Objective. Sentinel node biopsy in patients with cutaneous melanoma improves staging, provides prognostic information, and leads to an increased survival in node-positive patients. However, frozen section analysis of the sentinel node is not reliable and definitive histopathology evaluation requires days, preventing intraoperative decision-making and immediate therapy. Photoacoustic imaging can evaluate intact lymph nodes, but specificity can be hampered by other absorbers such as hemoglobin. Near infrared multispectral photoacoustic imaging is a new approach that has the potential to selectively detect melanin. The purpose of the present study is to examine the potential of multispectral photoacoustic imaging to identify melanoma metastasis in human lymph nodes. Methods. Three metastatic and nine benign lymph nodes from eight melanoma patients were scanned ex vivo using a Vero LAZR© multispectral photoacoustic imager and were spectrally analyzed per pixel. The results were compared to histopathology as gold standard. Results. The nodal volume could be scanned within 20 minutes. An unmixing procedure was proposed to identify melanoma metastases with multispectral photoacoustic imaging. Ultrasound overlay enabled anatomical correlation. The penetration depth of the photoacoustic signal was up to 2 cm. Conclusion. Multispectral three-dimensional photoacoustic imaging allowed for selective identification of melanoma metastases in human lymph nodes.
2.2. Phantom. In order to verify whether multispectral photoacoustic imaging is able to differentiate the chromophores of blood from melanin, a phantom was developed. This was made of absorbing and scattering agar gel (2% in water) mimicking the optical properties of soft tissue. Embedded inside the phantom at 4 ± 1 mm depth were two 2% agar cylinders (diameter 2 mm, height 6 mm). One cylinder contained bovine hemoglobin (Hb) (Sigma-Aldrich, Zwijndrecht, the Netherlands) in a concentration of 15 g/L. The second cylinder contained B-16 melanin producing melanoma cells (2 × 10⁶ cells/mm³). The background consisted of 2% agar completely covering the 6 mm high cylinders with a cover on the top of 4 ± 1 mm. Multispectral volume scanning was performed using five distinct illumination wavelengths between 680 and 840 nm with 40 nm intervals. After image acquisition, the photoacoustic spectrum was obtained by selecting a 3D region of interest in each inclusion and calculating the mean values with standard deviations.

2.3. Human Lymph Nodes. Twelve human lymph nodes from eight melanoma patients undergoing lymphadenectomy were obtained from the surgical specimen. The experimental protocol was performed according to the Dutch guidelines for clinical research and patient’s informed consent was acquired prior to surgery. The lymph nodes were stored in phosphate buffered saline before and during imaging. Subsequently, histopathology examination was performed using two or more slides stained with hematoxylin and eosin and biomarkers (S100, HMB-45, Melan-A) when needed.

In order to obtain accurate reference spectra for both blood and melanin, multispectral images were acquired for each node. Five wavelengths were selected: 3 wavelengths (700 nm, 800 nm, and 820 nm) covered the near infrared range. Two additional near infrared wavelengths (732 nm and 757 nm) were selected based on the absorption spectrum of oxy- and deoxyhemoglobin; both show an increase between 732 nm and 757 nm, whereas the absorption spectrum of melanin shows a slight decrease. In addition, at least one hyperspectral slice was acquired of every node using 101 colors of light, ranging from 700 to 900 nm with intervals of 2 nm. The photoacoustic reference spectrum of blood was obtained by selecting the vessels on the images of benign lymph nodes. The selected regions were also examined with high-resolution ultrasound and histology. In the metastatic nodes, ultrasound and histological slides were used to select an area in the photoacoustic dataset with a high melanin concentration as reference for melanin. Calculation of both a mean and a standard deviation for every measured wavelength resulted in reference spectra for both chromophores.

2.4. Unmixing Algorithm. The raw photoacoustic data were first filtered with a median filter. A two-dimensional median filter (block size 5×5 pixels in the image plane; approximately 110 × 110 μm) was chosen because of the noncubical voxel dimensions: 22 × 22 × 200 μm (depth × width × slice thickness). Variations in local fluence are expected, as light is absorbed and scattered while travelling through the heterogeneous tissue. Variations in light distribution were corrected...
by area under the curve normalization, that is, divided by the integral over the wavelength range. This correction aims to preserve the spectral characteristics, while the influence of absolute light intensity is reduced. The normalized measured signal was then compared to the normalized reference spectra of both blood and melanoma per image voxel. The reference spectra were based on 1168 voxels for blood and 1349 for melanin, selected per wavelength from a 3D region of interest. In order to calculate the resemblance of the measured spectrum and the reference spectra, a statistical t-test was performed. The P value (ranging 0-1) was displayed in a resulting image. The P value was calculated for both blood and melanin for each of the five measured wavelengths. A value close to 1 indicates high resemblance; a value closer to 0 implies an increasing discrepancy with the reference. The five P values are combined by multiplication, resulting in a single
Table 1: Patient characteristics.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Node</th>
<th>Histology</th>
<th>Node longest diameter (cm)</th>
<th>Used as reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>Male</td>
<td>1</td>
<td>Benign</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>Female</td>
<td>2</td>
<td>Malignant</td>
<td>1.4</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>Female</td>
<td>3</td>
<td>Benign</td>
<td>1.0</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>Male</td>
<td>4</td>
<td>Benign</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>79</td>
<td>Female</td>
<td>6</td>
<td>Malignant*</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>62</td>
<td>Female</td>
<td>7</td>
<td>Benign</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>38</td>
<td>Male</td>
<td>9</td>
<td>Benign</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>Female</td>
<td>11</td>
<td>Malignant</td>
<td>1.2</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>62</td>
<td>Male</td>
<td>12</td>
<td>Benign</td>
<td>1.8</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Amelanotic melanoma.

3. Results

3.1. Phantom. Figure 1 shows the photoacoustic intensity maps of the phantom at 680 nm (Figure 1(a)) and 840 nm (Figure 1(b)). The position of the reference inclusions is schematically visualized in Figure 1(c). Both inclusions have a signal above background, especially at the superficial part. The signal intensity inside the inclusions decreases with depth as light fluence decreases deeper inside the phantom. Examination of the photoacoustic images alone does not allow for discrimination between blood and melanin. The information for discrimination lies in combination of multiple images, acquired at different wavelengths of light. The spectra derived from the multispectral images show different patterns for blood and melanin (Figures 1(d) and 1(e)). The effect of area under the curve (AUC) normalization is characterized in the difference between the two curves. The result of the recognition algorithm shows that the two inclusions can be distinguished (Figure 1(f)). Some matching pixels were found outside the inclusions, indicating some margin of error that could result in false positive or false negative pixel classifications.

3.2. Human Lymph Nodes. An overview of the measured nodes is provided in Table 1. Three of the twelve lymph nodes contained melanoma metastases according to histopathological examination, one of which was amelanotic. The reference spectrum of blood was based on over 8000 pixels of blood vessels in four tumor-negative lymph nodes from four different patients (Figure 2). The reference spectrum of melanin was based on over 2200 pixels of two tumor-positive lymph nodes from two patients. The spectral differences between blood and melanoma are best reflected in the gradual slope of the photoacoustic signal from melanoma compared to the increase in photoacoustic signal from blood in the wavelength range between 732 and 756 nm (Figure 2).

The ultrasound image of a tumor positive lymph node, node number 2 in Table 1, (Figure 3(a)) visualizes the node as a round structure, surrounded by hyperechogenic fatty tissue. The corresponding photoacoustic image shows strong signal at the surface of the node, nearby the detector, while lack of signal can be noticed at a larger distance from the surface. The strong optical absorption of the melanin deposits limited the light penetration to 2-3 mm. The result of the spectral unmixing procedure (Figure 3(c)) shows a green strip overlaying the ultrasound image. The green represents image elements spectrally according to melanin. In this figure, almost no...
elements were found with a spectral response corresponding to blood. This is in accordance with the histological slide (Figure 3(g)) in which melanin is found in the upper part of the node (dark brown) and no larger vessels are seen inside the nodal capsule.

A representative benign node, node number 3 in Table 1, shows larger vessels entering the hilum of the node (Figures 3(d)–3(f) and 3(h)). The ultrasound (Figure 3(d)) visualizes a bean shaped node with a brighter hilum surrounded by bright fatty tissue. The photoacoustic image (Figure 3(e)) shows vessel shaped structures at 16 mm depth and signal at the surface of the fatty tissue. The vessel shaped structures are recognized as blood in the algorithm-result imaging; the signal at the surface of the fatty tissue does not resemble the reference of blood nor melanin. Histopathology slides (Figure 3(h)) confirm the location of the blood vessels within the hilum of the node. Within 20 minutes, total volume imaging proved possible for the benign lymph nodes, where penetration was sufficient to examine the entire nodes, which were up to 2 cm in diameter (Figure 3).

Tumor-positive nodes have different melanin distribution, which may result in different photoacoustic signal distribution. In Figure 4, the photoacoustic signal of node number 11 from Table 1, containing tumor metastasis, is compared to the first tumor-positive lymph node (Figure 3), the melanin distribution is more scattered throughout the node (Figure 4(a)) resulting in a more diffuse distribution of green photoacoustic pixels (Figure 4(b)). Also the number of blood vessels seems higher in this node which is reflected in the corresponding photoacoustic images (Figures 4(b) and 3(c)). Direct comparison of the photoacoustic images and the
The presented reference spectra show similarity with known optical absorption spectra for hemoglobin (oxyhemoglobin + deoxyhemoglobin) and eumelanin \([8, 9]\). The gradual slope in the spectrum of melanin and the increase between 732 nm and 756 nm for hemoglobin is characteristic in both optical absorption and the reference spectra. However, the reference spectra should encompass the response in a variety of situations, which requires a multitude of human samples to be scanned in further studies.

Specific detection of melanin can be useful in intraoperative photoacoustic imaging of an excised sentinel node. This would permit an immediate node dissection if metastatic foci are demonstrated and would obvi ate the need for a second operation. Perhaps the sentinel node could even be analyzed \textit{in vivo}. A penetration depth of 2 cm in the absence of melanin deposits enables the analysis of the entire nodal volume. This is in contrast with the conventional pathology evaluation that samples less than 0.1% of a node.

When present, melanin absorbs most of the light and limits the depth of photoacoustics imaging under these circumstances. For clinical application this may be of limited importance, since the extent of tumor involvement per node is presently of less relevance. Also the presence of a blue dye, generally used for sentinel node detection, may alter the penetration depth of optical imaging. However, the impact on melanoma detection will be limited since the optical absorption of blue dye in the infrared region is limited and the optical absorption spectrum of the dye is clearly distinctive from both blood and melanin.

A false positive test result may be realistic because normal naevus cells are present in one-third of the skin draining lymph nodes. Additional research should reveal whether adequate reference spectra for these kinds of conditions can still be defined.

As photoacoustic imaging relies on optical absorption, the 1.8% to 8.1% amelanotic melanomas may challenge the sensitivity of the procedure \([10]\). An occasional false negative procedure will not have major detrimental consequences, because a strong point of photoacoustic scanning is that histological and immunohistochemical analysis afterwards remains possible. Therefore, a false negative procedure does permit a node dissection, albeit at a somewhat later date. Recent reports describing molecular based staging methods do not allow for this option, because these techniques destroy the tissue during the procedure \([11, 12]\).

For \textit{ex vivo} robust real time sentinel lymph node analysis, further research should initial focus on the accuracy of photoacoustic imaging for small lymph node metastases.

5. Conclusion

A method is proposed to detect melanoma metastases in human lymph nodes using multispectral photoacoustic imaging. The method was conducted on a set of lymph nodes containing tumor positive and tumor negative nodes. Separation of important optical absorbers like blood and melanin proved possible based on the spectral information. Lymph nodes could be analyzed in image planes and also as entire nodal volumes (3D).
In hybrid photoacoustic imaging, the high resolution ultrasound imaging can be used for orientation, as well as for diagnosis, as in conventional ultrasound. The two techniques are synergetic as they are based on different imaging contrasts; ultrasound is based on acoustic impedance which is sensitive to anatomy and photoacoustics on light absorption which is sensitive to molecular properties. The reflective mode hand-held system proved capable of 3D imaging of the entire lymph node. Further research should be directed towards the robustness of this technology for the detection of small melanoma lesions within human lymph nodes and its specificity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

Submit your manuscripts at http://www.hindawi.com