The study of skin aging has greatly advanced. The inner structure of the skin can now be studied using various methods. This chapter describes observations on the inner structure of the skin and its age-related changes using in vivo reflectance confocal microscopy (RCM). RCM is a noninvasive method and offers real-time observation.

The human skin is made up of the epidermis and dermis. Each layer has characteristic inner structures. These inner structures and their age-related changes have been observed using RCM. The epidermis is divided into four layers, which differ in thickness and cell shape. The cells of the epidermis are densely arranged. The epidermis and dermis are separated by a basement membrane and form a concave–convex structure known as the dermal papilla. The dermis consists of cellular and stromal components, which form fibrotic tissue.

The inner structures of the epidermis change with age. The depth from the skin surface to the lower end of the dermal papillae and the thickness of the basal layer become thinner with age, and the granular layer becomes thicker with age. The structure of the dermal papillae is evaluated by calculating its parameters. Decrease in number, increase in cross-sectional area, and decrease in the height of the dermal papillae have all been observed with aging. Finally, with age, fibrous structures in...
the dermis change from a cobweb-like pattern to being oriented in the same direction. Elucidating these inner structural changes caused by aging may further the understanding of skin aging.

**Introduction**

The human skin consists of three layers: the epidermis, dermis, and subcutaneous tissue [1]. On average, the epidermis is approximately 0.2 mm in thickness, and 95% of the cells in it are keratinocytes. Epidermal keratinocytes are at different stages of maturation and are densely arranged into layers at four different levels: the horny cell layer (10–20 layers), the granular cell layer (2–3 layers), the suprabasal cell layer (5–10 layers), and the basal cell layer (1 layer). The epidermis and dermis are separated by a basement membrane and form a concave–convex structure. This structure is known as the dermal papilla, and it is believed to play an important role in the skin physiology. The dermis is approximately 15–40 times thicker than the epidermis. The dermis is divided into three layers: the papillary layer, subpapillary layer, and reticular layer. However, its boundary is not clear compared with the epidermis. In addition, the dermis is divided into cellular components, such as fibroblast, and stromal components that form a fibrous structure. The main component of this fibrous structure is the collagen fibril. Thus far, these inner structures of the skin have been observed using skin biopsy [2–6]. However, skin biopsy is invasive and an ex vivo method that requires the tissue to be removed from the skin. Noninvasive methods that do not require the excision of the tissue for evaluating the inner structure of the skin are required. Recently, there have been advances in observational technology for examining the inner structure of the skin. These methods are noninvasive and include ultrasound imaging, magnetic resonance imaging (MRI), and optical coherence tomography (OCT). Ultrasound visualizes structural changes in the acoustic organization of the skin and is used to measure skin thickness and skinfold thickness. MRI visualizes proton density, relaxation time of protons, and blood flow and is used to observe tomographic images as well as the status of tissues. OCT utilizes the coherence of light and offers tomographic images of tissues. However, because of the inadequacy of spatial resolution, it is difficult to apply these methods for the direct measurements of the inner structure of the skin.

Previous studies have observed the inner structure of the skin using in vivo reflectance confocal microscopy (RCM) [7–10]. RCM is a noninvasive technique used for imaging living skin. Recent reports have suggested that the inner structures of the skin, such as cell size in the granular and the suprabasal layers, thickness of the epidermis and the basal layer, dermal papillae, and collagen bundles, change with age [11–14]. In addition, recent reports have suggested that the structure of the dermal papillae varies depending on the site [15], and it correlates with skin elasticity and the I-value, which represents intensity of light [16].

Skin aging is classified into intrinsic aging and photoaging, each exhibiting characteristic changes [17]. In intrinsic aging, decrease of the skin thickness [18] and lower proliferative rate of the epidermis have been reported. Conversely, in photoaging, increase of the skin thickness [18], increase in the size of corneocytes and wrinkle grade [20, 21], and higher proliferative rate of the epidermis have been reported [19]. These age-related changes may correlate with age-related changes of the inner structure of the skin. Therefore, an observation of the inner structure of the skin may be important in the understanding of skin physiology and aging.

This chapter describes observations on the inner structure and age-related changes of human skin using RCM. Focused around the authors’ recent research related to the structure of the dermal papillae, the findings of studies on the inner structure of the skin are presented here.

**RCM Apparatus**

Confocal microscopy can be used in vivo and is available in fluorescence or reflectance mode. Previously, the fluorescence mode, utilizing a
fluorescent probe, has been used to observe the morphology of tissues and depth distribution of substances such as organelles [22, 23]. However, recently, the reflectance mode, known as RCM, utilizing reflected light has been more widely used. RCM allows for the noninvasive observation of living tissue and can be applied to obtain information on the inner structure of the skin. This chapter will focus on RCM.

In contrast to conventional microscopy, in confocal microscopy, only a portion of the focus is brightly imaged, and it is operated by detecting single back-scattered photons from illuminated living tissue [24]. Because light other than that of the focal position is cut by a pinhole, information from only the focal position reaches the detector. Resolution occurs in the depth direction (the Z direction); thus, high contrast and resolution are obtained compared with conventional microscopy. Confocal microscopy can therefore be used to observe horizontal cross-sectional images, every few μm from the tissue surface to the depth direction, by moving a vertically objective lens in real time. However, because RCM utilizes reflected light, signals from deep within the tissues decrease due to scattering and absorption of light. With RCM, imaging is limited to a depth of 200–300 μm.

An expansive image can be obtained by scanning the sample in the horizontal plane (XY plane) keeping a fixed light or scanning light in the horizontal plane keeping the sample. Three-dimensional (3D) images are obtained by reconstructing images in the depth direction on a computer. Most of the light sources used in confocal microscopy are lasers. Lasers can be regarded as a point light source and have high luminance and stable output. The authors’ study utilized RCM apparatus with an 830 nm laser, Vivascope 1500/3000 (Lucid Inc., Henrietta, NY).

The contrast of confocal images of the skin is provided by the refractive index differences of intracellular substances, extracellular matrix components, and other microstructures from the background. In the skin, refractive index differences correspond to melanin in the epidermis, collagen and elastin fibers in the dermis, and erythrocytes in the dermal capillaries [25]. Melanin in particular provides strong contrast [26]. It has been reported that various inner structures and cells can be observed using RCM (Table 1).

Observations using RCM are shown in plane images in the depth direction from the skin surface. Figure 1 shows RCM observations of dermal papillae. In the skin surface, a fissured appearance of the horny cell layer and pores is observed (Fig. 1a). With deepening of the image, a bright spot begins to appear representing the upper end of the dermal papillae (Fig. 1b). Subsequently, the dermal papillae are observed as bright circles (Fig. 1c). The dermal papillae are clearly observed with RCM because the melanin that exists in the basal layer along the dermal papillae offers strong contrast. Pores are observed from the skin surface to the depth direction and can be distinguished from the dermal papillary bright circles.

### Imaging of Inner Structures Using RCM

#### Inner Structure

RCM enables observation from the skin surface to the dermis. The skin surface is not uniformly smooth and is engraved by a number of fine grooves called sulcus cutis. Small ridges surrounded by the sulcus cutis are called the crista cutis. On average, the thickness of the epidermis is approximately 0.2 mm and 95 % of the cells are epidermal keratinocytes. Keratinocytes divide at the lowest layer of the epidermis and migrate to the upper layer as they mature. Keratinocytes at

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different stages of maturity are arranged in layers and are classified from the depth to the upper layer as follows: the basal cell layer, suprabasal cell layer, granular cell layer, and horny cell layer. Keratinocyte layers and cell sizes can be observed using RCM. The basal cell layer has a thickness of 10–20 μm [11] and the cell forms are cubic and columnar. The diameter of the cells in the suprabasal cell layer is 20–30 μm [14]. In the suprabasal cell layer, lower cells are polygonal and upper cells are flattened. The size of the cells in the granular layer is 800–1000 μm² [11], and the cells are flat. The horny cell layer has a thickness of 20–30 μm [13], and the flat cells in the horny layer are stratified.

The epidermis and dermis are in close contact by the basement membrane structure. The boundary of the epidermis and dermis forms a concave–convex structure. The structure by which the dermis is projected toward the epidermis is referred to as the dermal papillary structure. RCM has shown that the thickness between the upper end and the lower end of the dermal papilla is 20–50 μm [13].

The dermis is located below the epidermis across the basement membrane. It is divided into cellular components, such as fibroblast, and stromal components which form fibrotic tissue. The stromal component is composed mostly of collagen fibers and also includes elastic fibers and
reticular fibers. These are referred to as the extracellular matrix. The dry weight of collagen fibers accounts for 70% of the dermis. Collagen fibers are very tough and are important as a support organization to keep the mechanical strength of the skin. Collagen fibers are formed to gather into a thin basic unit called a fibril, and the gathered fibers of the fibrils are thick and tough. In the upper dermis (the papillary layer and subpapillary layer), sparse, thin collagen fibers have been observed using RCM at a depth of 90–140 μm from the skin surface [13]. On the other hand, in the lower dermis (the reticular layer), well-developed thick collagen fibers, referred to as collagen fiber bundles or collagen bundles, have been closely observed.

**3D Reconstruction Using RCM Image Sequences**

RCM image sequences can be used for 3D reconstruction, and dermal papillary structures can be visualized. The 3D imaging can be obtained using Fiji-ImageJ software (NIH, Bethesda, MD) [30], which is an open-source program.

Figure 2 shows a 3D image obtained from RCM image sequences, taken every 1 μm from the surface of the horny cell layer down to 99 μm. The 3D image was rotated, and snapshots at the skin surface (a) and dermal side (b) were taken. The sulcus cutis, crista cutis, and pores in the skin surface (a) and the concavity–convexity structure of the dermal papillae on the dermal side (b) are seen. Tomographic images in the vertical direction show that the dermal papillae project into the epidermis (c).

**Parameterization of the Inner Structure**

For the evaluation of the dermal papillary structure, the parameters of various structures can be determined using RCM (Fig. 3). Evaluation methods used in the authors’ recent study are described below.

Parameters related to the dermal papillary structure include the number of dermal papillae (/mm²), the cross-sectional area (μm²) representing broadness of the dermal papillae, and the depth to the upper end of the dermal papillae (μm). In these evaluations, pores were distinguished from the dermal papillae and were excluded. In addition, parameters in the sun-exposed skin sites (cheek and nasolabial fold) were employed for correlation analysis.
Horizontal RCM images, 20–30 μm in depth from the upper end of the dermal papilla, were used for the calculation of these parameters. The number of dermal papillae (/mm²) was evaluated by counting the dermal papillae in the RCM image. The cross-sectional area (μm²) of the dermal papillae was calculated using ImageJ software (NIH, Bethesda, MD). The depth to the upper end of dermal papillae (μm) was determined by calculating the depth of the upper end of the dermal papillae from the skin surface.

Parameters of other inner structures have been determined using RCM, including the thickness of the horny cell layer, the thickness from the skin surface to the lower end of the dermal papillae, the thickness of the basal layer, and the height of the dermal papillae [11–13, 15].

### Age-Related Changes of the Inner Structure

#### Structure of the Epidermis
The inner structures of the epidermis can be observed using RCM, including the depth from the skin surface to the lower end of the dermal papillae, thickness of the basal layer, cell size in the granular layer, and thickness of the horny cell layer. These inner structures have been compared between younger and older skin. The depth from the skin surface to the lower end of dermal papillae, including sun-exposed and -protected skin sites, is thinner in older skin than that in younger skin [13]. The thickness of the basal layer, which is a sun-protected skin site, is also thinner in older skin than that in younger skin [11]. However, the thickness of the granular layer, which is a sun-protected site, is thicker in older skin than that in younger skin [11]. The thickness of the horny cell layer does not change with age [11, 13].

Considering decreased skin thickness of sun-protected sites and increased skin thickness of sun-exposed sites with age [18], the pattern of structural change in the epidermis may differ between sun-exposed and sun-protected sites.

#### Dermal Papillary Structure
The dermal papillary structure has been observed using RCM. Studies have suggested that there are age-related changes in the dermal papillae both in sun-exposed and sun-protected skin sites [11–14]. Using RCM, the authors investigated the
correlation between age and parameters of the dermal papillary structure. The results are described below.

In facial skin (cheek and nasolabial fold), age had a significantly negative correlation with the number of dermal papillae and a significantly positive correlation with the cross-sectional area of the dermal papillae. There was no correlation between the depth to the upper end of the dermal papillae and age. The number of dermal papillae decreased with age, and the density of the concave–convex structures of the dermal papillae became sparse. The cross-sectional area of dermal papillae increased with age and sharp dermal papillae become broader. These changes suggest flattening of the dermal papillae with age. In addition, it has been reported that dermal papillae decrease in height with age [12, 13], that the depth to the upper end of the dermal papillae increases with age [11], and that vitamin C increases the number of dermal papillae [31].

Age-related changes in dermal papillary structure were shown in the cheek skin of females in their 20s and 60s, using 3D reconstruction of RCM images. On the dermal side, females in their 20s had abundant hole shapes with bright circular patterns indicating dermal papillae. However, females in their 60s had few hole shapes with bright circular patterns. In both groups, pores were observed to pass through from the side of the horny cell layer to the dermal side and were distinguished from the dermal papillary structure. These results using 3D reconstruction of noninvasive RCM images are similar to previous reports in which observations were made by invasive methods [32].

**Fibrous Structure of the Dermis**

The stromal components of the dermis are fibrotic tissue. Fibrotic tissue is mostly composed of collagen fibers. The collagen fibers form three-dimensional fibrous structures with substrates containing glycosaminoglycan and elastin. These fibrous structures are visible with RCM [13]. Figure 4 shows the fibrous structures in the cheek skin of participants in their 30s compared with those of participants in their 50s. The fibrous structures were arranged in a cobweb-like pattern in participants in their 30s. However, the fibrous structures were arranged in the same direction in participants in their 50s. In addition, anisotropy and clearness of the fibrous structures were shown to change with age [16]. Changes in the level of degraded collagen [33], type I procollagen protein expression [34], and the shape of collagen fibers [35] by photoaging may be related to the changes of fibrous structures in the dermis.

**Conclusion**

With the development of new observational methods, age-related changes in the inner structure of the skin can now be noninvasively observed. RCM is a suitable method for the
noninvasive observation of the inner structure of the epidermis and dermis. Using RCM, the study of the inner structure of the skin has greatly advanced. In particular, the structure of the dermal papillae, which are believed to be involved in the skin physiology, has been observed using RCM. The structure of the dermal papillae changes with age, and it has been suggested that the dermal papillae flatten with age. In addition, the inner structure of the epidermis and fibrous structure of the dermis change with age. Understanding age-related changes of the inner structure of the skin will help to elucidate skin aging and skin physiology.

References

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