Fingerprinting of skin cells by live cell Raman spectroscopy reveals melanoma cell heterogeneity and cell-type-specific responses to UVR

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Abstract
Raman spectroscopy is an emerging dermatological technique with the potential to discriminate biochemically between cell types in a label-free and non-invasive manner. Here, we use live single-cell Raman spectroscopy and principal component analysis (PCA) to fingerprint mouse melanoblasts, melanocytes, keratinocytes and melanoma cells. We show the differences in their spectra are attributable to biomarkers in the melanin biosynthesis pathway and that melanoma cells are a heterogeneous population that sit on a trajectory between undifferentiated melanoblasts and differentiated melanocytes. We demonstrate the utility of Raman spectroscopy as a highly sensitive tool to probe the melanin biosynthesis pathway and its immediate response to ultraviolet (UV) irradiation revealing previously undescribed opposing responses to UVA and UVB irradiation in melanocytes. Finally, we identify melanocyte-specific accumulation of β-carotene correlated with a stabilisation of the UVR response in lipids and proteins consistent with a β-carotene-mediated photoprotective mechanism. In summary, our data show that Raman spectroscopy can be used to determine the differentiation status of cells of the melanocyte lineage and describe the immediate and temporal biochemical changes associated with UV exposure which differ depending on cell type, differentiation status and competence to synthesise melanin. Our work uniquely applies Raman spectroscopy to discriminate between cell types by biological function and differentiation status while they are growing in culture. In doing so, we demonstrate for the first time its utility as a tool with which to probe the melanin biosynthesis pathway.

Keywords
keratinocytes, melanoblasts, melanocytes, melanoma, Raman, UVR

1 | INTRODUCTION
Melanocytes are the pigment-producing cells found in the skin, hair and eyes. Their embryonic precursors are melanoblasts derived from a transient tissue known as the neural crest.1 Melanocytes manufacture melanin from tyrosine and phenylalanine using the enzymes phenylalanine hydroxylase (PAH), tyrosinase (TYR), dopachrome tautomerase (DCT) and tyrosinase-related protein 1 and 2...
(TYRP1 and TYRP2). They use their dendrites to export melanin-containing melanosomes to keratinocytes which arrange them above their nuclei to protect from ultraviolet radiation (UVR) induced DNA damage. Melanoma, the cancer of melanocytes, is the deadliest form of skin cancer with a rising worldwide incidence. Melanoma risk is increased by exposure to UVR from the sun and from tanning beds.

Mutations in melanocytes arise from DNA damage caused either directly by UBV irradiation or indirectly following UVA irradiation through reactive oxygen (ROS) species via photosensitiser-mediated processes. Solar UVR is composed of mainly UVA (320–400 nm wavelengths) with a lesser component of UVB (280–320 nm wavelengths) and a UVA/UVB ratio of about 20 depending on latitude and time of day. Melanocytes respond differently to UVA and UVB. UVA induces immediate pigment darkening through photo-oxidation of melanin that can be observed within minutes of exposure. UVB on the contrary induces epidermal melanocyte proliferation and activation of melanogenic enzymes resulting in a delayed tanning response occurring 2–3 days postexposure. UVA and UVB also cause ROS-mediated lipid peroxidation and widespread oxidative modification of proteins resulting in their proteasomal degradation.

Raman spectroscopy enables a non-invasive label-free analysis of the biochemical structures present within a sample. Laser light is used to excite a sample and changes in scattering provide information about the molecular structures present. Its utility has already been demonstrated in identifying and discriminating between the molecular components of skin, and it has shown promise in the automated diagnosis of skin neoplasms in vivo including melanoma. Although previous studies have examined normal and melanoma biopsy tissue and compared melanocytes and melanoma cells, none have examined melanocyte function, differentiation status or their UV response. Here, we use live single-cell Raman spectroscopy and principal component analysis (PCA) to biochemically fingerprint melanoblasts, melanocytes, keratinocytes and melanoma cells. We identify the principal biomolecules that underlie these fingerprints and demonstrate that Raman spectroscopy is a highly sensitive method of probing the melanin biosynthesis pathway and its response to UVR.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Mouse melan-a cells were cultured in RPMI 1640 (21875–034; Invitrogen) supplemented with 10% (v/v) FCS and 200mM TPA. melb-a cells were cultured in RPMI 1640 (21875–034; Invitrogen) supplemented with 10% (v/v) FCS, 40pM FGF2 and 20ng/ml mSCF. B16F10 cells were cultured in RPMI 1640 (21875–034; Invitrogen) supplemented with 5% (v/v) FCS. COCA cells were cultured in CnT-07 (CELLnTEC). Cells were incubated at 37°C in humidified air containing 5% (v/v) CO2.

2.2 | UV irradiation

Cells were seeded onto CaF2 disks in 24-well plates using phenol-free media. Cells were irradiated with 100 KJ/m² UVA, 1000 J/m² UVA and UVB or 100 J/m² UVB and spectra acquired at 1, 3, 6, 16 and 24 h postirradiation as well as from an untreated control. The UVA source consisted of an array of Philips TLR 36 W “blacklights” tubes. Wavelengths below 320 nm were filtered out using polyester film (No 130 clear; Lee Filters, UK, spectrally equivalent to Mylar). The spectral irradiance ranged from approximately 330–400 nm with a peak output at 365 nm and an intensity of around 60 Wm⁻². The UVA source consisted of an array of Phillips T140 UVB tubes, emitting a broad spectral irradiance ranging from 275 to 380 nm with peak output at 315 nm and an intensity of 5.8 Wm⁻². The UVA/UVB source consisted of an array of solar-simulating Q-Lab QUV UVA-340 tubes (Q-Lab, UK). Spectral irradiance ranges from 295 to 420 nm, with a peak output of 340 nm and an overall profile in the 295–365 nm range that is similar to noon summer solar output, and an intensity of 23 Wm⁻². Output spectra and intensities of all UVR sources were measured using a double monochromator spectrophotometer (model SR991-PC; Macam Photometrics, Livingston, UK). For UVA treatments, where the prolonged exposure times required could lead to heating of samples, tissue culture plates were placed on water-cooled metal plates.

2.3 | Raman spectroscopy

Raman spectra were acquired at 60x using a 785 nm laser (200 mW at source, ~10 mW at sample) at a spectral range of 600–1700 cm⁻¹ using an InVia Raman microspectrometer (Renishaw plc, Gloucestershire, UK) equipped with an environmental chamber (Okolab, Ottaviano, NA, Italy) maintaining a temperature of 37°C in humidified air containing 5% (v/v) CO2. Collection was at 2 s with nine accumulations (18 s total) at 100% laser power. A total of 18 spectra were collected across three independent runs with spectra from six individual cells collected each run. Cells were selected randomly by moving diagonally along the sample and collecting spectra every 10th cell to ensure spread across the disk and that no overlap occurred. Data collected were baseline corrected using a polynomial method order 3, smoothed using p = 0.001, λ = 105 then vector normalised. Principal component analysis was performed in MatlabR2018A (The Mathworks, NA, USA) using an in-house script.

2.4 | Cell viability

Viability was measured using Cell Titre Glo (Promega). CaF2 disks were transferred to a 24-well plate, along with control disks that had not been used for spectral acquisition. Diluted Cell Titre Glo was added to cells for 10 min before scraping and transfer to a
white 96-well plate. Bioluminescence was measured as relative light units using a Perkin Elmer Wallac 1420 Victor2 microplate reader.

2.5 Intracellular melanin measurements

Cells were UV-irradiated as described above. Subsequently, they were washed in PBS, trypsinised and counted, then pelleted at 1000 RPM for 3 min. 100 μl of melanin lysis buffer (90% 1 M NaOH and 10% DMSO) was added to each pellet before incubation at 80°C for 90 minutes. Lysed pellets were transferred to a 96-well plate and their absorbance read at 490 nm on a Perkin Elmer Wallac 1420 Victor2 microplate reader. A standard curve was obtained by diluting synthetic melanin (Sigma, Poole, UK) at 0–1 μg/ml to enable the final melanin concentration to be determined.

2.6 Statistics

All statistical tests were performed in SPSS or using the ‘R’ statistics package (http://www.R-project.org). The Raman data in Figures 2-4 were normalised individually for each peak across all cell types and conditions. A one-way analysis of variance (ANOVA) was performed across the six time points for each combination of cell type and UVA treatment followed by pairwise comparisons by Tukey’s Honestly Significant Difference test (TukeyHSD). Therefore, both a significant ANOVA p value and a significant TukeyHSD p value are required for a significant pairwise difference to be considered. For the RT-qPCR data in Figures 2 and 3, data were analysed using SPSS. A one-way analysis of variance (ANOVA) was performed across the three time points for each cell type and UVR treatment; this was followed by pairwise Dunnett’s post hoc tests. Significant differences between control and time were evaluated with *p values ≤ 0.05 as indicated on the graphs.

2.7 RT-qPCR analysis

Total RNA was extracted using the RNeasy kit (Qiagen, Crawley, UK) following the manufacturer’s instructions. Reverse transcription of 1 μg RNA was performed using oligo dT18, M-MLV reverse transcriptase and RNase OUT (Invitrogen, Thermo Fisher Scientific) to generate cDNA. For RT-qPCR, 10 ng cDNA was used along with 12.5 μl of 2x Power SYBR® Green master mix (Applied Biosystems, Warrington, UK) and 400 nM primers (Sigma, Poole, UK). Primer sequences are in Table S1. Reactions were performed on a BioRad CFX RT-qPCR machine using the following parameters: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold (Ct) values were calculated for each mRNA sample and compared to their respective actin control to determine gene expression changes using the comparative CT (2−ΔΔCt) method.

3 RESULTS

3.1 Live single-cell Raman spectroscopy can discriminate between skin cell types and melanocyte differentiation status

We modelled keratinocytes, melanoblasts, melanocytes and melanoma cells using the COCA, melb-a, melan-a and B16F10 cell lines, respectively (Figure S1a). Live single-cell Raman spectroscopy was used to acquire individual spectra allowing us to establish their biochemical fingerprints. We confirmed viability of the cells after imaging using a CellTiter-Glo assay (Figure S2a–c). The mean Raman spectra for each cell type (n = 9) appeared qualitatively distinct under normal culture conditions (Figure S1b). Principal component analysis (PCA) demonstrated that the main differences between the spectra were accounted for by cell type. We observed tight groupings for the melanoblasts, melanocytes and keratinocytes while melanoma cells were more heterogeneous in nature falling between the melanoblast and melanocyte groups on the PCA plot (Figure S1c). The PCA factor loadings (Figure S1d) were used to identify the principal Raman peaks responsible for the PCA groupings which we attributed to their likely biomarkers using the established literature (numbered 1–10 in Figure S1b and Table S1). Nine of the 10 peaks had been previously described and were consistent with the biochemical properties of the cell types examined including melanin biosynthesis (phenylalanine, tyrosine, melanin) photoprotection (β-carotene) or keratinocyte function (keratin/lipid) (Table S1). We identified a peak at 1333 cm−1 (Figure S1b) in melanocytes and melanoblasts not previously attributed to a known biomarker but consistent with the Raman spectra for the melanin precursor DOPA (Figure S3a,b).

Undifferentiated melanoblasts and keratinocytes did not exhibit melanin peaks while the pigmented melanocytes and melanoma cells did (Figure S1b). Phenylalanine was apparent in melanoblasts, keratinocytes and melanoma cells but not in melanocytes presumably because melanin synthesis depletes the phenylalanine precursor in melanocytes. We observed DOPA peaks in melanoblasts and keratinocytes consistent with previous reports that they can convert tyrosine to DOPA.

3.2 Immediate and temporal effects on melanin biosynthesis pathways after UV irradiation detected by Raman spectroscopy

Melanin biosynthesis proceeds through a stepwise conversion of phenylalanine through tyrosine and DOPA. As demonstrated above, melanocytes and melanoma cells are competent to perform all of these enzymatic steps while melanoblasts appear able to synthesise tyrosine but are unable to convert DOPA to melanin. Although the enzymatic pathways are well delineated, the immediate and short-term biochemical response to UV exposure are still poorly understood. We therefore focused on the melanin biosynthesis
pathway immediately after UV exposure by comparing log₂ fold change (Log2-FC) in mean Raman peak height (n = 18 spectra in all cases) from control for the biomarkers identified above (Figure 1A and Table S1) in response to irradiation from a UVA, UVB and a mixed UVA/UVB light source over a one to 24-h response period.

3.3 | Opposing effects of UVA and UVB irradiation on the melanin biosynthesis pathway in melanocytes

Melanocytes were the most responsive to UVR. We observed a rapid (within 1 h) and transient (up to 6 h) statistically significant increase in the phenylalanine peak and a rapid (within 1 h) and transient (up to 6 h) statistically significant decrease in the tyrosine peak after UVA exposure (Figures 1A and S4a). In parallel, we observed significant reductions in gene expression of Mitf, Dct, Tyr and Tyrp1 as measured by RT-qPCR at 16 and 24 h postirradiation (Figure 1D). Together, these results suggest a block in melanin synthesis and accumulation of the phenylalanine precursor in the 24 h post-UVA exposure. The changes in melanin levels detected by Raman after UVA irradiation were mirrored by similar trends in intracellular melanin measurement between 0 (control) and 1–3 h (Figure 1F).

We observed opposing effects on the melanin biosynthesis pathway of UVB compared to UVA in melanocytes. This was characterised by a statistically significant rapid (within 1 h) and sustained (up to 24 h) decrease in the phenylalanine peak and a rapid (within 1 h) and sustained (up to 24 h) statistically significant increase in the tyrosine, DOPA and melanin peaks (Figure 1B). Consistent with a rapid UVB response increasing melanin production independent of gene expression, we did not observe any accompanying changes in the gene expression levels of Mitf, Dct, Tyr and Tyrp1 as measured by RT-qPCR at 16 and 24 h postirradiation (Figure 1E). The changes in melanin levels detected by Raman after UVB irradiation were mirrored by similar trends in intracellular melanin measurement between 0 (control) and 1–3 h (Figure 1F).

The response of melanocytes to a mixed UVA/UVB light source appeared more nuanced with features of both the UVA and UVB responses (Figure 1C). However, the UVB response dominated with a rapid (1–3 h) and transient (up to 3 h) statistically significant reduction in phenylalanine and a delayed (after 1 h) and transient (<24 h) statistically significant increase in the melanin peak mirrored by similar trends in intracellular melanin measurement between 0 (control) and 1–3 h (Figure 1F).

3.4 | Similar effects of UVA and UVB irradiation on the melanin biosynthesis pathway in melanoma cells

While melanocytes appear to respond differently to UVA vs UVB, melanoma cells demonstrated similar responses to the three light sources. We observed a rapid (within 1 h) and sustained (at least 24 h) statistically significant increase in the phenylalanine peak post-UVA irradiation (Figure 2A) and a delayed (between 6 and 24 h) and sustained (at least 24 h) statistically significant increase in the phenylalanine peak post-UVB and UVA/UVB irradiation (Figure 2B,C). This was accompanied by a statistically significant decrease in the melanin peak between one and 24-h post-UVB and UVA/UVB exposure (Figure 2B,C). The reduction in the melanin peak was the most pronounced after UVB treatment (Figure 2B), and we observed a corresponding reduction in the gene expression of Tyrp1 at 16 and 24 h for UVB but not UVA suggesting a block in melanin synthesis (Figure 2D-E) similar to the one observed in melanocytes (melan-a) after UVA treatment above. The changes in melanin levels detected by Raman after UVA irradiation were mirrored by similar trends in intracellular melanin measurement between 0 (control) and 1–3 h (Figure 2F).

3.5 | Minimal effects of UVA and UVB irradiation on the melanin biosynthesis pathway in melanoblasts and keratinocytes

We observed a subdued UV response in the melanin biosynthesis pathway in melanoblasts. Following UVA exposure, we observed a delayed (between 16 and 24 h) statistically significant reduction in the phenylalanine and the tyrosine peaks (Figure 3A) while following UVB exposure we saw an immediate (between 1 and 3 h) and transient (up to 6 h) statistically significant reduction in the phenylalanine and tyrosine peaks (Figure 3B). The response to the mixed UVA/UVB light source had features of the UVA and UVB responses with immediate (within 1 h) and delayed (by 16–24 h) statistically significant reductions in the phenylalanine and tyrosine peaks (Figure 3C). Taken with the results from melanocytes and melanoma cells, these results suggest that while accumulation of phenylalanine may be due to a block in the melanin biosynthesis pathway, reductions in phenylalanine, detected by Raman spectroscopy, can occur both through rapid production of melanin depleting the phenylalanine precursor but may also occur independently perhaps through photodegradation as previously reviewed.

Consistent with activity of the melanin biosynthesis pathway downstream of tyrosinase (TYR) being the key determinant in the Raman spectra observed in cells of the melanocyte lineage, we observed a subdued UV response in keratinocytes very similar to the one observed in undifferentiated melanoblasts with a delayed (24 h) statistically significant reduction in phenylalanine post-UVA exposure (Figure 3D) and an immediate (from 1 h) and sustained (up to 24 h) reduction in phenylalanine and tyrosine post-UVB exposure (Figure 3E). Exposure to the UVA-UVB mixed light source resulted in an immediate (1 h) and transient (up to 3 h) reduction in tyrosine (Figure 3F). We observed inconsistent changes in the DOPA and melanin peaks in keratinocytes likely attributable to noise caused by the very low basal level of DOPA in the control samples (Figure 3D-F).
Differentiated melanocytes but not melanoblasts, melanoma cells or keratinocytes demonstrate a photoprotective responses to UVR

Melanocytes have been shown to preferentially absorb β-carotene in culture. Consistent with this, we observed a distinct β-carotene Raman peak only in melanocytes (Figure S1b, Table S1). In response to UVR, we observed a statistically significant increase in this β-carotene peak (between one and 24 h post-UV) in response to UVA, UVB and mixed UVA/UVB light sources (Figure 4B). We therefore hypothesised that β-carotene may be performing a cell-type-specific photoprotective function protecting proteins and lipids from oxidative damage. We investigated changes in the Raman peaks for lipids (1300 cm\(^{-1}\) peak) and amide I (protein peaks at 1650 cm\(^{-1}\)) in our Raman spectra as biomarkers of oxidative damage (Figure 4 A – D).

We observed a rapid (1–3 h) and statistically significant reduction in the amide I peak in melanoblasts in response to UVA and UVB (Figure 4A) which appeared absent or dampened in melanocytes (Figure 4B). Furthermore, we observed a rapid (within 1 h) statistically significant reduction in the lipid peak in response to UVA exposure (Figure 4B).

3.6 | Differentiated melanocytes but not melanoblasts, melanoma cells or keratinocytes demonstrate a photoprotective responses to UVR
and a rapid (within 1 h) statistically significant increase in the lipid peak in response to a mixed UVA/UVB light source (Figure 4A). No changes in the lipid peak were observed in melanocytes (Figure 4B). These results suggest differentiated melanocytes are better protected against the effects of UVR than melanoblasts. Consistent with a more heterogeneous population of cells in melanoma, no clear trends in the lipid or amide I peaks were observed in melanoma cells (Figure 4C). Lipid levels have previously been shown to increase in human keratinocytes post-UVA and UVB dual irradiation. Consistent with this, here we observed rapid (from 1–3 h) and sustained (up to 24 h) statistically significant increases in the lipid peak in response to UVA, UVB and our mixed UVA/UVB light source in keratinocytes (Figure 4D).

3.7 Melanoblasts, melanoma cells and keratinocytes but not melanocytes demonstrate changes in keratin levels on UV exposure

Raman peaks consistent with keratin were present in keratinocytes and melanoblasts. We observed a rapid (within 1 h) and sustained (up to 24 h) statistically significant increase in the Raman peak for
FIGURE 3  UVA and UVB responses in the melanin synthesis pathway in melanoblasts (melb-a). Cells were grown on CaF$_2$ disks and irradiated with 100 KJ/m$^2$ UVA, 100 J/m$^2$ UVB or 1000 J/m$^2$ UVA + UVB. Raman spectra (at 785 nm) were acquired at 1, 3, 6, 16 and 24 h postirradiation. (A–C) Time course of log$_2$ fold (F) change (Log2-FC) and pairwise Tukey's honestly significant difference (TukeyHSD) comparing response (to UVA, UVB or UVA/UVB) to 0 h control for phenylalanine, tyrosine, l-DOPA and melanin ($n$ = 18 spectra for each square). (D–F) Time course of log$_2$ fold (F) change (Log2-FC) and pairwise Tukey's honestly significant difference (TukeyHSD) comparing response (to UVA, UVB or UVA/UVB) to 0 h control for phenylalanine, tyrosine, l-DOPA and melanin ($n$ = 18 spectra for each square). Significance level for a one-way analysis of variance (ANOVA) is indicated at the end of each series (**p < 0.05, ***p < 0.01, ****p < 0.001, ****p < 0.0001)
keratin in keratinocytes after UVA treatment with similar rapid (1–3h) and transient (<6h) statistically significant increases after UVB and combined UVA/UVB treatments in these cells (Figure 4D). Melanoblasts displayed a significant reduction in keratin after individual UVA and UVB treatments but an immediate (from 1h) and sustained (up to 24h) statistically significant increase in keratin after exposure to the dual UVA/UVB light source (Figure 4A). Melanoma cells but not melanocytes also exhibited a keratin peak and demonstrated a delayed (6–24h) statistically significant increase after UVB treatment (Figure 4C).

4 | DISCUSSION

We demonstrate the utility of live Raman spectroscopy performed on cells grown in a controlled environmental chamber, as a unique tool to probe the melanin biosynthesis pathway and its immediate response to UVR and reveal rapid and opposing responses to UVA and UVB irradiation by melanocytes.

4.1 | Raman fingerprinting demonstrates that melanoma cells are phenotypically heterogeneous

Previous reports have discriminated between malignant melanoma cells and their wildtype counterparts using Raman spectroscopy.20,26 Our Raman spectra appear consistent with those previously reported for melanocytes and melanomas cells,22,26,41 while our spectra for melanoblasts and keratinocytes are consistent with their inability to synthesise melanin31. Our PCA of the Raman spectra of melanoblasts, melanocytes, melanoma cells and keratinocytes showed that melanoblasts, melanocytes and keratinocytes represent spectrally, and therefore biochemically, homogeneous populations. This is consistent with their known phenotypic heterogeneity and the reactivation of their embryonic transcriptome.42,43 Further study is warranted to address whether this method can be used to predict the position of a melanoma cell on the differentiation trajectory of melanoma subtypes44 which may allow exploitation of these properties to increase immune therapy efficacy.
4.2 Rapid and opposing effects of UVA and UVB irradiation on the melanin biosynthesis pathway in melanocytes

We show here the utility of Raman spectroscopy to detect and sensitively measure changes in the melanin biosynthesis pathway in response to UVR over a smaller time-frame than is possible with conventional methods. UVA exposure has long been understood to result in immediate pigmentation within a few hours of exposure by acting on pre-existing melanin and by de novo melanin synthesis.45,46 The reduction in melanin post-UVA and accumulation of phenylalanine observed here may be consistent with a rapid tanning response resulting in the export of melanosomes from the cytosol and a subsequent block in synthesis because of their loss leading to accumulation of phenylalanine, consistent with reports UVA induces a redistribution of melanosomes in the skin.47 However, we do not see the rapid accumulation of melanin overserved in human epidermal melanocytes.45 UVB has long been demonstrated to cause a delayed tanning response mediated through DNA damage-induced de novo tyrosinase production.48,49 We were surprised to see such a rapid and profound UVB-mediated response in the present study characterised by rapid and sustained increases in tyrosine, DOPA and melanin and reductions in phenylalanine suggesting that a previously overlooked mechanism independent of DNA damage acts immediately after UVB exposure in mouse melanocytes.

4.3 Melanocytes uniquely harbour β-carotene with a possible role in photoprotection

The photoprotective effects of β-carotene have been attributed to its free radical/reactive oxygen species (ROS) scavenging abilities and direct absorption of UV at 400 nm.50,51 We observe melanocyte-specific accumulation of β-carotene consistent with previous reports39 and report here that β-carotene levels increase significantly in response to UVA, UVB and a mixed UVA/UVB light source. The presence of β-carotene also appeared to correlate with an apparent stabilisation in the peaks of amide I and lipid in melanocytes, whereas these biomarkers appeared to change dramatically in response to UVR in the other cell types studied. Taken together, these finding suggest that melanocytes use β-carotene as part of a UVR protective mechanism shielding against UV-induced lipid peroxidation, DNA damage and protein oxidation as previously reported51.

5 | CONCLUSION

We have defined a reference biochemical signature for each of the cell types studied that can be attributed to their biological function. In doing so, we have demonstrated the utility of Raman spectroscopy as a tool with which to probe the melanin biosynthesis pathway with greater temporal resolution and sensitivity than conventional methods. We have used this approach to uncover hitherto undescribed immediate responses to UVA and UVB irradiation in melanocytes including a possible photoprotective role for β-carotene. The ability to probe biochemical status with high temporal resolution will be highly beneficial to continued research in the study of melanoma and the further development of sensitive screening and early detection methods.

AUTHOR CONTRIBUTIONS

Conceptualization: RLM, SLA, JGK and LA; Data Curation: RLM, EW and JGK; Formal Analysis: ELW and RLM; Funding Acquisition: RLM and SLA; Investigation: ELW; Methodology: RLM and JGK; Project Administration: ELW; Resources: RLM, SLA, JGK and LA; Software: JGK, RLM and ELW; Supervision: RLM; Validation: ELW; Visualisation: ELW and RLM; Writing–Original Draft Preparation: ELW and RLM; Writing–Review and Editing: RLM, ELW, SLA, LA and JGK.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be available after publication at https://www.research.lancs.ac.uk/portal/en/people/richard-mort(ed9f7471-68c9-4816-97a9-58f8f44cf1fd.html) and from the corresponding author on reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Figure S1.** Live Raman spectroscopy can discriminate between cell types and differentiation status. Raman spectra (n = 9 for each cell type) were acquired from melanocytes (melan-a), melanoblasts (MelbA), melanoma cells (B16F10) and keratinocytes (COCA). Cells were grown on CaF2 disks in phenol red free medium prior to acquisition at 785 nm. A principal component analysis (PCA) was then conducted. (a) Bright field images of the cell types used. (b) The mean Raman spectra (offset for clarity) for each cell type differed qualitatively. (c) A scatter plot of the principal component vectors PC1 (98.27%) and PC2 (1.05%) showing tight grouping

of melanoblasts, melanocytes and keratinocytes with a more heterogeneous melanoma population. (d) PCA loadings of the PC1 and PC2 vectors - the ten principal Raman peaks are labelled with arrows on (b). The y-axis separation of the PCA loading plots in b and d is for visual purposes only. Scale bar in A = 200 μm. Numbers in (b): 1 = Lipid, 2 = Phenylalanine, 3 = Phospholipids/Tryptophan, 4 = Lipid, 5 = DOPA, 6 = β-carotene, 7 = Keratin, 8 = Melanin, 9 = Tyrosine, 10 = Amide I.

**Figure S2.** Cell viability post Raman spectral acquisition. melan-a, melbA, B16F10 and COCA cells were grown on CaF2 disks in duplicate in phenol red free medium for 24 h before irradiation with 100 KJ/m2 UVA, 1000 J/m2 UVB or 100 J/m2 UVB before spectra acquired from one disk at 1, 3, 6, 16 and 24 h post radiation as well as from an untreated control. The other disk was maintained under normal culture conditions as a control. Cell viability was measured using Cell Titer Glo. (A) UVA, (B) UVB and (C) UVA and UVB. Data represented as mean ± 95% CI, N = 18.

**Figure S3.** Raman spectra of synthetic melanin and DOPA. The spectra of melanin and DOPA was acquired using a 785 nm laser at a spectral range of 600–1700 cm−1. Spectra was collected at 2 s with 9 accumulations (18 s total) at 100% laser power. Data collected was baseline corrected and smoothed using p = 0.001, λ = 105 before vector normalising.

**Figure S4.** Raman spectra of UVR treated cells. melan-a, melbA, B16F10 and COCA cells were grown on CaF2 disks and irradiated with 100 KJ/m2 UVA, 1000 J/m2 UVB or 100 J/m2 UVB before Raman spectra acquired using a 785 nm laser at a spectral range of 600–1700 cm−1, 100% laser power 2s with nine accumulations (18s total) at 24 h post irradiation and compare to an untreated control. The other disk was maintained under normal culture conditions as a control. Cell viability was measured using Cell Titer Glo. (A) UVA, (B) UVB and (C) UVA and UVB. Data represented as mean ± 95% CI, N = 18.

**Table S1.** Summary of Raman spectral peaks.

**Table S2.** Oligonucleotides used for RT-qPCR analysis.

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