Fibre optic confocal imaging (FOCI) of keratinocytes, blood vessels and nerves in hairless mouse skin in vivo


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ABSTRACT

Fibre optic confocal imaging (FOCI) enabled subsurface fluorescence microscopy of the skin of hairless mice in vivo. Application of acridine orange enabled imaging of the layers of the epidermis. The corneocytes of the stratum corneum, the keratinocytes in the basal layers and redundant hair follicles were visualised at depths greater than 100 µm. Cellular and nuclear membranes of keratinocytes of the skin were visualised by the use of acridine orange and DIOC5(3). Imaging of the skin after injection of FITC-dextran revealed an extensive network of blood vessels with a size range up to 20 µm. Blood cells could be seen moving through dermal vessels and the blood circulation through the dermal vascular bed was video-taped. The fluorescent dye 4-di-2-ASP showed the presence of nerves fibres around the hair follicles and subsurface blood vessels. Comparison was made between images obtained in vivo using FOCI and in vitro scanning electron microscopy and conventional histology. FOCI offers the potential to study dynamic events in vivo, such as blood flow, skin growth, nerve regeneration and many pathological processes, in ways which have not previously been possible.

Key words: Laser scanning confocal microscopy; fluorescent imaging; skin imaging; dermal blood vessels; dermal nerve fibres.

INTRODUCTION

Confocal microscopy allows visualisation of subsurface structure in translucent tissues without the need for conventional histological sectioning. This property enables imaging of subsurface cellular structure and blood vessels in whole tissue or living animals (Delaney et al. 1993, 1994a, b). There are several studies which have described real time in vivo confocal microscopy of skin using the reflected light tandem scanning microscope (TSM) (Corcuff et al. 1993; Corcuff & Leveque, 1993; Bertrand & Corcuff, 1994). This instrument illuminates the skin using a bright light source, such as a mercury lamp, and has been used for imaging of distinctive patterns of cellular organisation in the stratum corneum, and in the living layers of the epidermis. Exploration of cellular structures in living human skin in situ has been described using TSM (Corcuff et al. 1996). Laser scanning confocal microscopy (LSCM) has been used in reflectance mode (i.e. reflected light) to image human skin in vivo (Rajadhyaksha et al. 1995). This technique was able to distinguish between the layers of the epidermis, image papillary blood vessels, and even differentiate subcellular details within the living cells (Rajadhyaksha et al. 1995). However, there are limitations to reflectance techniques as they rely on differences in the reflectivity of structures in order to differentiate between them. Fluorescence LSCM can be used to visualise selectively stained structures in skin in vitro (Veiro & Cummins 1994), thus offering greater imaging potential. A high degree of selectivity and specificity, such as that offered by fluorescence imaging, could make LSCM a powerful research and diagnostic tool in dermatology. However, conventional LSCMs suitable for fluorescence imaging are limited in application because they are bulky, immobile, and require accurate alignment of large components. Fibre optic LSCM utilises an optical fibre to eliminate critical alignments associated with conventional LSCM whilst achieving equivalent resolution and offering greater flexibility for in vivo use (Delaney et al. 1993, 1994a, b). The design also enables miniaturisation, and a prototype confocal
Fig. 1. Conventional light microscopy and SEM views of hairless mouse skin. (a) Light micrograph of skin from hairless mouse showing epidermis, dermis and inactive hair follicles. The epidermis is 2–3 cells thick covered by a stratum granulosum and a keratinised stratum corneum at the surface. The inactive hair follicle contains sebum. The dermis contains few cells, and is comprised of thick bundles of collagen, blood vessels and adipose cells in the deeper layers. Toluidine blue; bar, 30 µm. (b) Light micrograph showing a tangentially-cut section of epidermis and dermis of hairless mouse skin at a higher magnification. Profiles of individual keratinocytes in the epidermis are apparent. Cross sections of sebum-filled hair follicles in the dermis and epidermis can be seen. This tangential image of the skin complements and helps clarify the LSCM images. Toluidine blue; bar, 45 µm.

endomicroscope with a 10 mm imaging head is already being trialed for applications, including imaging skin in our laboratory (Delaney et al. 1995). FOCI was used in this study for surface and subsurface fluorescence microscopy of hairless mouse skin in vivo after application of fluorescent dyes including acridine orange which preferentially stains nuclear materials, FITC-dextran which is a water-soluble nonselective
stain, DIOC5(3) which stains intracellular membranes, and 4-di-2-ASP which stains nerve fibres (Lichtman et al. 1987; Hanani, 1992; Robitaille et al. 1996). Confocal examination of the skin of hairless mice in vivo yielded images of nerves, dermal vessels and living cells of layers of epidermis and dermis at depths of up to 100 µm or more (Bussau et al. 1997). The images acquired were compared with and correlated with those obtained using conventional histological staining techniques (including toluidine blue, silver staining) and using scanning electron microscopy (SEM).

The feasibility and purpose of acquiring confocal images of the blood vessels and layers of the living skin form the subject of this article. Future applications and implications in the treatment of many pathological conditions of the skin and the potential to study dynamic events in vivo such as nerve regeneration and wound healing are also discussed.

MATERIALS AND METHODS

Hairless mice (male and female, 12–36 g, n = 6 for each dye) were anaesthetised with Avertin (2,2,2-tribromoethanol, 6.3 mg/25 g i.p.). For subsurface imaging, the fluorescent dyes acridine orange (0.02 mg/ml, Sigma, USA), 4-di-2-ASP (0.004 mg/ml, Molecular Probes, USA) and DIOC5(3) (0.005 mg/ml, Molecular Probes, USA) were applied to the animal’s haunches using a high pressure jet (Wright Dental Group, Dundee, Scotland) which delivered a metered volume (0.1 ml/jet) of which only a fraction penetrated the skin. Excess dye was wiped from the skin surface using paper towel. 4-di-2-ASP is a non-toxic, phosphostable dye that stains neuronal cells. DIOC5(3) is a cell permeant probe that stains preferentially for intracellular membranes. For imaging of the mouse skin surface, fluorescein (Sigma, USA) or acridine orange (Sigma, USA) was applied for 30 min (0.05% in sorbolene cream) and then washed off with water. FITC-dextran (Sigma, 50 μl of 100 mg/ml in saline filtered through a 45 μm microfilter, i.v.) was used for subsurface vasculature imaging. The animals were placed on a heated microscope stage and a cover slip with immersion oil was placed on the haunch using a tissue-stabilising micropositioner. The gentle pressure applied by the micropositioner onto the underlying tissue was sufficient to overcome respiratory movements to enable 3-dimensional imaging in vivo. An Optiscan F9000e dual channel confocal system fitted to an Olympus BH-2 microscope was used for laser scanning confocal microscopy (excitation 488 nm argon ion laser, detection above 515 nm). Lenses used were Olympus SPlan Apo 10 × 0.4NA and a 40 × 0.95NA (dry lenses). The fast scan rate of this instrument (4 frames/s and up to 16 frames/s in search mode and for nonsquare fields) achieved a reasonable signal strength and a high image quality in living animals. However, faster (i.e. video rate) acquisition by confocal microscopes in fluorescence mode is not practical, as, at faster rates, the signal-to-noise ratio is greatly decreased (Gan & Sheppard, 1997).
Images of moving blood cells through dermal vessels were transferred to a television screen and recorded on video cassette recorder (Panasonic FS90 VHS). This allowed the experiments to be video-taped continuously.

Estimation of depth of imaging is complicated by changes in refractive index (RI) from the oil under the cover slip (RI = 1.518, which is similar to the RI of the stratum corneum), to the deeper layers of the skin (RI ~1.35, which is similar to water). The depth of cells imaged is governed by the following equation:

\[ M = m/RI, \]

where \( M \) is real movement, \( m \) is stage movement and \( RI \) is the refractive index. For example, if the stage movement was 100 \( \mu \)m, thickness of skin imaged would approximately be 72 \( \mu \)m using an estimate of 1.4 for the mean RI of the skin.

At the end of each experiment, mice were killed by anaesthetic overdose and cervical dislocation and skin samples taken for conventional histology or scanning electron microscopy (SEM) as appropriate. Formalin-fixed paraffin embedded mouse skin was sectioned (5 \( \mu \)m thick) for silver staining (Naoumenko & Feigin, 1967). Glutaraldehyde-fixed mouse skin embedded in Epon–Araldite sections (1 \( \mu \)m) was stained with toluidine blue for conventional histology. Tissues were prepared for light microscopy and SEM following established methods (Barkla & Tutton, 1977). For light microscopy, full thickness skin was fixed in 3% formaldehyde, 4% glutaraldehyde and 1% picric acid, rinsed in 0.1 M cacodylate buffer, postfixed in \( \text{OsO}_4 \) in cacodylate buffer, placed in 1% uranyl acetate in maleate buffer, dehydrated in ethanol and embedded in Epon–Araldite. Tissues were embedded at both right angle and horizontal orientations, cut into 1 \( \mu \)m sections and stained with toluidine blue. For SEM, full thickness skin was fixed in 5% glutaraldehyde and postfixed in 2% \( \text{OsO}_4 \). Specimens were then critical point dried under \( \text{CO}_2 \) gas, thinly coated with gold, and viewed in a Hitachi scanning electron microscope at 60 kV.

RESULTS

Light microscopy

The epidermal layer of the hairless mouse was relatively thin and showed clearly the differentiation from germinal cells in the stratum basale adjacent to the dermis through the stratum spinosum, stratum granulosum and stratum corneum on the surface (Fig. 1a). The openings of mostly inactive hair follicles containing sebum projected onto the surface (Fig. 1a). Transverse section of sebum-filled hair follicles in the dermis and epidermis could be seen in higher magnification (Fig. 1b).

Scanning electron microscopy

SEM showed a flat surface epithelium punctuated by round projections of mostly inactive hair follicles (Fig. 2). Occasional follicles showed short hairs emerging onto the surface.

Topical dye application—acridine orange and fluorescein

Topical application of fluorescein or acridine orange in sorbolene cream yielded images comparable to
those obtained using SEM. Both showed squamous cell outlines, as well as the redundant hair follicles (Fig. 3). In the confocal images, surface squamous cell boundaries were clearly stained, and the redundant hair follicles appeared as ring-like structures (Fig. 3).

Staining with high pressure jet—fluorescein

Unlike acridine orange, fluorescein reproducibly stained the squamous cell boundaries in the stratum corneum (Fig. 4a, b). After application by high pressure jet, fluorescein enabled imaging of surface cells, which were comparable to those shown by scanning electron microscopy (Fig. 2) and by confocal imaging following topical application of fluorescein in sorbolene cream (Fig. 3).

Staining with high pressure jet—acridine orange

Acridine orange (by high pressure jet) stained cells in the skin more uniformly than fluorescein, as might be expected because acridine orange is more lipid soluble than fluorescein. Figure 5(a–d) shows confocal images of the cells in strata basale, spinosum and granulosum, redundant hair follicles and tertiary wrinkle structure of hairless mouse skin. Around
redundant hair follicles, keratinocytes could be imaged to depths estimated to be greater than 100 µm from the skin surface (stage movements of over 150 µm toward the lens from the first visible structure). The differences in diameters of keratinocytes within the plane of optical sectioning of cells in different layers of the skin were consistent with the flattening and broadening of keratinocytes as they differentiate. The ratio of the size difference between the cells in the different layers was consistent with the differences between cells (8–26 µm) in the tangentially sectioned, conventionally stained tissue (Fig. 1b).

Although patches of the surface exhibited clear staining of squamous cell boundaries with acridine orange (perhaps due to surface dye), the boundaries and contents of the corneocytes in the stratum corneum were not reproducibly stained. This lack of specific binding to, or penetration of, acridine orange into corneocytes may relate to permeability characteristics and lack of nuclei of corneocytes in the stratum corneum.

**Dermal blood vessels**

Blood vessels in the dermis were imaged confocally after injection of FITC-dextran (Fig. 6a, b). A characteristic pattern of vessels surrounding individual hair follicles was consistently observed in the skin of the mouse haunch (Fig. 6a). The sizes of the blood vessels (up to 20 µm) corresponded to those of arterioles, venules and capillaries. Blood cells could be seen moving through dermal vessels after i.v. injection of FITC- dextran which could be video-taped via a video cassette recorder. FITC-dextran fluorescently labelled the plasma, and blood cells appeared as dark moving silhouettes through the vessels (Fig. 6b). This demonstrates the feasibility of microvascular imaging of the skin in vivo, and also the ability of FOCI to image structure within the dermis noninvasively.

**Staining with high pressure jet—4-di-2-ASP and silver stains**

The nontoxic, phosphostable 4-di-2-ASP dye which labelled nerve fibres red was applied to stain living dermal nerve terminals in subsurface skin of hairless mice in vivo. This fluorescent dye showed the presence of nerve fibres around the hair follicles and dermal blood vessels. Confocal images of vessels (in contrast to those of nerves) were seen to contain moving blood cells through the blood vessels in animals previously given i.v. injection of FITC-dextran which labelled the plasma green. Within the plane of optical sectioning, nerve fibres that project to the skin surface were seen as a dot whereas those that run horizontally were seen as fine lines (Fig. 7a). Cross sections of formalin-fixed hairless mouse skin were stained with a silver stain to demonstrate nerve fibres which stained dark blue-black (Naoumenko & Feigin, 1967) (Fig. 7b).

**Staining with high pressure jet—DiOC5(3)**

At depths of up to 45 µm DiOC5(3) showed clearly defined keratinocyte cell membranes and nuclear membranes. Endoplasmic reticulum could not be detected (Fig. 8).
This study demonstrates the viability of fluorescence laser scanning fibre-optic confocal microscopy as a technique for imaging skin in vivo. Images were obtained from the stratum basale, stratum spinosum, stratum granulosum and stratum corneum of the hairless mouse skin. The technique enabled resolution of subcellular structures in the epidermis in vivo. Blood vessels in the dermis were also imaged, demonstrating the feasibility of imaging through the intact epidermis and some of the dermis as well (Bussau et al. 1997). The results of the present study showed that the cellular micro-architecture of the skin can be imaged by FOCI in vivo, offering a unique and dynamic view compared to conventional histology. For instance, blood cells appearing as dark silhouettes
could be seen moving through dermal vessels injected intravenously with FITC dextran; this was in contrast to images of nerve fibres stained with 4-di-2-ASP which appeared motionless against the background of blood vessels in the same optical plane.

In the hairless mouse, the in vivo confocal data would indicate that each of the layers in the skin is usually 1 cell thick, and that the overall thickness of the epidermis is in the order of 40–60 µm, and somewhat thicker around redundant hair follicles. In considering the structures observed confocally, it is important to recognise the major differences between these images, and those obtained conventionally.

With in vivo confocal microscopy, subsurface imaging of various layers is achieved by optical sectioning, which is noninvasive and nondestructive. This may make in vivo confocal microscopy ideal for identifying changes in cells within a particular layer which affect only a small percentage of these cells. As neither fixing nor physical sectioning of the tissue is performed for in vivo confocal imaging, conventional staining techniques are of little relevance.

The plane imaged by confocal optical sectioning is virtually parallel to the surface of the tissue (tending to image 1 cell layer at a time). This view is not well documented in the literature, which typically features orthogonal sections of the skin.

Since this is a novel method of imaging, there are few established techniques for staining the tissue. Factors such as removal of dye by vascular or lymphatic networks, degradation of dye by enzymatic processes, or permeability characteristics of individual cells and structures (i.e. skin barrier function) will affect the staining pattern in vivo. In fact optimising and extending specific methods of staining in vivo probably represents one of the most important ways in which the usefulness of in vivo confocal microscopy can be expanded. In particular, the use of dyes which specifically target cellular processes may allow the study of dynamic events in vivo, such as skin growth, wound healing and the many pathological processes to which it is prone, in ways which have not previously been possible.

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REFERENCES


