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Standardization of *In vitro* Macrophotography for Assessment of Cutaneous Responses

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Abstract

The increased popularity of commercially available 3-dimensional human skin equivalents in recent years has allowed for assessment of melanogenesis modulated by compounds topically applied to the skin or directly incorporated from the medium. These skin equivalents provide a suitable model for elucidating the mechanisms of action of various factors that modulate skin pigmentation or other properties of the skin. As such, researchers need to objectively quantify cutaneous responses at the macroscopic level. A simple method to standardize macrophotography images is reported that can quantify cutaneous responses in human skin equivalents of Asian, Black or African American, and Caucasian or White racial/ethnic origin. Macrophotographs are analyzed using the Commission Internationale de l'Eclairage L*a*b* color space system in combination with a personal computer and image editing software. Pigmentation changes monitored over a 9 day period showed a high correlation with melanin content evaluated in Fontana-Masson stained sections. These results indicate the feasibility of using a macrophotography setup in a sterile tissue culture environment to objectively assess *in vitro* cutaneous responses in human skin equivalents. This serves as an adjunct tool to biochemical and morphological methods to effectively quantify changes in pigmentation over time.

INTRODUCTION

In recent years, 3-dimensional human skin equivalents have become more frequently used to monitor the modulation of pigmentation by melanogenic inhibitors or stimulators as well as to evaluate the photoprotective properties of melanin (1–4). In efforts to evaluate the regulation of pigmentation in human skin, this physiologically relevant *in vitro* model is being used to assess pigmentation, epidermal histology, melanin synthesis, melanosome translocation and distribution to keratinocytes (1,3,5–8). Although the morphology of human skin equivalents can be microscopically observed using an inverted microscope, there is a growing need to monitor potential color changes at the macroscopic level. A straightforward method is required to readily document observable macroscopic differences in culture with minimal perturbation and risk of contamination.

The purpose of this study was to develop a protocol using macrophotography to monitor melanogenesis within the sterile environment of a tissue culture laminar flow hood using commercially available digital cameras. Recent advances in charge coupled device (CCD) chips have improved the photosensitivity of diodes measuring light intensities within the visible region. Color filters on the photosensitive diodes capture the three primary colors that correspond to red, green, blue (RGB) spectral band values (9). Those values can be

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mathematically transformed to a color space that more readily mimics the human eye perception known as the Commission Internationale de l'Eclairage (CIE) $L^*a^*b^*$ color space system (10). The CIE $L^*a^*b^*$ color space correlates with the human eye response and has been used extensively in studies evaluating cutaneous responses (11–16). Improvements in digital camera resolution and their reasonably inexpensive cost have made using macrophotography a practical option in addition to biochemical and morphological methods. In this study, the technological improvements in digital photography were critical in allowing us to demonstrate the correlation between pigmentation responses and melanin content analyzed histologically.

Previously, we reported a computer assisted digital image evaluation (CADIE) technique that could be used for the objective assessment of skin color changes as an adjunct tool to visual and optical observations in clinical and scientific evaluations (11). That technique allowed the discrimination of small color changes in human skin of various racial/ethnic backgrounds and correlated well with diffuse reflectance spectroscopy. A parallel approach is now described (termed Macrophotography for Assessment of Cutaneous Responses, MACR) that can be applied to *in vitro* studies using reconstructed skin samples of different phototypes and racial/ethnic origins. Within an *in vitro* setting, diffuse reflectance instruments (such as colorimeters and spectrophotometers) are not practical because of the probe contact required and the potential for contamination of samples within the sterile culture plates. Simplicity, adaptability and cost efficiency are the main advantages to this approach. These advantages make standardized macrophotography an attractive tool that provides objective measurements while avoiding the drawbacks of diffuse reflectance instruments and minimizing disturbances that could lead to sample contamination.

MATERIALS AND METHODS

In vitro reconstituted 3-dimensional human skin equivalents

Human skin equivalents of Asian, Black or African American (hereafter termed Black) and White or Caucasian (hereafter termed White) racial/ethnic origin were purchased from MatTek Corp (Ashland, MA, USA). These human skin reconstructs are provided in kits of 24 each and are composed of melanocytes and keratinocytes grown in 12 mm diameter, 3.0 μ m sterile culture plate inserts. According to the manufacturer, the substrate on which the cells are cultured consists of a chemically modified, collagen-coated, 9 mm ID single well tissue culture plate insert that will not degrade for months under normal tissue culture conditions. The substrate of the tissue culture plate insert, transparent when wet, is designed for live cell imaging and microscopy applications. Tissue samples placed on culture stands in 6-well plates were maintained at the air/liquid interface using 5 ml pre-warmed LLMM phenol red-free medium changed every 2 days at 37 °C, 5% CO₂ according to the manufacturer's instructions. Previously, Yoon et al. (3) discussed other commercially available human skin equivalents that could also be used.

Melanin content

At day 0 and after 9 days of culture, tissue samples were fixed in 10% buffered formalin, and then were embedded in paraffin. Three μ m thick sections mounted on silane-coated glass slides were stained using the Fontana-Masson method (17) which correlates well with eumelanin content (18). Tissue sections were assessed for melanin content in arbitrary units (a.u.) by evaluating integrated density using ScionImage software (Scion Corp, Frederick, MD, USA) from 10 random images taken with a Leica DMRB/DMLD microscope (Leica Microsystems, Bannockburn, IL, USA) as previously described (7,18,19).

Camera settings

A commercial “point and shoot” camera, Canon PowerShot A720 IS (Canon Inc., Tokyo, Japan) with a 5.8–34.8 mm lens containing a 1/2.5-inch type CCD with approximately 8.3 million pixels was used. The camera was set to the Manual Mode using the macro setting with My Colors Mode off and internal settings (contrast, sharpness and saturation) set to normal. Photographs were taken with the following settings: focal length = 5.8 mm, aperture = f/8, shutter speed = 1/80 sec, ISO = 80, White Balance = Fluorescent H (daylight balanced fluorescent). Digital images were acquired in JPEG format under the largest image size (3264 × 2448) with the highest quality (Superfine). All images were taken in the sRGB color space with a gamma value of 2.2 according to the IEC 61966-2-1:1999 (20) standard adopted by the camera manufacturer. It is important to keep in mind that standard transfer functions are embedded in many “point and shoot” cameras, which is a limitation of this method when trying to compare data across different cameras. The large number of commercial digital cameras available allows each individual to select the most appropriate equipment based on their needs. The critical point to note about compact digital camera selection is the quality of the macro mode. An extensive review of digital cameras is beyond the scope of this report, but several comprehensive reviews are available on the web (i.e., www.cnet.com, www.dpreview.com, www.imaging-resource.com, etc.).

Macrophotography setup

Skin equivalents in 6-well plates (with covers in place) were photographed at a constant distance of ~3 cm from the surface to the camera in a tissue culture hood under controlled conditions (see Figure 1). A small tabletop copy stand CCS-305 (TableTop Studio LLC, Carpinteria, CA, USA) with a 12" × 9" base and a mounting pole that extends up to 12" was assembled inside the tissue culture hood to perform macrophotography. The mounting pole was set to the lowest possible position and was locked in place. The tripod socket of the camera was attached to the lowest position on the vertical arm of the camera bracket. The two desk lamp units were positioned at 45 degrees to the plane of the camera viewfinder. To minimize camera shake, the camera was set to a 10-second self timer and was hand stabilized after the shutter button was released. The lighting system consisted of two desk lamp units, each with 15-watt compact full spectrum daylight 5000K fluorescent lamps (Longstar Industry Co., Ltd., Shanghai, China). In addition, a flat-panel 6" × 8" GEPE Slim Lite 5000 Illuminator G-2003 (The Gepe Group, Zug, Switzerland) containing 2 daylight cold cathode 5000K fluorescent lamps was placed on top of the base of the copy stand below the 6-well plate containing the tissue samples. This slim illuminator provided diffuse daylight balanced lighting in conjunction with the two desk lamp units. All lights other than the daylight balanced bulbs within the tissue culture hood were turned off during image acquisition to maintain accurate color fidelity. During each photographic time point, an additional image was taken of an empty sterile culture plate insert to control for standardized color reproduction.

MACR standardization and quantification

Using the step-by-step color standardization and quantification technique previously reported (11), one can analyze cutaneous pigmentation responses of in vitro tissue samples using the CIE L*a*b* color space system (10) and standard image editing software (Adobe Photoshop CS, Adobe Systems, San Jose, CA, USA) on a personal computer. For standard color reproduction, a Kodak Gray Scale (Q-13) can be used to normalize all the images as previously described (11). Briefly, the midtone neutral density step labeled M can be cropped down to scale to be included in each image or in a separate image taken immediately before the target image under the same conditions. Prior to image quantification, each image can be normalized to the midtone on the Kodak Gray Scale by creating a uniform color swatch of the neutral density step in a similar fashion as depicted in Figure 2. Then to eliminate any potential overall

color cast one can click on Image>Adjust>Curves. Within the Curves window, select the middle eyedropper (gray) and move the eyedropper over the swatch created and click OK. In addition, during each photographic time point the user should use an empty culture plate insert, white reflectance standard and/or a Kodak Gray Scale to monitor color fluctuation over time and to control for standard color reproduction.

Figure 2 shows the sequence of steps used in the MACR technique. The center region of each tissue sample was measured in triplicate using the MACR technique and then was compared to untreated samples prior to treatment for each type of skin sample. It is important to avoid any glare or specular reflection as well as to monitor environmental variables such as lighting inconsistency. In this setup, light is transmitted through the sample as opposed to being simply reflected off the sample. This diffusely transmitted and reflected illumination provides superior images of the tissue samples without shadows when the camera is so close to the object of interest.

RESULTS

Reproducibility

The macrophotography imaging system (Figure 1) was tested for reproducibility by evaluating an empty culture plate insert for standardized color reproduction. The results showed that the culture plate insert standard imaged had a standard deviation of $\leq 1.2\%$ for RGB values recorded by the photosensitive diodes of the camera's CCD chip, photographed on the same day or over several days (data not shown).

Macrophotography Cutaneous Response Assessment (MACR)

Using the CIE $L^*a^*b^*$ color space system and the MACR technique, pigmentation differences were monitored over time from day 0 through day 9 by calculating the chromametric ΔL values. L^* is defined as the light intensity from 0 (black) to 100 (white) (10). Therefore, ΔL represents the difference between the average L^* values of the samples at day 9 relative to day 0 for Asian, Black and White tissues, (i.e., ΔL values become more negative as the tissues become darker). Increases in cutaneous pigmentation response over time are clearly visible (Figure 3a). Increases in visual pigmentation (with the corresponding negative increases in ΔL values) were quantified in the three tissue types (Figure 3b). Rapid gains in pigmentation and differences in the rate of response were found in tissues with greater degrees of melanin content. At day 2, it was hard to discriminate any cutaneous response difference either visually or instrumentally between the tissue samples and their respective controls at day 0. However, from day 4 on, clear, quantifiable color differences were discriminated between the tissues, particularly in the more pigmented skin equivalents.

Melanin content

Representative images of Fontana-Masson stained tissue sections show increased melanin content in all 3 tissue types from day 0 to day 9 (Figure 4a). The MACR chromametric ΔL values at day 9 correlated with the increasing melanin content measured in the 3 types of skin equivalents (Figure 4b). There was an excellent correlation between melanin content and MACR chromametric ΔL (R^2 at day 9 of 0.9964) for increasing melanin content in White, Asian and Black tissues. The relatively high correlation coefficient for melanin content versus visual pigmentation (assessed by ΔL) underscores the feasibility of using a standardized macrophotography setup to monitor cutaneous responses *in vitro*.

DISCUSSION

In order to objectively and accurately monitor *in vitro* cutaneous responses, a macrophotography imaging system (termed MACR) was developed that would allow a relatively simple technique to discriminate small color differences. This method expands the applicability of using digital image intensity values based on the sensitivity curves of CCD sensors in digital cameras. There are advantages and limitations to using macrophotography to assess response changes. Three important advantages are the lack of direct probe contact from diffuse reflectance instruments with the samples, the color accuracy and the minimized risk of contamination using the setup in a tissue culture hood. It is important to note that although inverted microscopes allow for morphological assessment of tissues, they are not suited for color analysis. Inverted microscopes use tungsten-halogen bulbs while this macrophotography setup uses daylight balanced bulbs which allows for true color accuracy. Macroscopes provide another alternative, but these are expensive and not common in most laboratories. In addition, microscopes and macroscopes are usually located outside of tissue culture hoods which increases the risk of tissue sample contamination. The potential for contamination using other techniques depends on the sterility of materials used in each lab, the precautionary measures taken by lab personnel and their individual aseptic techniques. However, using the macrophotography setup described in this study we have never had contamination problems with our tissue cultures.

Researchers need to account for the varying degrees of CCD sensor sensitivities for the red, green and blue detectors because that may limit the ability to discriminate very faint cutaneous responses, especially in skin equivalents with less melanin content. Therefore, this is one limitation that must be considered when first implementing a macrophotography setup, switching between cameras, comparing results across different labs or comparing results with studies using different cameras. Commercial “point and shoot” cameras do not provide spectral data that allow one to derive chromophore concentrations in the same manner that diffuse reflectance instruments do. For this reason we restricted our analysis to the CIE $L^*a^*b^*$ color space which closely approximates the response of the human eye. To evaluate pigmentation, we used only the L^* parameter because evidence regarding the b^* parameter in the literature to evaluate skin pigmentation is somewhat muddled and not as consistent as the use of L^* . The b^* variable has been used to study bruise color changes (21), to evaluate the decrease in tooth yellowness (22) as well as to evaluate pigmentation (23–25). Although b^* has been correlated with the perception of tanning (23), it has also been identified as the least sensitive parameter in determining the difference in facial pigmentation amongst Caucasian, Hispanic and African-American individuals (25). There is a significant relationship between L^* and MED, but not for the b^* parameter (24). The b^* parameter has been more useful in the vector representation of the L^* and b^* plane to quantify pigmentation defined as the individual typology angle (ITA). Previously, in skin types 2–3.5 we found that the ITA provided comparable results to L^* (26) and did not prove more sensitive than other variables.

Future improvements to CCD sensors for hyperspectral imaging will allow one to map out the chromophore concentrations of all pixels in an entire image (27). Camera variables need to be standardized to each individual setup and internal controls (i.e., empty culture plate insert, white reflectance standard of known values) should be implemented to determine reproducibility of all conditions. Under standardized conditions, a macrophotography imaging system serves as an inexpensive and effective tool to examine cutaneous responses of human skin equivalents *in vitro*.

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Figure 1.

Image of the standardized macrophotography setup placed inside the tissue culture hood. [1] 3-dimensional human skin equivalents in 6-well plate, [2] flat panel illuminator with daylight 5000K lamps, [3] small tabletop copy stand, [4] adjustable mounting pole and camera bracket, [5] "point and shoot" camera, [6] 2 desk lamp units with daylight 5000K lamps at 45° angle to tissue sample.

Figure 2.

Sequence for cutaneous response quantification of human skin equivalents using the MACR technique. Critical items in the screenshot sequence are highlighted in red for emphasis. **Step 1:** Open the Adobe Photoshop program. Click on Window > Show Info. Open each JPEG image using the embedded profile. Zoom in on the sterile culture plate insert. **Step 2:** Using the elliptical marquee tool, select a circle two-thirds the diameter of the culture plate insert while avoiding the borders. Create a uniform color swatch within the circle using Gaussian blur filter (This minimizes any errors caused by CCD noise or artifacts). **Step 3:** Using the color sampler tool (5×5 pixel sample size average), click 3 separate times within the circle in a random fashion to obtain triplicate measurements of which an average can later be calculated.

Click on each eyedropper in the info palette to change color space values to L*a*b* Color and record values. Repeat until all tissue samples and controls are measured. Transfer recorded values to a spreadsheet for data analysis.

Figure 3.

Development of pigmentation in cultured 3-dimensional human skin equivalents over time.

a) Macrophotographs of human skin equivalents from Asian, Black and White racial/ethnic origin tissues at 0, 2, 4, 6 and 9 days (left to right). **b)** Pigmentation in human skin equivalents by MACR-assessed ΔL values.

Figure 4.

Melanin content in human skin equivalents analyzed by Fontana-Masson staining at days 0 and 9. **a)** Representative sections from Asian, Black and White racial/ethnic origin tissues, scale bar = 100 μm . **b)** Melanin content increases from day 0 to day 9 in all three tissue types (left) and correlation between melanin content and MACR-assessed ΔL values (right).