Pump-probe imaging of pigmented cutaneous melanoma primary lesions gives insight into metastatic potential

Francisco E. Robles,1 Sanghamitra Deb,1 Jesse W. Wilson,1 Christina S. Gainey,1 M. Angelica Selim,2 Paul J. Mosca,3 Douglas S. Tyler,4 Martin C. Fischer,1 and Warren S. Warren1,*

1Department of Chemistry, Duke University, Durham, North Carolina 27708, USA
2Department of Pathology, Duke University Medical Center, Durham, NC 27705, USA
3Department of Surgery, Duke University Medical Center, Durham, NC 27705, USA
4Department of Surgery, University of Texas Medical Branch, Galveston, TX 77555, USA

*warren.warren@duke.edu

Abstract: Metastatic melanoma is associated with a poor prognosis, but no method reliably predicts which melanomas of a given stage will ultimately metastasize and which will not. While sentinel lymph node biopsy (SLNB) has emerged as the most powerful predictor of metastatic disease, the majority of people dying from metastatic melanoma still have a negative SLNB. Here we analyze pump-probe microscopy images of thin biopsy slides of primary melanomas to assess their metastatic potential. Pump-probe microscopy reveals detailed chemical information of melanin with subcellular spatial resolution. Quantification of the molecular signatures without reference standards is achieved using a geometrical representation of principal component analysis. Melanin structure is analyzed in unison with the chemical information by applying principles of mathematical morphology. Results show that melanin in metastatic primary lesions has lower chemical diversity than non-metastatic primary lesions, and contains two distinct phenotypes that are indicative of aggressive disease. Further, the mathematical morphology analysis reveals melanin in metastatic primary lesions has a distinct “dusty” quality. Finally, a statistical analysis shows that the combination of the chemical information with spatial structures predicts metastatic potential with much better sensitivity than SLNB and high specificity, suggesting pump-probe microscopy can be an important tool to help predict the metastatic potential of melanomas.

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Localized melanoma is effectively treated with aggressive wide excision, but many patients will ultimately recur and develop distant metastatic disease, which is associated with extremely poor prognosis [1,2]. Over the past several years, major advances have been made in the systemic treatment of advanced melanoma with the resulting FDA approval of several effective agents. These have included both BRAF pathway targeted therapies and immune checkpoint inhibitors [3]. Although these agents are not yet approved for the adjuvant therapy of high-risk melanoma—that is, for the control or eradication of micrometastatic disease—interferon alfa-2b is approved [4], and studies of novel targeted agents in the adjuvant setting are currently under way. The relative risk of recurrence and metastasis of clinically localized melanoma can be roughly estimated based on staging information such as primary tumor thickness and ulceration status, as well as sentinel lymph node metastasis [1]. Yet for melanomas of similar stage, it is not possible to reliably determine, based on characteristics of the primary tumor, which lesions will ultimately recur and develop distant metastatic disease, which is associated with extremely poor prognosis [1,2].
melanin pigments in the primary site of invasive melanomas to assess their metastatic potential.

In the event that a lesion is found to be malignant after initial histopathological evaluation, the most important prognostic factor associated with metastatic potential is tumor thickness. Generally patients with tumor thickness > 1 mm, or 0.76 to 1 mm with one or more adverse prognostic features such as ulceration or mitotic activity, undergo a sentinel lymph node biopsy (SLNB) for definitive pathologic staging of the regional lymph nodes [5–8]. It is estimated that this procedure improves the 5-year survival rate from 73% based on nodal observation to 78% [9]. However, SLNB has substantial drawbacks, including high cost and potential complications (e.g., lymphedema, infection, seroma, lymphatic fistula, hematoma, and neuropraxia) with an aggregate complication rate of 10-39% [7,10,11]. Moreover, only a small percentage (c.a. 16%) of patients undergoing SLNB are found to have nodal metastasis [7,9], meaning the majority of patients do not derive oncologic benefit from the invasive procedure. More importantly, SLNB does not identify the majority of patients who go on to die of metastatic disease (out of the patients who had their lymph nodes biopsied and later died, only 34% had a positive SLNB and the remaining 66% had a negative SLNB) [8,9]. An additional limitation is that for thin lesions (<1 mm) without adverse prognostic features SLNBs are not routinely performed as they would virtually always be negative [1]. However, the National Cancer Institute’s Surveillance, Epidemiology, and End Results (SEER) reports that this group of patients, which make up well over half of all melanoma cases [12], have a 15% death rate [13]. Thus, additional markers from the primary lesion (thick and thin) are still needed to help identify patients that have micrometastatic disease and will truly benefit from additional, more aggressive detection/treatment strategies.

Here we investigate the potential of pump-probe molecular imaging to quantitatively differentiate between metastatic and non-metastatic melanocytic melanomas based on melanin pigment chemistry in the primary tumor site as a means to (1) aid in identifying patients who would otherwise be left with untreated metastatic disease under the current paradigm and (2) reduce the number of expensive and invasive SLNB procedures performed on patients with no nodal involvement. Our approach consists of imaging thin unstained biopsy slides with pump-probe microscopy, an approach that has already shown significant promise in differentiating melanomas from cutaneous melanocytic nevi [14,15], as well as conjunctival melanoma from primary acquired melanosis [16]. Pump-probe microscopy provides subcellular resolution of the chemical composition of endogenous pigments by probing their electronic excited state dynamics [14,17]. A very important advantage of this approach is that molecular signatures of melanins do not degrade over time—we have shown that Jurassic-aged eumelanin has the same pump-probe features as its modern counterpart [18]—which enables us to use archived tissue samples in retrospective databases, an approach that is not possible with immunohistochemistry methods [19].

We examine pump-probe images of primary lesions for characteristics that correlate with the presence of metastasis in sentinel lymph nodes or history of recurrences. This work yields novel insight into the biochemical composition and structure of melanin in metastatic melanomas. Further, a statistical analysis reveals that chemical and structural features of melanin are promising surrogate biomarkers of metastatic disease that have the potential to help improve current clinical practice.

2. Materials and methods

2.1 Biopsy samples

Unstained biopsy slides from patients with invasive melanomas that have undergone SLNB were imaged (N = 29). Ten SLNB were positive for metastasis and two patients, originally with negative SLNB, later developed recurrences. A board-certified dermatopathologist identified regions in the primary lesions indicative of melanoma using H&E stained slides,
and adjacent unstained slides were imaged with a pump-probe microscope. All protocols were approved by the Institutional Review Board of Duke University. Properties of the analyzed samples are tabulated in Appendix 1 (Table 1).

2.2 Optical system

The pump-probe system consists of a commercial laser-scanning microscope (Zeiss, LSM-510) equipped with a custom laser setup that produces two ultrafast pulses. The center wavelengths of the pulses are 730 nm (pump) and 810 nm (probe)—this combination has been found to provide good contrast between different types of melanins [20]. The pulses are focused onto the sample using a 20X objective (0.8NA) with less than 0.65 mW total power to avoid any possible effects that may alter the melanin pigment chemistry (e.g., photobleaching) [20]. The nonlinear signals are detected using a custom-built lock-in amplifier (adapted from Saar, et al. [21]) with a time constant of 100 µs. See Ref [14] for more details.

Images are acquired by raster scanning the beams across the sample multiple times; each time with a different pump-probe time delay to ascertain the pigments’ excited state dynamics. For each field of view (420µm x 420µm), a stack of 34 images is collected. Figure 1(a) illustrates the image acquisition process along with representative pump-probe responses of four pixels. A total of 174 pump-probe image stacks were acquired.

2.3 Pigment type quantification: Geometrical PCA description of pump-probe signals

To quantify pump-probe signals, we first apply principal component analysis (PCA). Signals used in the algorithm were restricted to those consisting of melanin pigment expression by segmenting out regions with surgical ink and hemoglobin via visual inspection and phasor analysis [22] of the pump-probe images. The results, illustrated in Fig. 1, show that 98% of the variance from a total of ~2 million transient responses can be described by only three principal components (PCs): These results are similar to those found in previous work, where 128 handpicked regions were used to determine the PCs [14].

![Fig. 1. Image acquisition process and PCA representation of pump-probe signals. (a) A stack of 34 images is collected, with each image containing a different pump-probe time-delay (~1.5 to 4 ps). Inset shows the dynamics of four pixels in the composite stack. (b) Melanin dynamics measured from ~2 million spatial pixels are used to compute the PCs. Inset shows the variance captured by each PC. (c) The delays are projected onto the three PCs, which constitutes a three-dimensional space that may be described in spherical coordinates. (d) Cumulative histogram of the angles of the spherical coordinate projection of the 2 million spectra. (e) Phasor plot of the same data. (f) Example dynamics of the data—their mapping onto the PC angle and phasor space is demarcated on (d) and (e), respectively.]

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By themselves, the resulting PCs hold little physical meaning, making interpretation of the scores (projection of the PCs onto the measured transient responses) very difficult without reference standards or a model. Previously [14,23,24], we assumed that the pump-probe signals resulted solely from a linear combination of eu- and pheo-melanin (based on similarities to sepia eumelanin and synthetic pheomelanin), which permits quantification of the transient signals using an inverse approach. More recent work, however, has generated a richer picture: pump-probe imaging is sensitive to many more properties of melanin, including type (eu- and pheo- melanin), oxidation, aggregate size, and metal content [20]. While such sensitivity is valuable in capturing detailed molecular information, it presents a significant challenge in modeling the behavior, particularly since different physical properties can exhibit similar dynamics. Phasor analysis has been used to visualize the chemical information without the use of a model, but the bipolar nature of pump-probe signals leads to nonlinearities across phasor space [22], prohibiting quantitative analysis.

As an alternative, we develop a geometrical approach that leverages the fact that the projections of the 3 PCs onto the data span a three-dimensional space. By describing the space in spherical coordinates, the azimuth (θ) and elevation (φ) angles capture all of the chemical information (direction of the vector), and the radius, R, describes the signal strength (or concentration). Construction of a two-dimensional histogram based on the angles yields a plot similar to phasor analysis in that it provides an intuitive visualization of the pigment chemistry, does not require reference standards or a physical model, yet it is linear (with respect to the distribution’s endmembers) and quantitative (Figs. 1(d)-1(e)). The resulting cumulative PC histogram shows that the data lie mostly along the equator of the sphere indicating there are two major endmembers in the melanin distribution (Fig. 1(d)). The first endmember, around θ = 0 (signifying a positive contribution from PC1), is dominated by excited state absorption: this type of transient behavior has been observed for eumelanin void of metals, or with large molecular weight aggregates (>100 kDa) [20]. At the other extreme (~θ = 2.5; i.e., primarily a negative contribution from PC1) the excited state dynamics are dominated by ground state bleaching. This behavior has been observed in eumelanin with high levels of metal content, small aggregate size, and also in synthetic pheomelanin [20]. The elevation angle shows minor deviations, capturing more subtle changes in the pigments’ dynamics. These have not been documented using melanin standards, but have been observed in tissue [14,20], and further justify the use of an unsupervised quantitative approach. (We note that a small contribution to the elevation angle may result from small shifts in the pump-probe time-delay calibration.) For comparison, the corresponding phasor plot is also shown in Fig. 1, along with four points that lie within the distribution to illustrate how the dynamics map with each transformation. While phasor analysis maps these points on a curved path with nonlinear spacing, the geometric PC representation preserves linearity, enabling a straightforward quantitative analysis.

The PC representation of pump-probe signals can also be used to visualize the delay stacks by serving as a basis for false-color rendering. In this case, a hue-saturation-value (HSV) scheme is used, with the hue set by the azimuth angle as illustrated in Fig. 1(d), the value set by the radius, and the saturation set to a constant value of 1. The saturation can be used to encode the elevation angle, but here we use the grayscale for superposing co-registered confocal images, which give additional structural information. Figure 2 shows several examples of the resulting images, along with corresponding bright field photomicrographs of the unstained and H&E stained tissue sections. (Individual pump-probe images are stitched together in MATLAB based on the recorded location of the sample translation stage.)
Fig. 2. Representative pump-probe images and photomicrographs of unstained and H&E stained tissue sections. Primary tumors from patients with positive (a-c) and negative (d,e) SLNB. Scale = 100 µm.
2.4 Pigment structure quantification

To quantify image structure, we use principles of mathematical morphology [25–27], previously introduced as a means of differentiating melanomas from melanocytic nevi [15]. The method applies a two-dimensional autocorrelation (AC) to individual pump-probe images and extracts various parameters from the resulting distributions, including image signal to noise ratio (used as a metric of degree of pigmentation), anisotropy, and entropy. The method, like other techniques used to quantify structure [25–27], requires the input to be in grayscale. For this purpose, we can again leverage the PC description of the pump-probe dynamics to derive various grayscale images that reveal spatial structures from melamins with similar chemical composition. This is achieved by weighting each pixel in the magnitude (R) image by its corresponding angular coordinates in the PC sphere. For example, a grayscale map is computed by weighting pixels based on the azimuth angle: Pixels with $\theta = 0$ are multiplied by 0, while pixels with $\theta = \pi$ are multiplied by 1 (we denote this scale as $\theta^+$). A different grayscale image can be produced by reversing the scale ($\theta^-$). If this were a simple two-chromophore system (e.g., eu- and pheo-melanin), the two resulting images would effectively correspond to concentration maps of the two species. A total of five gray scale images are produced ($\theta^+, \theta^-, \theta^+, \phi^-, \phi^-$, and R), then their autocorrelations are computed (Fig. 3).

In total, 29 parameters are derived which combine structure and pigment chemistry (see Appendix 2, Fig. 8). Specifically, the parameters include the average and standard deviation of $\theta$ (individual distributions for each specimen are shown in Appendix 3, Fig. 9); mean and standard decitation of the three PCs, normalized by the total signal intensity; entropy of the spherical coordinates (R, $\theta$ and $\phi$); the variance accounted for by the top 3 PCs of each individual image; and finally the SNR, anisotropy and entropy of all five gray scale images (computed based on mathematical morphology).

![Fig. 3. Quantification of image structure. (a) Selected pump-probe image for illustration and (b) its corresponding 2D PC histogram, with arrows demarcating the different weightings used to derive the grayscale images shown in (c). (d) Corresponding 2D autocorrelation of each grayscale image used to quantify structure. Scale bar = 100µm.](image_url)
3. Results

3.1 Pigment distribution

Pigment distributions in primary lesions from patients with and without metastatic disease exhibit marked differences (Fig. 4). (The same behavior is observed if grouping is based on SLNB alone, or if recurrences are included in the positive group.) Pigment from the metastatic group shows a bimodal distribution, with peaks at both extremes of the PC histogram. Interestingly, individual samples from this group generally fall into one of the two regions: Three of the samples are shifted towards \( \theta = 0 \) and the remaining towards \( \theta = \pi \) (see Appendix 3). This result suggests the presence of at least two distinct phenotypes that drastically affect pigment chemistry and that are indicative of metastatic disease. Pigment from the non-metastatic group, on the other hand, tends to be grouped closer towards the center of the range, with wider pigment diversity.

![Fig. 4. Pigment distribution. Two-dimensional PC histogram of melanin in primary melanomas from patients with positive (a) and negative (b) SLNB. (c) Histogram of the azimuth angle for each group.](image)

3.2 Pigment structure

Using the Bonferroni correction to assess statistical significance, we find that the most significant parameters are associated with the entropy of the autocorrelation, which describes pigment texture (see Appendix 2). Images with fine, “dusty” melanin texture decorrelate faster than coarser granules, and thus contain an autocorrelation with lower entropy. Primary lesions from patients with metastatic disease, on average, exhibit a finer melanin texture (lower AC entropy) compared to those without metastasis. The parameter with the highest statistical significance is \( \theta \)- AC entropy (Fig. 6(c)), which yields a sensitivity (SE) of 77\% and specificity (SP) of 70\%, and an area under the curve (AUC) in a receiver operation characteristic analysis of 0.8, if grouping is based on SLNB alone. If recurrences are included in the positive group, the same parameter remains the most significant, and yields a SE and SP of 70\% with an AUC of 0.74.

3.3 Multivariate analysis

Next, we consider a multivariate analysis. As a first step, we determine the most relevant parameters using forward sequential feature selection in a wrapper fashion [28–31] with data drawn from individual images (this enables us to capture the most amount of variation across the data). Once features are selected, we test the method’s predictive power using the leave-one-out method with each specimen (rather than each image) treated as independent to properly decouple training and test sets.

The feature selection process begins by randomly selecting 50 of the 174 pump-probe images to set aside for validation (holdout method), and then using the remaining 124 images as input for a feature selection algorithm. To evaluate the performance of each feature, we further stratify the sub-data set using 10-fold cross-validation with training and classification based on support vector machine learning using a Gaussian radial basis function kernel (see Fig. 5). The process identifies six features, shown in Fig. 6, that minimizes the misclassification error (MCE). Adding more features does not improve the model; in fact, the
MCE increases, as illustrated in the inset in Fig. 5(a). This phenomenon is known as the ‘curse of dimensionality,’ and highlights the importance of the feature selection process. The selected parameters that together provide the lowest MCE are a mixture of average pigment chemistry and structural values (Fig. 6). Note that some of the selected parameters are not highly statistically significant on their own, but are important in combination with other parameters to achieve the best classification. Figure 6(g) plots two of the selected parameters to illustrate their complementary information (correlation $r = 0.72$) and Fig. 6(h) more directly shows how the combination helps to separate the two groups. The last step of the feature selection process is to cross validate with the 50 images that were withheld from the selection algorithm. The results yield 16.0% misclassification error, with a sensitivity of 80.0% and specificity of 83.3%. The whole procedure was repeated 100 times to allow for different random instances of the cross-validation, and the results only show a variance in misclassification error of 2.4% (Fig. 5).

Finally, using the selected features, we test the predictive power using the leave-one-out method with all images from a single specimen treated as the ‘test set’ and the remaining for training. In this manner, the test set is completely independent from the training set. After evaluating every lesion, the results yield similar performance values with SE of 78.8%, SP of 79.6%, and misclassification error of 20.7%, with training/testing based on SLNB results; but lower values—SE = 68.4%, SP = 76.7%, and MCE = 27.2%—if the process includes patients’ history of recurrences in the positive group.

3.4 Improving imaging speeds by critically sampling the nonlinear dynamics

Sampling many pump-probe time-delays is paramount for differentiating the various melanin dynamics, but the process is time consuming. With the current system, acquisition of one pump-probe stack (with 34 time-points) takes ~18 minutes. This acquisition time can be...
reduced by choosing a subset of time points that continue to support separation of the molecular dynamics. To achieve this, an error metric must be minimized, which requires knowledge of all the independent molecules or an appropriate model. For this task, we can in fact choose the 3 PCs as a model, since all of the measured dynamics are effectively described by the three vectors. Note that because there are 3 PCs, the system can be critically sampled with only three points. After estimating the PCs, the subsequent quantitative analyses remain unaltered from that described previously.

To select the optimal sub-set of time-delays, we utilize the determinant criterion, which consists of computing the determinant of the variance matrix from the down-sampled PC vectors [32]. The maximum of all permutations yields the optimal combination. Here we down sample to 12, 6, and 3 pump-probe time-delays, which corresponds to speed improvements of a factor of ca. 2.5, 5, and 10, respectively. The results (Fig. 7) reveal that the most critical points are those near time zero, when the pump and probe pulses are overlapped. Figure 7 shows representative down-sampled pump-probe images with their corresponding 2D PC histograms, clearly demonstrating that the selected points produce high-fidelity, quantitative pump-probe images. Figure 7(e) shows the detrimental effects of inadequately choosing one time-point. We also repeated the multivariate statistical analysis: The MCE using the leave-one-out method for all three down-sampled sets (12, 6, and 3 points) is ca. 28.5% if training/testing is based on SLNB only, and 34.5% if recurrences are included.

![Down-sampled pump-probe images](image)

**Fig. 7.** Optimization of the number of measured pump-probe time-points. (a) Original image stack and 2D PC histogram with 34 measurements. Down-sampling to 12 (b), 6 (c) and 3 (d) measurements. The lines on the inset show the selected points using the determinant criterion. Note that there is little change on the images and PC histograms. (e) Illustration of the effects of choosing an unoptimized time-point. Scale bar = 100µm.

### 4. Discussion

The results described here shed new light on the metastatic potential of melanocytic cutaneous melanomas. The process begins by interrogating the ultrafast excited state dynamic properties of endogenous melanin pigments. Quantification of the signals is realized via a mathematical interpretation instead of fitting to a set of reference standards or a physical model—this is paramount given the vast sensitivity of the technique to many different properties of melanin. The results show that all the dynamics can be captured by three PCs. Though there is no direct physical meaning of the PCs, a geometrical representation of the projections using spherical coordinates yields an intuitive format to view and quantify pigment chemistry. With this representation, pump-probe signals with similar features cluster together and trace a linear path from one endmember to another.

The geometrical PC description of the pump-probe dynamics also enables quantification of spatial structures in unison with the chemical information. This allows us to ascertain and parameterize, for example, the spatial structure of melanins with high metal content and small aggregate size. A final added benefit of the PC description is that the orthonormal vectors can be used as a mathematical model to help optimize the data acquisition process. We showed that quantitative images with high-fidelity could be obtained with only 3 carefully chosen
pump-probe time-delay measurements (plus a calibration point), reducing the acquisition time by nearly an order of magnitude.

Analysis of melanin chemistry from primary lesions reveals two distinct populations among patients with metastatic disease. This suggests that two or more phenotypes are affecting melanin chemistry, both of which are also indicative of aggressive behavior. Evidence of the expression of multiple molecular phenotypes has been linked to aggressive melanoma cells in various studies [33–35]. There is also a growing body of literature suggesting the existence of two modes of tumor invasion, each relying on distinct motility mechanisms [36–38]. A connection has been demonstrated between one of these modes and microphthalmia-associated transcription factor (MITF), which might provide some clues as to why such pigment differences are observed within the metastatic group [39]. While genetic information was not procured here, it is clear that future work should identify the mutations responsible for the observed bimodal distribution.

The full potential of the pump-probe method is realized by incorporating image structure. Quantitative analysis of the pigment spatial distribution reveals that the texture of melanins in primary lesions that have metastasized have a finer, “dusty” quality compared to non-metastatic melanomas. Reports that correlate fine, “dusty” melanin texture to malignant melanomas (compared to nevi) are well documented [40–44]; but, to our knowledge, none have investigated the relation to metastatic potential. The fine melanin texture in aggressive tumors may arise from loss of the extra cellular matrix (ECM) structure and other phenotypes that promote perfusion, ultimately allowing the pigment to diffuse into the observed dusty state. In particular Durko et al. [45] showed that type I collagenase (MMP-1), which degrade the ECM, play an important role in the onset of invasive melanoma. Seftor et al. [33] showed that VE-cadherin, which promotes the generation of permeable tubular networks, is more highly expressed in aggressive melanomas. The finer dusty melanin quality could also be a result of a less pigmented phenotype: Pinner et al [46]. showed that disseminating (metastatic) cells have high levels of Brn2 reporters, which repress MITF, and are hence poorly pigmented. Note that melanin structure can be evaluated using conventional bright-field imaging, but it is the combination of melanin chemistry and structure, uniquely available with pump-probe microscopy, that enables identification of metastatic disease with relatively high sensitivity and specificity.

With the developed predictive model we are unable to identify the two patients in the data set that had an initial negative SLNB but later developed recurrences. In these cases, it is possible that scattered microscopic disease, extending beyond the margins of excision, later acquired the ability to metastasize. Thus, the analyzed regions were biopsied prior to expressing any of the biomarkers indicative of metastatic disease. This is a limitation of both pump-probe microscopy and current clinical practice.

5. Conclusion

In conclusion, pump-probe imaging yields novel insight into the metastatic potential of melanocytic cutaneous melanomas, which can provide clinicians with an additional tool for staging without disrupting current clinical practice. Though the sample set in this study is limited, the results show that melanin pigment and structure can help improve the sensitivity and specificity of current clinical markers for identifying metastatic disease. Future work will focus on improving our understanding of the genetic mutations responsible for, or that accompany, the observed behaviors in a prospective manner to test clinical relevance. Additional technological advances will seek to reduce the complexity and cost of the instrumentation by, for example, using a single broadband laser to supply both the probe and pump beams.
| Case no. | SLNB   | Breslow Depth (mm) | Mitotic Rate | Ulceration |
|---------|--------|--------------------|--------------|------------|
| 1       | Negative | 0.90               | 1            | No         |
| 2       | Negative | 0.90               | 3            | No         |
| 3       | Negative | 1.01               | 0            | No         |
| 4       | Negative | 1.05               | 0            | No         |
| 5       | Negative | 1.20               | 0            | No         |
| 6       | Negative | 1.35               | 1            | No         |
| 7       | Negative | 1.40               | 1            | No         |
| 8       | Negative | 1.48               | 2            | No         |
| 9       | Negative | 1.50               | 4            | No         |
| 10      | Negative | 1.63               | 3            | No         |
| 11      | Negative | 1.88               | 1            | No         |
| 12      | Negative | 2.00               | 8            | No         |
| 13      | Negative | 2.10               | 1            | No         |
| 14      | Negative | 6.00               | 5            | No         |
| 15      | Negative (recurred) | 15.00     | 0            | No         |
| 16      | Negative  | 0.35               | 4            | Yes        |
| 17      | Negative (recurred) | 1.39       | 0            | Yes        |
| 18      | Negative  | 1.65               | 2            | Yes        |
| 19      | Negative  | 1.39               | NA           | NA         |
| 20      | Positive  | 0.41               | 0            | No         |
| 21      | Positive  | 0.79               | 0            | No         |
| 22      | Positive  | 1.11               | 2            | No         |
| 23      | Positive  | 9.00               | 2            | No         |
| 24      | Positive  | 0.90               | 1            | Yes        |
| 25      | Positive  | 3.50               | 4            | Yes        |
| 26      | Positive  | 0.90               | NA           | NA         |
| 27      | Positive  | 1.15               | NA           | NA         |
| 28      | Positive  | 3.50               | NA           | NA         |
| 29      | Positive  | 8.00               | NA           | NA         |

NA: information not available
Appendix 2

Fig. 8. Boxplot of all quantitative features. *p-val<0.1, **p-val<0.001, using Bonferroni correction.
Appendix 3

Fig. 9. Azimuth angle histogram of all lesions.

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