**Quantitative characterization and comparative study of feather
melanosome internal morphology using Surface Analysis**

Lorian Cobra Straker^{1, 2}

¹Smithsonian Institution, National Museum of Natural History, Division of Birds,
E-600, MRC 116, 10th & Constitution Ave, NW, Washington, DC 20560, USA.

²Universidade Federal do Rio de Janeiro, Instituto de Biofísica Carlos Chagas
Filho, Laboratório de Ultraestrutura Celular Hertha Meyer, Ilha do Fundão,
Rio de Janeiro, RJ, 21949-900, Brazil.

Email: StrakerL@si.edu

Telephone: +1 (202) 489-2503

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Abstract

A successful feather development implies in a precise orchestration of cells in the follicle, which culminates in one of the most complex epidermal structures in nature. Melanocytes contribute to the final structure by delivering melanosomes to the barb and barbule cells. Disturbance to the tissue during the feather growth can damage the final structure. Here, melanosomes seen in an unusual outgrowth on the barb cortex of a flight feather are reported and compared to commonly observed melanosomes embedded in the cortex. Transmission Electron Microscopy in scanning-transmission mode (STEM) generated images coupled with Secondary Electron detection. The two classes of melanosomes were registered on images combining transmitted and secondary electron signals. Image processing allowed surface analyses of roughness and texture of the internal morphology of these organelles. Results showed that the two classes of melanosomes are significantly distinct internally, indicating that different physiological processes up to feather maturation could have occurred. Surface analysis methods are not regularly used in cell biology studies, but here it is shown that it has great potential for microscopic image analysis, which could add robust information to studies of cell biology events.

1. Introduction

The avian feather is the result of a precise organization of specialized cells and intense cellular signaling in the follicle (Feo and Prum, 2014; Maderson et al., 2009; Alibardi, 2007a), which makes it one of the most complex epidermal appendages within vertebrates (Lucas and Stettenheim, 1972). The follicular tissue is composed of several longitudinal columns of keratinocyte cells identified as part of barb ridges and barbule plates (Feo and Prum, 2014; Maderson et al., 2009; Yu et al., 2002; Watterson, 1942; Strong, 1902). Additionally, in the in-between spaces, supportive cells and wedge cells create a physical support for the developing feather, which guarantees a characteristic final morphological format of barbs and barbules (Alibardi, 2007b).

Similarly to the supportive and wedge cells (Alibardi, 2007b), melanocytes (melanophore *sensu* Watterson, 1942; pigment-cell *sensu* Strong, 1902) are also present between the barb ridges and barbule plates. Prior to keratinization, these specialized pigmented cells will distribute the melanosomes to the keratinocytes starting at the barbule cells' end (Maia et al., 2012; Lucas and Stettenheim, 1972; Watterson, 1942; Strong, 1902). Although it is still unclear the cellular mechanisms or signaling which triggers the internalization of the melanosomes, their distribution and arrangement inside the keratinocyte is believed to be driven by depletion attraction, a result of the self-assembly keratin matrix shown in iridescent feathers during keratinization (Maia et al., 2012).

Bird feather melanosomes are mainly composed of eumelanin or phaeomelanin. Jimbow et al. (1979), studying the morphogenesis of melanosomes, states that its content correlates to the external shape: rod shape (eumelanin) or sphere shape (phaeomelanin). Despite the apparent lack of diversity in bird melanosomes, they do vary in length and in diameter (Li et al., 2014). Likewise, the internal structure of the melanosome greatly influences the visual signal emitted by the feather, for example, hollow melanosomes when present produce the iridescent effect of the plumage (Shawkey et al., 2015; Eliason et al., 2013). Therefore, melanosome morphology (external and internal) and its content are important elements for feather coloration and plumage pattern and diversity (Prum and Williamson, 2002; Durrer and Villiger, 1967). Additionally, melanized feathers are known to be more resistant to wear (Bonser, 1995) or bacterial degradation (Goldstein et al., 2004), and the melanosome morphological diversification is a correlate to the physiological shift that could be related to the origin of pinnate feathers (Li et al., 2014), which suggests that melanosomes may have a deeper role in bird physiology and evolution. Thus, careful observation of melanosome ultrastructure and internal characteristics are important aspects to further understand their physiological role in birds (Jimbow, 1979).

During a comprehensive morphological study of feather microstructures (unpublished data), different microscopy techniques were used to analyze and describe the microstructure characteristics of seabird flight feathers. A colored

flight feather of Leach's Storm petrel (*Oceanodroma leucorhoa* (Vieillot, 1818)) was sampled for general description of barb microstructures, including its melanosomes. The presence of an unusual small outgrowth of the barb cortex and the clustered melanosomes of this sample was not observed in any of the other species in the initial study. In order to further investigate the characteristics of the morphological alterations of the barb cortex, especially the melanosomes, a specific study was made.

The present study reports the morphological differences between more commonly observed melanosomes (hereafter, regular) of this flight feather rami and the cluster of unusual melanosomes (hereafter, modified) present in an outgrowth of the lateral cortex. Moreover, this study presents the use of Surface Analysis (roughness and texture) as a method to characterize and compare classes of organelles in different states by using the two classes of melanosomes as an example.

2. Material and Methods

2.1. Sample preparation and observation

Pennaceous barb samples were cut from the mid-portion of the external vane of the 9th primary flight feather of a Leach's Storm petrel (*Oceanodroma leucorhoa* (Vieillot, 1818)) specimen (MN36771) housed in the ornithological collection at the Museu Nacional, Rio de Janeiro, Brazil.

Samples were prepared for Transmission Electron Microscopy following the protocol of Shawkey et al. (2003) with some modifications related to time. After initial wash, barbs were cut into samples of 2 or 3 pieces and dehydrated in acetone 100%; infiltration was carried out in increasing concentrations of Spurr resin and Absolute Acetone solutions (3:1, 2:1, 1:1, 2:1, 3:1 v/v), with two last steps in Spurr 100%. During dehydration and infiltration, samples were left on a test tube rotatory incubator, and the solutions were changed every 4 days to assure that there was no more air inside the pith cells of the ramus medulla (Fig. 2A, inset). Finally, samples were embedded in resin blocks. Perpendicular ultra-thin sections were cut and collected using 200 mesh copper grids and stained with 5% uranyl acetate for 40 min followed by lead citrate for 5 min. Observation was done in a Phillips EM301 transmission electron microscope in scanning-transmission mode (STEM) at HV = 120 kV. The microscope was equipped with a secondary electron detector above the sample, so final images were combined images of transmitted and secondary electrons signals. Images were analyzed for surface roughness and texture (see below).

Additional samples of barbs of the same feather and further sectioning of the same material above were observed in Scanning Electron Microscopy (SEM) and in Transmission Electron Microscopy (TEM) to verify some of the findings using STEM. TEM (Jeol 1200 EX, at HV = 80 kV) images were used to understand the internal characteristics of regular melanosomes and their overall shape, which were then corroborated by cryofractured barbs (preparation following Adnet et al., 2013) observed in a SEM microscope (Phillips XL 30, at

HV = 12 kV and WD = 8 mm) (Supplementary Material online). The surface features found in the STEM images (see below), were verified on further sections observed in a SEM Zeiss Auriga (FIB-SEM) at HV = 0.5-3.0 kV and WD = 4-8 mm (Supplementary Material online).

Moreover, to understand if the Leach's Storm petrel coloration is primarily produced by eumelanin pigment, UV-VIS spectrophotometry was used for reflectance measurements of skin specimens (housed in the Smithsonian Institution). Measurements were compared to measurements of European Barn swallow's (*Hirundo rustica*) plumage: throat (phaeomelanin) and breast (eumelanin) (McGraw et al., 2004a; McGraw et al., 2004b). The equipment for the reflectance readings were: an Ocean Optics USB-2000 spectrophotometer and a DT-1000 Deuterium Tungsten Halogen Light Source, calibrated with a D65 illumination reference (Milton Roy Co., NY, USA), and the Ocean Optics SpectraSuite (v. 1.0) software. Three measurements from each sample were averaged to create the reflectance spectrum graph within the avian visible spectrum range (300 - 700 nm) (Supplementary Material online).

2.2. Image processing and analysis

Image processing and analyses was done in ImageJ (version 1.48). Profile surface plots were generated to create 3D surface plots, using the Interactive

3D Surface Plot plugin¹. The 3D surface plots showed the distribution of pixel values from the 8-bit images (0-255) in a three dimensional space meshed with the original image, enhancing the sense of topography and structural irregularities. Specific regions of interest (ROI) in the melanosome structure were selected and cropped from the original images for measurement and image analyses (Fig. A.1, see appendices in Supplementary Material online). Surface measurements included roughness and texture parameters, using Roughness Calculation plugin² and GLCM Texture plugin³, respectively. The latter plugin analyzed images by using the Grey Level Co-occurrence Matrix generating texture calculations of the second order (Haralick et al., 1973). Here, four of the generated parameters are used to characterize the cropped ROI image surfaces: Contrast, Entropy, Angular Second Moment and Inverse Difference Moment. These parameters are related to the distribution of the pixel values in the image and the relationship of neighboring pixels, thus, each parameter describes the surface in a different context. High values for Contrast indicated that an image had steep peaks and valleys, due to greater weights given during calculations to the neighboring pixels of greater value differences; higher values for Entropy indicated that the surface had random pixel distribution; higher values for Angular Second Moment (or Orderly) indicated that pixels were distributed in an orderly fashion or that some neighboring pixel combinations were more common in the image, thus, the higher the orderliness the smoother the surface is; and finally, higher values for Inverse Difference

¹ <http://rsb.info.nih.gov/ij/plugins/surface-plot-3d.html>

² <http://rsb.info.nih.gov/ij/plugins/roughness.html>

³ <http://rsb.info.nih.gov/ij/plugins/texture.html>

Moment (or Homogeneity) indicated that pixel values fluctuated less throughout the image, hence, a higher frequency of similar pixels in the co-occurrence matrix.

Two different datasets were created with the selected cropped ROI images. The dataset shown in Table 1 was based on higher magnification images, with ROI images of 392x392 pixel dimensions (Fig. A.1), and proved to be the best for surface characterizations and understanding of the relationships between roughness and the surface texture parameters. The other dataset (Table A.2) was based on a larger number of ROI images (Fig. A.3) but with a reduced size (96x96 pixels). This latter dataset was used to analyze the texture parameters in a quadratic discriminant analysis between regular and modified melanosomes.

Surface measurements were analyzed using R (version 3.1.2). Each surface texture parameter (Contrast, Entropy, Orderly and Homogeneity) was analyzed relative to R_q (root mean square of roughness). A principal component analyses (PCA) ('FactoMineR' package; Lê et al., 2008) was generated to test which surface measurements better explained the variation in regular and modified melanosomes. Further, a quadratic discriminant analysis (qda) ('klaR' package; Weihs et al., 2005) was used to evaluate if the texture parameters were sufficient to distinguish regular and modified melanosomes, the two datasets

were used comparatively. A variable indicating melanosome class (R = regular, A = modified) was assigned to each individual in all analyses.

It is important to point out that even though intermediary melanosomes were observed (Fig. 1C-D and Fig. A.3A), they were not part of the surface analyses. Hence, for comparative purposes, imaging, measurements and analyses were based on the two extreme states - regular and modified (Fig. 2B-C).

3. Results

3.1. Morphological observations

The dark color of the Leach's Storm petrel flight feathers is produced by melanosomes scattered throughout the cortex of the barbs; rarefied in the lateral cortex, but dense in the barbules (Fig. A.5A). SEM cryofracture images (Fig. A.6A-B) and TEM images of longitudinal sectioning (Fig. A.5B) showed that the melanosomes present in the barb cortex were of rod-shape (~200 nm width, ~1000 nm length), correlating with the results of feather black color and melanosome shape (Li et al, 2014). Additionally, spectrophotometry reflectance measurements of flight feathers strongly suggested that its dark color is primarily produced by eumelanin pigmentation, due to the characteristics of its reflectance curve, and its similarity to the known pigment/coloration reflectance spectrum of European Barn swallow's plumage (McGraw et al., 2004a; McGraw et al., 2004b) (Fig. A.6C). Also, TEM images showed that the melanosomes of Leach's Storm petrel were heavily filled with electron dense material (Fig. A5C),

and that the melanosomes were longitudinally orientated to the sagittal plane of the barb, ergo, their round image shape (Fig. 1A-B and D; 2A; A.5A).

Furthermore, an unusual outgrowth to the lateral cortex was visible in the STEM micrographs (Fig. 1A, 2A). The outgrowth had an amorphous shape protruding roughly 3 μm from the cortex surface (Fig. 1A, 2A). Internally, its matrix did not have the same electron density, nor did it had the same melanosome distribution pattern, as the barb cortex (Fig. 1B-D). The melanosomes in the amorphous outgrowth structure were morphologically different from those observed in the cortex. They were clustered, fused and randomly orientated to the cuts. The random layering resolved that most of these melanosomes were also of rod shape (Fig. 1B-D and 2A-B). Additionally, the micrographs showed that regular melanosomes (embedded in the cortex) had a consistent internal structural pattern (Fig. 1B, 1D, 2C and Fig. A.1B, A.3B), which was not observed for the modified melanosomes (Fig. 1B-D and Fig. A.1A, A.3). Moreover, some melanosomes in the outgrowth could be characterized as being in an intermediary state (Fig. 1C-D and Fig. A.3A), indicating that the modified melanosomes might have been similar to the regular organelles at some point during development.

3.2. Surface analysis

The surface plot (Fig. 2E, inset) showed that regular melanosomes had a more uniform distribution of pixel values over their surfaces, suggesting greater surface smoothness. Similar pattern was present in sections observed in SEM (Fig. A.7). In contrast, modified melanosomes showed greater variation in pixel

values, thus greater irregularity of the surface (Fig. 2D, inset). Complementarily, and confirming the surface plots, 3D surface plots (Fig. 2D-E) showed that the regular and modified melanosomes are very different internally. The rendered images revealed that both surfaces had peaks and valleys, but modified melanosomes had more pronounced structures in a much less orderly fashion.

The roughness and surface texture measurements confirmed the imaging analysis above, and revealed finer differences. The roughness measurements (Table 1) indicated that regular melanosomes had a smoother surface (mean $R_q = 62.60$ nm) than the modified melanosomes (mean $R_q = 117.97$ nm). Roughness was positively related to two surface texture parameters, contrast and entropy, but negatively related to the other two, inverse difference moment (homogeneity) and angular second moment (orderly) (Fig. 3A). Also, the PCA analysis (Fig. 3B) showed that its first two dimensions explained 98.58% of the total inertia of the dataset, of which 96.62% is related to the first dimension. It also showed that surface texture parameters generated by the GLCM method were strongly related to the two classes assigned to the melanosomes. The vector and individual factor maps (Fig. 3B) revealed that modified and regular melanosome surfaces were very distinct from each other (Fig. 3B, confidence ellipses), suggesting that the texture of their surfaces are explained by different parameters. The regular melanosome surface was better explained by homogeneity and orderly, and negatively related to R_q . In contrast, the modified melanosome surface data variation was less well explained by orderly and homogeneity and better explained by the R_q values and by the contrast and

entropy surface texture parameters, indicating that a profile of its surface would show greater amplitude and irregularity for its pixel values distribution.

Finally, the discriminant analyses partition plots (qda) (Fig. 4), using the larger dataset of smaller dimensional images (96x96 pixel) (Table A.2 and Fig. A.3), indicated good assignment of classes relative to texture parameters. The relation between orderly and homogeneity had 1.7% of individuals misassigned to their correct classes (Fig. 4), contrasting with homogeneity and contrast plot, which had a rate of 17.2% of individuals misassigned in terms of their true class group (Fig. 4). However, such results must be addressed cautiously, because small dimension images carry less information than larger images, which could create less realistic co-occurrence matrices for surface texture parameters.

The purpose of this analysis was to demonstrate that even images with a reduced amount of GLCM data could still have enough data to distinguish the regular melanosomes from the modified ones. These results showed that smaller images carry textural information similar to larger images, since the partition plots for both datasets discriminated the two classes of melanosomes, thus, confirming the textural distinction between regular and modified melanosomes based on its internal structure. Nonetheless, larger images (392x392 pixels) generated a more robust co-occurrence matrix in GLCM analysis, as shown by the quadratic plots that presented better results by not returning any misassignment on any of the plots (Fig. A.4).

4. Discussion

4.1. Sample limitation

To the best of this author's knowledge, no information on this type of outgrowth on feathers has ever been reported. Furthermore, limitation on sample size was related to the unique finding of this abnormal structure in a pool of 48 feather samples that comprised 23 genera of the Procellariiformes order (comprehensive morphological study cited in Section 1), indicating a rareness of such abnormalities in feather structures, and thus, very unlikely to be spotted and reported.

Moreover, finding the abnormalities here reported was not expected prior to sample preparation, which restricted microscopy techniques. Continuous attempts to re-sample and observe the cortex outgrowth were unsuccessful, indicating it was locally restricted. However, it was possible to verify the surface features of regular melanosomes observed in the STEM micrographs, using SEM imaging of further sections. Images and surface plots (Fig. A.7) corroborated the STEM surface analysis, resolving that the internal structure of regular melanosome was of a uniform and smoother surface as indicated by the lower R_q roughness values. Therefore, regular melanosomes should have a highly ordered internal structure.

Most importantly, sample limitations prompted for unconventional ways of image analysis and extraction of structural information embedded in the image with surface analysis methods.

4.2. Biological significance of the outgrowth

The cortex outgrowth, shown here, should be discussed in light of the developing feather. As such, any abnormality observed on the mature feather is a result of some modification during the developmental stages inside the follicle (Allibardi, 2007b). Ergo, melanocytes must produce and deliver the melanosomes to the feather cells before keratinization (Maia et al., 2012; Lucas and Stettenheim, 1972; Durrer and Villiger, 1967; Strong, 1902).

The unusual finding of this type of outgrowth on the feather barb can incite several different biological interpretations of precisely what could have caused it. However, the great dissimilarity between the two classes of melanosomes allows some biological observations regarding it. The amorphous morphology and the different internal electron density could be indications of a tumor-like or inflammatory-like cellular growth (Fig. 1 and 2A). This reasonable interpretation, although speculative, is based on the fact that the feather tissue, during development, is vulnerable to infections as any other living tissue. Because of its restricted nature, it is possible that melanocytes and melanosomes may have had an important role in a prompt immune-response during this feather's development.

Moreover, melanocytes are known to participate in inflammatory or microbial infection immune-responses in several organisms (Mackintosh, 2001), including reptiles (Johnson et al., 1999), which are closely related to birds. Additionally, the melanosome has been shown to be is a specialized cellular structure from the lysosomal lineage (Orlow, 1995), and melanocyte phagosomes carrying antigens do fuse with melanosomes, suggesting a lysosome-like function (Le Poole et al., 1993). Furthermore, follicle melanocytes differ from dermis melanocytes partially due to the differences of their melanosomes (Lucas and Stettenheim, 1972), indicating a possible explanation to why melanized feathers are more resistant to microbial degradation than non-melanized feathers (Goldstein et al., 2004).

4.3. Significance of melanosome's internal structure

In respect to the morphological characteristics of the melanosomes observed embedded in the outgrowth and in the cortex, it is possible to discuss some biological significances of their differences. Transmission electron micrographs of the mature feather parts show melanosomes as opaque and electron-dense structures embedded in the keratin matrix of barb or barbule cells (Fig. A.5A-B). At high magnifications, it is possible to see that the interior is not a homogenous or fully compact structure (Fig. A5C). Nevertheless, the electron-density of the melanin does not allow further characterizations of the mature melanosome. Durrer and Villiger (1967), on describing the production of melanosomes, show

that the pre-melanosome contains many small round vacuoles. During melanogenesis, melanin will be deposited and will accumulate in and on these small round vacuoles (Durrer and Villiger, 1967: fig. 5). Accordingly, the internal structural organization of the melanosome is formed during melanogenesis. Jimbow (1979), further characterizes in great detail the differentiation process of eu- and phaeomelanin melanosomes. He shows that both types have similar initial development, corroborating Durrer and Villiger (1967), but differentiation occurs due to internal organization of the vesiculo-globular bodies (small round vacuoles *sensu* Durrer and Villiger, 1967). Phaeomelanin melanosomes have a un-orderly internal organization of its vesiculo-globular bodies, which does not produce support for elongation of the melanosome, and consequently it matures in a spherical shape. Eumelanin melanosomes, on the other hand, have vesiculo-globular bodies highly ordered in rows, due to their attachment to filamentous proteins, which support and promote elongation of the melanosome into a rod shape structure (Jimbow, 1979: fig. 4).

Additionally, the characterization of the internal structural organization of the two classes of melanosomes was enhanced due to the topographic perspective added by the secondary electron detector in the STEM microscope, embedding surface parameters into each pixel value of the image, which was then extracted and analyzed. The 3D surface plots and the surface analyses for roughness and surface texture showed that the interior of a regular melanosome is orderly and homogeneous correlated with the lower roughness measurements (Fig. 3A, red), as expected of an eumelanosome, following

Jimbow's (1979) description. On the other hand, the irregular and heterogeneous internal structure of the modified melanosome (Fig. 2B, 2D) translated into increasing values of contrast and entropy (Table 1), correlating with higher roughness values (R_q) (Fig. 3A, blue); a result not expected of an eumelanosome.

Overall, there were stronger evidences showing that the regular and modified melanosomes studied are two states of the same melanosome type, than there were to determine which type of melanosome they are (eu- or phaeomelanin). On the one hand, the similar shape (rod) and the presence of intermediary melanosomes in the same sample (Fig. 1C-D and Fig. A.3A) supports the hypothesis that they are of the same type, and thus, the higher roughness and textural values in the modified melanosomes of the outgrowth would have been a disruption of its internal structural organization during maturation. On the other hand, the elongated rod shape of the two classes studied (regular melanosomes measuring ~ 1000 nm, Fig. A.6B) and the reflectance spectrophotometry data (Fig. A.6C) do suggest that the melanosomes of Leach's Storm petrel are eumelanosomes (following Jimbow, 1979). However, this should be view with caution, for shape correlates with color (Li et al., 2014), but does not confirm molecular content (McGraw et al. 2004b). Therefore, only a chemical analysis would give a definite evidence of the nature of these two classes and, thus, resolve the question if the Leach's Storm petrel melanosomes imbedded in the cortex are indeed eumelanossomes.

4.4. Broader impact

The rich histological and cellular environment of the developing feather has been used as a model tissue in several developmental studies (e.g. Widelitz et al., 1999), but morphological aspects of the cortex or melanosomes of a mature feather have never been used as testimonial pieces to understand potential events during development. This, and the growing interest in using feathers to report past events in the biological timeline of birds (toxicology and isotope studies) (e.g. Thompson et al., 1998), show how important feathers are to different studies on avian biology. Moreover, these observations and basic quantification analyses of modified and regular melanosomes show that further research is needed to better understand the roles of melanocytes and melanosomes in bird physiology, including feather development and feather degradation.

Finally, surface analysis methods are not regularly used in cell biology studies (e.g. Murata et al., 2001), but in this study it was essential to morphologically characterize the two classes of melanosomes, showing the great potential it has for microscopic image analysis. It adds robust information and could have positive impact as a quantitative resource to cell biology studies of other organelles.

5. Conclusions

This study reported on the internal structural morphology of feather melanosomes using quantitatively methods. The presence of an amorphous outgrowth on a feather barb made characterization and comparison between regular and modified melanosomes possible. Although distinction of the two classes was clearly seen in the micrograph images, image processing and surface analysis were essential to characterize and quantify their morphological differences. Quantification of roughness and texture parameters provided sufficient information and support to state that the two classes of melanosomes were significantly different, re-enforcing the hypothesis that the presence of modified melanosomes in a mature feather is an indication of a cellular immune-response during development.

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8 Captions

8.1 Figures

Table 1: Roughness and Surface Texture measurements from the cropped ROI images (392x392 pixel dimension) of Fig. 2B and 2C (for ROI references see Fig. A.1 on supplemental material online). Rq = root mean squared values for 2D roughness parameter; Orderly = Angular Second Moment; Homogeneity = Inverse Difference Moment; A = modified melanosomes; R = regular melanosomes. (392x392 pixel dimension).

Figure 1: Micrographs of a flight feather barb with two distinct melanosome morphologies. **A:** General view of the lateral cortex (cx) with an amorphous outgrowth presenting clustered melanosomes, ROIs (squares) highlight following amplifications, medulla (m) (bar = 2 μ m); Inset: Cross-section image (SEM) of a flight feather barb: arrow heads indicate the lateral cortex, between which is the medulla (bar = 100 μ m). **B, C** and **D:** Amplifications of the ROIs in 1A (1, 2 and 3, respectively) showing the lateral cortex (cx) with scattered regular melanosomes (arrows) and parts of the amorphous outgrowth with clustered modified melanosomes (asterisk); intermediary melanosomes are observed (ellipses) in C and D, revealing a gradual

disruption of the internal structural organization from regular melanosomes to modified melanosomes; (bar = 500 nm).

Figure 2: Micrographs and surface plots of a flight feather barb with two distinct melanosome internal morphologies. **A:** General view of the lateral cortex with regular (thin arrow) melanosomes in the cortex (cx) and modified (thick arrow) melanosomes clustered in an amorphous outgrowth (asterisks), m = medulla (bar = 2 μ m). **B** and **C:** High magnification of the two classes of melanosomes highlighted in 2A (square): modified melanosomes (B) from the outgrowth and regular melanosomes (C) from the cortex show distinct internal structural organization (bar = 100 nm). **D** and **E:** 3D Surface plots showing the internal structure in a three dimensional space relative to pixel value distribution in 1B and 1C image areas (667.4x779.1 nm and 666.8x778.8 nm, respectively), insets show the surface plots used to generate the 3D plots.

Figure 3: Correlation and PCA analyses of roughness and surface texture measurements. **A:** Scatterplots of surface texture parameters against surface roughness (Rq) show positive correlation between Contrast and Entropy with Rq, and negative correlation between Orderly and Homogeneity with Rq; red = regular melanosomes; blue = modified melanosomes. **B:** The PCA of all surface parameters shows that variation is mainly explained by the 1st Dimension; regular melanosome surface

characteristics (R, red dots) are especially explained by Orderly and Homogeneity on their pixel spatial distribution, and modified melanosomes characteristics (A, black dots) are more influenced by Entropy, Contrast and Rq parameters (see Individual factor map); confidence ellipses show spatial confidence of the variables in relation to the assignment of the two classes A (modified) and R (regular).

Figure 4: Quadratic Discriminant Analysis (qda) of the larger dataset based on the images of smaller dimensions (96x96 pixels). The six plots, show that the four surface texture parameters (Contrast, Orderly, Homogeneity and Entropy) are sufficient to confidently distinguish the two types of melanosomes (R = regular; A = modified); best results shown in the Orderly-Homogeneity plot (0.017 error rate), followed by Contrast or Homogeneity in relation to Entropy (0.034 error rate) and Contrast-Orderly plot (0.052 error rate); worst results to Orderly-Entropy and Contrast-Homogeneity plots (0.121 and 0.172 error rates, respectively).

8.2 Appendices

Figure A.1: Micrographs of the two distinct classes of melanosomes observed in the barb (bar = 500 nm). **A:** Modified melanosomes (mod) of the outgrowth (same micrograph as in Fig. 1B), with the indications of 10 regions of interest (ROIs), of which three ROIs are highlighted as an example (squares): 3, 4 and 7; these ROIs were cropped and amplified (3m,

4m and 7m, respectively). **B**: Regular melanosomes (reg) of the cortex (same micrograph as in Fig. 1C), with indication of 3 ROIs, which were cropped and amplified (1r, 2r and 3r, respectively). Measurements of surface parameters were based on the ROI images: Roughness parameter: Rq = root mean square of roughness; Texture parameters: C = contrast; E = entropy; O = orderly (Angular Second Moment); H = homogeneity (Inverse Difference Moment).

Table A.2: Table of Roughness and Surface Texture measurements extracted from ROI images of 96x96 pixel dimensions used in the quadratic discriminant analysis (Fig. 4). Rq: root mean squared values for 2D roughness parameter; Orderly: Angular Second Moment; Homogeneity: Inverse Difference Moment; A = modified melanosomes; R = regular melanosomes.

Figure A.3: Micrographs of the two regions of the barb indicating several regions of interest (ROIs) for surface analyses; (bar = 500 nm). A: Region of the barb outgrowth where modified melanosomes (mod) and intermediary melanosomes (int) are closely clustered; cropped images 13m, 27m, 24i and 28i exemplify the ROIs (Table A.2) that generated surface plots for the qda analysis (Fig. 4), and show the distinctions between modified (13m' and 27m') and intermediary (24i' and 28i') melanosomes. B: Different region of the barb where modified (mod) and regular (reg) melanosomes are located;

cropped images 2r, 3r, 5r and 11m exemplify the ROIs (Table A.2) that generated surface plots for the qda analysis (Fig. 4), showing differences between modified (11m') and regular (2r', 3r' and 5r') melanosomes. Regular and intermediary melanosome surfaces have similarities relative to modified melanosomes, but are still different between one another (compare ROIs and Surface plots).

Figure A.4: Quadratic Discriminant Analysis (qda) of the smaller dataset (Table 1) based on the images of larger dimensions (392x392 pixels). The six plots, show that the four surface texture parameters (Contrast, Orderly - Angular Second Moment, Homogeneity - Inverse Difference Moment and Entropy) are sufficient to confidently distinguish the two types of melanosomes (R = regular; A = modified); different to the larger dataset, here, the analysis correctly discriminated all individuals in all combinations assigning them to their correct class group, most likely due to the more informative matrices generated from the larger images.

Figure A.5: Melanosome morphological characteristics from cross-section and longitudinal sectioning of flight feather barbs. **A:** Distribution of melanosomes (arrow) rarefied in the ramus cortex (cx) and densely concentrated in the barbules (asterisk), ramus medulla (m) (bar = 10 μ m). **B:** Longitudinal section of the ramus showing that the melanosomes (arrow) are elongated and longitudinally orientated on the sagittal plane of the barb

(bar = 5 μm). C: High magnification of the melanosome reveals high concentration of electron dense material, but also that it has some internal structuring (bar = 200 nm).

Figure A.6: Melanosome morphology and UV-VIS spectrophotometry suggest feather coloration is based on eumelanin pigmentation. **A:** General view of barb ramus morphology from cryofracture SEM and ROI (square) amplified in B: dorsal crest (d), distal barbules (dbrb), proximal barbules (pbrb), medulla (m), ventral crest (head arrow), ramus lateral cortex (arrow) (bar = 100 μm). **B:** Detailed view of the lateral cortex of the ramus after cryofracture revealing sectioned melanosomes imbedded in the cortex matrix (arrow) with ~200 nm width, and intact melanosomes (arrow head) of rod shape and ~1000 nm length, medulla (m) (bar = 1 μm). **C:** UV-VIS reflectance curves of Leach's Storm petrel's (*Oceanodroma leucorhoa*) wing feathers (black) and European Barn swallow's (*Hirundo rustica*) plumage: throat (dark gray) and breast (light gray); the swallow's chestnut throat is primarily phaeomelanin pigmentation, and its black breast is primarily eumelanin pigmentation (McGraw et al., 2004a); spectra curve characteristics suggest that the petrel's flight feather color is based on eumelanin pigment, as it maintains a constant reflectance along the spectrum, while phaeomelanin spectrum curve rises constantly in the visible part of the spectrum (400-700 nm).

Figure A.7: SEM micrographs of ultrathin sections to verify surface features on regular melanosome found on the lateral cortex of the barb ramus. **A:** General view of the ultrathin section, dorsal crest (d), distal barbules (dbrb), proximal barbules (pbrb), medulla (m), lateral cortex (arrow head), ROI (rectangle) for melanosome analysis (bar = 20 μm). **B:** Regular melanosomes (arrow) embedded in the lateral cortex (cx) have a smooth internal structural organization (bar = 1 μm). **C:** High magnification of two regular melanosomes analyzed (ROI: square), as examples, to verify surface features; surface texture analysis revealed mean values similar to the STEM samples: Orderly = 4.3775e^{-4} , Contrast = 218.629, Homogeneity = 0.080, Entropy = 7.989 (bar = 200 nm). **D:** 3D Surface plot of melanosomes in C, pixel values distributed in a three dimensional space reveals a smooth and ordered internal structure.

Table 1

Roughness and Surface Texture Measurements						
Selection	Type	Rq	Orderly	Contrast	Homogeneity	Entropy
17_10	A	96.708	1.044E-04	860.371	0.043	9.457
17_9	A	125.096	7.367E-05	1409.617	0.035	9.825
17_8	A	119.199	7.978E-05	1406.906	0.034	9.737
17_7	A	122.68	7.566E-05	1588.142	0.033	9.774
17_6	A	140.336	7.007E-05	1710.097	0.03	9.862
17_5	A	117.781	9.755E-05	1219.488	0.036	9.532
17_4	A	102.495	9.936E-05	1282.403	0.035	9.524
17_3	A	118.868	8.237E-05	1507.024	0.033	9.696
17_2	A	123.414	1.112E-04	1027.602	0.039	9.413
17_1	A	113.112	9.521E-05	1398.921	0.034	9.566
18_1	R	63.517	3.91E-04	348.628	0.066	8.178
18_2	R	66.174	3.614E-04	390.964	0.063	8.251
18_3	R	58.105	4.733E-04	277.322	0.075	7.98

Figure 1

Figure 2

Figure 3

Figure 4

Partition Plot

Table A.2

Roughness and Surface Texture Measurements						
Selection	Type	Rq	Orderly	Contrast	Homogeneity	Entropy
B16-6	A	79.055	2.376E-04	918.161	0.043	8.565
B16-7	A	76.142	2.289E-04	750.283	0.046	8.6
B16-8	A	69.671	2.413E-04	659.974	0.054	8.554
B16-9	A	68.493	2.553E-04	840.672	0.048	8.503
B16-10	A	74.624	2.174E-04	919.404	0.045	8.653
B16-11	A	74.268	2.22E-04	812.691	0.047	8.638
B16-12	A	72.283	2.43E-04	697.766	0.052	8.552
B16-13	A	77.306	2.961E-04	573.51	0.058	8.346
B16-14	A	87.317	2.011E-04	1006.234	0.043	8.723
B16-15	A	102.928	1.754E-04	1052.794	0.044	8.86
B16-16	A	84.069	1.895E-04	906.933	0.043	8.777
B16-17	A	92.001	2.015E-04	935.897	0.045	8.722
B16-18	A	95.679	1.668E-04	984.42	0.046	8.899
B16-19	A	67.256	2.12E-04	670.242	0.053	8.681
B16-20	A	68.918	3.164E-04	567.545	0.055	8.295
B16-21	A	82.865	2.284E-04	710.92	0.046	8.611
B16-22	A	74.819	3.062E-04	590.008	0.056	8.331
B16-23	A	70.107	2.21E-04	595.12	0.054	8.62
B16-24	A	73.338	2.883E-04	495.033	0.059	8.388
B16-25	A	67.827	3.41E-04	518.154	0.06	8.23
B16-26	A	82.61	3.267E-04	578.408	0.059	8.272
B07-1	A	78.513	2.332E-04	512.719	0.053	8.598
B07-2	A	56.826	4.403E-04	377.508	0.065	7.987
B07-3	A	59.212	3.96E-04	434.739	0.059	8.092
B07-4	A	55.71	3.388E-04	486.906	0.055	8.24
B07-5	A	65.357	2.26E-04	549.724	0.052	8.614
B07-6	A	56.732	3.024E-04	569.302	0.051	8.344
B07-7	A	60.162	2.664E-04	611.815	0.051	8.467
B07-8	A	56.364	3.009E-04	578.793	0.052	8.345
B07-9	A	59.778	3.156E-04	530.982	0.054	8.307
B07-10	A	59.601	2.692E-04	453.107	0.059	8.44
B07-11	A	64.909	2.652E-04	606.275	0.051	8.468
B07-12	A	67.163	2.658E-04	567.644	0.051	8.48
B07-13	A	62.046	2.285E-04	541.974	0.051	8.605
B07-14	A	58.846	2.771E-04	475.888	0.055	8.421
B07-15	A	71.231	2.863E-04	630.267	0.053	8.408

Table A.2

B07-16	A	57.732	3.153E-04	565.224	0.053	8.302
B07-17	A	71.269	2.306E-04	580.897	0.049	8.587
B07-18	A	52.865	3.065E-04	575.946	0.054	8.328
B07-19	A	60.537	2.776E-04	529.504	0.053	8.423
B07-20	A	67.694	2.454E-04	516.177	0.057	8.546
B07-21	A	58.034	3.808E-04	430.28	0.056	8.121
B07-22	A	73.938	2.489E-04	596.977	0.053	8.523
B07-25	A	65.363	3.607E-04	499.554	0.058	8.191
B07-26	A	59.407	3.926E-04	394.867	0.064	8.108
B07-27	A	59.16	3.271E-04	409.901	0.059	8.272
B16-5	R	66.53	3.225E-04	639.077	0.049	8.278
B16-4	R	66.972	3.417E-04	646.221	0.05	8.228
B16-3	R	64.412	4.225E-04	497.015	0.058	8.034
B16-2	R	66.163	3.882E-04	520.929	0.055	8.1
B16-1	R	61.318	4.156E-04	440.187	0.06	8.037
B15-3	R	98.434	9.411E-04	164.997	0.095	7.254
B15-2	R	98.982	1E-03	114.882	0.116	6.883
B15-3	R	101.408	1E-03	102.219	0.119	6.874
B11-3	R	54.046	6.282E-04	285.032	0.072	7.644
B11-2	R	46.901	8.083E-04	205.441	0.082	7.386
B11-1	R	52.269	5.472E-04	323.04	0.068	7.777
B9-4	R	55.272	5.43E-04	393.233	0.064	7.777
B9-3	R	60.004	3.944E-04	576.667	0.052	8.099
B9-2	R	61.645	3.289E-04	696.463	0.046	8.262
B9-1	R	67.781	4.271E-04	507.015	0.057	8.02

A

3m

Rq=118.868
C=1507.024
E=9.696
O=8.237E-05
H=0.033

4m

Rq=102.495
C=1282.403
E=9.524
O=9.936E-05
H=0.035

7m

Rq=122.68
C=1588.142
E=9.774
O=7.566E-05
H=0.033

B

1r

Rq=63.517
C=348.628
E=8.178
O=3.91E-04
H=0.066

2r

Rq=66.174
C=390.964
E=8.251
O=3.614E-04
H=0.063

3r

Rq=58.105
C=277.322
E=7.98
O=4.733E-04
H=0.075

Partition Plot