IFSCC 2025 full paper (IFSCC2025-764)

# An Image-Based Method for Assessing Depigmenting Agents in Reconstructed Human Pigmented Epidermis Models

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## 1. Introduction

Melanin, the principal determinant of skin color in humans, is produced by melanocytes and transferred to neighboring keratinocytes [1], and it elicits a strong photoprotective mechanism against ultraviolet (UV) radiation. In this regard, alterations in melanin production and distribution may lead to various pigmentation disorders including melasma, post-inflammatory hyperpigmentation, pityriasis alba and solar lentigines, common cosmetic concerns [2].

The demand for effective depigmenting agents is increasing globally. Therefore, the need for accurate, reproducible, and non-invasive methods to quantify melanin production is essential for cosmetic research. Today, *in vitro* evaluation of melanin content is predominantly performed by chemical extraction of melanin followed by spectrophotometric quantification. While this technique is well-established, it presents strong limitations, including high variability, potential underestimation of changes in melanin content due to an intrinsic low sensitivity, as well as irreversible destruction of samples.

To address these drawbacks, in the present work we propose an innovative image-based quantification technique by machine-learning to measure melanin quantity in Reconstructed Human Pigmented Epidermis (RHPE) models. By applying this method to stereomicroscopic and histological images and using kojic acid, a well-known tyrosinase inhibitor [3], as a depigmenting control, we sought to establish a reliable, non-destructive and sensitive alternative to classical melanin extraction.

## 2. Materials and Methods

### RHPE Generation

Cell culture inserts with polycarbonate membranes (Thermo Fisher Scientific, Pittsburg, USA) were used for RHPE generation. In brief, the inserts were placed in 24-well plates containing 1.5 mL of coculture medium combining 95 % of EpiLife medium (Thermo Fisher Scientific, Pittsburgh, PA, USA) supplemented with human keratinocyte growth factor (HKGS; Thermo Fisher Scientific, Pittsburgh, PA, USA ) and 5 % of Melanocyte Growth Medium 2 (PromoCell, Heidelberg, Germany). Subsequently, 500.000 cells/cm2 in ratio 1:20 of Human epidermal Melanocytes (HEMn-DP, Thermo Fisher Scientific, Pittsburg, USA) to Human Epidermal Keratinocytes (HEKn; PromoCell, Heidelberg, Germany), respectively, were seeded onto the insert with 500 µL of the above-mentioned coculture medium. After 72 h, the models were raised to air-liquid interface (ALI). The medium was replaced by EpiLife medium supplemented with 1.5 mM calcium, 10 ng/ml KGF, 50 µg/ml of vitamin C and 50 µg/ml of gentamicin. The medium was renewed every other day. On day 9, the medium was replaced by coculture medium with test compounds and renewed every day. On day 17, melanin was extracted and quantified as described below. Pictures were also taken at day 17 with the stereomicroscope Leica EZ4 W (Leica Microsystems, Wetzlar, Germany) and quantified as described below.

### Melanin Extraction and Measurement

Samples were submerged in 400 µL Solvable solution (Perkin Elmer; Milan, Italy), incubated for 1 h at 80 ºC and vortexed until dissolution of RHPE. Separately, a fresh stock solution of synthetic melanin (Sigma; St. Louis, MO, USA) at 1 mg/ml in Solvable solution was prepared as standard for the interpolation of melanin concentration. Melanin was then read by absorption spectrophotometry at 490 nm in 96-well plates on a SpectraMax M2 (Molecular Devices; Sunnyvale, CA, USA) plate reader.

### Histological Staining

Samples were fixed for 24 h with 4% paraformaldehyde and then dehydrated with progressively increasing concentration of ethanol, followed by two baths of xylene before paraffin embedding. Later, five µm-thick cross sections of paraffin-embedded specimens were placed onto glass slides (VWR, Mississauga, ON, USA). Samples were deparaffinized and rehydrated with three washes of xylene and decreasing ethanol concentration and water. Subsequently, hematoxylin-eosin staining was performed. Samples were stained with Harris´s hematoxylin (VWR, Mississauga, ON, USA) and washed with tap water. Later, specimens were differentiated with 1% acid ethanol and rinsed with tap water. Then, samples were stained with eosin Y (Sigma; St. Louis, MO, USA). Next, slides were dehydrated with increasing ethanol series and 3 washes of xylene and mounted with DPX (Sigma; St. Louis, MO, USA). Fontana Masson

staining was performed using a commercially available kit (#ab150669; Abcam, Cambridge, UK), following the manufacturer´s instructions. Images were taken with a DMI1 microscope (Leica, Wetzlar, Germany) combined with Flexacam C3 camara (Leica, Wetzlar, Germany).

### Melanin Image Quantification

Images obtained with the stereomicroscope, as well as images of the Fontana-Masson staining, were analyzed using the machine learning tool for bioimage analysis Ilastik [4]. Segmented binary masks were then used to calculate the percentage of melanin-positive area relative to total tissue area.

### Statistical Analysis

Data was analyzed using GraphPad version 10.4.2 (Graphpad Software, La Jolla, CA, USA). Quantitative comparisons between treatment groups and methods were assessed using paired t test. The alpha nominal level is set at 0.05 in all cases.

## 3. Results

### Classical Melanin Extraction

As expected, melanin extraction (**Figure 1**) showed that kojic acid significantly reduces melanin content by 29.1 % when compared to untreated RHPE controls (70.9 ±1.4 % of Control; ****p<0.0001). This result aligns with known literature on kojic acid's inhibitory effects on melanogenesis and validates its use as a positive control [3]. Importantly, this assay provided a baseline quantitative measurement of melanin concentration in the RHPE model, serving as a reference point for comparison with the image-based approaches described below.

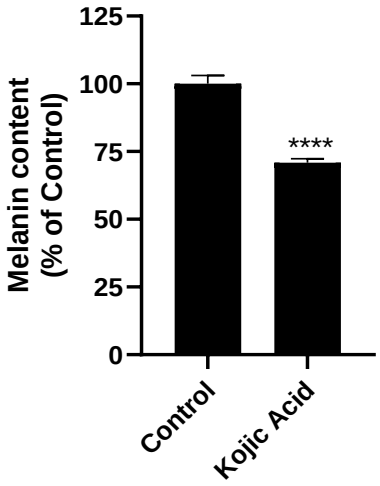



**Figure 1.** Melanin measurement on RHPE by melanin extraction. Paired t test, ****P<0.0001 vs control (n=6).

## Stereomicroscopic Image Quantification

Surface images of RHPE captured at day 17 showed visible differences in pigmentation between control and kojic-treated tissues (**Figure 2**). The control samples exhibited a brown coloration, while the kojic-treated tissues appeared lighter. Binary segmentation using Ilastik highlighted a substantial quantitative decrease in pigmentation in the kojic acid-treated group with a 40 % reduction in melanin-positive area (59.6 ± 5.3 % of control; ****p<0.0001).

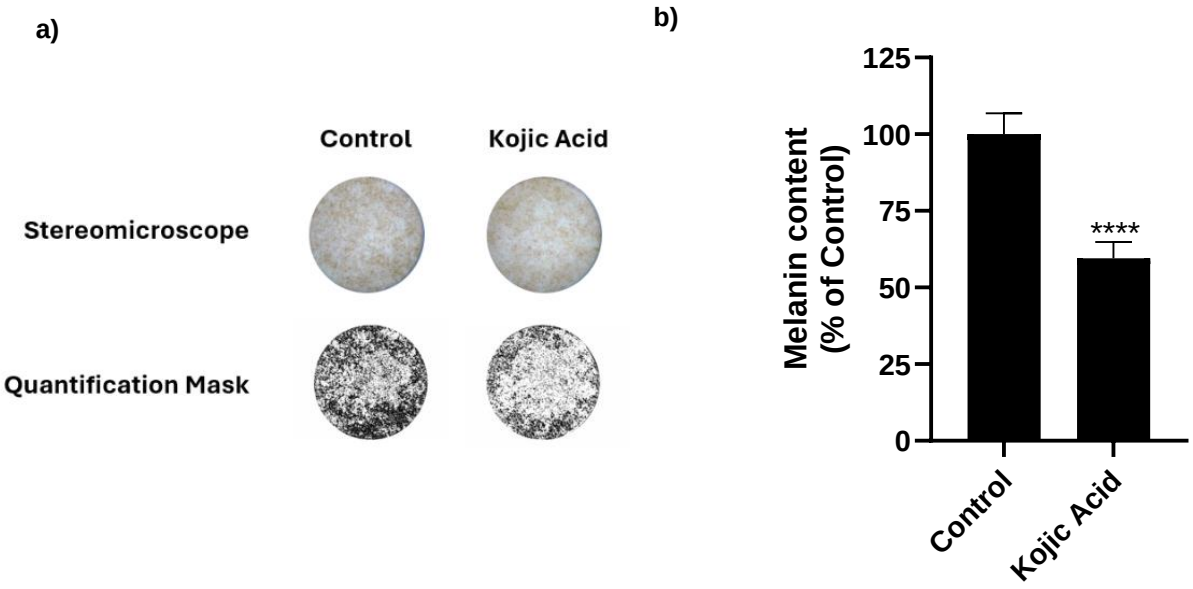
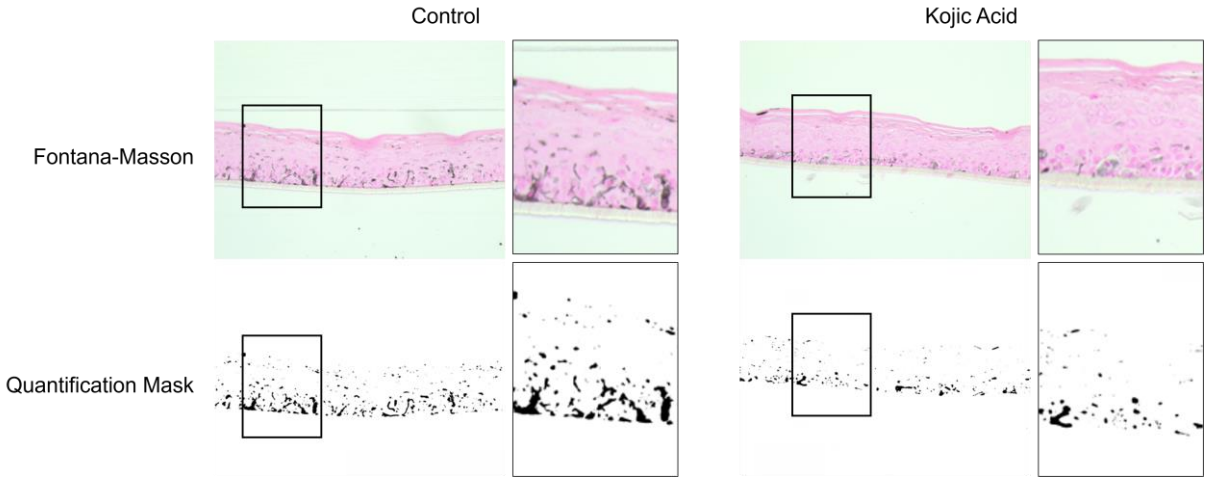



**Figure 2.** Melanin measurement on RHPE by image quantification of stereomicroscopic images. **a)** Images from RHPE were captured with a stereomicroscope and quantification masks were produced by pixel classification with the machine learning tool Ilastik. **b)** Quantification of positive-melanin areas was analyzed for control and kojic acid (250 µM) stimulated RHPEs. Paired t test, ****P<0.0001 vs Control (n=6).

## Fontana-Masson Histology Quantification

In order to inspect melanin content and distribution microscopically within the epidermis, Fontana-Masson staining was performed as described under material and methods on the RHPE models (**Figure 3**). Further analysis by binary segmentation of Fontana-Masson stained images revealed a 34 % reduction in melanin content following kojic acid treatment (66.3 ± 11.1 % of Control; *** p<0.001). This data is consistent with the findings shown above for kojic acid impact on melanin content when measured by chemical extraction of melanin or by image analysis of stereomicroscopic images.

**a)**




**b)**

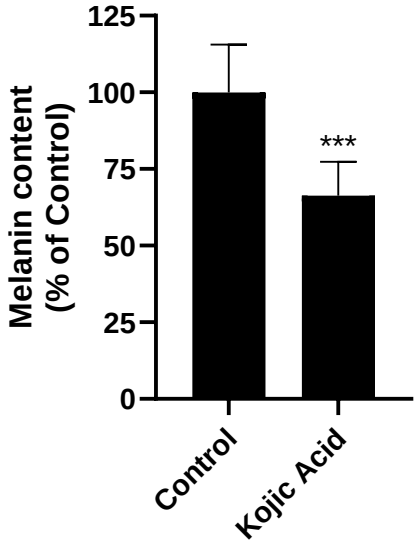



**Figure 3.** Melanin measurement on RHPE by image quantification of Fontana-Masson histological images. **a)** Fontana-Masson Images and quantification masks produced by pixel classification with the machine learning tool Ilastik. Boxes show magnification of the selected areas. **b)** Quantification of positive-melanin areas was analyzed for control and kojic acid (250 µM) stimulated RHPEs. Paired t test, ***P<0.001 vs Control (n=6).

## 4. Discussion

With the aim of validating image analysis as a reliable method for measuring melanin content, stereomicroscopic images and images of Fontana-Masson histologies were captured from RHPE models and then analyzed using pixel classification with the machine learning tool Ilastik

[4]. From the same samples, the classical melanin extraction method was also used to measure melanin, allowing therefore direct comparison among the three methods.

The 40% reduction by kojic acid observed via stereomicroscopy surpassed the 29% measured by chemical extraction, suggesting that image analysis is more sensible than chemical bulk melanin extraction. Similarly, histological analysis revealed a 34% decrease in melanin upon kojic acid stimulation, again showing more sensitivity than total melanin extraction. Together, these results suggest that image-based methods may be of great interest to detect false negative whitening agents that classical melanin extraction would fail to capture.

Besides improving melanin detection sensitivity, quantification of melanin using image-based methods has the major advantage of preserving the integrity of the sample, allowing therefore the use of the sample for additional assays, such as immunohistochemistry or gene expression profiling, thereby maximizing data output from limited tissue availability. Moreover, automated pixel segmentation minimizes human bias, increasing thus reproducibility and allowing scalability for high-throughput screening. Similarly, the use of open-source platforms like Ilastik [4] ensures accessibility and transparency. A further advantage of analyzing pigmentation in histologically reconstructed skin samples is that it allows to observe in situ the distribution of melanin along the different cell layers. This observation can help to better understand the mechanism of action of a cosmetic ingredient and its cellular impact at the level of melanin synthesis by the melanocyte, its transfer to the keratinocyte and its distribution to the outermost layers. Taking together, the findings of the present work present exciting opportunities for the study of the efficacy of cosmetic ingredients that modulate the content of melanin.

## 5. Conclusion

The present study serves to validate a novel image-based method for melanin quantification in RHPE models. The analysis of both stereomicroscopy and Fontana-Masson images, with machine-learning tools showed strong agreement with the classical melanin extraction method, with the added benefits of enhanced sensitivity and sample preservation, strongly suggesting that the adoption of these techniques may help to improve in the efficacy tests for whitening cosmetic actives

## 6. References

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