CONFOCAL MICROSCOPY IN THE DIAGNOSIS OF MELANOMA

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Abstract - Melanoma is the most deadly form of skin cancer of melanocytic origin. The tumor has a high malignant potential and early metastasis. Prognosis is directly linked to the stage of the disease. Diagnosing thin melanoma at an early stage offers patients their best chance for survival. The crucial innovation in the early recognition of melanoma was the development of in vivo examination of the skin in high-resolution, by confocal microscopy. Confocal microscopy and its modifications provides a “virtual biopsy”, owing to melanosomes and melanin, which are a source of endogenous contrast. Confocal scanning laser microscopy (CLSM) provides visualization of microanatomical structures and cellular detail in real time (pigmented keratinocytes, melanocytes, melanosomes and melanophages) in the epidermis, dermoepidermal junction and superficial dermis at a resolution equivalent to the resolution of conventional microscopes.

Key words: Melanoma, melanocytic skin tumor, laser, dermoscopy, confocal microscopy, in vivo CSLM, sensitivity, specificity.

INTRODUCTION

Melanoma represents an aggressive tumor that early metastasizes and is resistant to medical therapy (Lazar et al., 2010). In the past several decades, the incidence of cutaneous melanoma has increased more rapidly than any other cancer. This “melanoma epidemic” has stabilized over the last decade and has even begun to fall in younger cohorts. The recent trends in incidence may reflect effective public education campaigns for primary prevention via sun protection and avoidance (Schaffer et al., 2004). However, the incidence rates continue to increase in western, eastern, and southern Europe, particularly in older men and women (De Vries and Coebergh, 2004). Also, the mortality of melanoma has increased, showing a consistently higher mortality in men than in women (MacKie et al., 2002; De Vries et al., 2003; Lindholm et al., 2004).

Tumor thickness is the most important factor in survival prognosis. Early diagnosis and surgical excision of the primary cutaneous melanoma in specialized institutions leads to better surveillance (Džodić, 2010). “The timely diagnosis and surgical treatment of melanoma are the only approaches to date that have increased survival”. This statement in the Journal of the American Academy of Dermatology by Sober and his coworkers in 1980, unfortunately, still holds true today (Sober, 1980). In the absence of effective treatment approaches of advanced disease, no agent has consistently been shown to
produce a benefit to overall survival (Schaffer et al., 2004).

The ability to recognize the varied clinical “faces” of melanoma (including those of thin, “featureless” and amelanotic melanoma) continues to evolve and improve with each passing day as a result of the implementation of imaging technologies in oncological dermatology. The introduction of dermoscopy in 1989 provided a noninvasive method of visualizing subsurface structures of the epidermis and papillary dermis that are not discernible to the naked eye (Schaffer et al., 2004). In a recent systematic review of the diagnostic accuracy of dermoscopy in detecting melanoma, a 10-27% higher sensitivity than clinical diagnosis by the naked eye has been reported (Mayer, 1997). In the predermoscopy era, the ABCD(E) clinical rule and good clinical visual inspection were the main diagnostic tool whose sensitivity values ranged between 65%-80%, depending on the expertise of the clinician (Schaffer et al., 2004; Miller and Ackermann 1992; Wolf et al., 1998). After the implementation of dermoscopy, there was a significant reduction in the benign:malignant ratio of excised melanocytic lesions from 18:1 to 4:1 (pre- and postdermoscopy eras, respectively) (Carli et al., 2004). Dermoscopy is a rapidly evolving field and it has become an important tool for the diagnosis of skin tumors (Braun et al., 2009). However, the presence of benign lesions with dermoscopic aspects indistinguishable from melanoma, melanomas lacking specific dermoscopic and/or clinical features, the presence of hypo- or amelanotic melanoma also represent great diagnostic challenges (Menzies et al., 1998; Menzies et al., 1996; Carli et al., 2004; Puig et al., 2007). The existence of so-called “featureless” melanoma (unrecognizable both clinically and dermoscopically) varies from 1-15%, and definitively implies the need for diagnostic improvement and new preoperative diagnostic tools (Carli et al., 2002; Pizzichetta et al., 2004).

In vivo confocal scanning laser microscopy (CslM) is a novel technique enabling the noninvasive imaging of the skin – the epidermis, papillary dermis and upper part of the reticular dermis. In vivo CSLM represents the missing link between dermoscopy and histological examination by producing horizontal sections of the skin with precise correspondence to the dermoscopic feature at cellular-level resolution and correspondence to histological findings (Rajadhyaksha et al., 1999; Pellacani et al., 2005). The application of confocal microscopy and its modification provide a “virtual biopsy”, owing to the presence of melanosomes and melanin, which are a strong source of endogenous contrast. In addition, CSLM provides visualization of microanatomical structures and cellular detail in real time (pigmented keratinocytes, melanocytes, melanosomes and melanophages) at a resolution that is equivalent to the resolution of conventional microscopes (Busam et al., 2001a).

With its reflectance mode, confocal microscopy (RCM) can visualize the skin in vivo and in freshly biopsied skin without fixing, sectioning, and staining (necessary for the preparation of conventional histological slides). The resolution enables the imaging of the nuclear, cellular, and tissue architecture of the epidermis and the underlying structures, including the connective tissue, inflammatory infiltrates, tumor cells, capillaries, and even circulating blood cells (Rajadhyaksha et al., 1995).

**Historical Development of Confocal Scanning Laser Microscopy**

Marvin Minsky described the first confocal microscope in 1957 (Minsky, 1957). A tandem scanning confocal microscope was designed by Petranin in 1968 to optically section tissue in real time (Petran et al., 1968). Research on in vivo imaging of human skin ultrastructure with confocal microscopy began in the early 1990s (New et al., 1991 Corcuff and Leveque, 1993, Veiro and Cummins, 1994). Rajadhyaksha et al. constructed a laboratory prototype use of a laser light source providing high illumination power and deeper penetrating wavelengths of near infrared to improve imaging capabilities. In 1995, Rajadhyaksha et al. first reported the foundations of laser scanning RCM (Rajadhyaksha et al., 1995). They reported the ability to image high-resolution nuclear and cellular level detail in normal human skin in vivo with good correlation to conventional histology. In 1999,
the same authors reported improved resolution, contrast, depth of imaging, and field of view with further advances in confocal instrumentation (Rajadhyaksha et al., 1999).

Langley and colleagues (2001) presented the first study of benign and malignant melanocytic lesions by in vivo confocal microscopy. Busam et al. (2001a) investigated the feasibility of recognizing the cellular constituents of pigmented skin lesions, such as pigmented keratinocytes, melanocytes, and melanophages, by CSLM. The same authors, in 2002, demonstrated that intraepidermal melanoma can be recognized by CSLM through analysis of the intraepidermal growth patterns of melanocytes using the same criteria as established for conventional histology (Busam et al., 2002).

Principles of confocal microscopy

A confocal microscope consists of a light source, a condenser, an objective lens and a detector. The light source, illuminated point, and detector aperture are in optically conjugated focal planes (i.e., confocal planes). This configuration allows the collection of light from the single in-focus plane and rejection of light from all out-of-focus planes (Gonzalez and Gilaberte-Calzada, 2008). The light source illuminates a small three-dimensional spot (voxel) within a sample, such as skin, from which reflected light is collected to produce a pixel. The illuminated spot is then scanned horizontally over a two-dimensional grid to obtain a horizontal microscopic section. This process is known as “optical sectioning” of the image in a series of horizontal planes stacked vertically to produce an image pixel by pixel, with an axial thickness of 2-5 µm (Gareau et al., 2008). This property enables a confocal microscope to look at a slice in the body of a thick semi-transparent sample, whereas, conventional microscopes visualize all the planes contemporarily (Branzan et al., 2007). The optical section is oriented parallel (i.e. en face) to the skin surface in unlike the conventional orthogonal sections of histopathology that are oriented perpendicular to the skin surface. The numerical aperture of the objective lens determines image resolution, which means that there is an inversely proportional relationship between high-resolution images and small apertures (less light), and low resolution through larger apertures (more light). Factors affecting the depth of penetration of light include the wavelength of illumination, power of illumination, reflectivity of the superficial layers, and scattering properties of the dermis. Longer wavelengths will penetrate deeper because of decreased light scatter, but the resolution decreases as the wavelength increases (Gonzalez and Gilaberte-Calzada, 2008). Confocal images are resolved in grey scale. White represents total light reflected and black represents no reflection at all (Rajadhyaksha et al., 1995). CSLM can be used in either reflectance (in the clinical field) or fluorescence mode (in research).

Optical reflectance microscopy relies on the natural variations in refractive indices of tissue microstructures for contrast. In human skin, melanin is the strongest endogenous contrast source for confocal imaging, resulting in a bright appearance of basal keratinocytes and melanocytes. Although melanin absorbs in the near-infrared spectrum (700 to 1064 nm), its high refractive index (1.7) compared to the epidermis (near water 1.34) determines a great dispersion of reflected light. According to Mie’s theory, more light is reflected when the tissue contains structures of a size similar to the wavelength of the light source (Vand de Hulst, 1981). Other sources include keratin, mitochondria and cytoplasmic organelles, chromatin in the nuclei, and collagen in the dermis (Rajadhyaksha et al., 1995; Rajadhyaksha et al., 1999).

A water immersion objective lens is used because the refractive index of water (1.33) closely matches the 1.34 refractive index of the living epidermis. It also minimizes spherical aberrations and reduces loss of resolution and contrast when imaging deep in the skin. Typically, water-based gels are used as the immersion media reducing irregularities in refraction. The numerical aperture of the objective lens determines the axial resolution and the amount of light detected (Rajadhyaksha et al., 1995; Rajadhyaksha et al., 1999).
**Fluorescence confocal microscopy** is based on the same optical principles as its reflectance counterpart. Fluorescent CSLM achieves contrast by the dynamic distribution pattern of the dye emission. CSLM in fluorescence mode relies on the differential distribution of endogenous or exogenous fluorescent molecules (fluorophores or fluorescent dyes such as fluorescein, toluidine blue, methylene blue, or Yaroslavsky stains) (Yaroslavsky et al., 2007) to provide highly specific contrast in tissue (Delaney, 1994). A laser light source, at an appropriate wavelength, is used to excite the fluorophore. The excited fluorophore emits a fluorescence signal at a longer wavelength, which is then detected and displayed in gray scale according to the fluorescence intensity. An area of great potential for fluorescence imaging is in the ability to target specific subcellular structures or proteins of interest, depending on the combination of wavelength of light and fluorophores used. Some of these dyes distribute selectively to specific tissue elements (e.g. nucleus, cytoplasm, stroma), and others can be chemically or immunologically targeted to specific molecular ligands. Recent studies on dual-mode reflectance and fluorescence confocal laser scanning microscopy in murine models have yielded promising results for tracking melanoma progression. (Gareau et al., 2007; Li et al., 2005). The present commercially available confocal laser microscope uses a deeper penetrating near-infrared wavelength of 830 nm (diode laser) or wavelengths of 785 nm (near-infrared), 658 nm (red) or 445 nm (blue). Up to three lasers are integrated in one device (www.lucid-tech.com/imaging Products.). A 30X objective lens of NA 0.9 that is routinely used provides a field of view of 0.5 mm, lateral resolution of 0.7 µm, and optical section thickness of 3 µm, which is comparable with that of conventional histology sections (Rajadhyaksha et al., 1995; Rajadhyaksha et al., 1999).

The maximum depth of imaging is 350 µm, dependent on the examined tissue, with good visualization of the epidermis, papillary dermis, and upper reticular dermis. To enable visualization of larger areas of tissue with varying magnification, similar to that in histopathology, a two-dimensional sequence of images is captured and software-stitched into a mosaic of contiguous 500 x 500 µm images (Nehal et al., 2008). This mosaic represents an overview image with 5-fold magnification. The imaging procedure for one single lesion requires approximately 5-15 minutes (Hofmann-Wellenhof et al., 2009). Live images may be captured either as single frames or as sequential frames to form videos to demonstrate dynamic events (Nehal et al., 2008).

**Confocal scanning laser microscopy features of normal skin**

CSLM imaging of human skin in vivo has shown good correlation with conventional histology (Ra-
When imaging the skin in real time, starting from the surface and progressing deeper, most superficial images are obtained from the stratum corneum. The structures are well visualized in all strata. The epidermis/dermis boundary seems to provide more information than other layers (Gerger et al., 2004). Some of the main characteristics of CSLM features of normal skin are presented in Table 1.

Confocal scanning laser microscopy features of melanocytic skin lesions

Differential CSLM diagnosis of melanocytic lesions requires analysis of the architectural and cellular aspects of the skin, including melanocyte and keratinocyte shape and distribution. (Pellacani et al., 2005). Because melanin provides a strong contrast in RCM, melanocytes can be easily assessed, enabling enhanced diagnosis and differentiation of malignant from benign melanocytic lesions, detection of local recurrences after surgical excision, and in vivo tumor follow-up (Curiel-Lewandrowski et al., 2004). The first study to systematically investigate the use of in vivo CSLM in diagnosing pigmented skin lesions (PSLs), was that of Langley et al. in 2001 (Langley et al., 2001). Busam et al. described the morphological features of melanocytes, pigmented keratinocytes, and melanophages in vivo (Busam et al., 2001a). CSLM enables the recognition and position of melanocytes within the epidermis. Therefore, it may allow immediate recognition of a pigmented lesion as melanocytic in origin. Once the melanocytes are recognized, the ability to analyze their growth pattern within the epidermis should in principle allow a preliminary diagnostic evaluation and a decision on the need for a biopsy or excision (Busam et al., 2001a).

Reflectance confocal microscopy features of melanoma

Pigmented and amelanotic melanomas exhibit remarkably similar features when assessed by RCM (Langley et al., 2001; Busam et al., 2001b; Curiel-Lewandrowski et al., 2004; Tannous et al., 2002). These features may be evident because of the presence of melanosomes or melanin-filled premelanosomes in the cytoplasm (Hofmann-Wellenhof et al., 2009). Another useful advantage of RCM is that it enables the noninvasive detection of amelanotic melanoma (Busam et al., 2001b). RCM allows for the recognition of abnormal intraepidermal melanocytic proliferation in clinically amelanotic melanomas that is distinct from features of normal skin.

A useful advantage of RCM is that it enables the identification of abnormal intraepidermal melanocytic proliferation, granules and dendritic structures in clinically amelanotic melanomas. This is the most important advantage over dermoscopy, since dermoscopy cannot provide the diagnosis of amelanotic melanoma with that level of certainty. Intraepidermal melanoma (in situ) can be recognized by CSLM through analysis of the intraepidermal growth patterns of melanocytes using the same criteria as established for conventional histology. The intraepidermal melanoma component shows atypical epithelioid melanocytes at all layers within the epidermis distributed in a pagetoid fashion as well as intraepidermal melanocytic nests with discohesion of cells (Busam et al., 2002; Pellacani et al., 2005). These include structural changes in the spinous and granular layers, keratinocyte disarrangement and loss of intercellular demarcation (disruption of the “honeycomb pattern”). Enlarged atypical cells with pleomorphic morphology, variable refractivity and angular nuclei may be found in several layers of the epidermis (pagetoid dissemination) (Langley et al., 2001). Recognition of early melanoma in situ is also a very important advantage over dermoscopy.

It needs to be borne in mind that this is not sufficient for the diagnosis of melanoma. This feature can be seen in acral, congenital and Spitz nevi, as well as in recurrent nevi or after trauma (Weedon, 1997; Cochran et al., 1997). Especially inflamed nevi, such as a nevus with associated eczematous features, may show an increased number of intraepidermal Langerhans cells simulating pagetoid melanocytosis (Busam et al., 2005). On the other hand, regular
dermoepidermal architecture, and the absence of pagetoid infiltration and atypical cells are suggestive of benign lesions. At the dermoepidermal junction level, non-edged papillae were observed in 90% of melanomas and in 41% of nevi, whereas edged papillae were predominantly present in nevi. Study has highlighted the importance of the identification of pagetoid infiltration that resulted the most relevant discriminating parameter (Pellacani et al., 2007).

Confocal imaging for tumor margin mapping

In vivo CSLM (“virtual biopsies”) has the potential to guide invasive sampling of complex cutaneous neoplasms, and promises to be useful for mapping the margins of a skin lesion prior to and during surgical procedure. RCM may improve the presurgical and intraoperative margin detection for cutaneous neoplasms with ill-defined borders. This may help in the early detection of clinically barely visible or nonpigmented melanomas, as well as lentigo maligna melanoma, and may facilitate preoperative noninvasive assessment of their margins (Busam et al., 2001b). Confocal imaging may also potentially be used for intraoperative assessment of the deep margins of resection during Mohs microscopically controlled surgery (Rajadhyaksha et al., 2001; Chung et al., 2004).

Sensitivity and specificity CSLM in diagnosing melanocytic tumors

Studies have shown the high sensitivity and specificity for detecting melanocytic skin tumors with in vivo confocal imaging (Busam et al., 2001a; Langley et al., 2001; Gerger et al., 2004; Gerger et al., 2005; Pellacani et al., 2005; Gerger et al., 2008a; Gerger et al., 2009). The diagnostic applicability of RCM in melanocytic skin tumors, determined by evaluating sensitivity, specificity, as well as positive and negative-predictive value (PPV, NPV), has been described in several studies (Busam et al., 2001a; Langley et al., 2001; Gerger et al., 2005; Pellacani et al., 2007; Langley et al., 2008; Gerger et al., 2008b) (Table 2). Taking into account all RCM studies dealing with the diagnostic accuracy in melanocytic skin lesions, sensitivity and specificity of approximately 90% and 86%, respectively, could be reached (Hofmann-Wellenhof et al., 2009).

Advantages of confocal scanning laser microscopy

The main advantage of CLSM is the offer of a unique opportunity to image thin sections of living tissue at a resolution equal to that of conventional microscopes used to view histological slides. Cellular and architectural details can be examined without

Table 1. The layers of the epidermis - main characteristics of CSLM* features of normal skin

<table>
<thead>
<tr>
<th>Skin layer</th>
<th>Depth from stratum corneum (μm)</th>
<th>Keratinocyte diameter (μm)</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stratum corneum</td>
<td>Skin surface</td>
<td>15–30</td>
<td>Bright polygon cells with dark outlines forming islands separated by wrinkles (dermatoglyphs)</td>
</tr>
<tr>
<td>Stratum granulosum</td>
<td>15–20</td>
<td>20–35</td>
<td>Bright granular cytoplasm and dark oval or round nuclei “honeycomb pattern” or “cobblestone pattern”</td>
</tr>
<tr>
<td>Stratum spinosum</td>
<td>15–25</td>
<td>20–100</td>
<td>Similar pattern to the stratum granulosum but smaller cells with less refractive cytoplasm</td>
</tr>
<tr>
<td>Stratum basale</td>
<td>7–12</td>
<td>50–100</td>
<td>Highly refractive basal cells arranged as aggregates (horizontal optical sectioning at the suprapapillary plates) or form bright rings (“edged papillae”) that increase in size at deeper levels (horizontal optical sectioning through the dermal papillae)</td>
</tr>
</tbody>
</table>

*CSLM - Confocal scanning laser microscopy
## Table 2. Sensitivity and specificity observed in various clinical trials.

<table>
<thead>
<tr>
<th>Study</th>
<th>Observers</th>
<th>Type of study</th>
<th>Number of RCM* images per lesion</th>
<th>Algorithm (CSLM** instrument model)</th>
<th>Sample size</th>
<th>Melanoma No.</th>
<th>Nevi No.</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerger, 2005.</td>
<td>5 independent observers, no prior training in RCM</td>
<td>Blinded to the clinical, dermoscopic and histopathological diagnoses</td>
<td>20 per lesion</td>
<td>Logistic regression analysis</td>
<td>27( all histologically verified)</td>
<td>90</td>
<td>30 (30 histologically verified)</td>
<td>88.15</td>
<td>97.6</td>
</tr>
<tr>
<td>Pellacani, 2005.</td>
<td>2 independent observers</td>
<td>Blinded to the clinical, dermoscopic and histopathological diagnoses</td>
<td>More than 100 capture images per lesion</td>
<td>Major et minor criteria</td>
<td>88</td>
<td>88</td>
<td>72.3</td>
<td>72.3</td>
<td></td>
</tr>
<tr>
<td>Pellacani, 2007.</td>
<td>2 expert observers</td>
<td>Blinded to the clinical, dermoscopic and histopathological diagnoses</td>
<td>More than 100 capture images per lesion</td>
<td>Major et minor criteria</td>
<td>215</td>
<td>136</td>
<td>215</td>
<td>136</td>
<td>97.3</td>
</tr>
<tr>
<td>Langley, 2007.</td>
<td>1 observer with experience in RCM and dermoscopy</td>
<td>Dermoscopy performed initially by same investigator</td>
<td>1 observer with experience in RCM and dermoscopy</td>
<td>Overall evaluation</td>
<td>378</td>
<td>89</td>
<td>83</td>
<td>97.3</td>
<td></td>
</tr>
<tr>
<td>Gerger, 2008.</td>
<td>2 independent observers</td>
<td>Retrospective manner</td>
<td>2 observers</td>
<td>Decision tree</td>
<td>2050</td>
<td>97.5</td>
<td>97.6</td>
<td>99</td>
<td>92.4</td>
</tr>
</tbody>
</table>

*RCM - Reflectance confocal microscopy
**CSLM - Confocal scanning laser microscopy
having to excise and process the tissue as is done in standard histology. The whole procedure can be done in a few minutes “at the bedside”. The current commercially available technology is already advancing toward handheld confocal microscopes that are less expensive, less robust and easier to use on humans. In addition, CSLM’s features are easy to learn and use. Reflectance confocal microscopy, similar to dermoscopy, images lesions in an en face plane, thus enabling direct correlations with dermoscopic images.

Consequently, the RCM can provide subsampling of the region of interest on the dermoscopic image; pointing the cursor at a dermoscopic structure on the digital image will focus the RCM to scan the corresponding location (Scope et al., 2007). Such a direct comparison between the two techniques could be achieved by selecting lesions by naked-eye examination and then applying both methods. However, the RCM specificity was more than two-fold superior to dermoscopy for light-colored lesions. The improved diagnostic accuracy of RCM with light-colored lesions may be explained by the fact that melanin appears very bright under reflectance microscopy, even in very small quantities. CSLM had a relatively higher sensitivity over dermoscopy with similar specificity. These results suggest that dermoscopy and CSLM are complementary (Langley et al., 2008). Confocal microscopy seems useful for second-level examination of clinically and dermoscopically equivocal lesions (Guitera et al., 2009).

**Limitations of CSLM**

A limitation in the current state of RCM technology is that the imaging is restricted to a depth of 350-500 μm due to tissue-induced scattering and aberrations, which corresponds to the papillary dermis and, depending on skin thickness, the superficial reticular dermis. Therefore, assessment of microanatomical structures in the reticular dermis or tumor invasion depth cannot be evaluated reliably. The presence of refractive structures may also decrease contrast and make melanocyte visualization difficult. This might be improved by testing the different immersion media and illumination sources (Hofmann-Wellenhof et al., 2009). The grayscale (black/white) contrast also lacks specificity for organelles and ultrastructure (Nehal et al., 2008). Nuclear features (e.g. mitoses) cannot be assessed accurately by CSLM (Branzan et al., 2007). Recently, novel confocal line-scanning microscopes have demonstrated the imaging of nuclear and cellular morphology in human epidermis. Multimodal confocal microscopy and spectrally encoded confocal microscopy (SECM) may thus offer enhanced diagnostic potential and application (Boudoux et al., 2005). As 5-10 minutes are required for the CLSM diagnosis of a single lesion, the method is clearly not to be applied for the first-level examination (Guitera et al., 2009), but only after clinical and dermoscopic examination.

**CONCLUSION**

CSLM represents an opportunity for the noninvasive evaluation of skin lesions in histological detail. It may be employed as a guide for performing biopsies by helping to determine which areas have features suspect for malignancy and reducing sampling error, or as an adjunct to Mohs surgery and therapy by mapping out the margins or extent of involvement prior to excision or other therapies. In addition, CSLM can be used to monitor the progression or resolution of lesions, providing for reduced number of unnecessary biopsies. In the future, CSLM might be used as an in vivo follow-up of the natural changes in melanocyte nevi due to aging or sun exposure, as already established with digital dermoscopy (Dobrosavljevic et al., 2009). Although it is currently too expensive and laborious to be considered as a screening tool for melanoma, with minor advances CSLM may become practical for assessing pigmented lesions that have been selected as suspect on the basis of clinical examination and/or dermoscopy (Busam et al., 2005).

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