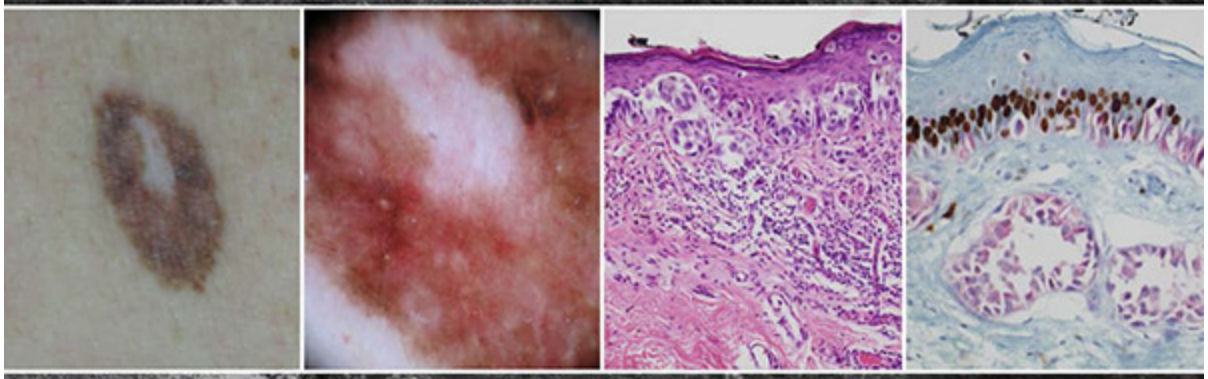
REFLECTANCE CONFOCAL MICROSCOPY OF CUTANEOUS TUMORS

Second Edition



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Reflectance Confocal Microscopy of Cutaneous Tumors

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Preface

The last fifteen years have witnessed an explosion of knowledge in the field of dermatology. A major reason for this push relies on the tremendous advances produced in the field of skin imaging and the development of novel, noninvasive tools to examine, diagnose, measure, delimit, and follow-up different skin pathologies. Dermoscopy was the harbinger of these techniques, enabling the examination of topographical skin details with an unprecedented level of detail and architectural resolution. However, other techniques caught on quickly, including the main subject of this book. Indeed, the application of reflectance-based confocal microscopy to generate contrast and detail and thus visualize the skin in vivo in a noninvasive manner was a completely innovative application back in the 1990s, but one that lacked reference points at the time, particularly on how to compare confocal microscopy findings with conventional histology, which is a hundred-year-old technique considered the gold standard for the diagnosis of virtually every skin condition. Fast-forward to 2008, when a few dozen eager groups had adopted reflectance confocal microscopy as their tool of trade and were generating comparative maps of findings by reflectance confocal microscopy and comparing them to histology. In addition, the idea that confocal imaging was not invasive had supported pioneering efforts to use it to delimit surgical margins and also to study the response of different skin conditions to the treatment. It was in this context that we realized that a comprehensive atlas of skin findings as visualized by confocal microscopy would be a terrific tool. That was a task worth our time; hence, Melissa Gill, Allan Halpern, and I delved into preparing what would become the first edition of this book. And what an overwhelming response we had! This book was rapidly embraced by the growing community of reflectance confocal skin "imagers" around the world, and the number of publications, images, and references to this application of confocal imaging grew exponentially. As a testimony of the growing interest in the application of this technique, as many studies were included in the National Institutes of Health (NIH) search engine PubMed during 2015 as in all the previous years combined, when "skin reflectance confocal microscopy" was used as the searching term. This indicated that not only the potential audience of this book had grown exponentially: the amount of new information, images, and comparisons had grown equally, which was a major force behind producing a second, updated edition of the *Atlas*. And these events bring us to this point. With the invaluable help and collaboration of some of the pioneers and most renowned experts in the field, it is with great pleasure that I introduce the second edition of the book *Reflectance Confocal Microscopy of Cutaneous Tumors: An Atlas with Clinical, Dermoscopic and Histological Correlations*. This new edition contains a section of the state of the art regarding technology, edited by one of the earliest pioneers in the use of this technology, Milind Rajadhyaksha. It also includes an extensive section on how normal tissues look when seen under the light of the reflectance confocal microscope, edited by Marco Ardigò. Two major sections

CHAPTER 1

Fundamentals of reflectance confocal microscopy

Bjorg Larson, Milind Rajadhyaksha, and Sanjee Abeytunge

INTRODUCTION: HISTORY OF TECHNIQUE AND PRINCIPLE OF OPERATION

In 1957, Marvin Minsky submitted a patent for a confocal microscope, which he had developed for imaging in brain tissue. The microscope images a thin optical slice in thick tissues without the need for physical sectioning. This is achieved by placing a pinhole in front of the detector to accept only that light that is emitted from the focus of the objective lens while rejecting light from the out-of-focus plane. A schematic for the confocal microscope is shown in Figure 1.1. The confocal microscope is by design a scanning microscope, meaning only a single point is imaged at a time, and the focus is scanned across the sample, usually in a raster scan pattern, to build up an image. In Minsky's original design, the sample was placed on a vibrating sample holder. Other early confocal microscopes made use of white light sources and spinning pinhole disks to scan the light across the sample. Lasers now provide an inexpensive and bright monochromatic light source, and spinning disks have been replaced by scanning mirrors in many applications to steer the beam across the sample.[1,2]

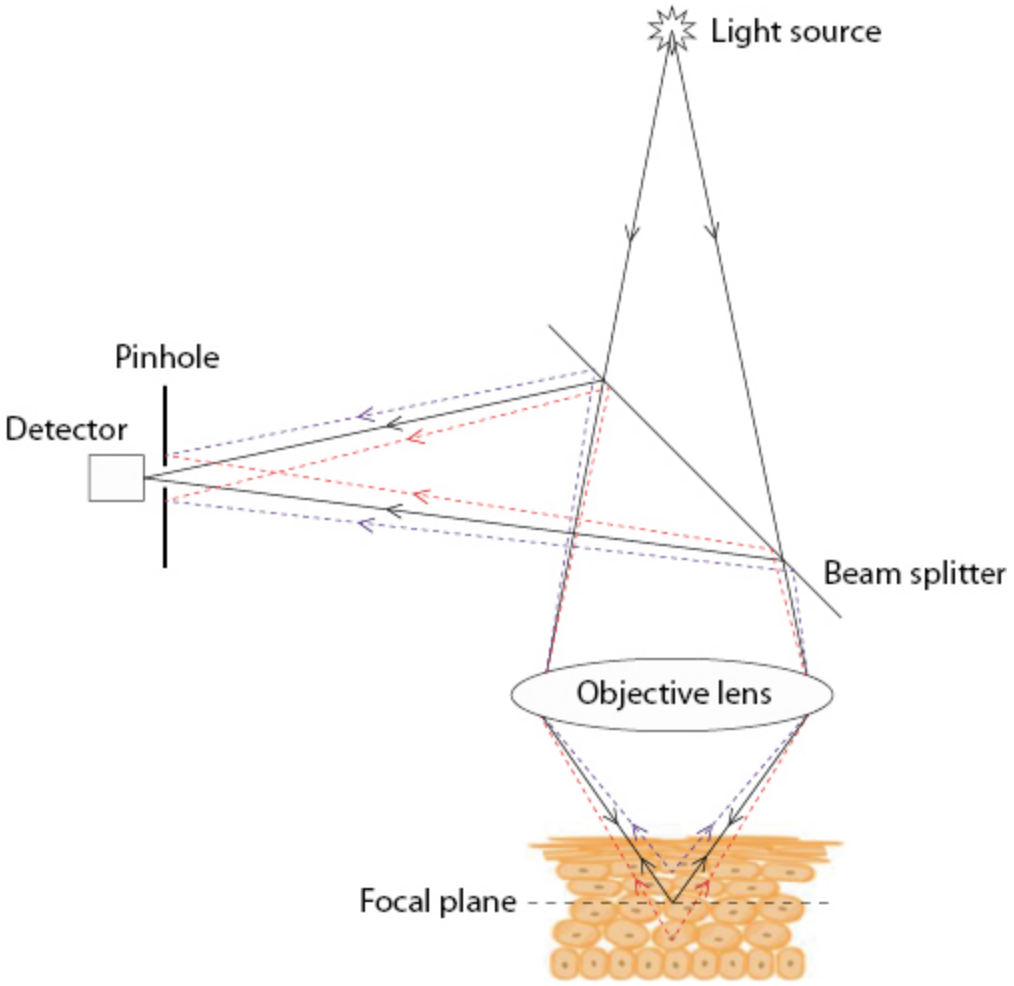

Figure 1.1 The confocal microscope. The light is focused onto the tissue by the objective lens. Light that is reflected by the tissue is collected by the objective lens and imaged onto the pinhole. Light that scatters from outside the focal plane is rejected by the pinhole.

DEVELOPMENT FOR CLINICAL USE

Confocal microscopy was quickly adopted for use as a research tool in imaging biological samples, and the technique was then adapted for use in human skin, first using white light sources[3–6] and then adapting the technique with laser light sources.[7,8] In the 1990s, Noran Inc. (Madison, Wisconsin) made available a series of video-rate confocal microscopes. The first was based on a broadband light source and tandem spinning disk technology, which was later redesigned to better reach more parts of the skin for in vivo imaging. Later, Noran introduced an acousto-optical scanning microscope with laser illumination that provided both reflectance and fluorescent imaging. In 1997, Lucid Inc. (Rochester, New York, now Caliber ID, Andover, Massachusetts) produced a confocal microscope using a laser light source and rotating polygon mirror. Lucid introduced a microscope design, the VivaScope 1000, that could increase the field-of-view of the microscope by stitching 500 μm square images together into a mosaic. The VivaScope 1000 produced 1.5 mm mosaics of skin in vivo.

Current instruments available from Caliber ID include the VivaScope 1500 for imaging in vivo, which can mosaic up to 8 mm areas, the VivaScope 2500 for imaging excised tissue and can mosaic up to 20 mm areas, and the VivaScope 3000, a handheld confocal microscope, with which video-mosaicking can be performed. Mosaicking is an approach to increase field of view, as is necessary for clinical and pathological examinations, and this is further described below. Most available instruments offer both live video-rate imaging as well as the ability to save single images and mosaics.

METHODS: A REVIEW OF TECHNIQUES USED IN THIS ATLAS

Principle of operation and scanning techniques

Traditional wide-field microscopes illuminate and image a large volume of tissue. For this reason, imaging tissue with traditional microscopy requires that the tissue be physically sectioned to a thickness of

5–10 μm to eliminate the out-of-focus tissue image. The current gold standard for diagnosing cutaneous tumors is histopathology. The process is as follows: the tissue is excised from the patient, fixed or frozen, sectioned using a microtome, placed on a glass slide, and stained with hematoxylin and eosin (H&E) to provide contrast between cell and nucleus. The tissue can then be imaged using a wide-field microscope. Confocal microscopy provides optical sectioning and endogenous reflectance contrast, which eliminates the need for tissue processing, and can even be performed directly on patients, in vivo.

Synopsis

- Reflectance confocal microscopy provides noninvasive optical sectioning and endogenous contrast of skin in vivo.
- Imaging parameters of RCM are chosen to provide resolution and section thickness comparable to that of traditional histopathology.
- Image mosaics allow the coverage of large areas of tissue.

In a confocal microscope, the sample is illuminated with a point-source of light which is focused onto the sample by the condenser lens. The illuminated spot in the tissue is imaged by the objective lens onto a point detector. In the case of reflectance confocal microscopy, the objective lens serves as both the condenser and objective lens, focusing the light onto the tissue and imaging the reflected light onto the detector. The point detection is achieved by placing a pinhole in front of a detector (typically an avalanche photodiode or a photomultiplier tube). Only light that is reflected or scattered back from the focal plane of the objective lens is accepted through the pinhole. Light scattered back from outside the focal plane is rejected by the pinhole. Figure 1.1 shows the schematic layout of the confocal microscope. Because light outside the focal plane is rejected, the microscope images an optical section at the focal plane. In Figure 1.2, the axial response of the microscope is shown. The strongest signal is at the focal plane, and drops off with distance from the focal plane. The optical section is measured as the width of this axial response curve at half the maximum value. In Figure 1.2 the optical section is ~2 μm as shown by the arrow.

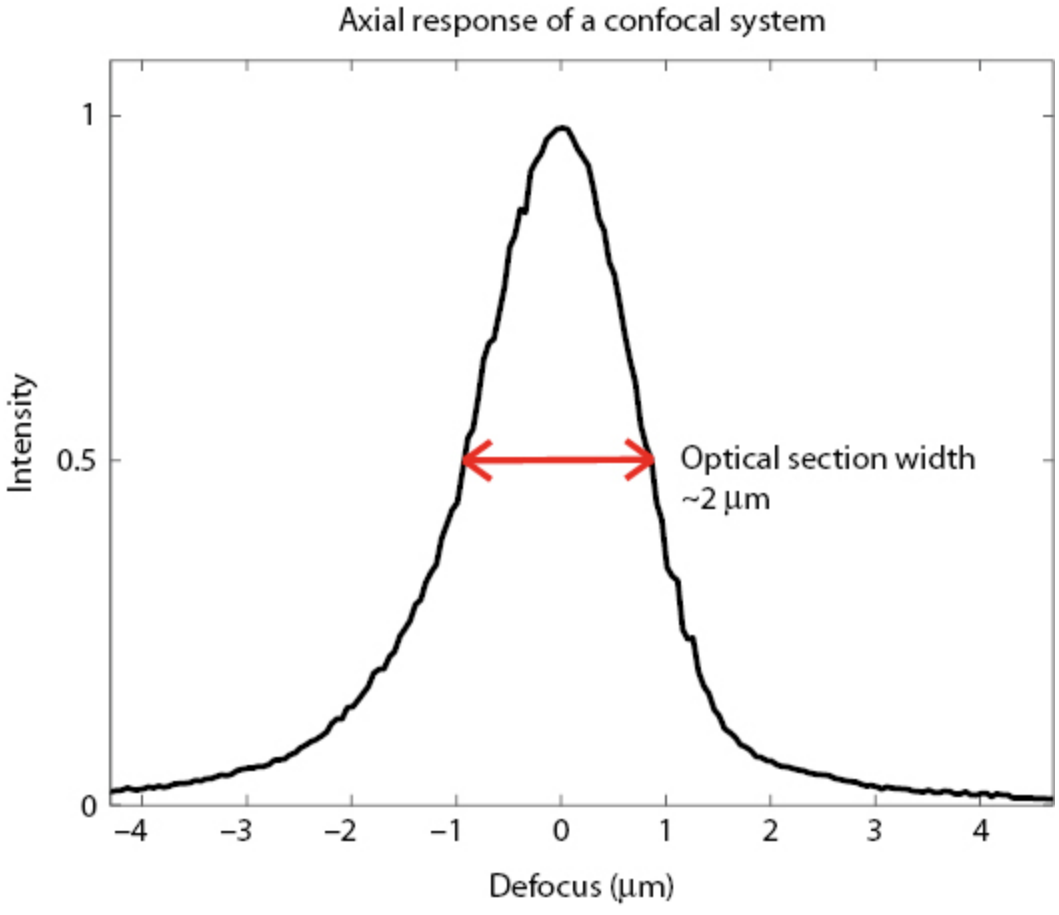

Figure 1.2 Axial response or depth response of a confocal microscope. Because the pinhole rejects light scattered from outside the focal plane, the maximum signal is collected from the focal plane (defocus of 0). Away from the focal plane the signal drops off. The optical section is measured as the width of the axial response at half of the maximum signal. In this figure, the optical section is ~2 μm.

In most commercially available confocal microscopes, the light is scanned across the sample in a raster-scan pattern using two scanning mirrors. This can be done using a rotating polygon mirror or a resonant galvanometric scanner for the fast axis scan. The resonant galvanometric scanner can be made very small, and it is often the method used in smaller, handheld devices. A standard galvanometric scanner is often used for the slow axis scan. Figure 1.3a shows a schematic of the scanning process.

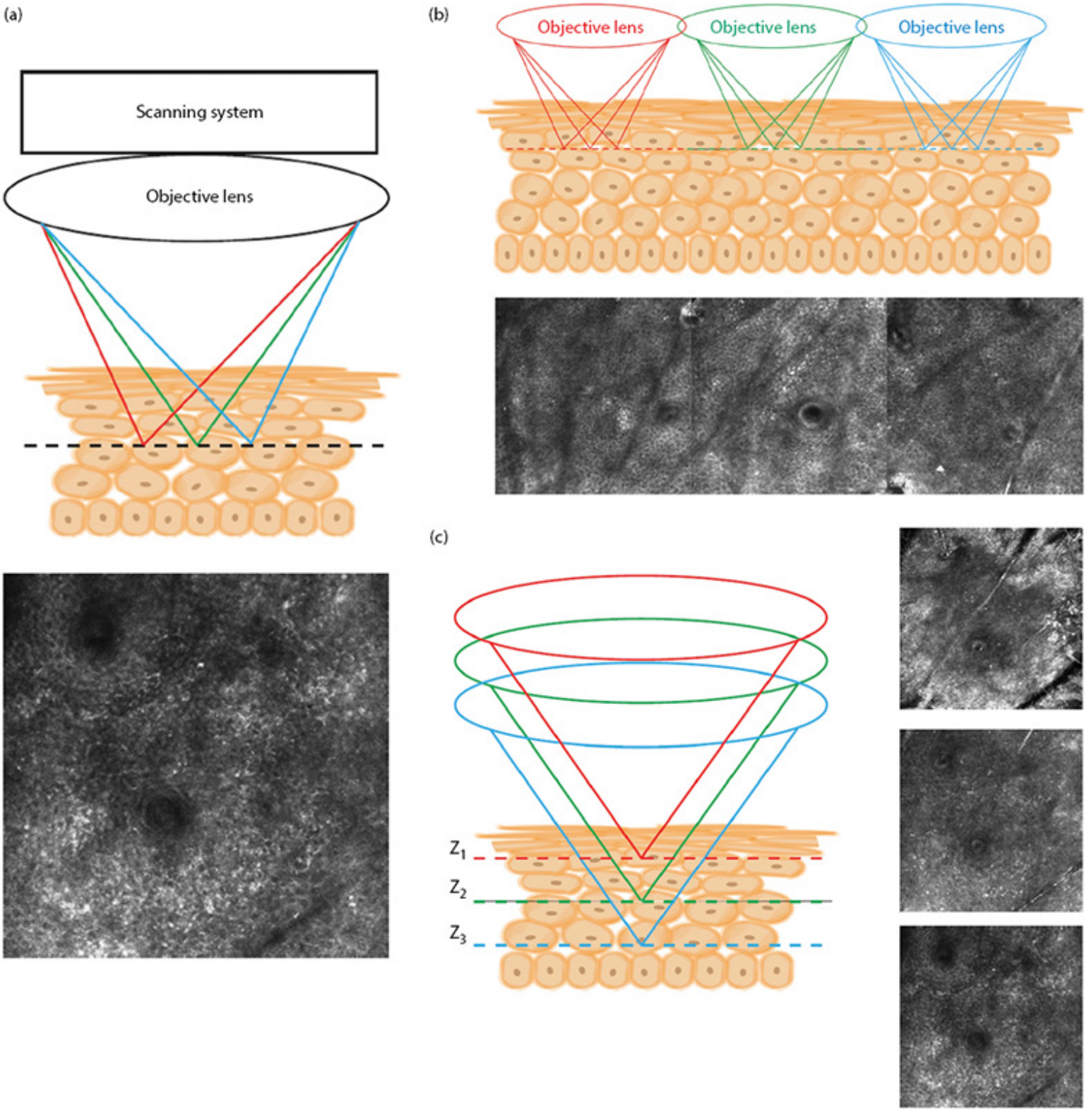

Figure 1.3 (a) A scanning system produces a raster scan of the tissue to produce a single image. Typically the scanning system is composed of galvanometric scanners or rotating polygon mirrors. (b) A mosaic is produced by moving the objective lens across the tissue to collect many images. In this way a larger area of tissue may be imaged. (c) A z-stack is produced by moving the objective lens closer to the tissue, collecting images at successive depths. In this way the depth of the lesion may be imaged.

Imaging of human skin can be done either directly on the patient or on excised tissue. When imaging in vivo, either oil or gel is placed on the skin to match the index of refraction of the stratum corneum. A cover slide may be attached to the skin using adhesive to provide a flat imaging surface. Few fluorescent dyes are approved for use in humans, but reflectance contrast can be enhanced by the use of acetic acid. Details on imaging in vivo can be found in Chapter 2.

When imaging in excised tissue, the tissue is pressed onto a large glass slide to provide a flat surface for imaging. The tissue may be stained with fluorescent dyes to increase contrast or for multimodal imaging, described in more detail in Chapters 6 and 7.

Mosaicking and video-mosaicking

Because the field of view of the confocal microscope is small compared to the lesion, stitching many images together into a mosaic is a method of covering large areas of tissue while maintaining the high resolution of the microscope.[9] Mohs excisions can be as large as 30 mm in diameter, while the field of view of the confocal microscope is generally 0.5–1 mm. While imaging excised tissue, the ultimate size of tissue that can be imaged using the mosaicking technique is only limited by the range of the sample stage and by time considerations, as the imaging time is generally proportional to the area of the tissue. Mosaicking of excised tissue is described in detail in Chapter 7. Figure 1.3b illustrates the technique of mosaicking, and Figure 1.4b shows a mosaic made up of 11×9 images, or an area of 5.5 mm $\times$ 4.5 mm.

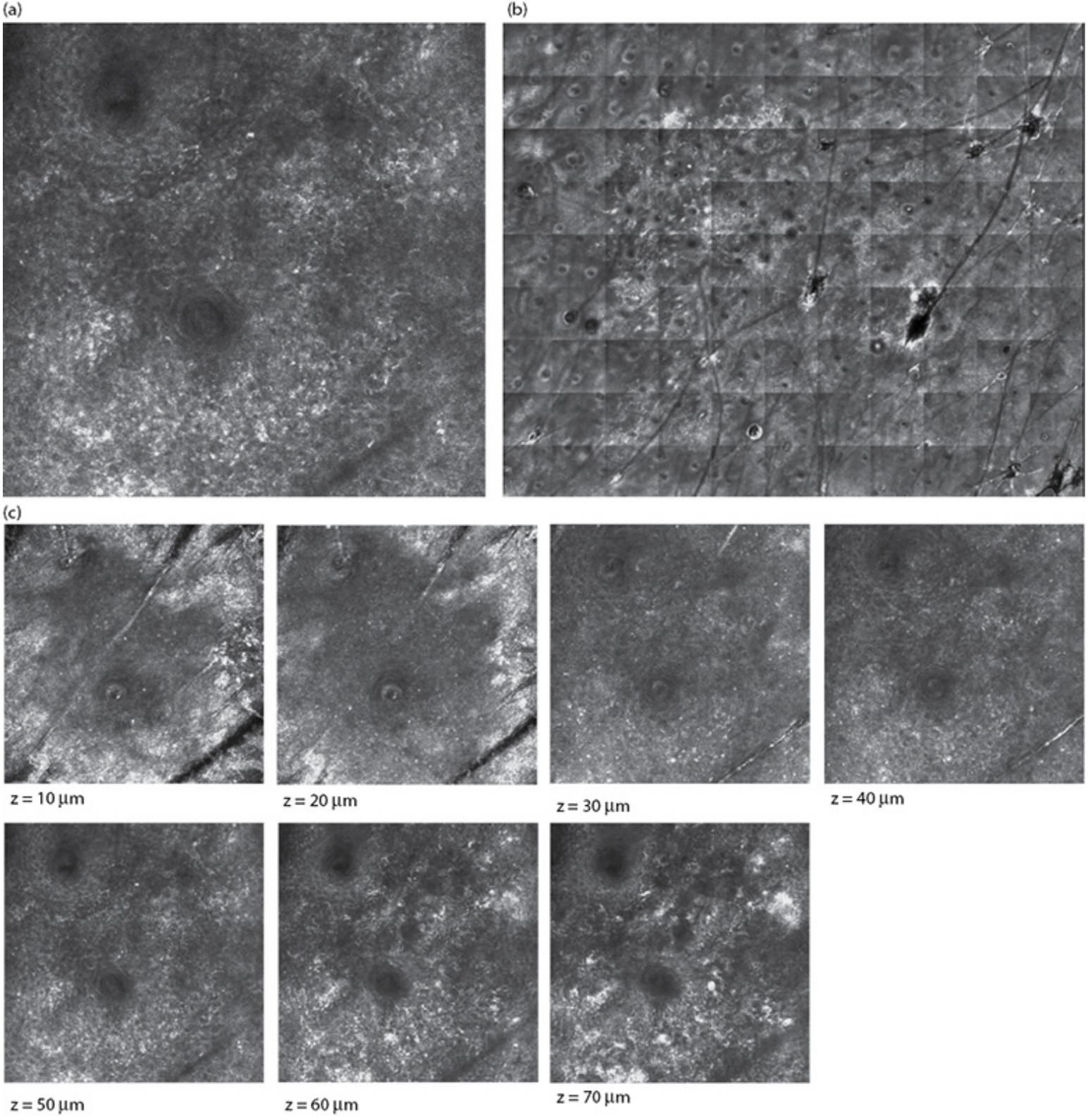

Figure 1.4 In vivo imaging of melanoma with follicular infiltration. (a) A single confocal image, taken at a depth of 50 µm below the skin surface. (b) A 5.5 mm × 4.5 mm mosaic of human epidermis, near the dermal-epidermal junction. (c) A z-stack of seven images ranging from a depth of 10 to 70 µm. Each image is separated by a depth of 10 µm.

When imaging in vivo, there are two mosaicking methods to consider. The first is a traditional mosaic in which the microscope head is attached to the cover slide, which is attached to the skin via adhesive. In this case, the size of the mosaic is limited by the size of the cover slide as well as the range of movement of the objective lens within the head of the microscope. The current version of the VivaScope 1500 has a mosaic size of 8 mm × 8 mm. Another method for mosaicking in vivo is video-mosaicking.[10,11] Using a handheld confocal

device, the objective lens is slowly moved across the lesion while recording a video. The video frames are then stitched together into a mosaic. The mosaic size is not physically limited in this case.

Z-stacking

In addition to the lateral extent of the lesion, skin lesions have extent in depth, and may extend to the dermal-epidermal junction, 250 μm beneath the surface of the skin. A technique called z-stacking is used to produce a series of images at different depths at a single position on the skin. The objective lens may be

moved closer to the skin by increments, so that the first image is near the surface of the skin and each successive image is deeper in the lesion. In the coordinate system of the reflectance confocal microscope (RCM), the *z*-axis is the optical axis, or depth axis, and thus a "z-stack" is a set of images taken at varying positions along the *z*-axis. In this way a lesion may be traced in depth. Figure 1.3 illustrates the technique of z-stacking, and Figure 1.4c shows a z-stack with 10 μm between images.

IMAGING PARAMETERS

Resolution and optical sectioning

The resolution of an optical system is the distance between two points that can be distinguished from each other. An optical system that is not limited by aberrations in the lenses is said to be diffraction limited. The diffraction limit of the lateral resolution in a point-scanning RCM is given by $r = 0.46\lambda/NA$, where r is the minimum distance between two points that can be resolved, λ is the wavelength of the light source, and NA is the numerical aperture of the objective lens, which is a measure of how sharply the lens focuses the light. For 830 nm laser illumination and a 0.8 NA objective lens, this gives a resolution limit of 0.48 nm. In a practical sense, however, the actual lateral resolution may be larger and is determined by the pixel size as determined by the scanning system. Typical pixel size is 0.5–1 μm for a point-scanning RCM.

Because the primary feature of confocal imaging is the optical sectioning it provides, it is the axial response of the RCM that is of most importance in imaging scattering human tissue. The optical section thickness of the RCM is often defined as the width of the axial response, as shown in Figure 1.2. A wide-field microscope, with no sectioning capability, has an axial response width that is essentially infinite, as compared to the finite axial response width of the RCM. The section thickness of the RCM is proportional to the axial resolution, $z = 2\lambda/(NA)^2$, but is also determined by the pinhole size. The section thickness increases with increasing pinhole size. Typical optical section width for RCM is 2–4 μm.

Features of RCM

- Reflectance contrast is provided by natural variations in index of refraction of cellular features.
- Optical sectioning of 1–5 μm eliminates the need for physical sectioning of tissue.
- High-resolution images show cellular and nuclear morphology.

Because both the lateral resolution and optical section thickness are proportional to the source wavelength, wavelength must be chosen with resolution in mind. However, in highly scattering tissue such as skin, the imaging resolution and optical sectioning degrades with depth as the light is scattered through the skin. Shorter wavelengths scatter more readily than longer wavelengths, and so the depth to which imaging is possible increases with increasing wavelength. As a result, there is a trade-off between resolution and depth of imaging. In highly scattering tissues such as skin, using a near-infrared wavelength such as 830 nm allows imaging to a depth of approximately 100–200 μm, sufficient for reaching the dermal-epidermal junction in skin, while maintaining sufficient lateral resolution.

Frame rate

The human visual system is capable of processing images at a rate of 10–12 frames per second. For live RCM video imaging in vivo, a video that runs at a rate higher than this will appear as smooth motion. The RCM frame rate is determined by the type of scanners used. To obtain a frame rate of 10 frames per second, with images of 1000 × 1000 pixels, the fast scanner must run at a rate of 10 kHz. This is generally achieved by either a spinning polygon with mirror faces or a resonant galvanometric scanner. Resonant galvanometric scanners have the advantage that they can be made very small and are therefore used in small handheld confocal devices.

Confocal line scanning

The success of RCM in imaging skin cancers has led to the development of smaller RCM devices to make daily clinical use more convenient. In addition, a smaller device could reach areas of the skin that may be inaccessible to a large bulky microscope head. While handheld confocal devices have been developed based on the traditional confocal microscopy design (e.g., the VivaScope 3000) a technique called confocal line-scanning is now being translated into the clinic. The traditional confocal design utilizes "point-scanning" in which the light is focused to a point and scanned across the sample in a two-dimensional raster scan. In line-scanning, the light is focused into a line and is scanned across the sample in only one dimension. This reduces the complexity of the optics and is therefore suitable for designing very small handheld devices for use in the clinic.

The line-scanning design also has the potential to increase the frame rate. The line-scanning technique does have drawbacks, as the background rejection is less efficient and results in an increase in section width and loss of image contrast.[12–16] However, a technique called divided-pupil line-scanning can recoup some of this loss of contrast by separating the illumination and detection paths.[17–23]

MODES OF CONTRAST

Contrast refers to the changes in the amount of light that is detected, thus providing contrast between physiologically different structures such as nucleus and cytoplasm. In reflectance contrast imaging, light reflected or scattered from the sample is detected and used to provide image contrast. In fluorescence imaging, a fluorescent dye is added to the sample. The dye can be targeted to specific sites on the cell. The illumination light excites the dye molecule, which then emits light of a longer wavelength as it relaxes to its ground state. Filters placed in front of the detector separate the strong reflectance from the weak fluorescent signal, allowing only the fluorescent signal to be detected. Because the fluorescent light emanates only from the targeted site, contrast is high in fluorescence imaging.

Reflectance contrast

In reflectance contrast imaging, variations in index of refraction of the physiological structures of the tissue cause more or less light to be reflected. Melanin is a major source of contrast in skin, as it has a large index of refraction (~1.7) compared to the average of ~1.3.[24,25] But even small changes in the index of refraction can provide contrast.[26,27] An image of typical normal epidermis will appear with dark nuclei and bright surrounding cytoplasm, indicating a difference in index of refraction between nucleus and the surrounding cell.

If the natural variability of index of refraction does not provide the needed contrast to distinguish between organelles, microstructures, and types of cells, the contrast may be enhanced by applying contrast agents such as acetic acid or aluminum chloride, both of which compact the chromatin and result in the appearance of bright nuclei.[28,29] Other possible contrast agents include microspheres or nanoparticles. When imaging in vivo, an applied contrast agent must be either nontoxic or used in low concentrations, while still enhancing imaging by being detectable by the imaging system.

Imaging contrast may be reduced by sources of noise in the system. Speckle noise arises in reflectance confocal imaging due to the coherent nature of the laser illumination used. Speckle noise is essentially a random interference pattern superimposed on the image that results in a salt-and-pepper-like appearance. Speckle noise can be reduced by using a larger detector pinhole, though because enlarging the pinhole also results in a loss of optical sectioning, a balance is found between optimizing optical sectioning and speckle noise reduction.[30]

Fluorescence contrast

In fluorescence imaging, a fluorescent dye is added to the tissue. The dye may be nonspecific, or targeted to a specific site on the cell. Fluorescence images have high contrast and do not suffer speckle noise. However, few dyes have been approved for use in humans. For that reason, fluorescence imaging is currently being used primarily in imaging excised tissue from Mohs and other surgeries.[9] Methylene blue is one of a few dyes that are approved for use in humans, and it may offer enhanced contrast,[31] especially when used in conjunction with reflectance imaging. In other in vivo studies with intraepidermal injection of fluorescein, the images changed with time as the dye was taken up by the cells, but did offer promising images.[32,33]

In excised tissue, acridine orange is used, as it is bound to nucleic acid, and produces images of bright nuclei. These fluorescence images can be combined with reflectance images of the same tissue, which show bright cytoplasm. The images are falsely colored blue and pink and overlaid to produce “digital H&E” images that mimic traditional H&E histopathology slides. This multimodal technique is described in more detail in Chapters 7 and 8.

Limitations of RCM

Depth of imaging is limited to 100–200 μm in highly scattering skin tissue.

SUMMARY AND CONCLUSIONS

The optical sectioning and endogenous reflectance contrast offered by reflectance confocal microscopy has been leveraged for use in human skin in vivo. Imaging parameters such as resolution and section thickness have been chosen to mirror traditional histopathology parameters, while the techniques

of mosaicking and z-stacking offer larger fields of view and imaging in depth. Resolution and optical sectioning are dependent on factors such as wavelength and choice of objective lens, and must be balanced with depth of imaging and considerations of speckle noise. Endogenous reflectance contrast is due to variations in index of refraction of cellular structures, and may be enhanced by the application of contrast agents. Reflectance confocal microscopy provides imaging of human skin in vivo that is comparable to that of traditional histopathology (Table 1.1).

Table 1.1 Comparison of confocal and histologic parameters

Parameter	*Confocal*	*Histology*
Wavelength (λ)	Single wavelength between 400 and 1064 nm	Broadband white light, 400–700 nm
Maximum imaging depth	50–100 μm at λ = 488 nm 150–250 μm at λ = 830 nm 300–400 μm at λ = 1064 nm	—
Section thickness	1–5 μm Noninvasive, optical	5 μm Physical
Lateral resolution	0.1–1 μm	0.1–4 μm
Numerical aperture (NA)	0.7–1.4	0.1–1.4
Immersion media	Water or oil	Air or oil
Magnification	40–100×	1–100×
Field of view	0.5–0.2 mm	20–0.2 mm
Contrast mechanism	Endogenous reflective microstructures	Exogenous absorbing dyes
Contrast agents/stains	Melanin Keratin Collagen	Hematoxylin and eosin (H&E) Methylene blue Toluidine blue

Source: Adapted from Gonzalez S, Gill M, Halpern A. eds. Reflectance confocal microscopy of cutaneous tumors. An atlas with clinical, dermoscopic and histological correlations. London, UK: *Informa Healthcare*; 2008.

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