Introduction to Confocal Microscopy

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Conventional microscopy requires viewing a thin-cut "section" of fixed or frozen tissue, and therefore cannot be used to view thick tissue samples or for *in vivo* investigations. *In vivo* microscopy requires a virtual, rather than a physical, section of the specimen. Confocal microscopy, developed and patented by Marvin Minsky in 1955, uses optical imaging to create a virtual slice or plane, many micrometers deep, within the tissue. It provides very-high-quality images with fine detail and more contrast than conventional microscopy. In addition, the imaging technique allows for reconstruction of virtual 3-dimensional (3-D) images of the tissue when multiple sections are combined.

ADVANTAGES OF CONFOCAL MICROSCOPY

- High-resolution, high-contrast images.
- Reconstruction of 3-D images.
- Absence of artifacts induced by conventional microscopy (e.g., shrinkage, loss of fat, no blood flow).
- *In vivo* microscopy to a skin depth of about 200 μm.

LIMITATIONS

- Depth of imaging is limited by optical penetration and signal-to-noise ratio. *In vivo* confocal microscopes can generate high-resolution images of the entire epidermis and a superficial layer of dermis.
- Photobleaching of fluorescent probes and phototoxicity of live samples are no worse with confocal microscopes than with conventional fluorescence microscopes. However, multiphoton fluorescence microscopes can nearly eliminate photobleaching compared with either confocal or conventional imaging.
- High cost relative to conventional microscopy or dermoscopy.

WHAT IS CONFOCAL MICROSCOPY? A SIMPLE EXPLANATION

In conventional microscopy, a tissue section is placed on the microscope stage and the entire field of the specimen is simultaneously illuminated by light and visualized. Although the brightest and highest intensity is at the focal point of the objective lens of the microscope, there is illumination of other parts of the sample, resulting in background "noise," which compromises the quality of the image. Both conventional and confocal microscopy can use reflected light or fluorescent light to image the specimen. However, in confocal microscopy, a beam of incoming light (the excitation beam) is focused through the microscope objective on a small spot *inside* the tissue, which can be almost as small in diameter as the wavelength of light itself—about 0.5 μm. The same objective gathers the reflected or fluorescent light coming back from the tissue, but unlike conventional light microscopy, this light is projected (like a slide projector) and not directly viewed. In conventional light microscopy, although only a small field of tissue is illuminated at one time, some of the reflected or fluorescent light scatters, which could blur or obscure the image. Confocal microscopy overcomes this problem using a small pinhole aperture in a screen that allows only the light emitting from the desired focal spot to pass through. Any light outside of the focal plane (the scattered light) is blocked by the screen. In optical terms, the pinhole is placed in a conjugate focal plane as the tissue specimen (hence the designation "confocal"). A sensitive light detector, such as a photomultiplier tube, on the other side of the pinhole is used to detect the confocal light. This technique allows the specimen to be imaged one "point" at a time.

To create an image of the specimen, the focal spot is rapidly and serially scanned in the *X*–*Y* plane, which is why confocal microscopes yield horizontal virtual-sectioned

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images. As the scanning progresses, signal from the detector is fed to a computer that collects all the "point images" of the sample and serially constructs the image one pixel at a time. Because the sample is not actually sectioned, it is possible to image a "stack" of virtual, confocal image planes that can later be used to make tomographic images, similar to the reconstructions of magnetic resonance imaging or computed tomography scanners in medicine. Images are displayed on a video monitor and what results is a sharp, high-contrast image that could not be acquired by conventional light microscopy.

FLUORESCENCE CONFOCAL MICROSCOPY

Fluorescence confocal microscopy is most commonly used for dermatologic research of *in vitro* or *ex vivo* studies. In general, fluorescence microscopy uses dyes that fluoresce when stimulated by light ("fluorophores") and are added to the specimen depending on the purpose of the imaging. Fluorescence microscopy is generally much more sensitive than light microscopy. Fluorophores that specifically target and identify subcellular structures such as the cytoplasm, sarcoplasmic reticulum, nuclei, and mitochondria have been designed. Fluorophores improve sensitivity and specificity by increasing the signal-to-noise ratio and by allowing better and sharper detection of the target. The excitation light in fluorescence confocal microscopy is usually provided by a laser at a wavelength that will also excite a specific fluorophore. In some instances, more than one fluorophore can be used at the same time, and by switching the excitation light or by observing at different emission wavelengths, different parts of the specimen can be distinguished. When the excitation laser hits the target tissue, it generates high intensities of fluorescence at a well-defined focal point. Both the laser light (the excitation beam) and the resultant emission fluorescence pass through the same objective—a special mirror called a dichroic mirror that reflects the incoming, higher-energy (but shorter-wavelength) laser light, but allows the lower-energy (higher-wavelength) fluorescent light to pass through to the light detector (Figure 1). As described above for reflectance confocal microscopy, a pinhole is also used in fluorescence confocal microscopy to eliminate scattered light. The end result is that light is collected from a highly focused point. Images of the scanned specimen can then be reconstructed point by point. One limitation of fluorescence microscopy is the phenomenon of photobleaching; fluorophores tend to irreversibly fade or react when exposed to excitation light. Various strategies are being explored to minimize this problem.

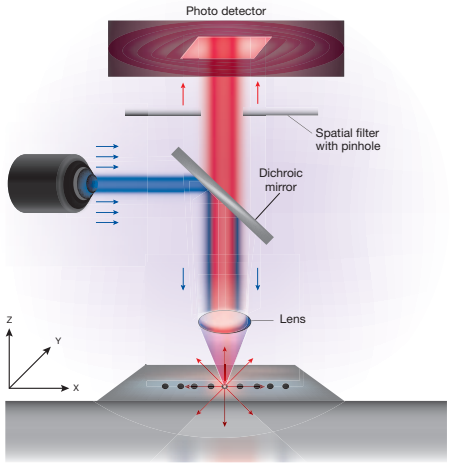

Figure 1. A simplified view of confocal microscopy.

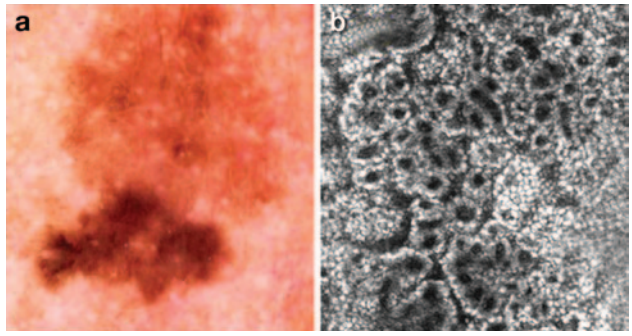

Figure 2. *In vivo* RCM image showing features of benign nevus. (**a**) An 8-mm-diameter lesion suspicious of melanoma with focal broadening of the pigment network. (**b**) Dark focus with regular bright-pigmented cells around dark dermal papillae at the dermoepidermal junction. Diagnosis: lentiginous junctional nevus with mild dysplastic features (adapted from Guitera *et al.*, 2009). RCM, reflectance confocal microscopy.

Most confocal microscopes use this laser-scanning strategy and can be applied to the field of dermatology. For example, *in vivo* reflectance confocal microscopy (RCM) has been used to delineate pigmentary changes in skin due to aging or specific stimuli, such as ultraviolet radiation (UVR). In the study, investigators were able to detect UVR-induced pigmentary changes in the skin of pigmented guinea pigs, a common animal model for studying human pigment biology (Middelkamp-Hup *et al.*, 2006). The changes could be detected well before a tanning response was clinically visible and included increase in melanocyte size, dendricity, and number and pigmentation of keratinocytes in the irradiated epidermis.

Confocal microscopes that do not require laser scanning include the spinning-disk confocal microscope. Spinning-disk confocal microscopes use an alternative design, specifically a series of moving pinholes on a disk, called the Nipkow disk, to scan and obtain the confocal images. Another nonlaser strategy uses a modulator that creates a moving pattern of light focused in the virtual plane, which is then projected onto a CCD camera in which each little pixel on the camera chip acts somewhat like a pinhole.

LIVE REAL-TIME MICROSCOPY

In vivo RCM, as mentioned above, generates optical sections within the depth of intact tissue and is a useful tool for studying the skin surface, epidermis, and superficial dermis because it provides cellular resolution. Melanin acts as a natural contrast agent for RCM, which has been shown to improve melanoma diagnostic accuracy by identifying both malignant features in apparently benign lesions and benign features in clinically appearing malignant lesions.

Blood flow, inflammatory cells, and dermal collagen fibers are also seen. The practical limitations of RCM at present are its limited field of view and cost compared with regular microscopy or dermoscopy. However, there is a high-quality RCM inside every DVD or CD player; therefore, RCM may eventually be clinically practical. Guitera *et al*. (2010) used *in vivo* RCM to define unique features that can distinguish lentigo maligna from benign macules on the face (Figures 2–4). Two major positive diagnostic features for malignancy were identified (i.e., nonedged papillae and round, large pagetoid cells >20 μm) and four minor features were shown, including three or more atypical cells at the dermoepidermal junction in 0.5 × 0.5 mm^2, follicular localization of atypical cells, and nucleated cells in the dermal papillae. One negative diagnostic feature for malignancy was a broadened honeycomb pattern, which was seen in benign lesions and normal epidermis.

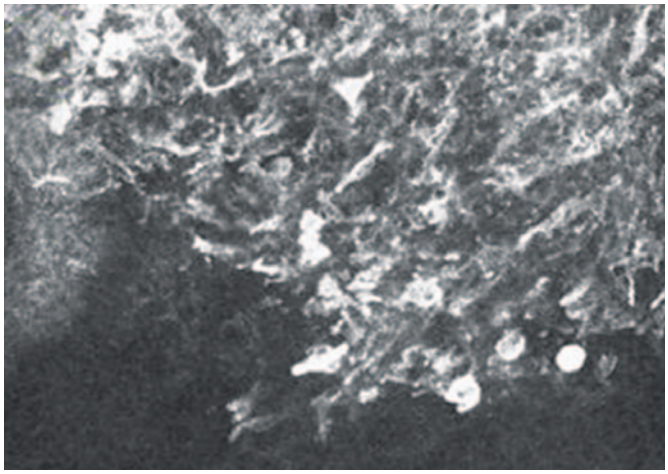

Figure 3. *In vivo* RCM image showing features of lentigo maligna. Atypical cobblestone pattern with small, bright, nucleated cells of the epidermis of a lentigo maligna of the cheek (adapted from Guitera *et al*., 2010). RCM, reflectance confocal microscopy.

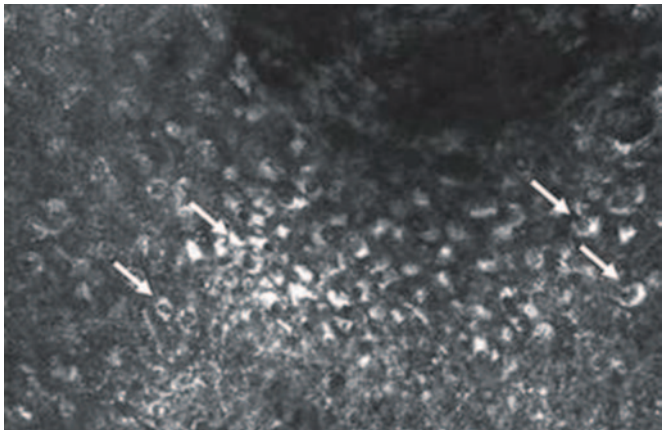

Figure 4. *In vivo* RCM image showing features of lentigo maligna. Numerous atypical cells (both dendritic and large, roundish cells) at the dermoepidermal junction of a lentigo maligna of the cheek (adapted from Guitera *et al*., 2010). RCM, reflectance confocal microscopy.

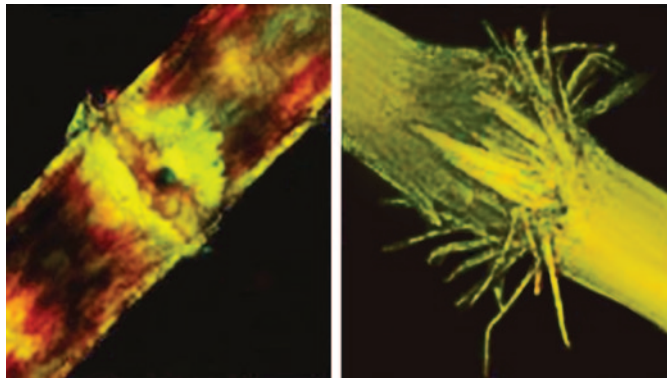

Figure 5. Confocal microscopy showing hair autofluorescence. (Left) trichoschisis; (right) trichorrhexis nodosa–like fracture (adapted from Liang *et al*., 2006).

OTHER CONSIDERATIONS

The advantages of confocal microscopes compared with conventional microscopes include sharper, more detailed images; ability to image a virtual section inside live cells or tissues; ability to track dynamic events such as blood flow and cell migration; and ability to image a stack of virtual confocal image planes that can later be used to make tomographic or 3-D images of cells or tissues, similar to reconstruction of magnetic resonance or computed tomography images. The disadvantages include cost and limited depth (typically up to about 200 μm) when imaging intact tissues. Fluorescence confocal microscopes have become a mainstay for biological research, used to image dyes, immunofluorescence probes, and green fluorescent protein.

MOVING FORWARD

Confocal microscopy started a modern revolution, but it is not the only way to see virtual sections inside cells or tissues. Two other forms of specialized microscopy that image virtual sections include deconvoluted and multiphoton imaging microscopes. Deconvolution uses mathematical models and computation to improve imaging. Multiphoton fluorescence microscopes use very brief, intense laser pulses at long wavelengths of light that can penetrate more deeply to excite fluorescence only at the focal spot. Multiphoton microscopes are still at an early stage of development for *in vivo* skin imaging, but appear to be promising. Collagen, elastin, cell cytoplasm, and nuclei are easily and specifically seen with multiphoton microscopy of skin *in vivo*. Scanning confocal electron microscopy (SCEM) is another scanning technique using concepts similar to those in laser scanning confocal microscopy (LSCM), by which 3-D images can be acquired. The main difference is that high-energy electrons are used instead of light; SCEM is lethal and cannot be used to image living cells. In SCEM, a focused electron beam illuminates the sample, and depth resolution is obtained by placing the collection system for scattered electrons symmetrical to the illumination system (Zaluzec, 2007). As with other electron microscopes, resolution is higher than in optical microscopy.

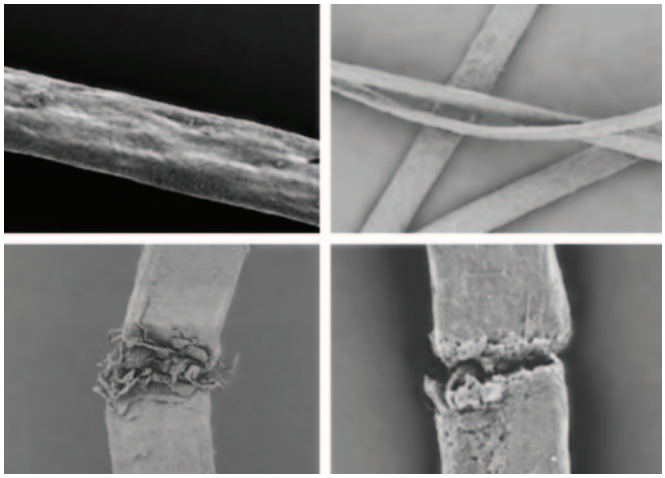

Figure 6. Scanning electron microscopy. (Upper left) Grooved, irregular hair surface. (Upper right) Flattened hair, resulting in a ribbon-like appearance. (Lower left) Trichorrhexis nodosa–like fracture. (Lower right) Trichoschisis. The original magnification in millimeters is indicated by the length of the row of dots (adapted from Liang *et al*., 2006).

Liang *et al*. (2006) used both SCEM and LSCM to characterize hair abnormalities in trichothiodystrophy patients, who show fragile hair that breaks easily owing to decreased sulfur content (Figures 5 and 6). Structural features of breaks, autofluorescence of the hair, and z-slices of autofluorescent hair shafts were analyzed by confocal microscopy. The scanning electron microscopy enabled a detailed examination of hair shaft abnormalities. Strands of hair, 12 mm long, were fixed to a standard aluminum specimen stub with a carbon adhesive tab. Gold coating revealed visualization of the eroded cuticular layer. Using various microscopy techniques, the investigators were able to characterize structural and molecular hair abnormalities in trichothiodystrophy patients.

SUMMARY

Confocal microscopy is a technique in optical imaging that uses point illumination via a spatial pinhole to eliminate out-of-focus signals. The excitation light in confocal microscopy is usually provided by a laser to generate high intensities of fluorescence or reflectance from the focal spot. Fluorescence confocal microscopy is the most used in dermatology to analyze *ex vivo* and *in vitro* samples. Reflectance confocal microscopy can be used for real-time microscopy and uses melanin as a natural contrast agent. Confocal microscopy has many advantages, including increasing the optical resolution and contrast of an image of a specimen; facilitating reconstruction of 3-D images; enabling collection of serial optical sections from thick specimens; and enabling *in vivo* imaging without the artifact induced by tissue processing (Pawley, 2006). In addition to LSCM, 3-D images of nonliving samples can also be acquired by SCEM, where an electron beam is used for illumination, resulting in higher resolution compared with confocal microscopy. Limitations of confocal microscopy include the depth of imaging within thick samples and cost compared with conventional microscopes. The problems of fluorescent probe photobleaching and phototoxicity inherent in conventional fluorescence microscopy are also present with confocal microscopy. Multiphoton microscopy is an alternative strategy for fluorescence microscopy, which offers higher resolution, somewhat greater depth of imaging, and minimal photobleaching. Technologies for microscopy are promising and are still being improved.

QUESTIONS

1. Features of confocal microscopy include which of the following?
   - A. Formation of the focal point of the objective lens on a pinhole to decrease "noise."
   - B. Increase in the optical resolution and contrast of the image.
   - C. Ability to reconstruct a 3-D image of the specimen.
   - D. Ability to collect serial optical sections from thick specimens.
   - E. All of the above.
2. Which microscope uses a series of moving pinholes on a disk?
   - A. Programmable array microscope.
   - B. Spinning-disk confocal microscope.
   - C. Scanning transmission electron microscope.
   - D. Phase-contrast microscope.
3. What is the role of a photomultiplier tube?
   - A. It collects fluorescence at the dichroic mirror.
   - B. It provides the excitation light.
   - C. It scans the emitted light.
   - D. It detects the emitted light.
4. What may be the consequence of using two different fluorescent dyes?
   - A. Photobleaching.
   - B. Phototoxicity.
   - C. Chromatic and spherical aberration.
   - D. Less-detectable photons.

Answers to the questions and an opportunity to comment on the article are available on our blog: http://blogs.nature.com/jid_jottings/.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Answers and a PowerPoint slide presentation appropriate for journal club or other teaching exercises are available at http://dx.doi.org/10.1038/jid.2012.429.

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