm. Our study detailed all of the corneal cells with clear cell nuclei and cell boundaries from sections parallel to the surface of the cornea. Moreover, cellular stereoscopic images, the relationships with neighboring cells, and the interfaces of different layers were also displayed distinctly with 3D construction of 2PH imaging.

Our results clearly showed three different cellular layers and two interfaces in the corneas of C57BL/6 mice: the epithelium, the stroma, the endothelial layers, Descemet’s membrane, and the anterior limiting lamina. The literature has been controversial regarding whether there is an anterior limiting lamina and Descemet’s membrane in the mouse cornea. Our in vivo cornea findings clearly showed the anterior limiting lamina. This result is consistent with some of the previous findings. In fixed cornea sections, we did not detect the anterior limiting lamina either; this may be explained on the basis that the anterior limiting lamina is very thin originally in vivo and became thinner after fixing such that it could not be observed by light microscopy. Our results displayed a Descemet’s membrane thicker than the anterior limiting lamina in the mouse cornea, in strong agreement with previous studies. There is no doubt that the human corneal epithelium consists of five or six layers of epithelial cells: two or three superficial cell layers, two or three layers of wing cells, and a monolayer of columnar basal cells. The penetration of vital fluorescein from the surface of the cornea into the corneal stroma in our experiment demonstrated that the wing cell layer plays the role of a barrier in the corneal epithelium, in agreement with previous studies. Our study showed clearly for the first time that there are seven or eight layers of keratocytes and also showed the morphology of the keratocytes. The morphology of the keratocytes is similar to that of humans after digestion of stromal collagen, as observed previously by scanning electron microscopy. The corneal endothelium of mice is similar to that of humans, with minimal morphological variations.

To demonstrate the significance of obtaining corneal images in vivo, we compared images from the in vivo cornea and the conventional corneal whole mount, which was the representative ex vivo sample. The results showed that the entire thickness of the cornea in whole mount appeared thinner than in vivo; the size of the nucleus in all cells appeared smaller, and the density of the corneal cells increased in whole mount compared to in vivo. This could be explained by shrinkage, a distortion that occurred when the cornea was fixed.

Many commonly used stains do not stain living cells because they cannot cross live plasma membrane. The vital dyes, which are either small or are pumped actively into live cells, can stain living cells without killing them. Therefore, we applied vital dye in our experiment to obtain morphological and component information on the entire cornea in the living mouse. The CellMask Orange plasma membrane stain used in our experiment is a convenient marker of cell boundaries and provides excellent and rapid plasma membrane staining in live cells, and the staining pattern is also maintained after fixation with formaldehyde. In combination with the cell-permeant nuclear counterstain Hoechst 33342, corneal cells can be

**FIGURE 8.** Representative 2PH image sequences (A) and reconstructed 3D images (B) from in vivo and ex vivo cornea. Cornea stained with plasma membrane stain (red) and nucleus (blue). The gallery of representative 2PH image sequences (A) shows the x and y dimension from the endothelium to epithelium with a 2-μm interval. Three-dimensional images (B) reconstructed, respectively, from the upper photograph; the white dashed line indicates the boundary between epithelium and stroma. Scale bar: 100 μm (A).
visualized in the living mouse. Numerous studies have applied CellMask Orange plasma membrane stain and Hoechst 33342 to stain live cells27–29; however, the safety of these vital dyes has rarely been studied. Therefore, the safety on these vital dyes need to be studied more.

In summary, we acquired morphological and structural information on the whole cornea and its components with plasma membrane- and cell-permeant nuclear counter live cell fluorescent probes in C57BL/6 mice in vivo, using 2PH laser scanning microscopy. Our study details all of the corneal cells with clear cell nuclei and cell boundaries at sections parallel to the surface of the cornea, as well as cellular stereoscopic images, the relationships with the neighboring cells, and the interfaces of the different layers. Our study clearly shows the anterior limiting lamina and Descemet’s membrane in the mouse cornea and, for the first time, shows that there are only three or four layers of cells in the epithelium and seven or eight layers of keratocytes in the stroma in mice. These results are necessary primarily to contribute important insights into the anatomy and pathology of the cornea in mice.

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![Image](https://example.com/image.png)


