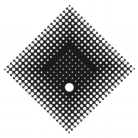
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Transcriptome analysis of the effects of polarized photobiomodulation on human dermal fibroblasts

Nicholas Tripodi[1,2,3], Fotios Sidiroglou[2,4], Vasso Apostolopoulos[1,3], Jack Feehan[1,3,5]*

[1] Institute for Health and Sport, Victoria University, Melbourne, VIC Australia

[2] First Year College, Victoria University, Melbourne, VIC Australia

[3] Australian Institute for Musculoskeletal Science (AIMSS), Victoria University, The University of Melbourne and Western Health, St. Albans, VIC Australia

[4] Institute for Sustainable Industries and Liveable Cities, Victoria University, Melbourne, VIC Australia

[5] Department of Medicine-Western Health, Melbourne Medical School, The University of Melbourne, St. Albans, VIC Australia

* Corresponding Author: jack.feehan@vu.edu.au

Abstract

Background: Photobiomodulation (PBM), the therapeutic use of light, is used to treat a myriad of conditions, including the management of acute and chronic wounds. Despite the presence of clinical evidence surrounding PBM, the fundamental mechanisms underpinning its efficacy remain unclear. There are several properties of light that can be altered in the application of PBM, of these, polarization—the filtering of light into specified plane(s)—is an attractive variable to investigate.

Aims: To evaluate transcriptomic changes in human dermal fibroblasts in response to polarized PBM.

Results: A total of 71 Differentially Expressed Genes (DEGs) are described. All DEGs were found in the polarized PBM group (P-PBM), relative to the control group (PC). Of the 71 DEGs, 10 genes were upregulated and 61 were downregulated. Most DEGs were either mitochondrial or extracellular matrix (ECM)-related. Gene Ontology (GO) analysis was then performed using the DEGs from the P-PBM vs. PC group. Within biological processes there were 95 terms found ($p < 0.05$); in the molecular function there were 18 terms found ($p<0.05$); while in the cellular component there were 32 terms enriched ($p<0.05$). A KEGG pathways analysis was performed for the DEGs found in the P-PBM vs. PC group. This revealed 21 significantly enriched pathways ($p<0.05$). Finally, there were 24 significantly enriched reactome pathways when comparing the DEGs of the P-PBM vs. PC groups ($p<0.05$).

Discussion and Conclusions: The P-PBM DEGs were almost always down regulated compared to the comparator groups. This may be explained by the P-PBM treatment conditions decreasing the amount of cellular stress, hence causing a decreased mitochondria and ECM protective response. Alternatively, it could point to an alternate mechanism, outside the mitochondria, by which PBM exerts its effects. Additionally, PBM appears to have a more widespread effect on the mitochondria than previously thought, opening up many new avenues of investigation in the process.

Keywords: Photobiomodulation; low level light therapy; polarized light; fibroblasts; wound healing; RNA-seq; transcriptome

1. Introduction

Chronic wounds are a major burden on the health systems globally, with an estimated prevalence of 2.21 persons in every 1000 [1]. There are a number of treatments for chronic wounds including, debridement, standard and bioengineered dressings, and anti-microbial agents [2, 3]. Given the high prevalence, there have been several investigations into new, low-cost and minimally invasive therapies to aid in the management of these conditions, one of these being phototherapies [4]. There are numerous clinical applications of light therapy in use today, none more so than that of photobiomodulation (PBM). PBM is used to treat numerous of conditions in clinical practice—from wound healing to sports injuries [5]. Despite a significant body of clinical knowledge surrounding PBM, the fundamental mechanisms underpinning its efficacy remain unclear [5, 6]. Currently the leading mechanistic model centres on mitochondrial Cytochrome c Oxidase (CcO), oxygen and nitric oxide (NO) [7]. In this model the red and infrared photons emitted during PBM interact with the chromophore CcO, in the process dislodging NO molecules, leaving oxygen to bind with CcO in their absence. This is thought to lead to an increase in overall ATP production by the mitochondria, subsequently responsible for the clinical effects seen with PBM [7, 8]. However, there has been no direct photonic interactions observed between CcO and PBM, and recent evidence suggests that PBM can exert its effect in the absence of CcO [9, 10], raising questions surrounding the fundamental mechanisms of PBM.

Regardless of the fundamental mechanisms underpinning PBM, there have been cellular effects resulting from its use in *in vitro* studies across a number of settings [4], however one area of significant study is the treatment of wounds. When examining the specific effects of PBM on wound healing, many PBM studies have investigated the effect of PBM on fibroblasts, due to their critical role in the process. Fibroblast survival and proliferation are crucial in the process of wound closure [11], and so have been widely investigated in the PBM field. While a range of fluences (Joules/cm^2) have been shown to increase these metabolic parameters, there are many conflicting findings, particularly when it comes to viability, highlighting the need for more stringent experimental parameters [4]. For example, PBM can influence multiple genes related to cell proliferation and wound healing such as vascular endothetial growth factor (VEGF) and genes related to collagen production (COL1AI, COL4A1, COL5A1) [4]. However, similar studies have also shown PBM to have no effect on these genes [12, 13], while some even decrease their expression [14]. Additionally, *in vitro* proliferation assays have been further established via work showing increased cellular migration brought on by PBM [4]. Given ATP production in the mitochondria is at the heart of the proposed mechanisms of PBM [6, 15], how it affects functional measures of

mitochondrial substrate and energy production have been reported. Again, these studies demonstrate that a range of fluence are able to increase both ATP production and mitochondrial membrane potential [4]. In addition, genes related to mitochondrial energy metabolism, have shown that PBM contribute to genes influencing the function of complexes I, IV and V, and hence energy production [16]. However, there remains debate around the illumination dose needed to illicit the maximum amount of mitochondrial function [17].

Beyond the fundamental mechanisms of PBM, there is conjecture surrounding the optimal method of delivering PBM both *in vitro* and clinically [10]. There are many variables that can be altered during the application of PBM, and include: beam area, irradiation time, fluence, power, polarization, wavelength, pulse parameters and treatment number, all which may modulate treatment outcomes [10]. Of these, polarization—the filtering of light waves whose electric field vectors move in a specific plane or planes—presents as an interesting variable to investigate [5, 18]. There is a small but growing body of research demonstrating polarization of light may provide additional biological efficacy in PBM [5, 19, 20]. This is thought to occur due to the polarized light having a greater level of tissue penetration, compared to equivalent non-polarized PBM [18]; however, further research is required to determine the therapeutic mechanisms of polarized light. Hence, the aim of this work was to profile the transcriptome of human dermal fibroblasts using RNA-seq to provide novel insights into how polarization of PBM affects gene expression.

2. Methods

2.1. Setting

All experiments were undertaken in standard laboratory conditions, in a PC2 facility at a public university in Victoria, Australia.

2.2. HFF2 fibroblast cell culture and experimental treatments

The human caucasian foetal foreskin fibroblast (HFFF2) (Cell Bank Australia NSW, Australia) cell line was used for all experiments. The cells were cultured according to the manufacturers recommended protocol, documented in previous works [21]. Due to the scattering of light that occurs in standard, clear-walled plates, cells were plated at $4x10^4$ cells per well in 500 µL of growth media in black-walled, 24-well plates (Eppendorf, Germany) [22]. To induce oxidative stress, the cells were treated with 0.5 µM of hydrogen peroxide (H_2O_2), twenty-four hours after seeding [23]. Immediately after peroxide treatment, the cells were exposed to PBM at a fluence of 1 J/cm^2 (A full description of the light parameters used is presented in Table 1). Three treatments were used to compare effects, with four technical replicates used in each.

The treatments were polarized PBM (P-PBM); non-polarized PBM (NP-PBM); and a no-light control (positive control - PC), with all exposed to the (H_2O_2) stressor. 24 hours post irradiation, the RNA extraction was performed as described below.

2.3. Light Source

The light source used for experimental treatment was a fiber coupled 670 ± 5 nm BWF laser diode (B&W Tek, Delaware, USA) (Table 1). The fluence dose used in the treatments was calculated as described previously [21]. A linear, 25 mm glass filter (Edmund Optics, New Jersey, USA) was used to polarize the laser diode (Supplementary Figure 2). The laser output power was appropriately adjusted in both polarized and non-polarized treatment setting to ensure consistent light treatment parameters across all experimental wells. [18, 21].

Table 1: Laser system and fluence parameters

Manufacturer	B&W Tek
Model	BWF1
Emitter	Laser Diode
Class	III B
Pulse Mode	Continuous wave
Wavelength	670 nm
Distance from target	80 mm
Target spot size	1.9 cm^2
Power at target site (mW)	11.2
Exposure Duration (sec)	169
Total Fluence per site (J/cm^2)	1

2.4 RNA Sequencing

RNA was extracted with an RNeasy mini-kit according to the manufacturer's instructions (Qiagen, USA), and immediately stored at -80°C until sequencing. RNA sequencing was performed by the Micromon genetics facility (Monash University, Melbourne, Australia). RNA quality was assessed via Agilent Bioanalyzer electrophoresis and Qubit fluorometer (Invitrogen, USA). A minimum of 2 μg of total RNA underwent library preparation and sequencing. Secondary quality control pf the RNA was performed using the AATI fragment analyzer prior to sequencing to asses for possible degradation of the samples during transport and/or preparation (Invitrogen, USA).

2.5 Statistical Analysis

Raw files were analysed using the RNAsik pipeline [24] utilising STAR aligner [25] with the Genome Reference Consortium Human Build 38 (GRCh38; Homo sapiens) genome reference. Feature Counts was employed to quantify the reads [26] producing the raw genes count matrix and various other quality control metrics. Raw counts were then analysed with Degust [27], which performed the normalisation using trimmed mean of M values [28], and differential expression analysis using limma/voom [29]. Differentially expressed genes (DEGs) were obtained using a False Discovery Rate (FDR) ≤ 0.05. Functional enrichment analysis (GO, KEGG and reactome pathways) was performed using STRING-db [30], where the data were exported and plotted using either SR plot and ggplot packages. Enrichment groups were considered significant at $p<0.05$.

3. Results

3.1 RNA Quality Control

The RNA integrity number of all samples was ≥9.9, representing high sample quality (Supplementary Figure 1). The mean Phred score was 36 across the samples, indicating >99.9% accuracy across sequencing reads (Supplementary Figure 1). Additionally, the size of each RNA library, distribution of p-values and normalized expression were all within acceptable limits across all samples (Supplementary Figure 1). The fourth NP-PBM was excluded as it was an outlier in the MDS analysis.

3.2 Screening Analysis of DEGs

There were a total of 71 (from 16280) DEGs when each experimental group was compared only to the control group (FDR <0.05). All these DEGs were found in the PPBM group, relative to the PC group (Figure 1). Of the 71 DEGs, 10 were upregulated and 61 were downregulated (Table 2).

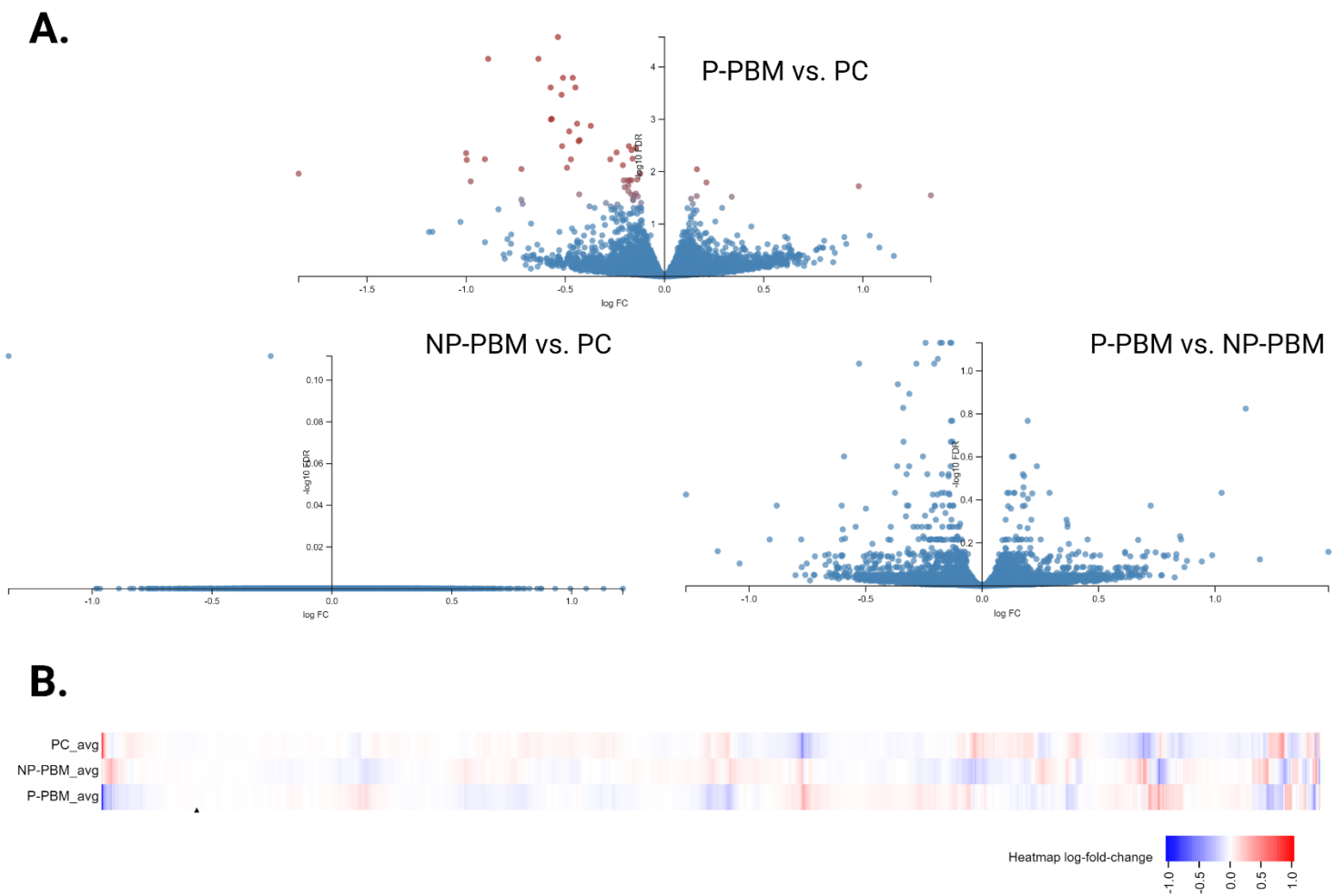

Figure 1: A. Volcano plot analysis of all genes analyzed across all groups. **B.** Heat map analysis of all genes analyzed across all groups. Figure sourced from the Degust bioinformatics platform.

Table 2: Full list of both upregulated and downregulated DEGs.

Group Comparison	Upregulated DEGs	Downregulated DEGs	
P-PBM vs. PC	AC048341.2	ACTC1	MT-CYB
	AKR1B1	AMOTL2	MT-ND1
	AKR1C1	C1orf198	MT-ND2
	CLU	CLDN1	MTND2P28
	LAMB3	COL1A1	MT-ND3
	MIR199A1	COL4A1	MT-ND4
	PCNA	COL4A2	MT-ND4L
	PHLDA3	COL5A1	MT-ND5
	S100A4	CTGF	MT-ND6
	Z74021.1	CYR61	MT-RNR1
		DCLK2	MT-RNR2
		DDAH1	MT-TC
		DIO2	MT-TE
		FZD7	MT-TH
		GOPC	MT-TI
		IGFBP3	MT-TS2
		LAMA4	MT-TV
		LDLR	MT-TW
		LMO7	MT-TY
		LMOD1	NRBP2
		MARCKS	P3H2
		MIR100HG	PCNA
		MRVI1	SMAD3
		MSRB3	SSBP4
		MT-ATP6	SULF1
		MTATP6P1	TAF10
		MT-ATP8	THBS1
		MT-CO1	THBS2
		MTCO1P12	TPM1
		MT-CO2	TXNDC5
		MT-CO3	

3.3 Network Pathway Analysis

There were two main gene association clusters found on network pathway analysis using the DEGs from above. The first involved mitochondrial genes associated with energy production, whilst the second involved genes associated with the ECM and collagen production (Figure 2).

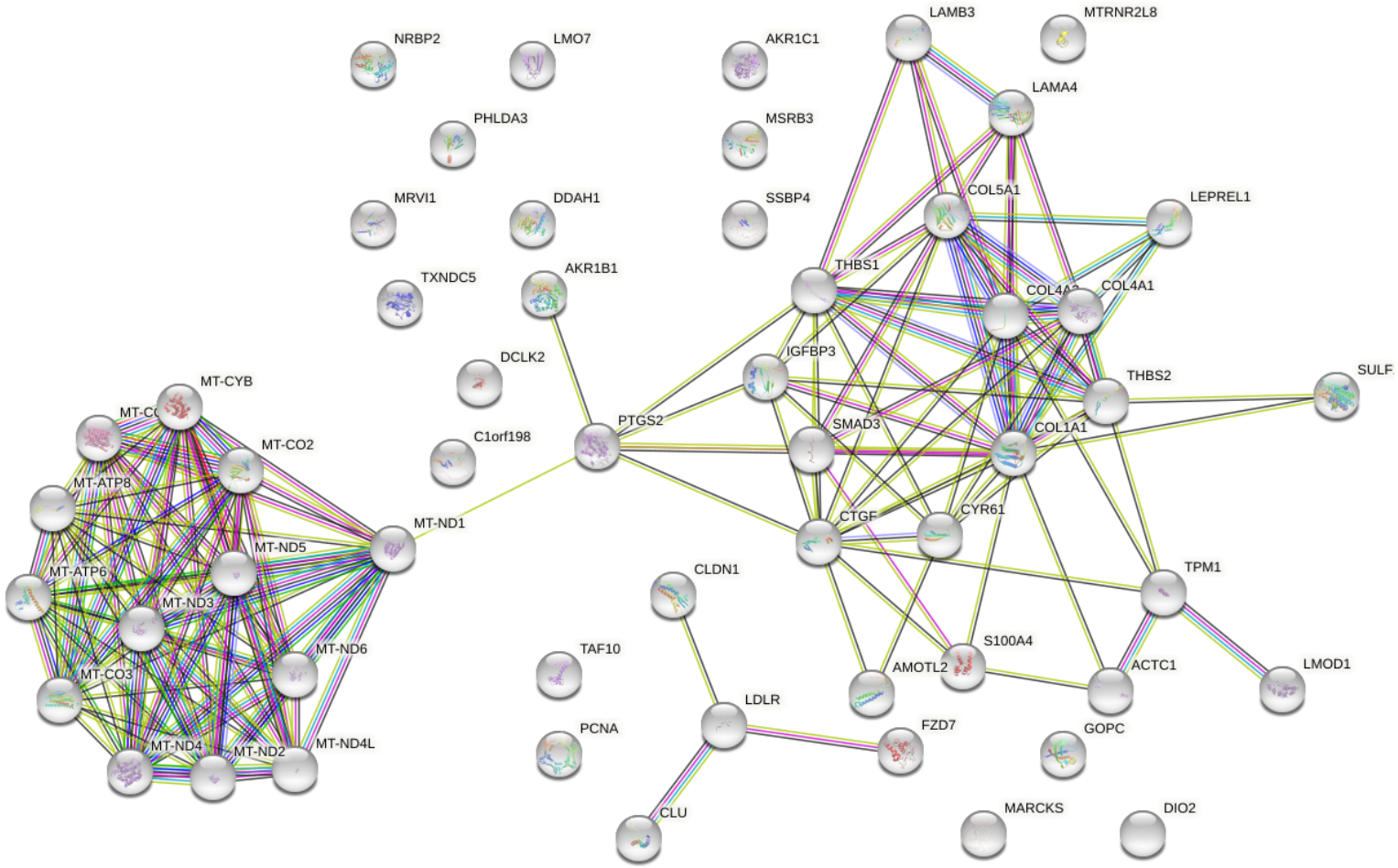

Figure 2: StringDB Network Analysis using DEGs. Light blue lines indicate known interactions from curated databases; Pink lines indicate experimentally determined known interactions; Dark green lines indicate gene neighbourhood predicted interactions; Red lines indicate predicted interaction from gene fusions; Dark blue lines indicate gene co-occurrence predicted interactions; Light green lines indicate text mining interactions; Black lines indicate co-expression interactions

3.4 Functional Enrichment Analysis

Gene Ontology (GO) analysis was performed using the DEGs from the P-PBM vs. PC group. In the biological process ontology there were 95 significant terms found ($p < 0.05$); in molecular function there were 18 terms ($p<0.05$); and in the cellular component ontology there were 32 terms found ($p<0.05$) (Figure 3).

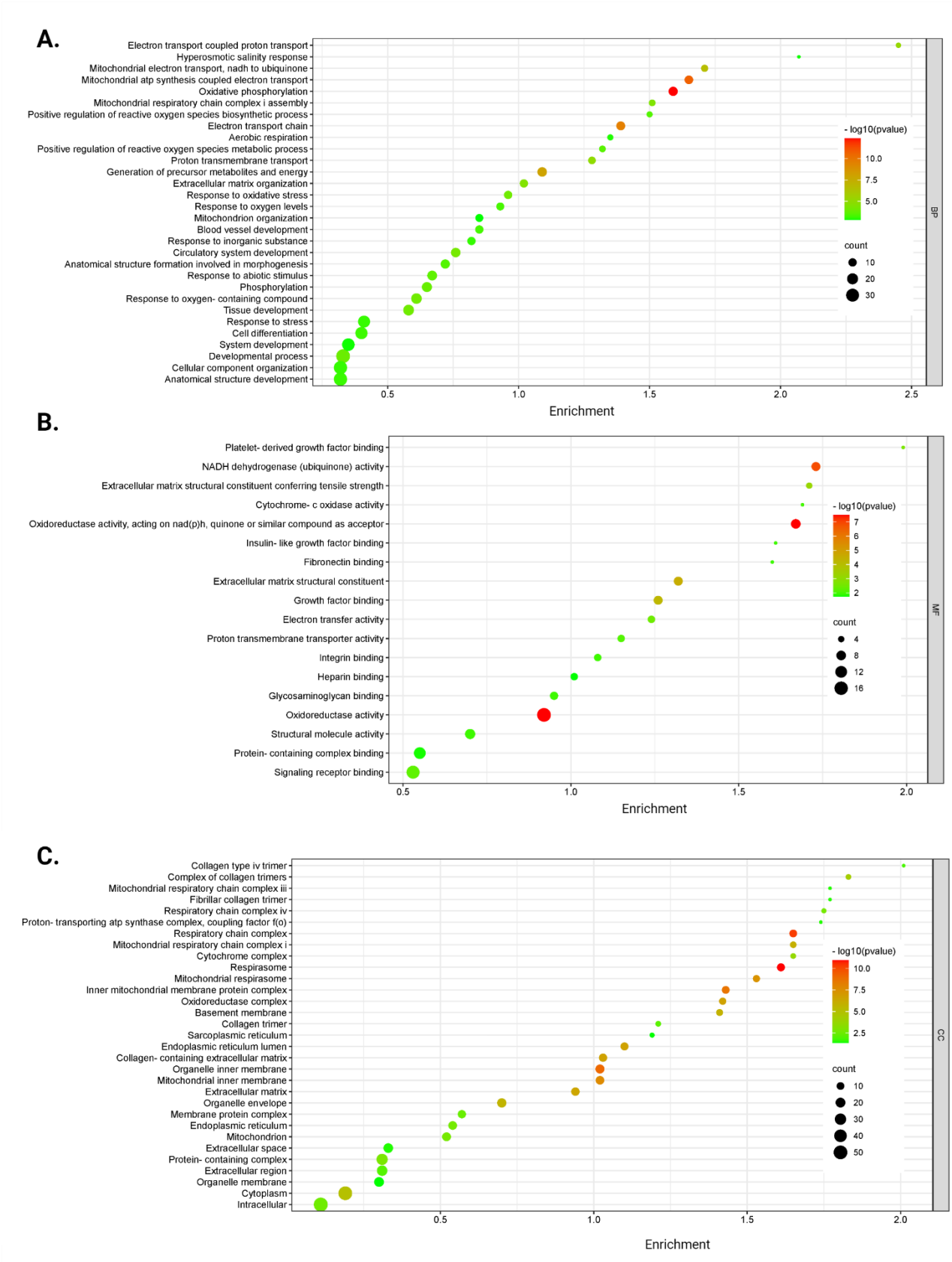

Figure 3: A. Top 30 Biological Process (BP) GO terms. **B.** Significantly enriched Molecular Function (MF) GO terms. **C.** Top 30 Cellular Component (CC) GO terms. Figure created with https://www.bioinformatics.com.cn/en

A KEGG pathways analysis was performed for the DEGs found in the P-PBM vs. PC group. This revealed 21 significantly enriched pathways ($p<0.05$) (Figure 4).

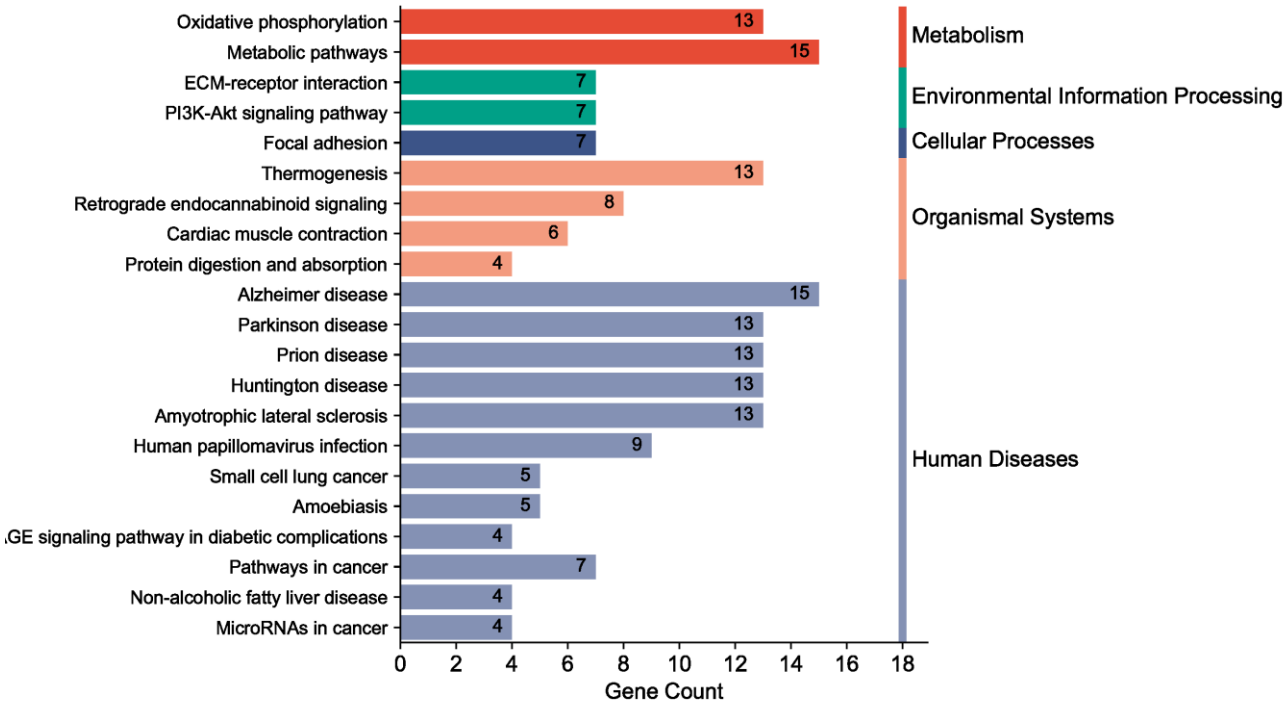

Figure 4: KEGG pathway analysis using DEG count. Figure created with https://www.bioinformatics.com.cn/en

Finally, there were 24 significantly enriched reactome pathways found when comparing the DEGs of the P-PBM vs. PC groups ($p<0.05$) (Figure 5).

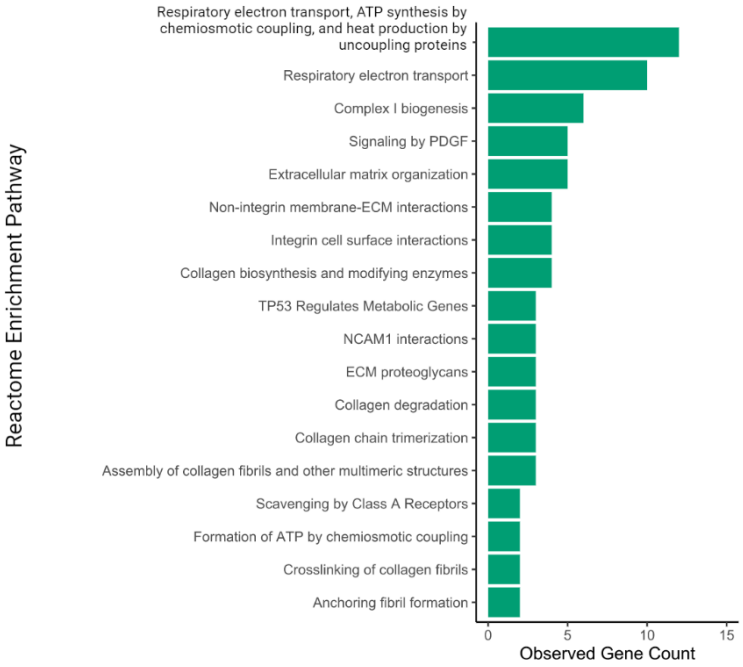

Figure 5: Reactome pathway analysis using DEG count. Figure created using ggPlot.

4. Discussion

PBM is a commonly employed intervention across multiple areas of clinical practice, often producing tangible clinical benefits. Despite this widespread use, there remains conjecture around the fundamental biological mechanisms responsible for the clinical effects observed [10]. As such, the transcriptome of human dermal fibroblasts were profiled following their exposure to oxidative stress, in response to both polarized and non-polarized PBM. The overarching results demonstrated that, P-PBM can influence the expression of multiple genes, mostly associated with the mitochondria and ECM, which relate to a number of important ontological and functional pathways.

The current leading mechanistic model of PBM centres on the mitochondria. To our knowledge, this is the first study which has investigated the mitochondrial transcriptome of human dermal fibroblasts in response to PBM. Interestingly, all the mitochondrial DEGs were downregulated when exposed to P-PBM. Previous research demonstrated that in healthy cells, and cells grown in ischaemic and diabetic models, PBM produces an upregulation in genes encoding for enzymes involved in ATP synthase and complexes I and IV [16]. That said, the previous works analyzed nuclear mitochondrial-related genes, as opposed to the

specific mitochondrial genes analyzed in this study. Given how susceptible the mitochondrial genome is to oxidative damage [31], in addition to the known cellular protective effects of PBM [21, 32], we propose that the downregulation of mitochondrial DEGs may have been caused by PBM ameliorating some of the effects brought on by the addition of an oxidative stress-inducing agent—H_2O_2. Recent findings have also cast doubt on the CcO-NO-ATP model of PBM as the sole mechanism underpinning its effect, demonstrating that PBM increased cellular proliferation and other metabolic parameters similarly in cells both with and without CcO [9]. Taken together with our findings, it appears that PBM fundamentally influences mitochondrial function, but it may be that it influences other areas of the mitochondria equally, or more so than CcO.

Currently, much research has focused on the efficacy of PBM in the treatment of dermal wounds [33-35]. Fibroblasts play a key role in this, by being stimulated from a mostly dormant state, in response to factors released in response to tissue damage [36]. They play an integral part in the integrity of the ECM in healing tissue by increased tensional forces brought about by their contractile capacity [37]. Importantly, one of fibroblasts chief functions is to produce the collagen matrix—the main structural component of connective tissue, which ultimately helps form focal adhesion complexes, which have important regulatory and structural functions [38]. The collagen-related and other ECM-related DEGs in this study, were universally downregulated, which conflicts with some, but not all of the findings relating to ECM-related gene expression in PBM exposed fibroblasts [4]. Several studies within the field, have demonstrated that collagen, and other ECM-related genes can either be upregulated, unchanged, or downregulated by PBM within acceptable fluence levels [4, 13, 22]. This is likely due to experimental inconsistencies, chiefly being, irradiation timings and cellular growth conditions [4, 10, 39]. It appears that the timing of PBM in response to cellular stress or damage is important, as the known protective effects of PBM, such as apoptosis inhibition [21, 40, 41], may more effectively inhibit cellular damage when applied closer to the initiating cellular stressor. This raises important clinical implications for the treatment of both acute and chronic dermal wounds, with timing of PBM application in relation to these conditions remaining underexplored.

This work has identified several functional ontological pathways which are influenced by PBM and relate to both cellular metabolism and wound healing. All the mitochondrial DEGs which were downregulated contribute to the ontological processes and pathways concerned with oxidative phosphorylation, ATP synthesis and the electron transport chain. More specifically, the Mitochondrial respiratory chains I, III, and IV cellular component ontological pathways, as well as the Reactome pathway Complex I biogenesis were significantly enriched with the downregulated DEGs, further supporting the notion that PBM can influence

multiple parts of the mitochondria, not only CcO [9]. Furthermore, there were multiple significantly enriched pathways associated with the ECM and wound healing processes including ECM organisation, structure, and interactions, collagen formation and biosynthesis, and integrin binding and interactions. Taken together these pathway analyses demonstrate that PBM has a strong influence on multiple areas of mitochondrial energy production, and pathways associated with wound healing, revealing many avenues for further research.

Interesting among the findings of this study, was the superiority of polarized light over equivalent non-polarized PBM. Work by ourselves and others has demonstrated that when compared to non-polarized, otherwise matched PBM, P-PBM can increase cellular viability and proliferation, decrease apoptosis, increase mitochondrial membrane potential and increase functional outcomes post-spinal injury in mice [5, 18, 21, 42]. The present results follow this trend, with P-PBM demonstrating the most profound influence on gene expression. The mechanisms underpinning these changes in PBM efficacy that polarization can affect are not fully understood, but currently it is thought that polarized light may present a way to better penetrate biological tissue through minimizing light attenuation, possibly through reduced light scattering, and therefore, be able to exert its effects more efficiently [5, 18]. This effect may be further enhanced when the plane of polarization is aligned to the tissue histological orientation [18]. Despite these findings, further research is required to determine the exact biophotonic interactions at play.

4.1 Limitations and future research

Although the methodological processes of this project being stringently controlled, there are some limitations we would like to acknowledge. One of the NP-PBM replicates was excluded from the analysis due to it being an outlier. This may have influenced the magnitude of gene expression in the NP-PBM group, however, the results in this study reflect our previous work demonstrating that P-PBM has a greater effect on cellular metabolic and regenerative function compared to NP-PBM and experimental controls [21]. Furthermore, the *in vitro* model of wound healing that was employed in this study, may not fully reflect the clinical treatment of wounds, with them often undergoing multiple exposures to PBM. These findings open many exciting avenues for future research. Firstly, the numerous significantly enriched ontological pathways found could be further explored to confirm if they translate to functional cellular changes. Secondly, these experiments could be replicated with other wavelengths and intensities, as well as being translated to 3D *in vitro* cell cultures, animal studies and clinical translation studies to determine the full scale of effects that P-PBM and NP-PBM can have on wound healing. Finally, it is important to note that changes in gene expression

doesn’t necessarily reflect changes in downstream protein expression, hence, these could be further explored in future research.

5. Conclusion

PBM is a widely used therapy for a number of clinical conditions, including wounds, however, both the exact fundamental mechanisms underpinning its effects, as well as the optimum irradiation conditions remain unclear. The leading mechanistic theory of PBM is centred on increasing the efficiency of mitochondrial CcO. This study has shown that PBM, specifically when polarized, can have a more generalzsed effect on mitochondrial energy production, affecting multiple mitochondrial complexes, not only complex IV, which aligns with more contemporary PBM research. Additionally, this work supports other fundamental and clinical literature by identifying that PBM can strongly influence the pathways that influence the ECM and therefore wound healing. Further research should explore the cellular and molecular pathways identified herein, to continue to build a better understanding of the fundamental mechanisms of PBM.

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Conflict statement

The authors have no conflicts of interest to declare.

Author contributions

Nicholas Tripodi: Validation, methodology, Formal analysis, resources, data curation, writing – original draft, Writing- review and editing, project administration.

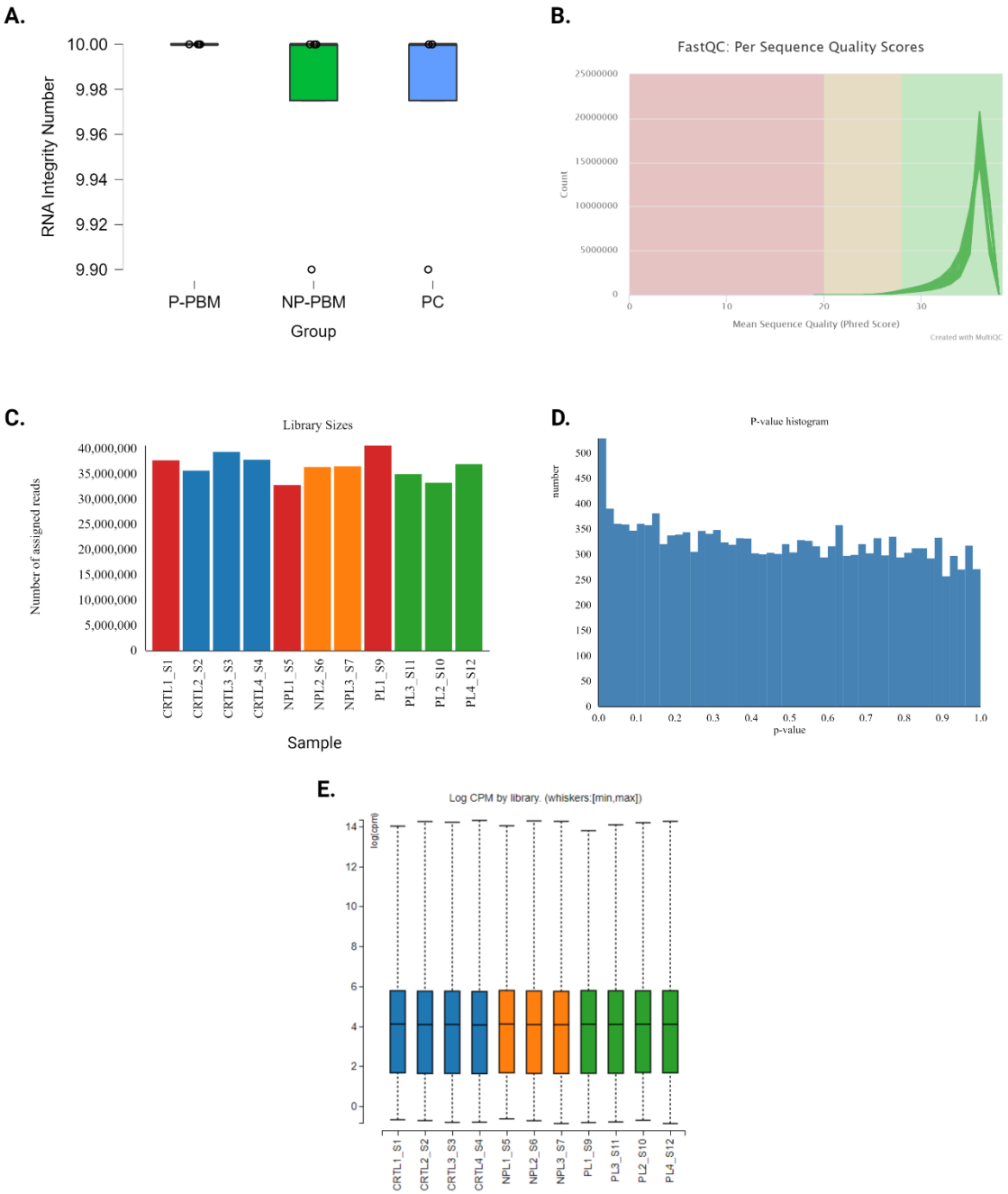
Fotios Sidiroglou: Formal analysis, Supervision, Writing – review and editing

Vasso Apostolopoulos: Formal analysis, Writing – review and editing, resources, supervision, and project administration

Jack Feehan: Validation, methodology, Formal analysis, resources, data curation, writing – original draft, Writing- review and editing, supervision, and project administration.

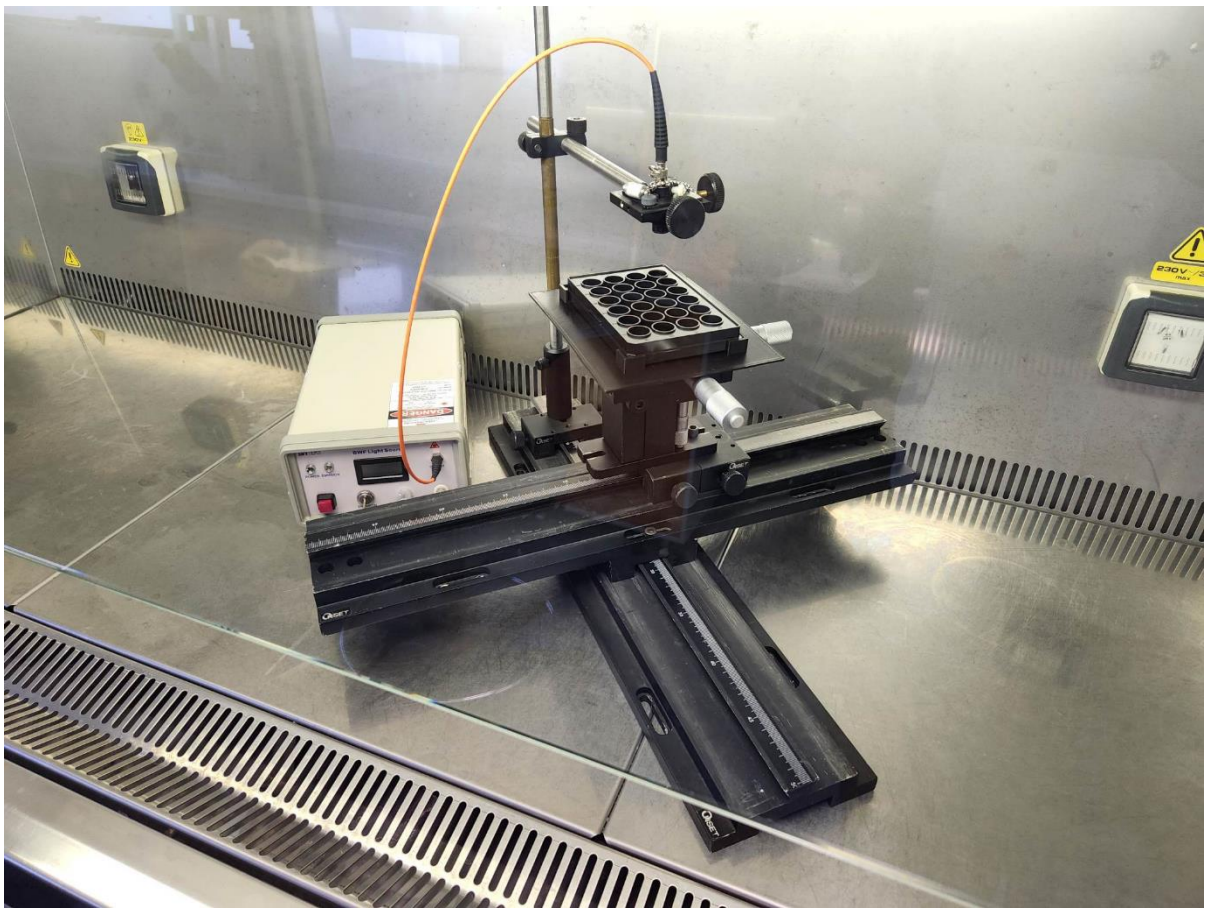
Appendix

Supplementary Figure 1 – RNA Quality Control



Supplementary Figure 1: A. RNA Quality Assessment; **B.** Phred quality score of analysis of base calling; **C.** Sequencing library size distribution; **D.** Distribution of p-values throughout the sample; **E.** Normalized expression intensity. P-PBM: Polarized Photobiomodulation; NP-PBM: Non-Polarized Photobiomodulation; PC: Positive Control.

Supplementary Figure 2 – Image of PBM Irradiation Procedure



Supplementary Figure 2: Custom stage built for PBM irradiation

Supplementary Figure 3 – List of Abbreviations

ATP	Adenosine Triphosphate
BP	Biological Process
CC	Cellular Component
CcO	Cytochrome C Oxidase
DEGs	Differentially Expressed Genes
ECM	Extracellular Matrix
GO	Gene Ontology
H_2O_2	Hydrogen Peroxide
HFFF2	Human Caucasian foetal foreskin fibroblast
J/cm^2	Joules per Centimeter Squared
KEGG	Kyoto Encyclopedia of Genes and Genomes
MF	Molecular Function
NO	Nitric Oxide
NP-PBM	Non-Polarized Photobiomodulation
PC	Positive Control
PBM	Photobiomodulation
P-PBM	Polarized Photobiomodulation
RNA	Ribonucleic acid
VEGF	Vascular Endothelial Growth Factor

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