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Quantitative photoacoustics to measure single cell melanin production and nanoparticle attachment

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Abstract

Photoacoustics can be used as a label-free spectroscopic method of identifying pigmented proteins and characterizing their intracellular concentration over time in a single living cell. The authors use a microscopic laser irradiation system with a 5 ns, Q-switched laser focused onto single cells in order to collect photoacoustic responses of melanoma cells from the HS936 cell line and gold nanoparticle labeled breast cancer cells from the T47D cell line. The volume averaged intracellular concentration of melanin is found to range from 29–270mM for single melanoma cells and the number of gold nanoparticles (AuNP) is shown to range from 850–5900 AuNPs/cell. Additionally, the melanin production response to UV-A light stimulus is measured in four melanoma cells to find a mass production rate of 5.7 pg of melanin every 15 minutes.

1 Introduction

A technique of biomolecule quantification is used in this study which can be carried out in the living cell exploiting the photoacoustic (PA) effect. Photoacoustics is a laser-induced ultrasound mediated by optical absorption and a sample can be investigated based on its optical properties. Photoacoustic (PA) imaging has been used in the past both on a microscopic and macroscopic scale to identify structures with pigmented proteins like hemoglobin or melanin[1]. More recently, there is an increasing interest in quantitative photoacoustics which can measure the content of these pigmented proteins[2, 3, 4, 5].

Cook et al have been able to use PA imaging to detect the presence and quantify the amount of nanoparticles in histological samples[6]. However, their use of high fluences on the order of 10^2 mJ/cm² in nanosecond pulses cannot be translated directly to viable tissue samples or cells. Recently, Zhang et al imaged the presence of cytochrome in cells using photoacoustic microscopy[7]. They were able to calculate a relative proportion of different types of cytochromes based on spectral PA response but no measurements or calculations were made to infer a numerical concentration of cytochromes. Additionally, Viator et al have used photoacoustics in the past to determine epidermal melanin content and port wine stain depth but both measurements were done on a macro-scale and not on a single cell level[8, 9].

With the exponential increase in genomic and proteomic data, there is intense interest in mathematical modeling of cell biological processes. For such approaches to be useful, quantification of protein levels in single cells is frequently necessary. Biological cells translate mRNA into proteins and bio-molecules in their cytosol. This mRNA level can be used to infer a protein level within the cell. However, RT-qPCR methods of quantifying mRNA, though widely used, do not always correlate to protein concentrations due to post-translational modification[10]. Moreover, a cell must be lysed to perform any type of PCR, making it a terminal measurement. Most RT-qPCR techniques do not have single cell sensitivity and require the combined mRNA pool from many cells (100 or more) resulting in an average measure of mRNA content of a large group. This hides variation within the cell population which could be potentially valuable information. Though photoacoustics has been used to measure relative oxygenation of hemoglobin in real time, no effort has yet been made to quantify changing protein concentrations in single cells over time[11].

Wicks et al demonstrated that human melanocytes can increase melanin content through a rhodopsin mediated cascade within one hour after exposure to UV-A light[12]. However, they did so by lysing many cells in order to harvest enough melanin for measurement with an optical density test. The measurements were averages of populations and terminal experiments because the cells had to be lysed. In this study, we have used photoacoustics to investigate the process of melanin synthesis in single melanoma cells with a non-destructive technique.

The photoacoustic method of protein quantification is first calibrated and then used to measure variations in melanin expression of single melanoma cells in the HS936 cell line and EpCAM expression on single breast cancer cells. The system is also used to obtain a PA spectrum of single melanoma cells from 470–650nm which is proportional to the optical absorption spectrum. Finally, a UV light source is used to induce melanin growth in the HS936 melanoma cell line. The melanin synthesis is measured in HS936 cells following induction by UV light source. Thus, we demonstrate label free quantification of changing concentrations of intracellular protein in single cells.

2 Materials and Methods

2.1 Experimental Setup

As shown in Figure 1a in portions labeled 1 and 3, light delivered by a 1mm optical fiber (Thorlabs, Newton, NJ) was collimated using a 1 in diameter aspheric lens (Thorlabs, Newton, NJ) into the camera port of an Olympus BX50 WI microscope (Central Valley, PA). The 7mm beam diameter after collimation was blocked with a 400 μm aperture. This 400 μm aperture created a 20 μm image of itself at the focus of a 20X water-immersion Olympus objective with 0.95NA and a 2mm working distance(Central Valley, PA). This resulted in a 20 μm beam spot in the center of the field-of-view. A camera (Model: MU035, Amscope, Irvine, CA) was installed in one of the eye ports of the microscope and was used to visualize the cells. Figures 1b and c are captured frames from the live camera feed demonstrating that the cross-hairs correspond to the position of the beam spot. The samples were positioned under the beam spot with the translation stage native to the microscope being used.

The energy of every laser shot, measured with a Coherent PowerMax (Santa Clara, CA) energy meter, was monitored by measuring the energy of a partial reflection at the laser head which was used to calculate the proportional energy out of the objective. This energy out of the objective was used to calculate the radiant exposure during irradiation. A Q-switched Nd:YAG laser with a 5 ns pulse and a tunable OPO laser system (OPOTEK, Carlsbad, CA) was used for exciting PA emission. All samples were submerged in either 1X PBS (Thermo Fisher Scientific, Waltham, MA) or DI water, depending on the experiment, in a 85mm diameter dish Petri dish (Thermo Fisher Scientific, Waltham, MA) with the ultrasonic transducer placed in the fluid. The ultrasonic transducer (Olympus, Central Valley, PA) had a focus at 12.5mm and a center frequency of 20MHz. Since the geometry of the microscope and the transducer did not allow for the irradiated spot to be exactly at the focus, the sample was placed 1mm farther than the focal point of the ultrasonic transducer.

The signals were amplified with a Stanford Research Systems (Sunnyvale, CA) digital amplifier with 350MHz bandwidth and captured with a 2024 Tektronix Oscilloscope (Beaverton, OR) with 200MHz bandwidth. All acquired signals were computationally low pass filtered with a sharp cut-off at 50MHz. This cut-off frequency was determined through the frequency domain analysis of noise and signal components (data not shown). Additionally, acoustic frequencies above 50MHz in water (30 μm wavelength) are not meaningful as the 5 ns laser pulse convolves the optical information in the PA emission to a spatial average over about 75 μm . This can be inferred from the findings of Choi et al, who show that the duration of the laser pulse must be one-tenth the stress confinement time[13].

2.2 Calibration

Varying concentrations of Direct Red 81 (Sigma Aldrich, St. Louis, MO) dye were irradiated under microscopy to ensure that peak-to-peak voltage (V_{p-p}) of the photoacoustic wave increased linearly with increasing concentration of dye. Concentrations ranging from 120–4000 μM of dye in DI water were irradiated with single pulses at 532 nm. As shown in Figure 2, a 9-channel array fluid chamber was made with 150 μm , 20mm $\times$ 20mm thick glass substrate (Thermo Fisher Scientific, Waltham, MA). Strips of 70 μm thick, 1mm wide dual adhesive tape (3M, Austin, TX) were placed approximately 1mm apart from one end of the glass substrate to the other. Finally, a 15 μm thin layer of polyethylene (Dow, Saginaw, MI) was placed on top and adhered to the dual layered adhesive. Different concentrations of dye were placed within each chamber with a micropipette and the ends of the chamber were sealed with nail polish. The chamber array was then submerged in a large 85mm diameter Petri dish containing 50mL of DI water and irradiated with the laser.

Additionally, Direct Red 81 dye concentrations ranging from 120–4000 μM were also tested with an HR2000 Ocean Optics (Dunedin, FL) spectrometer in order to determine their individual absorption coefficients. Dye solutions were placed between two standard microscope slides (Thermo Fisher Scientific, Waltham, MA) with 150 μm thick cover slips (Thermo Fisher Scientific, Waltham, MA) as spacers. The known path length through the dye (150 μm) and light attenuation at 532 nm with dye solution as opposed to DI water in place, as measured by the spectrometer, was used to calculate the absorption coefficients of the dye concentrations. Knowing the absorption coefficient of the dye and response from the

microscopic photoacoustic system would help create a functional relationship between these two values. The analysis of the data from this calibration protocol would then allow for a calculation from photoacoustic response to absorption coefficient, which can then be used to infer a concentration through the molar extinction coefficient of the sample.

2.3 Cell Preparation

The HS936 (melanoma) and T47D (breast cancer) cell lines were grown on the base of a T25 flask and removed with 5mL TrypLE (Life Technologies, Carlsbad, CA) when they reached 70–90% confluency. The cells were centrifuged out of the TrypLE at $140\times g$ for 10 minutes and resuspended in 3mL of growth media as specified by ATCC for the cell line. Neutravidin coated AuNPs (Nanopartz, Loveland, CO) were conjugated to biotin conjugated EpCAM (Pierce Antibodies, Rockford, IL) by combining 25 μL of stock AuNP solution with 1 μL of stock EpCAM solution in 600 μL of 1X PBS. After 1 hour, the nanoparticles were centrifuged out of suspension at $800\times g$ for 8 minutes and resuspended in 500 μL of 1X PBS. T47D breast cancer cells were incubated in growth media for 30 minutes with 112.5 μL of AuNP conjugated with anti-EpCAM solution. The excess nanoparticles were then washed off by centrifuging the suspension at $140\times g$ for 10 minutes and resuspending the AuNP labeled cells in the 3mL of growth media as specified by the ATCC for the T47D cell line. Melanoma cells were not conjugated to AuNPs.

Glass disks 150 μm thick, 12mm diameter (Thermo Fisher Scientific, Waltham, MA) coated with poly-L-lysine (Sigma Aldrich, St. Louis, MO) were placed in larger 85mm diameter dishes. The cells were plated by placing 65 μL of about 1×10^6 cells/mL suspension on each glass disk. The large 85mm diameter dish containing the glass disk remained undisturbed for 1 hour for the cells to settle and attach to the disk surface. After 1 hour the large dish was filled with approximately 50mL of 1XPBS submerging the plated cells at the bottom of the dish. This dish was then placed under microscopy and the cells on the disk were irradiated with laser light while an acoustic transducer (refer Section 2.1) placed in the fluid detected the emitted PA signals. The glass disks and 85mm diameter dishes did not produce any baseline PA signals.

2.4 Repeatability of PA Measurements

Melanoma—Ten isolated melanoma cells visibly expressing pigment (darker cells) were irradiated with 50–75mJ/cm² of 532 nm for four courses of irradiation over 9 minutes in order to measure the precision of the method along with any photo-bleaching or thermally-induced necrotic effects. Each course of irradiation involved 5 shots within 5 seconds and the PA signal from 5 shots were averaged. This was repeated every 3 minutes for four times per cell. The peak to peak voltage, V_{p-p} , is used as an indication of melanin content in the single cell and is measured for each irradiation course. The average V_{p-p} from each course of irradiation was normalized with average radiant exposure. Cell morphology was continually monitored to detect signs of thermally induced necrosis.

Breast Cancer with AuNPs—T47D breast cancer cells with membrane-bound gold nanoparticles (AuNPs) were also investigated with this technique. However, their optical absorption properties change after exposure to high-energy pulses of light in this range of

radiant exposures[14]. Five single cells were irradiated with the same parameters as the melanoma cells mentioned previously. The morphology of the cells was also visually inspected. Theoretically, the intensity of the PA signals from the AuNPs should change with each successive measurement.

2.5 Photoacoustic Emission Spectrum

A melanoma cell chosen at random was irradiated with 470–650nm in increments of 20 nm with one pulse from each wavelength. The V_{p-p} was normalized by the radiant exposure for each wavelength. This was repeated for five different cells. Then the photoacoustic emission spectrum from each cell was scaled to 1 by dividing through by the largest value in the spectrum. The unity scaled emission spectra were then averaged together to obtain a relative PA emission spectrum over 470–650nm.

2.6 Population Distribution of Protein Content

Melanoma—Single cells of melanoma plated on the poly-L-lysine disk were randomly chosen and irradiated with one pulse of 50–75mJ/cm² of 532 nm light. The V_{p-p} from each pulse irradiation was normalized with the radiant exposure for that pulse. This value was used as an indication of the melanin content of the single cell. A group of cells (n=25) was tested in order to create a histogram which could represent the population distribution.

Breast Cancer with AuNPs—Breast cancer cells were plated and then targeted with anti-EpCAM conjugated AuNPs. Single cells (n=25) were randomly chosen and irradiated with the same radiant exposure as the melanoma cells. Though the PA signal strength would start changing after one pulse, the initial pulse would provide the necessary data. The V_{p-p} normalized with the radiant exposure for each pulse was used as a measure of the EpCAM content of the cancer cell and a histogram was used to visualize the population distribution.

Negative Control—Single cells of breast cancer with no AuNPs were also irradiated as a negative control to verify that there was no baseline signal which would shift the measured PA responses. Since the T47D breast cancer cell line is not naturally pigmented, this was used as a control for both melanoma cells and labeled breast cancer cells. Additionally, it further verified that there were no other PA signals than those emitted by the absorbers (AuNP or melanin) of interest.

2.7 UV-A Induced Melanin Production

Melanoma cells from the HS936 cell line in growth media were incubated with 30 μ M all-trans retinal for 30 minutes, as done in Wicks et al. They demonstrated that human melanocytes incubated with retinal produce more melanin after UV-A irradiation when compared to those cells with no added retinal[12]. Therefore, HS936 cells were incubated with retinal to exaggerate the instantaneous melanin synthesis as a result of UV-A exposure. The cells were suspended in the recommended growth media by ATCC at a concentration of about 500,000 cells/mL, placed in the fluid chambers described in Section 2.2 and allowed to settle onto the polyethylene side of the fluid chamber (refer to Figure 2). The ends of the chamber arrays were sealed with nail polish and the chamber array was submerged in the

85mm Petri dish with 50mL of DI water. The cells were sealed into the chamber array to retain sterility and decrease the amount of growth media needed to complete the experiment.

A cell was chosen at random and the melanin content was measured with the microscopic PA technique by irradiating the cell with 5 pulses with the radiant exposure normalized V_{p-p} from each pulse average together. Following which, the cell was exposed to a continuous UV light through a DAPI filter (Olympus, Central Valley, Pennsylvania) with near equal radiance from 350–400nm for 10 minutes with an intensity of 300mW/cm². After exposure to UV, the cell was irradiated with 4 laser pulses within 5 seconds and the V_{p-p} from each was divided by the corresponding radiant exposure. This was done every 15 minutes for 1 hour in order to measure a change in the melanin concentration or content within the cell.

3 Results

3.1 Calibration with Direct Red 81 Dye

The initial calibration of the microscopic PA system was completed to ensure linearity of response in the regions of interest and also to characterize the limitations of the system with optical absorption parameters. The photoacoustic measurements of peak-to-peak voltage, V_{p-p} , (in mV) normalized with the radiant exposure (normalized voltage) were compared with the spectrometric measurements of absorption coefficient (μ_a).

As seen in Figure 3, both photoacoustic and spectrometric values are linear across the measured range as indicated by the high coefficients of determination even though the linear fits were forced to go through the origin. Though the photoacoustic technique is most likely linear even beyond the measured region, the known region of linearity corresponds to a ~35:1 dynamic range. The lowest V_{p-p} response from the microscopic PA system corresponds to a μ_a of 6.16 cm⁻¹ as inferred from the spectrometric measurements.

$$V_n = 0.27 C_{dye} \quad (1)$$

$$\mu_a = 43 C_{dye} \quad (2)$$

$$\mu_a = 159 V_n \quad (3)$$

In the previous equations, V_n denotes normalized voltage in mV/mJ/cm², C_{dye} stands for the concentration of Direct Red dye in mM, and μ_a stands for the absorption coefficient in cm⁻¹. Equations 1 and 2 describe the linear fits in Figure 3a and 3b, respectively (p<0.001). Equations 1 and 2 can be used to derive Equation 3 which directly relates normalized voltage to the absorption coefficient of the irradiated sample.

3.2 Repeatability of PA Measurements

Figure 4a plots the relative PA response of single cells of melanoma or AuNP labeled breast cancer. All cells were irradiated with 5 pulses every 3 minutes in order to determine the invasiveness (bleaching effects and/or cell death) and precision of the microscopic PA

technique. The final PA emission values for each cell type were scaled to 1 by dividing through by the largest value for each group. This allows us to pool data from different cells which have different melanin or AuNP content. Each point is the average relative response of melanoma cells (n=10) or breast cancer cells (n=5) along with the standard deviation as error bars.

The data from AuNP labeled breast cancer reaches significance ($p=0.003$) with a single-factor ANOVA test but the data from melanoma cells do not ($p=0.43$). This implies that the AuNPs bleached with high-power pulsed laser light since the V_{p-p} for the AuNP labeled breast cancer cells drops over time. However, the melanin in the melanoma cells did not bleach since the V_{p-p} for melanoma cells does not change significantly. Additionally, the standard deviation bars denote that the precision of the system is about $\pm 10\%$. There are no error bars for the point at $t = 0$ for the AuNP labeled breast cancer cells since it was the largest response ubiquitous for all cells.

Figure 4b and c are captured frames of the camera feed where single cells under the target are being irradiated. The morphology of the irradiated cells, which was tracked with the live camera feed and evaluated qualitatively, did not change over the many courses of irradiation.

3.3 Photoacoustic Emission Spectrum

Since the microscopic PA technique did not significantly bleach the melanin inside of melanoma cells, the PA emission of five different melanoma cells over 470–650nm was acquired. The PA spectra of each cell was scaled to 1 and then averaged with spectra from other cells to produce Figure 5. The error bars are the standard deviation for the measurements. The solid red line is the best fit described by the model in Equation 4 which has been used by others[15, 16]. In this case, λ_0 is 470 nm while k_m is computed from the gathered data and describes the exponential dependence on the wavelength of the melanin absorption spectrum. The value of relative absorption ranges from 0 to 1. The calculated value of k_m from the spectral data was 2.3 (R-squared = 0.73) and agrees well with values which range from 2.1–2.4 reported by others for eumelanin[17, 18, 19].

$$Relative\ Absorption = \exp \left[-k_m \frac{\lambda - \lambda_0}{\lambda_0} \right] \quad (4)$$

The dashed black line is the absorption spectrum of eumelanin as gathered by other researchers[20, 21]. Previous literature suggests that melanin in melanoma cells may be exclusively eumelanin[22]. Eumelanin is largely responsible for the black and brown hues, while pheomelanin is responsible for red hues and neuromelanin is only found in dopaminergic neurons[23, 24]. A photoacoustic spectrum of AuNP labeled breast cancer cells was not attainable as PA signal from the AuNPs change with every successive pulse of irradiation.

3.4 Population Distribution of Protein Content

For Figure 6a, 25 melanoma cells were irradiated with one pulse of 532 nm and the V_{p-p} was normalized with the radiant exposure for the pulse. A histogram of absorption coefficients (cm^{-1}) was created using the normalized V_{p-p} response along with Equation 3 to understand the population distribution of melanin content, the main PA emitter, inside of melanoma cells. As is apparent in Figure 6a, the distribution is not Gaussian and skewed to the left. For Figure 6b, a similar procedure was followed with 25 AuNP labeled breast cancer cells. A histogram of absorption coefficients was generated to display the population distribution of EpCAM content, since the AuNP labels were targeted to the EpCAM surface antigen.

Breast cancer cells which did not have bound AuNPs were not found to produce any detectable PA emissions with the current experimental setup. This demonstrates that AuNPs were the only PA emitter on the breast cancer cell. Melanin was verified as being the primary PA emitter for the melanoma cells through the photoacoustic emission spectrum in Figure 5.

3.5 UV-A Induced Melanin Production

Melanoma cells which had been pre-incubated with retinal were exposed to UV-A and their instantaneous melanin synthesis was measured with photoacoustics. As seen in Figure 7, all cells produced more melanin after a 10 minute exposure to UV-A light. Cells 2 – 4 produced the most melanin directly after exposure while Cell 1 continued producing melanin even 30 minutes after exposure. Single-factor ANOVA tests on each cell reached significance with all p – values < 0.05 , demonstrating that melanin content in a cell changed after UV exposure.

Since the PA response of the melanoma cells was measured before exposure to UV-A, the results from this experiment further support the claim that the cells were not harmed by the radiant exposures used in this study. They continue to function and produce more melanin after the initial course of irradiation.

4 Discussion

As shown in Figure 3, the microscopic PA system has a wide linear range from 120–4000 μM of Direct Red 81 Dye which results in an absorption coefficient range from 6–170 cm^{-1} . Though that was the only range tested in this study, other researchers have demonstrated the linear range of photoacoustics is on the order of 10^2 – 10^3 :1 [13, 25]. The data on Figure 3 help demonstrate that the system created for this study was indeed linear over most of the transducer responses encountered.

Other than linearity of response, it was important to be certain that the technique was not harmful to the cells themselves or the melanin pigment. In Figure 4, both melanoma cells and AuNP labeled breast cancer cells were exposed to a total of 20 pulses over the course of 9 minutes. The PA response from the melanoma cells did not change significantly (for statistical results refer to Section 4) over that course of irradiation indicating that the melanin pigment was not being bleached. Additionally, the cells were visually inspected in between pulses to determine the occurrence of laser induced damage but none was seen in

either melanoma or breast cancer cells. A visual inspection was necessary since a dye based test for cell viability was difficult to implement as it would introduce optical absorbers into the environment.

Though the PA response from melanoma cells did not change, the response from AuNP labeled breast cancer cells dropped quickly indicating a bleaching effect with the nanoparticles. Though the results are not reported here, it was noted that when the radiant exposure of pulses exceeded 130mJ/cm², melanin could also be bleached and melanoma cells could be damaged depending on the melanin content of the cells.

The PA emission spectrum in Figure 5 of melanoma is in good agreement with the optical and PA absorption spectra of eumelanin, pheomelanin, and melanoma cells collected by others[20, 21, 15, 26]. Though it is possible that there are other optical absorbers in the melanoma cells, melanin seems to be the major PA emitter due to the likeness of the PA emission spectra to the optical absorption spectra of the two major types of melanin. Furthermore, this method demonstrates a technique to measure the optical absorption spectra of single cells using photoacoustic emission. However, the PA emission of AuNP labeled breast cancer cells was not optically characterized since the PA signal from AuNPs would change after a few pulses as demonstrated in Figure 4.

From Figure 6, it is apparent that the labeling of breast cancer cells with AuNPs made them equivalent emitters to melanoma cells as the photoacoustic response distribution between the two cell types is comparable. All cells in both cell types generated detectable PA responses and they ranged by almost 10 times.

The average melanoma cell emitted a PA response of about 0.6mV_{p-p}/mJ/cm² which is equivalent to approximately 95 cm⁻¹ as can be inferred from Equation 3. Equation 5 describes the functional relationship between $\mu_{a,cell}$ - the absorption coefficient of the melanocyte, f_{mel} - the volume fraction of melanosomes within the cell, and $\mu_{a,mel}$ - the absorption coefficient of a single melanosome[27]. The absorption coefficient of a single melanosome was calculated by Jacques and McAuliffe to follow Equation 6, which produces a $\mu_{a,mel}$ of 555 cm⁻¹ for 532 nm[28]. Using this value of $\mu_{a,mel}$ and Equation 5, we can infer a volume fraction of 0.171 or 17.1% when the absorption coefficient of the entire cell is 95 cm⁻¹.

$$\mu_{a,cell} = f_{mel} \cdot \mu_{a,mel} \quad (5)$$

$$\mu_{a,mel} = 1.7 \times 10^{12} \cdot \lambda_{nm}^{-3.48} \quad (6)$$

The molar extinction coefficient of eumelanin and pheomelanin at 532 nm is interpolated to be about 1100 cm⁻¹/mole/Liter from data gathered by other researchers[20, 21]. From the absorption coefficient of a melanosome (555 cm⁻¹) and the molar extinction coefficient, one can infer that the melanin concentration within a melanosome is about 505mM. However, the volume fraction of 0.171 makes the average intracellular concentration of melanin for the entire cell about 86mM. This is mathematically equivalent to dividing the measured

absorption coefficient of the entire cell, 95 cm^{-1} , by the molar extinction coefficient of melanin at 532 nm. This technique of calculating the average whole-cell concentration will be used for the rest of the study as it circumvents the possibility of natural variations of melanin concentrations in melanosomes and volume fractions. Light scattering from the single cells are not accounted for since simulations and experimental results from others have indicated that the forward scattered light intensity ($\theta = 2^\circ$) is 10–100 times higher than scattering in any other direction[29, 30, 31].

This demonstrates the utility of this microscopic PA system in measuring intracellular concentration of analytes in a single cell. Some of the darkest melanoma cells measured in this study, with the strongest PA emission, had a response of $1.9 \text{ mV}_{p-p}/\text{Radiant Exposure}$ implying a melanin concentration near 270mM. Overall, the melanin concentration in the HS936 cell line as measured by this system ranged from about 29–270mM and the absorption coefficient at 532 nm ranged from $32\text{--}300 \text{ cm}^{-1}$. Though this implies that most cells ($12 \mu\text{m}$ diameter) are optically thin, we have encountered optically thick cells under microscopy which were non-transparent. However, these cells are very infrequent (1 in 100 or 1000) and therefore, are not represented in a randomized study of 25 cells. This information on single cell optical absorption may be especially useful to those attempting to detect single cells of melanoma using photoacoustics[32, 33, 34, 35].

The AuNP labeled breast cancer cells had a similar range of absorption coefficients at 532 nm but a different concentration of nanoparticles due to the difference in concentration dependent extinction coefficients. An extinction coefficient of $5 \text{ cm}^{-1}/10^{11}$ nanoparticles/mL was determined with the spectrometer for the gold nanoparticles used in this study. The technique used to measure this value was similar to that followed for Figure 3b. Absorption coefficients of seven different concentrations of nanoparticle suspension were measured with an HR2000 Ocean Optics (Dunedin, FL) spectrometer and the slope of the linear fit ($R\text{-squared} = 0.98$ and $p < 0.001$) was used to determine the concentration dependent extinction coefficient (data not shown).

The average AuNP labeled breast cancer cell had an absorption coefficient of 110 cm^{-1} with a the range of absorption coefficients from $47\text{--}330 \text{ cm}^{-1}$. The effective concentration range is about $0.9 - 6.6 \times 10^{12}$ AuNPs/mL as inferred from the concentration dependent extinction coefficient. Most cells had a diameter of about $12 \mu\text{m}$ and therefore, a volume of 9×10^{-13} Liters or 9×10^{-10} mL, assuming the cells are spherical. From the concentration and the volume of a cell, a $850\text{--}5900$ AuNPs/cell range can be inferred with the average breast cancer cell having about 2000AuNPs/cell. These calculations assume a homogeneous distribution of nanoparticles within the cell since nanoparticle aggregation will change optical and ultrasonic properties causing an underestimation of nanoparticle load. Though this introduces some error in the measurement, this is the first reported instance of quantification of gold nanoparticles in single living cells which can be useful for those who use AuNPs to enhance photoacoustic detection[36, 37, 38].

Cook et al found concentrations of iron nanoparticles on histological (fixed) samples to be higher by an order of magnitude[6]. This discrepancy could be because Cook et al incubated

their samples with more than 100 times the concentration of nanoparticles for 44 times as long when compared to the protocol used in this study.

It is difficult to formulate a mathematical relationship between the AuNP concentration per cell and the EpCAM content. From Figure 6b, we can infer that the AuNP concentration was not saturated because there was a variation of photoacoustic response from different cells. Since there is no obvious connection directly linking the AuNP concentration to the number of EpCAM receptors, the label content per cell is only indicative of the EpCAM content in a relative sense.

Though all cells tested for melanin were from the same melanoma cell line, HS936, there was up to a 10 times difference in melanin concentration from the lightest to the darkest cell. This same trend was seen with EpCAM expression in the T47D cell lines as can be inferred from the range of intracellular concentrations of AuNPs. However, the skewed shape of both distributions imply that most of the cells (about 80%) contain similar amounts of melanin or EpCAM, but there are outliers (about 8%) which contain much higher concentrations of protein when compared to most of the population.

In Figure 7, four cells were pre-incubated with retinal and then exposed to UV-A light in order to induce melanin synthesis and measure the change over time. Wicks et al reported that healthy human melanocytes could increase their melanin content by about 20% on average in the first hour after UV-A exposure[12]. Cells 2–4 in Figure 7 demonstrate that quite well as they increase in melanin content by about 30% but do so during and directly after UV-A exposure.

This seems to be a much faster process than that reported by Wicks et al (1 hour) since most of the melanin synthesis for melanoma Cells 2–4 happens within the first 15 minutes. Cell 1, on the other hand, increases its melanin content by about 45% within the first fifteen minutes and then continues to double it by the end of the hour with a short period of stagnation 20 minutes after exposure. Based on the results of Wicks et al, we should expect a slow continuous production of melanin but there may be some unexplored mechanism which starts producing melanin during UV exposure and such activity could not be previously measured due to the lack of technology. However, differences with the results of Wicks et al and in between cells in this study may also be attributed to the fact that these are cancer cells which may behave erratically when compared to healthy melanocytes.

Cells 2–4 increase their melanin concentration by about 20–50mM in 15 minutes, while Cell 1 increases its melanin concentration by about 175mM in the 60 minute time course. The four cells produced melanin at the average rate of 37mM per 15 minutes. An average melanin mass production rate of 5.7 pg per 15 minutes can be calculated using the volume of a single cell, as previously calculated, and the molar mass of eumelanin which is 171 g/mole[39]. This is a fast production rate of about 0.6% of total cell mass added in 15 minutes considering that the mass of a single cell is approximately 1 ng, which can be calculated from the volume of a single cell and the average density of melanocytes measured by other researchers[40].

The current results demonstrate the ability to study time scales and quantity of melanin synthesis with response to environmental factors, like UV-A exposure, on a single cell level. Furthermore, the technique developed in this study was able to shed light on the range of melanin concentrations (29–270mM) in single HS936 melanoma cells and also quantify the number of AuNPs in breast cancer cells (850–5900 AuNPs/cell). The distribution of PA responses from both cell types indicated that the concentration of proteins did not fit the parameters of a normal distribution. Further studies need to be completed to investigate the time dynamics of melanin synthesis and see how UV-A exposure might effect the overall population distribution of melanin content in the melanoma cells as opposed to healthy melanocytes.

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Figure 1.

Part a) is a schematic of the single cell irradiation setup. Light exiting an optical fiber is collimated through a lens (1) and sent through an aperture in the camera port (3) and out of the objective in order to irradiate the cell (7). The PA signal generated is detected by a focused ultrasonic transducer (4), amplified (5), and sent to an oscilloscope (6). The cell was visualized throughout the procedure with a live camera feed to a computer (2). Parts b) and c) are captured frames from the live camera view demonstrating the laser beam position and diameter with 50 micron scale bars.

Figure 2.

The fluid chamber array contains different concentrations of Direct Red 81 dye used to calibrate the PA measurement. This figure shows the top and side views of the chamber.

Figure 3.

Part a) plots the PA response to increasing concentrations of dye where the V_{p-p} measured in mV was normalized with the radiant exposure. Part b) plots the calculated absorption coefficient, μ_a , from spectrometric measurements. From this data, a normalized voltage measurement can be used to infer an absorption coefficient.

Figure 4.

Part a) plots the relative PA response as single cells of melanoma or AuNP labeled breast cancer are irradiated with 5 pulses every 3 minutes. Each point is the average relative response of melanoma cells (n=10) or breast cancer cells (n=5) along with the standard deviation as error bars. Part a) demonstrates that while PA signal from melanin in melanoma cells did not change, the signals from the AuNPs on the labeled breast cancer cells did. Part b) and c) are captured frames from the live camera feed demonstrating cells which were subjected to irradiation.

Figure 5.

Part (a) Plots the relative PA emission of melanoma cells over 470–650 nm. The PA spectra of five different melanoma cells were scaled to 1 and then averaged to produce each point on the graph. The error bars are the standard deviation for each measurement. The continuous red line and black dashed-line represent a model fit and data collected by previous researchers, respectively (find citations in Section 3.3). Part (b) is an example of a photoacoustic signal from a single melanoma cell.

Figure 6.

Demonstrates the population distribution of absorption coefficient of single melanoma and breast cancer cells. For melanoma cells, this is proportional to the melanin content and for breast cancer cells, it is proportional to the AuNP label content. Since the AuNPs on the breast cancer are targeted to the EpCAM surface antigen, it is indicative of the EpCAM content of breast cancer cells for the T47D cell line.

Figure 7.

Demonstrates melanin synthesis response as function of UV light exposure. After 10 minutes of exposure to UVA radiation, the melanoma cells produce more melanin as indicated by the increase in the absorption coefficient as measured by the PA response. Each line follows a different cell as it was tracked for 60 minutes and each point is the average of 4 pulses. The bars shown are the standard errors of the means.