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Characterization of Pigmentation of an *In Vitro* Reconstructed Human Pigmented Epidermis

EPISKIN

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INTRODUCTION

In vivo, human skin pigmentation is the result of constitutive pigmentation, which is genetically programmed, and of facultative pigmentation, which is stimulated by ultraviolet light (UV). A very large variety of different skin phenotypes exists among human population.

A method, called Individual Typology Angle (ITA°) measurement, was proposed by Chardon *et al*. to quantify human skin pigmentation at clinical level. It is based on the L*a*b colour space, where the axe L* (Luminance) represents the level of grey, from black (value 0) to white (value 100), the axe a* represents the 'red-green' level and b* represents the 'yellow-blue' level. Using only L* and b* values, Chardon *et al*. designed a graph (*cf* Figure 1) where an individual skin colour is characterized by its Individual Typology Angle (ITA°).

In vitro, 3D reconstructed human epidermis models give the possibility to mimic and to study some parameters linked to *in vivo* pigmentation mechanisms. SkinEthic RHPE™ models are manufactured and sold since more than 10 years with 3 possible skin colour types: Light ("phototype II"), Tanned ("phototype IV") or Brown ("phototype VI").

This study presents the characterization of the pigmentation of these SkinEthic RHPE™ models based on ITA° measurements using a skin colorimeter as well as microscopic morphology analysis.

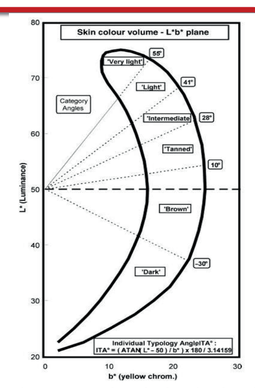


Figure 1. ITA° is calculated using the L* value (skin luminance) and the b* value (yellow component of skin).

MATERIAL AND METHODS

Culture of SkinEthic RHPE™ models: Reconstructed Human Pigmented Epidermis model from EPISKIN SA (Lyon, France) consists of a co-culture of keratinocytes and melanocytes at the air/liquid interface in the appropriate culture medium. A stratified and differentiated epidermis is obtained after 10 days of incubation (manufactured under ISO9001 quality system).

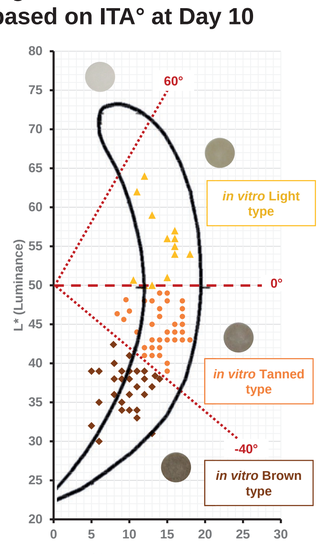
ITA° measurements: The colour type of models is measured using a photocolorimeter SkinColorCatch (DelfinTech). The values recorded are L* (luminance) and b* (yellow chrominance). ITA° was calculated using the formula ITA°= (ArcTangent ((L*- 50)/b*)) x180/π).

Microscopic morphology analysis: Models are fixed and treated with histology paraffin process. Morphology is observed with Hematoxylin-Eosin-Saffron (HES) staining, melanin pigments are observed with Fontana-Masson (FM) staining and melanocytes with L-DOPA staining.

RESULTS

Pigmentation control at the output of production at D10

Figure 2. Classification of the 3 types of *in vitro* SkinEthic RHPE™ models based on ITA° at Day 10

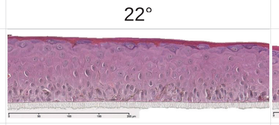
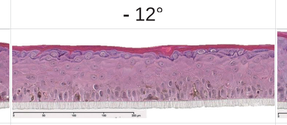
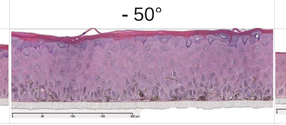
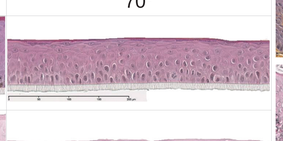
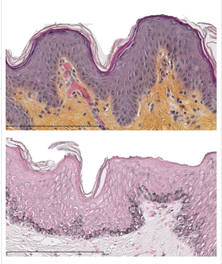
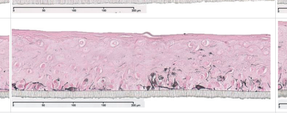
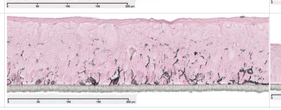


- ITA° measurements of 117 batches of SkinEthic RHPE™ (including 52 commercialized batches) were collected. With these data, our pigmentation control specifications for batch release were set as following:

Light type ("phototype II"):
0° < ITA° ≤ 60°
Tanned type ("phototype IV"):
- 40° < ITA° ≤ 0°
Brown Type ("phototype VI"):
ITA° ≤ - 40°
A tolerance of ± 5° is applicable to the specifications of all three colour types.

- ITA° correlates well with visual observation of tissues and enables to quantify their pigmentation. Batches are better characterized and followed.

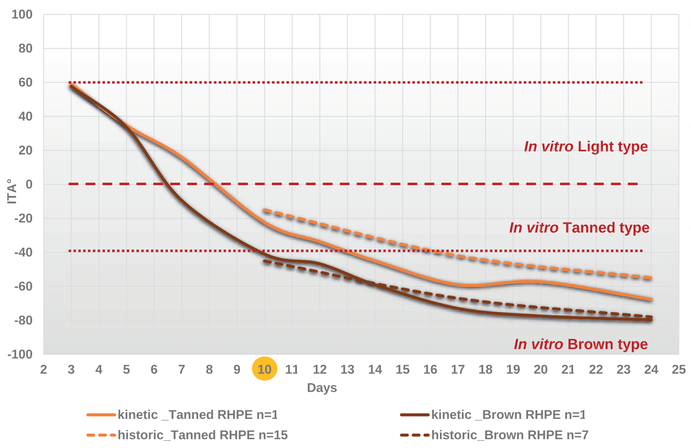
Figure 3. Pigmentation and morphology data of the 3 colour types of SkinEthic RHPE™ models at Day 10, of a SkinEthic RHE™ (negative control) at Day 10 and of an *in vivo* human skin biopsy (positive control)

	Light type	Tanned type	Brown type	Negative Control (SkinEthic RHE™)	Positive Control (*in vivo* human skin)
Macro Photo					
ITA°	22°	- 12°	- 50°	70°	
HES staining					
FM staining					

- As expected, the darker the colour type is (*cf* macro photo), the smaller the ITA° is. ITA° are within the specification defined in Figure 2.
- Morphology (*cf* HES staining) is similar for the three colour types.
- FM staining specifically black-marks melanin pigments: Light type has few of them, mainly located at the basal layer. Tanned type has more melanin pigments, located at the basal layer and ascending toward the suprabasal layer following a dendritic path ; a few capped keratinocytes are present. Brown type has the highest and most intense melanin pigments content from basal layer until *stratum corneum*, and several capped keratinocytes.

Kinetic characterization of pigmentation

Figure 4. Kinetic ITA° measurements of pigmentation of Tanned and Brown models from Day 3 to Day 24 of reconstruction.



- ITA° values are decreasing with time: models are darker as they get older
- Tanned model stays lighter than Brown model over days
- Historical data (dotted lines) are consistent with the single batch kinetic (full lines).

Figure 5. Pigmentation over days of Tanned and Brown models: macro photography of the reconstructed epidermis and photography of L-DOPA staining of whole tissues

Days		D3	D5	D7	D10	D12	D14	D17	D24
Tanned type	Macro photo								
	L-DOPA staining		/						
Brown type	Macro photo								
	L-DOPA staining		/						

- Visually, pigmentation increases over days until Day 24
- L-DOPA shows endogen intra-melasomal tyrosinase activity:
  - ✓ Tanned type: high density of melanocytes with well-elongated dendrites until Day 14. After, beginning of retraction of some dendrites
  - ✓ Brown type: slightly bigger, more deeply stained melanocytes than in Tanned model, beginning of retraction of some dendrites later (from Day 17)

Figure 6. Histological sections of models (HES staining) and pigmentation (FM staining) over days of Tanned and Brown models

Days		D5	D7	D10	D12	D14	D17	D20	D24
Tanned type	HES staining								
	FM staining								
Brown type	HES staining								
	FM staining								

- HES staining: Both models are in proliferation phase until Day 14 (high proliferation rate and beginning of differentiation). After, they enter into differentiation phase with progressive increase of the *stratum corneum* thickness.
- During the differentiation phase, keratinocytes and melanocytes reduce their proliferation rate, which could be correlated with the lower rate of pigmentation process observed in Figure 3. Keratinocytes from basal layer are differentiating first into spinous cells (suprabasal layer), then into granular cells (granular layer) and then dying to form the layer of *stratum corneum*.
- Fontana-Masson staining: all layers contain melanin pigments. More intense and concentrated melanin pigments are present in Brown model melanocytes compared to those in Tanned model, similarly to what is described in the literature about *in vivo* brown and tanned human skins. After that epidermis enters into differentiation phase from Day 14, less melanin pigments are seen in basal layer but they continue to accumulate in upper layers (until *stratum corneum*) in both types.

CONCLUSION

- Using ITA° measurement, pigmentation specifications were determined for Light, Tanned and Brown types. SkinEthic RHPE™ batches are currently checked with this method at Day 10, as a new Quality Control test.
- Light, Tanned and Brown models have functional melanocytes, producing melanin pigments consistent in quantity and intensity compared to *in vivo* skins of equivalent colour category.
- With a kinetic study with different endpoints, Tanned and Brown models were compared:
  Similarities: 1) increase of pigmentation over days, at similar rates 2) high density of melanocytes, with well-elongated dendrites 3) when epidermis enters differentiation phase, melanin pigments are less numerous in basal layer.
  Differences: 1) Brown model stays darker than Tanned model over days 2) highest quantity and intensity of melanin pigments, more melanocyte activity in the Brown model.
- These *in vitro* reconstructed human pigmented epidermis models could support efficacy studies, and can for example be used to develop *in vitro* testing methods of skin lightening or pigmentation-modulating of cosmetic ingredients.