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Optical Metabolic Imaging Identifies Tumor Cell-Cycle Status on a Single-Cell Level

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Abstract

The goal of this study is to validate fluorescence intensity and lifetime imaging of metabolic co-enzymes NAD(P)H and FAD (optical metabolic imaging, or OMI) as a method to quantify cell-cycle status of tumor cells. Heterogeneity in tumor cell-cycle status (e.g. proliferation, quiescence, apoptosis) increases drug resistance and tumor recurrence. Cell-cycle status is closely linked to cellular metabolism. Thus, this study applies cell-level metabolic imaging to distinguish proliferating, quiescent, and apoptotic populations. Two-photon microscopy and time-correlated single photon counting are used to measure optical redox ratio (NAD(P)H fluorescence intensity divided by FAD intensity), NAD(P)H and FAD fluorescence lifetime parameters. Redox ratio, NAD(P)H and FAD lifetime parameters alone exhibit significant differences ($p < 0.05$) between population means. To improve separation between populations, linear combination models derived from partial least squares - discriminant analysis (PLS-DA) are used to exploit all measurements together. Leave-one-out cross validation of the model yielded high classification accuracies (92.4 and 90.1% for two and three populations, respectively). OMI and PLS-DA also identifies each sub-population within heterogeneous samples. These results establish single-cell analysis with OMI and PLS-DA as a label-free method to distinguish cell-cycle status within intact samples. This approach could be used to incorporate cell-level tumor heterogeneity in cancer drug development.

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Keywords

quiescence; tumor dormancy; fluorescence lifetime; metabolic imaging; cell-cycle status; single-cell analysis

1. Introduction

The metabolism, drug response, and genetic expression of cells are heterogeneous within a single tumor, which affects cancer progression and response to treatment [1]. Specifically, quiescent cancer cells lack responsiveness to standard chemotherapies, introducing a major challenge to efficacy of cancer treatment [2–3]. Quiescent cell sub-populations promote tumor dormancy, which results in therapeutic resistance and later tumor recurrence. However, the mechanisms responsible for the emergence of quiescent cell populations and interactions between quiescent cells and the tumor or metastatic microenvironment are not well understood. This is largely due to the inability to distinguish quiescent cell populations from the bulk, proliferating population. Furthermore, quiescent cells can re-enter the cell cycle after years of remission, and initiate tumor recurrence [3]. Thus, it is essential to avoid misidentification of quiescent cells as apoptotic, despite their similarly reduced metabolic activity [3]. Robust methods to identify quiescent cells within the heterogeneous tumor mass are needed to develop improved cancer therapies that target these dormant, yet lethal, cell populations.

Flow cytometry is a standard measure of cell function, and is used to identify quiescent and resistant cancer cells in heterogeneous tumors. Flow cytometry applications are constrained to exogenous labelling using fluorescent antibodies or dyes to allow *ex vivo* sorting into pure cell populations. The use of these fluorescent labels is highly disruptive to cell physiology, limiting the applicability of flow cytometry [4]. Additionally, flow cytometry requires the dissociation of the sample into a single cell suspension *ex vivo*, and thus loses the microenvironmental and spatial context of each sub-population. Identifying and characterizing cell sub-populations within intact, heterogeneous tumors would inform on spatial relationships with supporting cells in the microenvironment. For example, interactions between dormant tumor cells and blood vessels, extracellular matrix, immune cells, and fibroblasts could be compared to those interactions with proliferating tumor cells within intact samples. Also, the use of flow cytometry prevents the study of dynamic changes in live tumors over time, because tumors must be removed and dissociated for analysis. Thus, a technology that could monitor dynamic changes over time, and identify tumor cell sub-populations within intact, living samples, could supplement current methods to provide a more complete picture of tumor heterogeneity.

Optical metabolic imaging (OMI) measures the autofluorescence intensities and lifetimes of the metabolic co-enzymes NAD(P)H and FAD. OMI is attractive for the study of tumor heterogeneity because it is non-invasive, does not use exogenous labels, can monitor dynamic changes within intact samples [5–8], including *in vivo* tumors [9–10], achieves cellular resolution, and is sensitive to cell metabolism [11]. OMI is sensitive to cell malignancy, cancer progression, and provides early measures of tumor cell drug response

[5–7]. The fluorescence intensities of NAD(P)H and FAD can be combined into the “optical redox ratio” (fluorescence intensity of NAD(P)H/FAD), which is sensitive to the relative amounts of electron donor and acceptor in a cell [12]. The redox ratio was established by Chance *et al.* [13] and has since been used for an array of applications in cancer, including studies of cancer progression, invasion, and drug response [5–8, 14]. Fluorescence lifetime imaging (FLIM) provides a complementary measurement to the redox ratio [9], and is sensitive to the enzyme binding activities of NAD(P)H and FAD [15]. Specifically, the protein-bound NAD(P)H lifetime is significantly longer than the free NAD(P)H lifetime, due to self-quenching in the free state [15, 19–23]. Conversely, FAD lifetimes are short and long in the protein-bound and free states, respectively [15]. Combined information from the fluorescence intensities and lifetimes of NAD(P)H and FAD provide a measure of the global metabolic activity in individual cells within intact samples [5, 13–18, 24], specifically on redox balance and enzyme binding activity. Previous studies have established that OMI is sensitive to cancer progression and drug response [5–7, 9].

The goal of this study is to use OMI to discriminate proliferating, quiescent, and apoptotic cell populations. We hypothesized that populations exhibiting varying cell cycle activity can be metabolically distinguished based on the NAD(P)H and FAD fluorescence lifetimes and redox ratio. Here, we demonstrate the feasibility of using OMI to identify sub-populations in an acute myeloid leukemia (AML) model, a well-defined model for observing cell-cycle status. Pure and co-cultured populations of each cell type were evaluated using OMI. The results illustrate that OMI can identify proliferating, quiescent, and apoptotic cell populations within heterogeneous samples. Therefore, this approach could be valuable in the development of new cancer therapies that target dormant and treatment-resistant cell sub-populations.

2. Materials and methods

2.1 Cell culture

Kasumi-1 cells (acute myeloid leukemia progenitors; ATCC) were suspended in standard RPMI 1640 culture medium with additives of 10% fetal bovine serum and 1% penicillin:streptomycin. Proliferation, quiescence, and apoptosis was achieved in separate cultures by: (1) refreshing standard RPMI media (no treatment, proliferation group), (2) substituting media supplemented with 250 nM JQ1 (a transcription inhibitor [25–27]; Bradner lab, quiescence group), or (3) substituting media supplemented with 2.1 μ M cytarabine (Ara-C, standard chemotherapy [27]; Vanderbilt pharmacy, apoptosis group). Cell seeding density was maintained at 2.5×10^4 cells per 35 mm glass bottom dish (MatTek). All imaging samples were overlaid with a coverslip immediately prior to imaging, to reduce motion artifact of suspended cells.

In a separate cohort, cell-cycle activity was validated with flow cytometry for each treatment group. Cell-cycle status was determined for apoptotic and proliferating populations using standard cleaved caspase 3 and Ki67 labeling, respectively. Cell-cycle status of the quiescent group was confirmed upon simultaneous Pyronin Y labeling of RNA content and Hoechst 33342 labeling of DNA content in proliferating and quiescent groups, based on lower RNA levels in quiescent cells compared with cells undergoing active proliferation [29]. Cells from

proliferation, quiescence, and apoptosis groups were seeded at a density of 2.5×10^6 cells per milliliter in 75-T tissue culture flasks. 72 hours after treatment, each culture was labeled with Ki67 antibody conjugated to FITC (proliferation; Life Technologies), cleaved caspase 3 (CC3) antibody conjugated to FITC (apoptosis; Life Technologies), Hoechst 3342 (quiescence; Sigma) and pyronin Y (quiescence; Sigma) to confirm cell-cycle status of each respective culture via flow cytometry. Population fluorescence thresholds, or gates, for cell sorting were established by autofluorescence flow cytometry (no antibody labeling) of cells from each population. Experts in flow cytometry established these gates based on standard practice. Percent of positively stained cells per sample was calculated as the ratio of cells exhibiting fluorescence intensities beyond established thresholds to total cell count.

2.2 Verification of Metabolism with Varied Cell Cycle Status

The dominant role of oxidative phosphorylation in leukemic progenitor cells was confirmed upon perturbation with cyanide. Proliferating Kasumi-1 cells were plated on 35 mm glass dishes at 2.5×10^4 cells per dish after a 72-hour incubation period. Following acquisition of NAD(P)H and FAD images, cell media was replaced with media supplemented with 4mM NaCN (Sigma) and imaged five minutes after media change [9]. Redox ratio measurements were normalized to untreated proliferating samples for comparison between treatment groups.

Unlike proliferating leukemic cells, quiescent leukemic cells rely on fatty acid oxidation, which is a feeder reaction for oxidative phosphorylation [28, 30]. JQ1 is known to induce quiescence in leukemic cells, forcing them to rely on fatty acid oxidation [25–27, 30]. To verify the metabolism of JQ1-treated (quiescent) cells, proliferating and quiescent cell groups were resuspended in media supplemented with 100 nM of etomoxir (Sigma), an inhibitor of fatty acid oxidation [30]. Following treatment, cells were incubated for 48 hours and imaged using fluorescence lifetime imaging [30]. As previously discussed, redox ratio comparison between treatment groups was possible following normalization to untreated proliferating samples.

2.3 Fluorescence Lifetime Imaging

FLIM was conducted on a multi-photon fluorescence lifetime microscope (Bruker Nano Inc.) built around a Nikon Ti:E inverted microscope using a $40\times$ (1.3NA) oil-immersion objective. A titanium:sapphire (Chameleon Ultra II, Coherent) laser was used for excitation of NAD(P)H (750 nm) and FAD (890 nm) autofluorescence. A 400–480 nm bandpass filter isolated NAD(P)H fluorescence emission. A 500 nm high pass dichroic mirror and a 500–600 nm bandpass filter isolated FAD fluorescence emission. Both NAD(P)H and FAD fluorescence emission were detected with a GaAsP photomultiplier tube. Fluorescence lifetime images were acquired using electronics enabling time-correlated single photon counting (TCSPC). Acquisition of $170 \times 170 \mu\text{m}$ (256×256 pixels) images required a 60-second integration time and pixel dwell time of 4.8 microseconds. Photobleaching of the sample was avoided during image acquisition by maintaining photon count rates at approximately $1\text{--}2 \times 10^5$ photons/second. Fields-of-view for NAD(P)H and FAD were the same for each image location within a sample. Four fields-of-view were acquired from each dish, and fields-of-view were selected to control for cell number per field-of-view across all

experimental groups. An average of 40–60 cells were included in each field-of-view, resulting in 150–250 cells from each dish. All reported data represent a single replicate. Three independent replicates were conducted for all experiments, each demonstrating agreement with observed trends in reported data. Average power was measured daily for all experiments and maintained at approximately 6.5–6.9 mW for NAD(P)H excitation and 7.4–7.7 mW for FAD excitation.

2.4 Image Analysis

Fluorescence lifetime fits were calculated using SPCImage (Becker & Hickl GmbH). Fluorescence contributions from background and cell nuclei were minimized via thresholding. The second harmonic generated signal from urea crystals at 900 nm excitation was used to measure the instrument response function, which was found to have a full width at half maximum of 220 ps. The measured instrument response function was deconvolved from the experimentally measured decay curve prior to curve fitting. All decay curves were fit to a two-component exponential model:

$$I(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2} + C \quad (1)$$

where $I(t)$ is the fluorescence intensity at time t following laser excitation, τ_1 and τ_2 are the short and long fluorescence lifetimes, α_1 and α_2 represent the short and long lifetime component contributions, and C represents background light contribution. A two-component decay curve was used to represent the free and protein-bound conformations of NAD(P)H and FAD [7]. Respective mean lifetimes of NAD(P)H and FAD were calculated as a weighted average of the short and long lifetimes ($\tau_m = \alpha_1 \tau_1 + \alpha_2 \tau_2$). Fluorescent bead (YG microspheres, Polysciences Inc.) measurements were acquired daily to validate fluorescence lifetime values. Bead lifetime measurements (1.9 ± 0.09 nanoseconds) were consistent with values reported in literature [8–9].

Photon counts from the NAD(P)H and FAD fluorescence lifetime images were integrated over the fluorescence decay time on a per-pixel basis to generate NAD(P)H and FAD intensity images. The redox ratio for each pixel per image was calculated by dividing NAD(P)H fluorescence intensity by FAD fluorescence intensity per pixel. The redox ratio was normalized to redox ratio measurements of a control, proliferating sample taken each day to account for variations in relative laser powers across days and excitation wavelengths.

A customized CellProfiler pipeline was used to segment individual cell cytoplasms (nucleus excluded). The steps of the pipeline were as follows: (1) threshold the low intensity nucleus from lower intensity background and higher intensity cytoplasm, (2) propagate to identify the cell boundary, and (3) subtract the nucleus from the cell boundary. This mask was applied to all cells in all images to compute a mean redox ratio, NAD(P)H τ_1 , τ_2 , α_1 , τ_m , and FAD τ_1 , τ_2 , α_1 , τ_m , for each cell cytoplasm in each image (9 total variables) [5].

2.5 Threshold for Classification Based on Individual Variables

Thresholds were used to classify cells as apoptotic, proliferating, or quiescent based on each of the 9 individual variables (redox ratio, NAD(P)H τ_m , τ_1 , τ_2 , and α_1 , FAD τ_m , τ_1 , τ_2 , and α_1). Two thresholds per variable were defined from receiver operator characteristic (ROC) analysis as a value that maximizes the true positive rate while minimizing the false positive rate between the low- and mid-range populations and the mid- and high-range populations. Classification accuracies and confusion matrices were calculated from the number of misclassified cells.

2.6 Partial Least Squares - Discriminant Analysis

To obtain maximal separation between groups with differing cell-cycle activity, partial least squares – discriminant analysis (PLS-DA) [32] was used to incorporate 7 fluorescence variables (NAD(P)H τ_1 , τ_2 , α_1 ; FAD τ_1 , τ_2 , α_1 ; redox ratio). This technique performs iterations of linear transformations on a matrix of samples with known classification. These transformations are then projected onto a vector comprised of classification values for each sample. The resultant equation represents an optimal boundary between all classes and provides information about the significance of each variable included in model construction, unlike other classification methods [32]. PLS-DA determines the contribution of each independent variable incorporated into the model, thus NAD(P)H and FAD τ_m were excluded from generation of our model. Weight centering of all groups was applied to minimize bias in separation boundaries influenced by uneven cell numbers. Weight centering was achieved by calculating the difference between individual values in the data set and the average of means across all groups [33]. Separate models were developed for two-group and three-group classification to overcome skewed weighting criteria by the training data set. In the case of three-group classification, one-vs-all PLS-DA was applied to calculate distinct separation boundaries for all three classes, ensuring appropriate transformation of the known training data set for satisfactory class representation [32–33]. Calculation of the Mahalanobis distance from each class mean to all data points ensured appropriate assignment of separation boundaries [33].

Images were acquired from pure samples of apoptotic, proliferating, or quiescent cells 72 hours post-treatment. Data from all three conditions were combined to form comprehensive datasets for PLS-DA model development and evaluation using leave-one-out cross validation (LOOCV) in the R software package. Each cell within these comprehensive datasets (n samples) was assessed by exclusion prior to PLS-DA analysis, partitioning the 1 excluded cell as the validation sample and the remaining $n-1$ observations as the training set. The PLS-DA model was then developed based on the $n-1$ training set data, and model classification performance was evaluated on correct classification of the 1 excluded cell. This approach was repeated n times, to classify all n cells in the comprehensive data set.

PLS-DA was also implemented in the R software package. Linear combination models based on the 7 fluorescence variables were separately generated for the two-group and three-group cases. Weights represent the relative contribution of the variable toward overall covariance of the training dataset in relation to its known class. Confusion matrices representing the number of misclassified cells per class from the validation set were

generated, with each matrix column and row corresponding to the true and predicted classes, respectively. Evaluation metrics (i.e. classification accuracy, sensitivity and specificity) were subsequently calculated for each model, based on the validation set. Generated PLS-DA models were evaluated using the posterior probability of class membership, which was calculated for individual validation set samples.

For experimental demonstration of these models, heterogeneous samples were generated by plating cells from each population at various proportions. Images were acquired immediately following plating. For the two-group demonstration set, proliferating and quiescent cells were plated at the following proportions: 100:0, 30:70, 50:50, 70:30, 0:100 (proliferating : quiescent). The three- group demonstration included plating proportions of 33:33:33, 50:25:25, 25:25:50 (proliferating : quiescent : apoptotic). Two- and three-group PLS-DA models generated from the entire training data sets were applied for single-cell identification within these mixed samples. Color-coded images were produced to demonstrate the model's ability to resolve each population within the co-cultures, with color assignments corresponding to the model-determined cell-cycle status of individual cells. Confirmation of cell-cycle status for each cell in each field-of-view was not feasible in the heterogeneous samples.

2.7 Statistical Analysis

A Wilcoxon rank sum test of the redox ratio and lifetime parameters (τ_m , τ_1 , τ_2 , and α_1) for NAD(P)H and FAD was used to assess significant differences between groups using these 9 individual variables alone. A Tukey multiple comparison test was used to correct for multiple comparisons across cell types. Error bars are representative of the mean $\pm$ standard error of the mean. Statistical significance was indicated with an α value less than 0.05.

3. Results

3.1 Validation of Homogeneous Populations

Pure populations of apoptotic, quiescent, and proliferating cells were generated by replacing standard cell culture media with media supplemented with cytarabine, the bromodomain and extra terminal (BET) family inhibitor JQ1, or no supplementary agent, respectively. Cell-cycle status for each population was validated by labeling with antibodies or cellular component dyes followed by flow cytometry (see methods). Histogram plots of the apoptotic group show significantly higher FITC-CC3 fluorescence from the labeled population compared to the negative control (unstained cells), and 97.5% of cells in the labeled group were positive for apoptosis (Figure 1A). Similar differences in fluorescence signal were observed in the proliferating group for FITC-Ki67 labeled vs. negative control, and 99.7% of cells in the labeled group were positive for proliferation (Figure 1B). Overlaid dot density plots of the labeled proliferating and quiescent groups show distinct separation between each population based on lower RNA content (Pyronin Y) in quiescent cells as expected, with 93.4% cells in the double-stained quiescent population exhibiting decreased Pyronin Y fluorescence intensity compared to the proliferating population (Figure 1C). This is comparable to previous studies reporting elevated Pyronin Y levels in proliferating populations [29]. Similarly, apoptotic populations can be distinguished with this double-

staining method as they exhibit extremely low Hoechst and Pyronin Y fluorescence [25]. Additional cohorts of quiescent populations labeled with Ki67 and cleaved caspase-3 yielded less than 5% of cells positive for either marker, further supporting a quiescent phenotype (data not shown).

3.2 Metabolic Perturbations in Proliferating and Quiescent Cells

A series of metabolic inhibitors were used to perturb dominant metabolic pathways in proliferating and quiescent leukemia cells. These perturbations verify that our autofluorescence measurements accurately reflect changes in active metabolic pathways in these cells. Cyanide perturbation assessed the metabolism of proliferating human acute myeloid leukemia cells. Cyanide inhibits oxidative phosphorylation, resulting in an abundance of NADH in the cytoplasm. The significant increase in the redox ratio after cyanide treatment (Figure 2A) agrees with the reported dominance of oxidative phosphorylation in leukemic cells [28]. Similarly, the reduced NAD(P)H τ_m is consistent with previous studies of cyanide treatment in cancer cells (Figure 2B) [5–7]. Trends in FAD τ_m were also comparable to previously reported data (Figure 2C) [9]; however, there was not a significant change in the FAD τ_m following addition of cyanide in these cells.

Quiescent Kasumi-1 cells rely largely on fatty acid oxidation (Figure 3). Etomoxir treatment increased the redox ratio of quiescent cells (Figure 3B). The increase in the redox ratio after etomoxir treatment in quiescent cells indicates that inhibition of fatty acid oxidation also inhibits downstream oxidative phosphorylation in quiescent cells. No significant change in the redox ratio was observed in proliferating cells treated with etomoxir, which indicates that these proliferating cells have less dependence on fatty acid oxidation [28]. Furthermore, changes in NAD(P)H τ_2 were not statistically different ($p > 0.05$) before vs. after etomoxir treatment in proliferating cells. Significant differences in NAD(P)H τ_m , τ_1 , τ_2 , and α_1 were observed ($p < 0.05$) between proliferating and quiescent cells prior to etomoxir treatment. Perturbation with etomoxir yielded significant increases in NAD(P)H τ_m , τ_1 , τ_2 , and α_1 ($p < 0.05$), similarly demonstrating contrast between proliferating and quiescent populations. Etomoxir resulted in significant decreases in FAD τ_m , τ_1 , τ_2 , and α_1 following treatment ($p < 0.001$, data not shown).

3.3 OMI of Proliferating, Quiescent, and Apoptotic Cells

OMI was performed in acute myeloid leukemia cells forced into apoptotic, proliferating, and quiescent states, to validate OMI as a method for classifying these cell states. Untreated (proliferating), JQ1-treated (quiescent), and cytarabine-treated (apoptotic) Kasumi-1 cells were imaged at 72 hours post-treatment. Two-photon fluorescence lifetime imaging revealed qualitative differences in the redox ratio, NAD(P)H τ_m , and FAD τ_m within proliferating, quiescent, and apoptotic cell populations (Figure 4). Quiescent cells exhibited a distinct decrease in redox ratio across all cells in comparison to both proliferating and apoptotic groups (Figure 4). Longer NAD(P)H and FAD mean lifetimes were observed in apoptotic cells than in proliferating and quiescent groups. Cellular morphology can also provide some discrimination between cells with varied cell-cycle status. In contrast to the apoptotic and quiescent groups, proliferating cells were characterized with a well-defined nuclear area (Figure 4). Quiescent cells exhibited a significant decrease in overall cell area (Figure 4)

compared to apoptotic populations ($p < 0.05$, $72.5 \pm 3.99 \mu\text{m}^2$ vs. $96 \pm 9.06 \mu\text{m}^2$) and modest, but not significant, differences compared to proliferating populations ($84.5 \pm 4.30 \mu\text{m}^2$).

Quantitative OMI analysis can distinguish proliferating, quiescent, and apoptotic samples on a population-level (Fig. 5). Each cell was treated as an independent measurement, across all 4 FOVs (40–60 cells per FOV) per dish and all 3 replicate dishes per experimental group (proliferating, apoptotic, quiescent). The redox ratio is increased in proliferating cells compared to apoptotic and quiescent cells, and is also increased for apoptotic cells compared to quiescent cells ($p < 0.001$, Figure 5A). Both NAD(P)H and FAD τ_m are highest in apoptotic populations ($p < 0.001$), but lack significance between proliferating and quiescent cells (Figures 5B and C). Proliferating cells exhibited higher NAD(P)H τ_1 and α_1 compared to apoptotic and quiescent cells ($p < 0.001$, Figure 5D,F). Like the redox ratio, NAD(P)H τ_2 is significantly different between all three groups ($p < 0.001$, Figure 5E). FAD τ_1 was only effective in discriminating quiescent cells from the other two conditions ($p < 0.001$, Figure 5G). Apoptotic cells exhibited significant differences in FAD τ_2 and α_1 compared to proliferating and quiescent cells ($p < 0.05$, $p < 0.001$, Figures 5H and I). Overall, significant differences exist between cell groups in Figure 5A–I, but there is still overlap between apoptotic, proliferating, and quiescent cells at the single-cell level. Furthermore, classification of cells to all three categories (apoptotic, proliferating, quiescent) based on any OMI variable alone yields poor overall classification accuracy (Table 1). Therefore, classification based on a combination of all measured OMI variables could improve separation between cell groups.

3.4 Classification Model with Partial Least Squares – Discriminant Analysis (PLS-DA)

PLS-DA was performed to maximize separation between cell-cycle status groups on the single-cell level. The first iteration of PLS-DA aimed to separate proliferating and quiescent cell populations. This two-group PLS-DA model (Table 2) was created from an initial training data set, consisting of measurements of individual cells from pure samples of quiescent and proliferating cells (Figure 6). The weights of each fluorescence variable (NAD(P)H τ_1 , τ_2 , α_1 ; FAD τ_1 , τ_2 , α_1 ; redox ratio) included in two-group PLS-DA model based on the entire training data provide a general sense of the influence of each variable (Table 2). Cross-validation using the leave-one-out approach demonstrated classification accuracy of 92.4% for the generated model. The two-group PLS-DA showed improved separation of proliferating and quiescent populations (Figure 6) compared to any single variable (Figure 5). A confusion matrix representing overall classification performance of individual cells shows low rates of misclassification across classes for the two-group model and resulted in a sensitivity and specificity of 96.1% and 84.7%, respectively, for discriminating between both groups (Table 3).

This approach was extended to include all three populations using one-vs-all multiclass PLS-DA, again with measurements of pure samples from apoptotic, proliferating, and quiescent cells for the initial training data set. Variable weights for PLS-DA model based on the entire three-group training data set are also reported (Table 2). Similarly, the three-group PLS-DA showed improved separation between proliferating, quiescent, and apoptotic populations on a per-cell basis (Figure 7) compared to any single variable (Figure 5),

yielding classification accuracy of 90.1%. A confusion matrix representing overall classification performance of individual cells shows low rates of misclassification across classes for the three-group model (Table 4). Model classification yielded sensitivities of 87.6% (apoptosis), 82.3% (proliferating), and 97.5% (quiescent), and specificities of 97.9% (apoptosis), 95.5% (proliferating), and 93.7% (quiescent) for discriminating indicated populations from all other sample populations.

3.5 Application of PLS-DA Models to Two- and Three-Group Co-cultures

The PLS-DA model accurately identified all cells in homogenous samples of each population not included in initial model training, supporting translation to heterogeneous samples. The two- and three-group PLS-DA were subsequently applied to mixed co-cultures of proliferating, quiescent, and apoptotic cells. Two-group PLS-DA identified proliferating cells and quiescent cells at each plating proportion (Figure 8A–E). The proportion of cells classified as proliferating : quiescent (38:62, 46:54, 64:36) are similar to their respective plated proportions (30:70, 50:50, 70:30) (Fig. 8F).

Next, the three-group model was demonstrated on mixtures of all three populations (Figure 9). The three-group model enabled identification of apoptotic, proliferating, and quiescent populations in mixed samples plated at varied proportions (Fig. 9A–C). All populations were resolvable regardless of plating proportion. The proportion of cells classified as proliferating : quiescent : apoptotic (35:37:28, 42:31:27, 19:34:47) are similar to their respective plated proportions (33:33:33, 50:25:25, 25:25:50) (Fig. 9D).

4. Discussion

Tumor heterogeneity is a significant challenge in new drug development and treatment planning in cancer [1]. There is a need for methods to assess tumor heterogeneity in intact samples without the use of contrast agents, so that improved therapies can be developed for heterogeneous tumors. Metabolism is an attractive measure of tumor heterogeneity, due to its fundamental role in malignant transformation and in drug resistance [1, 11, 14]. Our results demonstrate that OMI can discriminate cell-cycle status on a single-cell level in cell lines, without the use of contrast agents. OMI has been previously used to study single-cell response *in vivo* [5, 9–10, 14] and in live, three-dimensional cultures [8, 34–35]. The current study supports the use of OMI to quantify cell-cycle heterogeneity within these *in vivo* and three-dimensional *in vitro* samples. Therefore, the proposed methods can provide further insight into strategies to circumvent drug resistance.

To our knowledge, this study is the first to apply label-free imaging techniques and classification algorithms to classify the cell-cycle status of single cells. Previous studies have used fluorescent labels to characterize the metabolism of leukemic cells in different cell-cycle phases. Oxidative phosphorylation was reported as the primary energy source for leukemic progenitor cells, by observing levels of reactive oxygen species produced by these cells [28]. These findings are consistent with our studies, specifically with the increased redox ratio in Kasumi-1 cells upon inhibition of oxidative phosphorylation with cyanide ($p < 0.05$, Fig. 2). Oxidative phosphorylation is driven by metabolites produced in upstream pathways, which promote these cells to adopt different functional phenotypes. Similarly,

analysis of the oxygen consumption of leukemia progenitors in proliferating or quiescent states showed that proliferating leukemic cells use more glycolytic metabolism compared to quiescent cells [30]. Furthermore, perturbation with a fatty acid oxidation inhibitor (etomoxir) eradicated the quiescent cells [27], suggesting these cells rely more heavily on fatty acid oxidation. Our results with the same fatty acid oxidation inhibitor (Figure 3) are consistent with these previous findings [30], and validate the reliance of quiescent leukemic cells on fatty acid oxidation (in contrast to proliferating leukemic cells). Additionally, comparisons of the redox ratio, NAD(P)H and FAD lifetime parameters (τ_m , τ_1 , τ_2 , and α_1) across proliferating, apoptotic, and quiescent cells indicate that metabolism differs with cell-cycle status (Figure 5).

The redox ratio, NAD(P)H and FAD lifetime parameters (τ_m , τ_1 , τ_2 , and α_1) individually demonstrate poor classification accuracy in distinguishing proliferating, apoptotic, and quiescent cells *in vitro* (Table 1). To optimize single-cell identification, we applied PLS-DA to generate a linear combination model of 7 measured OMI variables (NAD(P)H τ_1 , τ_2 , α_1 ; FAD τ_1 , τ_2 , α_1 ; redox ratio). Our results show that this PLS-DA improves the separation of apoptotic, proliferating, and quiescent cells in pure samples (Figure 6 and Figure 7). Both the two- and three-group PLS-DA models exhibited increased classification accuracy compared to the classification accuracy with any metabolic variable alone (Tables 1, 3, and 4). These PLS-DA models combined with OMI enable visualization of tumor heterogeneity on a single-cell level.

The PLS-DA models were applied to OMI images of mixed cultures of proliferating, apoptotic, and quiescent cells. This mixed-culture sample did result in reduced sensitivity of the PLS-DA model to apoptotic cells compared to the pure samples. This reduced sensitivity is likely due to drug removal from the media for co-culture experiments [36]. Furthermore, the plated ratio does not directly reflect the imaged ratios of each cell-cycle group within mixed samples. We hypothesize that discrepancies between plating and imaged ratios could be due to secretions from apoptotic cells, which affect the cell-cycle status of other cells in the culture [36]. Additional discrepancies arise from sampling error for the small field-of-view of the microscopy images vs. the overall plated ratio. These discrepancies between plated and imaged ratios in co-culture are consistent with those observed in our previous studies [37], in which cell morphology was used for gold standard classification of individual cells in microscopy images (cell morphology is not sufficient to discriminate the cell cycle in the current study). These results suggest that OMI combined with PLS-DA can identify proliferating, apoptotic, and quiescent cells co-culture (Figures 8 and 9).

The methods demonstrated here have the potential to contribute to studies of tumor dormancy and drug resistance. Tumor dormancy has been identified as a primary source of resistance to conventional therapies, because non-proliferating tumor cells lack treatment response [1–2]. There are many theories on the mechanisms driving tumor dormancy. One predominant theory suggests that, at the single-cell level, quiescence within a cell sub-population of the tumor promotes the bulk mass to enter a state of dormancy [2–3, 38–39]. The OMI and PLS-DA approach used in this paper to identify and monitor quiescent tumor cells in the presence of both proliferating and apoptotic cells could be used to study interactions between dormant, proliferating, and apoptotic tumor cells, and the effect of

quiescence on tumor progression. Our methods could also be used to identify extracellular influences that initiate transitions between quiescence, proliferation, and apoptosis. Furthermore, these results support the use of cellular metabolic measurements to study the development of tumor dormancy.

With additional validation, our approach could be applied to universally examine cell cycle-driven tumor heterogeneity. These techniques could be translated to clinical samples, using blood samples for blood cancers, or using organoids derived from solid tumor biopsies. Our group has previously applied OMI to patient-derived organoids, to guide personalized treatment decisions [8, 34, 35]. The tools developed in this paper could further characterize heterogeneous cell populations within these organoids for more informed treatment planning. These tools could also be used to study tumor dormancy in mouse models of cancer. Overall, we have demonstrated OMI and PLS-DA as valuable tools for observing cell cycle status, which can be applied to future studies of tumor dormancy and treatment resistance.

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Figure 1.

Flow cytometry analysis confirms cell cycle activity associated with apoptotic, proliferating, quiescent and Kasumi-1 cell populations. Pure populations of (A) apoptotic cells individually labeled with cleaved caspase 3 (CC3) conjugated to FITC, and (B) proliferating cells individually labeled with Ki67 conjugated to FITC. Comparison to unstained controls (negative control, A&B) demonstrate that the stained populations are positive for CC3 (A) and Ki67 (B). (C) Similarly, pure populations of quiescent and proliferating cells were labeled with Pyronin Y (RNA content) and Hoechst 33342 (DNA content) simultaneously to demonstrate separation between the quiescent populations (low RNA content) and proliferating population (high RNA content). In (C) each dot is an individual cell.

Figure 2. Inhibition of oxidative phosphorylation affects proliferating cell metabolism. Quantitative measurement (mean $\pm$ SE) of the (A) redox ratio, (B) NAD(P)H mean lifetime (τ_m), and (C) FAD τ_m for proliferating Kasumi-1 cells ($n = 150$ – 250 cells per group) before and five minutes after addition of 4 mM cyanide. (* $p < 0.05$, *** $p < 0.001$)

Figure 3.

Inhibition of fatty acid oxidation affects quiescent cell metabolism. (A) Representative redox ratio images. (B) Quantitative measurement (mean $\pm$ SE) of the redox ratio for untreated proliferating and quiescent Kasumi-1 cells ($n = 150$ – 250 cells per group), as well as etomoxir-treated proliferating and quiescent cells ($n = 150$ – 250 cells per group). Kasumi-1 cells were forced into quiescence by treatment with JQ1 (250 nM) 72 hours before etomoxir (100 nM) or vehicle treatment. (***) $p < 0.001$

Figure 4. Lifetime and redox images. Representative redox ratio, NAD(P)H τ_m , and FAD τ_m images of Kasumi-1 cells forced into apoptotic, proliferating, and quiescent states, 72 hours post-treatment.

Figure 5.

Quantitative measurement (mean $\pm$ SE) of the (A) redox ratio, (B) NAD(P)H τ_m , (C) FAD τ_m , (D) NAD(P)H τ_1 , (E) NAD(P)H τ_2 , (F) NAD(P)H α_1 , (G) FAD τ_1 , (H) FAD τ_2 , and (I) FAD α_1 at the single-cell level for proliferating, quiescent, and apoptotic ($n = 150$ – 250 cells per group) Kasumi-1 cells at 72 hours post-treatment. Each dot is an individual cell. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 6.

Posterior probability of class membership predictions from two-group partial least squares – discriminant analysis. Classification of proliferating (triangles), and quiescent (circles) cells yields an overall classification accuracy of 92.4%. Model classification demonstrated satisfactory sensitivity and specificity for discriminating proliferating and quiescent populations. (sensitivity: 96.1%, specificity: 84.7%). Each symbol is an individual cell.

Figure 7.

Posterior probability of class membership predictions from three-group partial least squares – discriminant analysis. Classification of apoptotic (pluses), proliferating (triangles), and quiescent (circles) cells yields an overall classification accuracy of 90.1%. Model classification demonstrated satisfactory sensitivity/specificity for discriminating each respective population from remaining sample populations. (apoptotic: 87.6%/97.9; proliferating: 82.3%/95.5%; quiescent: 97.5%/93.7%). Each symbol is an individual cell.

Figure 8. Two-Group PLS-DA Model Demonstration. Representative images of heterogeneous mixtures of proliferating (green) and quiescent (blue) populations plated at various proportions ($n=150-250$ cells per mixture). (A) 100% proliferating, 0% quiescent, (B) 30% proliferating, 70% quiescent, (C) 50% proliferating, 50% quiescent, (D) 70% proliferating, 30% quiescent (E) 0% proliferating, 100% quiescent. (F) Graphical representation of differences between plated proportions and measured proportions for each mixture sample. These illustrations demonstrate the sensitivity of OMI to distinguish proliferating and quiescent populations in heterogeneous samples.

Figure 9.

Three-Group PLS-DA Model Demonstration. Representative images of heterogeneous mixtures of proliferating (green), quiescent (blue), and apoptotic (red) populations plated at various proportions ($n = 150\text{--}250$ cells per mixture). (A) 33% proliferating, 33% quiescent, 33% apoptotic, (B) 50% proliferating, 25% quiescent, 25% apoptotic, (C) 25% proliferating, 25% quiescent, 50% apoptotic. (D) Graphical representation of differences between plated proportions and measured proportions for each mixture sample. These illustrations demonstrate the sensitivity of OMI to quiescent populations in the presence of proliferating and apoptotic populations, at varying proportions.

Table 1

Overall Three-Group Classification Accuracy with Individual OMI Variables

Variable	Classification Accuracy
Redox Ratio	43.2%
NAD(P)H τ_m	44.6%
FAD τ_m	66.0%
NAD(P)H τ_1	27.2%
NAD(P)H τ_2	41.0%
NAD(P)H α_1	22.8%
FAD τ_1	31.3%
FAD τ_2	23.6%
FAD α_1	46.1%

Table 2

Variable Weights for Two-group and Three-group PLS-DA Models

Variable	Redox Ratio	NADH τ_1	NADH τ_2	NADH α_1	FAD τ_1	FAD τ_1	FAD α_1
Two-group PLS-DA weights	0.023	0.00035	0.0017	0.058	-0.000080	0.000073	-0.00092
Three-group PLS-DA weights:							
Proliferating vs. rest	0.011	0.0013	0.00070	0.049	0.00051	-0.00032	0.014
Apoptotic vs. rest	0.013	-0.0027	0.0021	-0.0093	-0.00063	0.00046	-0.025
Quiescent vs. rest	-0.023	0.0014	-0.0028	-0.039	0.00012	-0.00014	0.011

Table 3

Confusion Matrix: Two-Group Classification

	Actual		
		Proliferating	Quiescent
	Predicted		
	Proliferating	105	6
	Quiescent	19	251

Table 4

Confusion Matrix: Three-Group Classification

	Actual			
		Apoptotic	Proliferating	Quiescent
Predicted	Apoptotic	177	6	5
	Proliferating	19	93	1
	Quiescent	6	14	234