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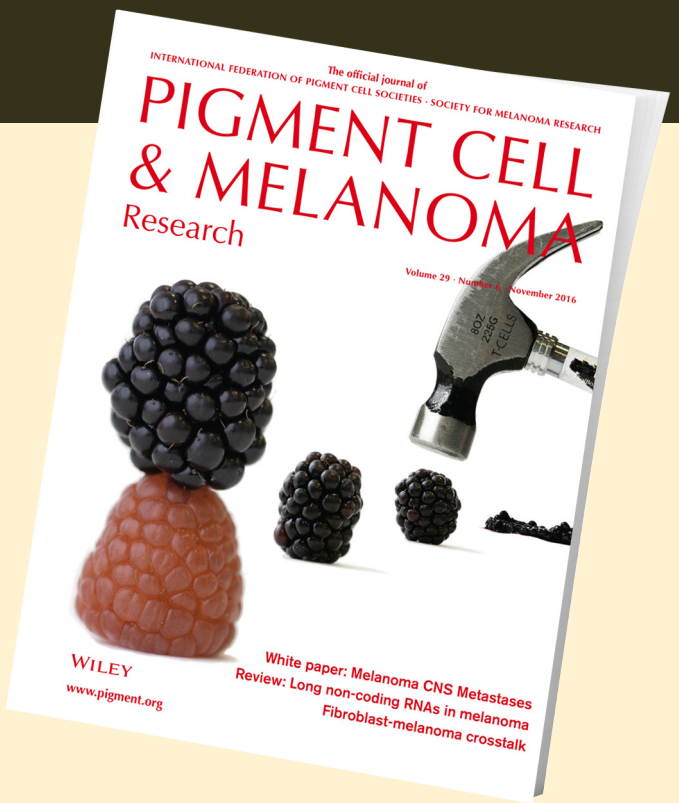

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Summary

Melanin quantification is reportedly performed by absorption spectroscopy, commonly at 405 nm. Here, we propose the implementation of fluorescence spectroscopy for melanin assessment. In a typical in vitro assay to assess melanin production in response to an external stimulus, absorption spectroscopy clearly overvalues melanin content. This method is also incapable of distinguishing non-melanotic/amelanotic control cells from those that are actually capable of performing melanogenesis. Therefore, fluorescence spectroscopy is the best method for melanin quantification as it proved to be highly specific and accurate, detecting even small variations in the synthesis of melanin. This method can also be applied to the quantification of melanin in more complex biological matrices like zebrafish embryos and human hair.

Introduction

The in vitro measurement of melanin is the basis of many studies involving differentiation of pigmented malignant melanoma cells, the protective role of compounds against UV light, and the development of treatments for pigmentation disorders such as vitiligo (Rosenthal et al., 1973). Also, in the cosmetic field, the development of new skin whitening agents created the necessity to accurately measure melanin in order to evaluate its significance (Watanabe et al., 1997).

There are several methods reported in the literature to assess the amount of melanin in biological samples. Electron spin resonance spectrometry (ESR) allows the measurement of electron spin resonance signals based on free radicals derived from melanin; this technique is highly specific, but it lacks sensitivity. Regarding photoacoustic spectroscopy, it has the advantage of being non-destructive and efficiently applicable to solid, gel, and liquid samples (Watanabe et al., 1997). High-performance liquid chromatography (HPLC) is also used in the quantification of melanin, particularly when it is necessary to distinguish between eumelanin and pheomelanin. This method is highly sensitive and accurate, allowing the quantification of different types of melanin present in samples, through the detection of their specific degradation products (Ito et al., 2013). As these techniques are not easily performed in most laboratories (they require very specific equipment and expertise), absorption spectroscopy is the most extensively used method to quantify melanin (Watanabe et al., 1997). Following solubilization of melanin pigments from cells or tissue samples in hot strong alkali (Soluene-350 or 1 M NaOH), total amount of melanin can be estimated spectrophotometrically by analyzing absorbance and comparing the data obtained with a standard curve of synthetic melanin or melanin isolated from *Sepia officinalis* (Wakamatsu and Ito, 2002).

Although it is the most popular method for melanin quantification, absorption spectroscopy presents major drawbacks and does not always provide the sensitivity and specificity required (Wakamatsu and Ito, 2002). Thus, the need for a reliable, simple, and economical method for melanin quantification is still a pertinent issue. Many years ago, Rosenthal et al. (1973) proposed the use of fluorescence spectroscopy to quantify melanin in cell cultures and tumors. Melanins do not fluoresce but, once they are subjected to oxidative conditions (heating in alkaline hydrogen peroxide solution), they acquire fluorescence. Sachs was the first to report that pigments of various origins become fluorescent after oxidation with hydrogen peroxide. Recently, other authors confirmed that degradation of melanin is accompanied by the

development of strong fluorescence (Kayatz et al., 2001). The method originally proposed by Rosenthal et al. (1973) has the potential to overcome the disadvantages of using absorption spectroscopy; according to the authors, soluble products are formed and the fluorescent signal of oxidized melanin is not affected by proteinaceous or lipid contamination. However, its application to melanin quantification in biological samples is still scarcely reported.

We revisited the fluorimetric method to optimize the oxidative conditions for accurate melanin quantification by fluorescence spectroscopy in melanoma cells. Subsequently, we compared the two methods regarding their limits of detection, linearity, specificity, reproducibility, and applicability in melanin quantification to demonstrate the advantages of using fluorescence spectroscopy. Two of the most frequent melanin standards (synthetic and *Sepia* melanin) and different pigmented and non-pigmented human cell lines were used in this comparison. The fluorimetric method was also successfully applied to the quantification of melanin in more complex pigmented samples: zebrafish embryos and human hair.

Results and discussion

Melanin production is the obvious main function of melanocytes, and its quantification is required for fundamental biology studies as well as for more applied research involving these cells. Figure 1 summarizes the protocol that we propose to quantify melanin by fluorescence spectroscopy in in vitro cell culture assays.

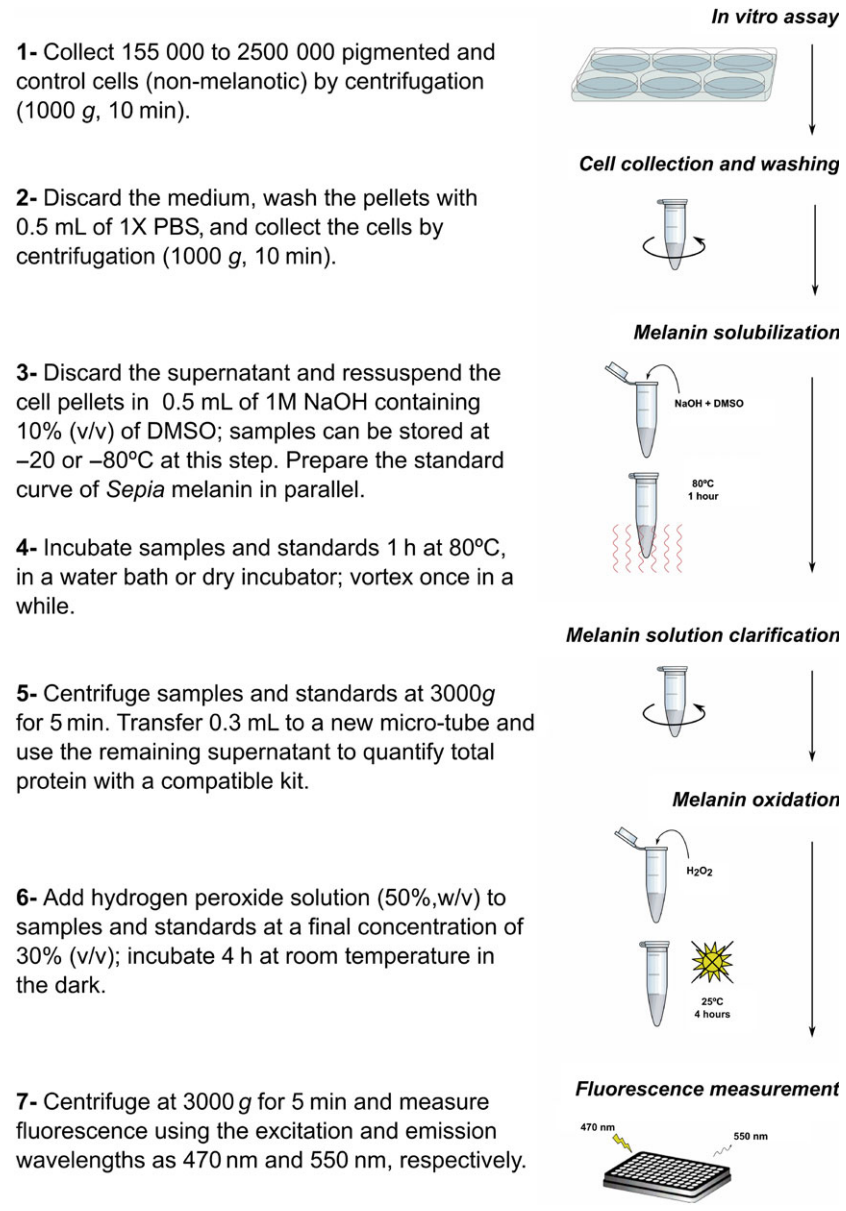

Figure 1. Schematic overview of the proposed protocol for melanin quantification by fluorescence spectroscopy in in vitro assays.

This protocol results from several optimization experiments where the fluorescence and absorption spectroscopy methods were extensively compared regarding their limits of detection, linearity, specificity, and reproducibility (Appendix S1). Regarding the parameters of sensitivity and linearity, fluorescence spectroscopy has an equal or slightly better performance than the traditional method – Table 1. However, as shown in the following section, the differences between the two methods become evident during the quantification of melanin in the presence of complex matrices like cell lysates (see also Appendix S1).

Fluorescence spectroscopy is the best choice for melanin quantification in in vitro-cultured melanoma cells

We have compared the performance of fluorescence- and absorbance-based methodologies in a typical in vitro assay to determine melanin production (Appendix S2). After incubation of several cell lines with a well-known inducer (forskolin, Fsk) and inhibitor (kojic acid, KA) of melanogenesis, their effects on melanin synthesis were assessed by both methods – Figure 2. The control cell lines were all non-melanin producers: BJ-5ta (human foreskin fibroblasts), NCTC2544 (human skin keratinocytes), and A-375 (amelanotic human skin malignant melanoma). The test cell lines were SK-Mel-1 and SK-Mel-23, established from metastatic sites of pigmented human skin melanomas (Houghton et al., 1987; Oettgen et al., 1968). The amount of melanin was normalized to total amount of protein in cell lysates, which is a frequently used alternative to counting cells for each experimental condition. The melanin contents presented in Figure 2 were calculated by interpolating the results with standard curves, generated by either the fluorescence or absorbance of *Sepia* melanin. Melanin contents were also calculated with standard curves of synthetic melanin (Figure S8, Appendix S4).

At first glance, the absorbance-based method overvalues the melanin concentration when compared to the fluorescence-based method. Another key outcome from the results shown in Figure 2 is that only by fluorescence spectroscopy, all control cell lines indicate no significant melanin production. With absorption spectroscopy, measurable amounts of melanin (which are melanocyte specific) are erroneously detected and, according to Figure 2B, there are no differences between the melanin produced by pigmented SK-MEL-1 cells and A-375 or NCTC2544 control cells. This result proves the high specificity of the fluorescence-based method as it erased the interference of other cell components from the detection/quantification of melanin.

The effects of the incubation of melanoma cells with an inductor or inhibitor of melanogenesis were also striking. After 72 h of incubation, as expected, the treatment with Fsk significantly increased the intracellular level of melanin in SK-Mel-23 cells when compared to cells treated with 0.2% (v/v) EtOH, the vehicle control. However, the pronounced depigmentation effect of KA (assessed by visual inspection) was only perceptible after oxidation of cell lysates and quantification of melanin by fluorescence. Concomitantly, the effect of the treatments on the melanin content of SK-Mel-1 was only detectable by fluorescence. Contrarily to SK-Mel-23 cells, visual inspection of these cells does not provide any insight regarding the effect of the compounds on melanogenesis. Nevertheless, the sensitivity of the method is so powerful that even small variations in the synthesis of melanogenesis can be detected and accurately quantified by fluorescence spectroscopy. Using absorption spectroscopy, these differences were neglected. On the one hand, according to the calibration curves generated (Appendix S1), absorbance-based quantification is much less sensitive to small variations in melanin. On the other hand, the contribution of non-melanotic material to absorbance background signal is so pronounced that melanin quantifications are overvalued and some variations can be masked. The contribution of non-melanotic material to melanin quantification is also clear by the analysis of the results for other cell lines. As mentioned before, NCTC2544 and A-375 cells have melanin contents similar to SK-Mel-1 despite of being unable to produce melanin.

Fluorescence spectroscopy can be used in the quantification of melanin in zebrafish embryos and human hair

To test the applicability of the proposed methodology on biological samples more complex than in vitro-cultured cells, fluorescence spectroscopy was used to quantify melanin in zebrafish embryos and human hair – Figure 3.

Table 1. Comparison of the fluorescence- and absorbance-based methodologies for melanin quantification, regarding sensitivity and linearity

	Detection limits				Range of linearity for melanin standards (μg/ml)	
	Melanins (μg/ml)		Cell Lines (cells/ml)			
	Synthetic	*Sepia*	SK-Mel-1	SK-Mel-23	Synthetic	*Sepia*
Fluorescence	0.098	1.563	310 000	310 000	1 – 100	5 – 100
Absorbance	0.391	1.563	310 000	630 000	1 – 100	5 – 100

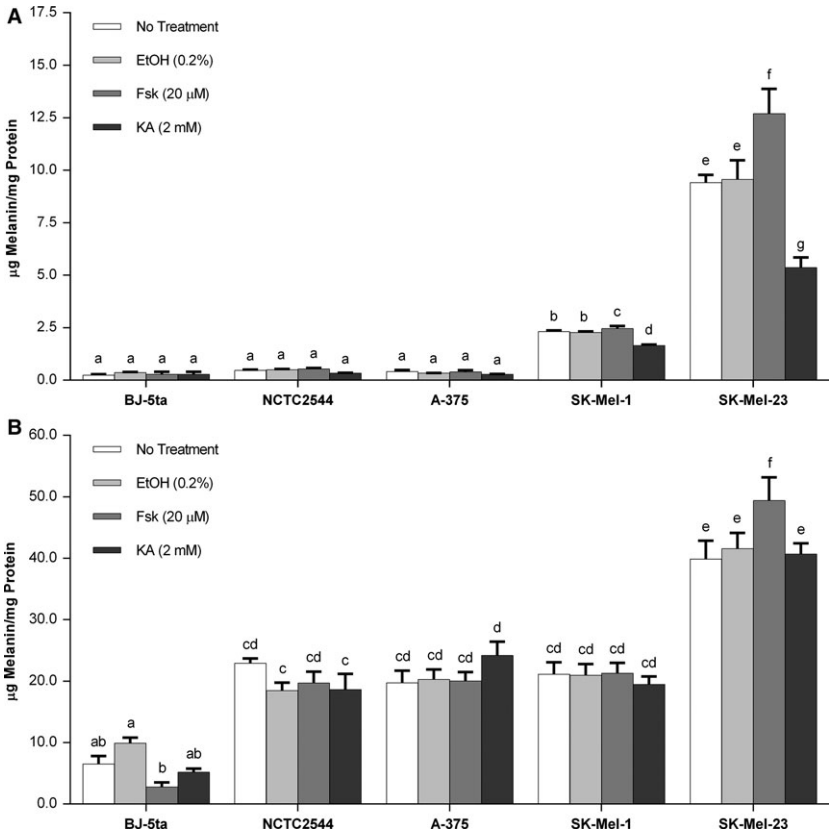

Figure 2. Effect of forskolin (Fsk) and kojic acid (KA) on intracellular melanin content of non-melanotic (BJ-5ta, NCTC2544), amelanotic (A-375, melanoma cells with no visible pigmentation), or melanized cells (SK-Mel-1 and SK-Mel-23, pigmented melanoma cells), after 72 h of treatment. Melanin content was calculated by interpolating the results with standard curves, generated by the fluorescence (after oxidation) (A) or absorbance (B) of *Sepia* melanin solutions of known concentration, prepared in 1M NaOH containing 10% (v/v) of DMSO. The results were normalized by total protein levels in each sample. Data were analyzed by two-way ANOVA, followed by post hoc Tukey's test. Means that do not share a letter are significantly different.

The protocol presented in Figure 1 (for cultured cells) was slightly adjusted in order to be applied to more complex samples, mainly in terms of NaOH concentration and incubation time during tissue disruption and melanin solubilization (Appendix S2).

Zebrafish *Danio rerio* is a powerful model system to study genetic mechanisms of vertebrate development, including the earliest events of pigment cells biology (Quigley and Parichy, 2002). More recently, zebrafish was also established as an in vivo model to evaluate the activity of melanogenic regulatory compounds, making the quantification of melanin in those in vivo models of the highest importance (Choi et al., 2007; Lin et al., 2011). Using fluorescence spectroscopy, a melanin production profile during zebrafish embryonic development was successfully drawn up to 120 h post-fertilization (hpf) – Figure 3A. For each measurement, only ten embryos were used (Appendices S1, S2). According to the literature, much higher number of embryos (at least 100 embryos/ml of NaOH) are commonly used to perform melanin quantification by absorbance spectroscopy (Baek and Lee, 2015; Choi et al., 2007). As expected, until 24 hpf, the embryos did not present quantifiable pigmentation (non-melanogenic control embryos). From 48 to 120 hpf, there is a steady increase in melanin production; the amount of melanin shows significant statistical difference in embryos collected at the three chosen time points. Also important to mention is that other pigments produced by zebrafish do not interfere in the quantification of melanin by this method (Appendix S1).

Fluorescence spectroscopy was also used to determine total melanin contents in hair samples of different colors – Figure 3B. Using hair concentrations above the detection limit (Appendices S1, S2), the measured melanin contents are according to the expected and shows an excellent correlation with the visual phenotypes: black hair has the highest amount of melanin followed by dark brown, reddish brown, medium blonde, and light blonde hairs. The total melanin content in red hair (described as containing similar amounts of eumelanin and pheomelanin) is similar to that of medium blonde hair and comparable to what was reported by others, using HPLC (Ito et al., 2011). HPLC is highly sensitive and, contrarily to absorbance and fluorescence spectroscopy, it also allows the analysis of melanin composition through the specific detection and quantification of the different degradation products of eumelanin and pheomelanin. According to the literature, H_2O_2 is efficient in the alkaline oxidation of both eumelanin and pheomelanin (Ito et al., 2011). However, it is still unknown whether their oxidation products exhibit similar spectroscopic properties, being properly accounted for the quantification of total melanin by the fluorimetric method here proposed. Although the results obtained offer some insight regarding the usefulness of

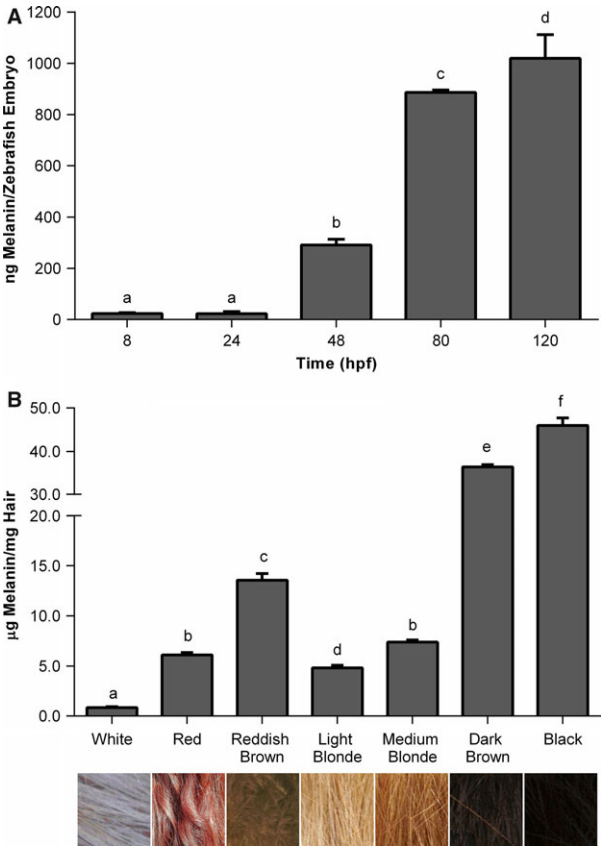

Figure 3. Quantification of melanin in biological samples by fluorescence spectroscopy. (A) Developmental changes in the accumulation of melanin in zebrafish embryos. (B) Contents of total melanin in human hair samples of various colors (visual phenotypes). Melanin contents were calculated by interpolating the results with standard curves, generated by the fluorescence (after oxidation) of *Sepia* melanin solutions of known concentration, prepared in 1M NaOH containing 10% (v/v) of DMSO. The results were normalized by number of embryos (A) or amount of hair (B) in each sample. Data were analyzed by one-way ANOVA, followed by post hoc Tukey's test. Means that do not share a letter are significantly different.

fluorescence spectroscopy in the quantification of samples containing pheomelanin, more studies are needed to understand the contribution of each type of melanin to the fluorescent signal. Therefore, the fluorescence-based method should be used in essentially eumelanic systems.

Final remarks

The quantification of melanin in cell cultures by fluorescence spectroscopy was successfully validated. The protocol proposed is easy to perform and highly reproducible. Compared with the traditional widespread methodology based on absorbance, fluorescence measurement of melanin oxidation products is more sensitive and accurate in the quantification of melanin in biological samples. However, in the absence of non-melanogenic components, absorption spectroscopy is also expected to perform well but the methods employed in the extraction of melanin are exhaustive and time consuming. Melanin oxidation followed by the fluorescence measurement does not require the previous isolation of melanin from samples. This work supports fluorescence spectroscopy as the best choice for routine total melanin quantification in complex biological matrices.

Methods

Please refer to Appendix S2 for Material and methods.

Acknowledgements

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Supporting information

Additional Supporting information may be found in the online version of this article:

Appendix S1. Optimization of the fluorescence-based protocol.

Appendix S2. Material and methods.

Appendix S3. Additional references.

Appendix S4. Figure S8.