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Rapid Assessment of Mitochondrial Complex I Activity and Metabolic Phenotyping of Breast Cancer Cells by NAD(p)H Cytometry

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Abstract

Cancer cells are known to display a variety of metabolic reprogramming strategies to fulfill their own growth and proliferative agenda. With the advent of high resolution imaging strategies, metabolomics techniques etc., there is an increasing appreciation of critical role that tumor cell metabolism plays in the overall breast cancer (BC) growth. In this report, we demonstrate a sensitive, flow-cytometry-based assay for rapidly assessing the metabolic phenotypes in isolated suspensions of breast cancer cells. By measuring the temporal variation of NAD(p)H signals in unlabeled, living cancer cells and by measuring mitochondrial membrane potential $\{\psi_m\}$ in fluorescently labeled cells, we demonstrate that these signals can reliably distinguish the metabolic phenotype of human breast cancer cells and can track the cellular sensitivity to drug candidates. We further show the utility of this metabolic ratio $\{\psi_m/\text{NAD(p)H}\}$ in monitoring mitochondrial functional improvement as well as metabolic heterogeneity in primary murine tumor cells isolated from tumor biopsies. Together, these results demonstrate a novel possibility for rapid metabolic functional screening applications as well as a metabolic phenotyping tool for determining drug sensitivity in living cancer cells.

Keywords

Breast cancer; Mitochondria; NAD(p)H; Metabolism; Metabolic Plasticity; Tumor Heterogeneity

1. Introduction

In the continuous quest to understand human cancer biology, it has been a common practice to resort to a number of preclinical research models. Human-derived breast cancer cell lines, transgenic animal models, patient-derived xenograft models are some of these reductionist tools that have significantly enhanced our mechanistic understanding of cancer cell behavior.^{1–3} These models have successfully elucidated the genetic basis of cancers and have

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Conflict of Interest

The author declares that he has no conflict of interest.

practically laid out the conceptual framework for how we address the challenges in multiple types of cancer in the past few decades.^{4–6} Despite the tremendous progress in the past few decades (discovery of oncogenes, tumor suppressors, cancer signaling pathways), cancer-related mortality and morbidity are still high since we still do not have clarity on how tumor genetics and tumor metabolism collectively determine the cancer phenotype and the clinical manifestations. Another confounding factor is the spatial and temporal heterogeneity commonly observed in growing tumors.^{7–10} Furthermore, recent studies have pointed out that several aggressive tumor cells display robust mitochondrial function challenging the original Warburg hypothesis that cancer cells upregulate aerobic glycolysis owing to mitochondrial respiratory defects.^{11, 12} It is therefore critical to integrate genetic and metabolic components of cancer growth as well as tumor heterogeneity metrics in all aspects of cancer research. Our laboratory has been focusing on redox-sensitive enzyme systems by developing tools to study a few of these enzyme systems at the single cell level and by developing a pipeline for translating basic discoveries to clinical applications.^{13–21} In this paper, we describe a simple, rapid assay for monitoring activity of an important redox enzyme system namely, the mitochondrial Complex I. Mitochondrial Complex I (NADH: Ubiquinone oxidoreductase) is the gate keeper between glycolytic and mitochondrial pathway and its dysfunction has been implicated in a number of neurological disorders including Parkinson's disease and Alzheimer's disease as well as in many human cancers.^{22, 23} Recently we demonstrated that mitochondrial Complex I function can significantly impact the metabolic plasticity potential (i.e, tendency to reprogram metabolic pathways) in breast cancer growth in preclinical xenograft models of human breast cancer.²⁴ Motivated by these studies, we set out to develop a reliable method to monitor mitochondrial Complex I activity in single living cells. Since the mitochondrial Complex I catalyzes the $\text{NADH} \rightarrow \text{NAD}^+$ oxidation, one can obtain information on the status of mitochondrial Complex I activity by monitoring the changes in fluorescence signal while the fluorescent NADH converts to non-fluorescent NAD^+ . In living cells, (NADH/NAD^+) is a vital redox pair that facilitates a number of electron transfer reactions including mitochondrial electron transport chain.^{19–21} Intracellular free NADH concentration is usually in the range of a few micromoles while the NAD^+ concentration can be 5–8 fold higher in concentration than the free NADH pool.²⁵ These ratios may vary significantly between the tissue types and may dynamically alter in pathology states. Mitochondrial and cytosolic compartments also display distinct redox ratios owing to the difference in the corresponding free and protein-bound NADH in these compartments. From the measurement point of view, the conventional approach has been to monitor the spectrophotometric absorbance (maximum absorbance at 350nm, UV region) or fluorescence of NADH (maximum emission at 450nm) in cell lysates or in isolated mitochondria. Our laboratory has been developing two-photon imaging strategies (near infrared excitation with deep tissue imaging capabilities) for realizing optimal mitochondrial complex I activity measurements in single cells.^{16, 17, 26, 27} However, these methods are less suited for high-through put measurements in cell suspensions. In this paper, we describe a flow cytometry-based assay for monitoring NAD(p)H oxidation kinetics in cell suspensions and we demonstrate its utility in: (a) assessing metabolic status of different human breast cancer cells, (b) determining drug specificity in human breast cancer cells, (c) tracking the effects of adaptive metabolism (metabolic plasticity) in human

breast cancer cells, and (d) measuring tumor cell metabolic heterogeneity in primary tumor epithelial cells isolated from preclinical animal models.

2. Materials & Methods

2.1 Cell culture, Generation of adaptive cell lines:

MDA-MB-231 and MDA-MB-436 are tumorigenic human breast epithelial cell lines derived from pleural effusions of female patients with metastatic adenocarcinoma.²⁸ It is well established in the literature that these two cell lines do not express the three breast-cancer associated receptors (“triple-negative”) estrogen receptor, progesterone receptor and HER2 receptor.²⁴ On the other hand, MCF7 cell lines are the estrogen-receptor positive breast cancer cells derived from pleural effusion of a female patient with metastatic adenocarcinoma.²⁹ All the three cell lines were originally obtained from ATCC and were cultured in DMEM with high glucose (4.5g/l) and 10% fetal bovine serum and antibiotics. For comparison in some experiments, non-tumorigenic epithelial cell line, MCF10A was used. With a motivation to generate isogenic cancer cell lines with selective adaptation to mitochondrial complex I modulation, we cultured the cell line, MDA-MB-231 in the presence of sub-lethal doses (100nM) of mitochondrial complex I inhibitor, rotenone continuously for 20 generations. More details can be found in reference 24. After 20 generations, the rotenone selection pressure was removed, and the cells were cultured in normal culture medium. Similar culture protocol by using the mitochondrial Complex III inhibitor, antimycin A resulted in metabolic modified cells as shown in Figure 4. For the sake of brevity, the following notation will be used to refer to these cells: (A) Parental: 231-P (B) Metabolically modified: 231–100R and 231–100A obtained by culturing the MDA-MB-231 cells under the selection pressure of either 100nM rotenone (100R) or 100nM antimycin A (100A).

2.2 Flow cytometry acquisition and data analysis

Flow cytometry analysis of living cells was performed in LSR II flow cytometer (BD Biosciences) equipped with 4 laser lines (488nm, 640nm, 405nm and 355nm UV). Prior to the live cell cytometry experiments, the cells in suspension were labeled with either a mitochondrial membrane potential probe (tetramethyl rhodamine methyl ester, TMRM) or a reactive oxygen species sensor (6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester, CM-H₂DCFDA). NAD(p)H signals were collected either in unlabeled cells or multiplexing with TMRM probe.¹⁵ For all the experiments reported in this paper, we primarily used the 355nm ultraviolet laser line for NAD(p)H measurements and the 561nm laser line for mitochondrial membrane potential probe (PE TXRed channel:TMRM) measurements. In some independent experiments, 488nm laser line was used for reactive oxygen probe (FITC channel: DCFDA). These three probes have minimal overlap in their excitation and emission spectra and there was no particular need to perform compensation during multiplexing. The raw data were obtained using the BD FACSDIVA software that was also used for standard flow cytometry tasks such as gating and analysis. The same software was used to export data statistics which in turn, were graphically plotted using the Origin 8.0 software (OriginLab Corporation, Northampton, MA). For each experiment, mean intensity values from NAD(p)H and TMRM channels were normalized with respect to

the control and/or untreated values. The ratio of (TMRM/NAD(p)H) was then computed for each condition. In the case of time-lapse kinetics, this ratio was plotted as a function of time as schematically depicted in figure 1b. Two specific parameters, α_1 and α_2 were computed as shown in figure 1b. α_1 is a measure of how glucose stimulus can induce NAD(p)H generation in the tricarboxylic acid (TCA) cycle in the mitochondrial matrix and α_2 is a measure of how mitochondrial complex I activity (NAD(p)H oxidation) leads to an increased mitochondrial membrane potential (Ψ_m). Increased mitochondrial complex I activity leads to the conversion of fluorescent NAD(p)H to non-fluorescent NAD⁺ which can be monitored by tracking the loss of signal in the NAD(p)H channel. Pharmacological inhibition of mitochondrial complex I (rotenone) is expected to block NAD(p)H oxidation. Since cells with intact mitochondrial function are expected to respond well to rotenone treatment, we can use the parameter α_2 as a measure of rotenone sensitivity of the cells and hence as a measure of their mitochondrial complex I activities.

2.3 Single cell imaging

High resolution images were obtained either on a Leica confocal microscope platform or on a Leica two-photon excitation microscope platform as described in prior publication.²⁴ 730nm excitation was used to excite in living cells and the resulting NAD(p)H signals were collected with a bandpass filter (440–490nm) using a 20X objective (NA 1.0, Leica microsystems). Image analyses were performed using the open-access software, ImageJ (National Institutes of Health, Bethesda, USA). For each experiment, 40–60 cells in each field-of-view were randomly selected and NAD(p)H signals were computed. Mitochondrial complex I inhibition was induced by 1 μ M rotenone treatment and the resulting accumulation of NAD(p)H signals were monitored. Mean values from multiple fields-of-view for each cell line were plotted as a function of time.

2.4 Animal experiments

The preclinical transgenic mouse (MMTV-PyMT) was obtained from a commercial vendor (Jackson Labs, Maine, USA) and was bred and maintained in-house within the animal vivarium in our institution. The PyMT adenocarcinoma mouse has a short tumor latency period (hyperplasia, mammary intraepithelial neoplasia and DCIS @ 4 weeks, early carcinoma @ 6–8 weeks and late carcinoma @ 8–12 weeks and lung metastasis >16 weeks).^{30, 31} Primary mammary epithelial cells were isolated from freshly excised animal tumors and cultured following the standard protocols.³² Briefly, the freshly excised tumor specimens (1–3 cm³) were washed in cold phosphate buffered saline before chopping with sterile scissors. Smaller fragments of the tissue were transferred to a conical tube containing tissue digestion medium (DMEM/F12 culture medium, antibiotics and 2 μ g/ml collagenase). This tissue digestion procedure was carried out at 37 °C on a rocking platform for 4 hours. The tube was then centrifuged at 800 rpm to remove the lipid components and the cell pellet was washed twice with cell culture medium (DMEM/F12 with 5% fetal bovine serum and antibiotics) before plating on tissue culture dishes. After three days, viable primary mammary tumor epithelial cells were clearly seen adherent to the culture plates. All flow cytometry experiments using suspensions of primary mammary tumor epithelial cells were performed within 5–6 passages after isolation. All animal experiments were performed in

strict accordance with the animal protocol as approved by institutional animal care and use committee.

2.5 Statistics

Data presented are Mean $\pm$ S.E from at least three independent experiments unless otherwise mentioned. Statistical significance was estimated based on Student's t-test ($p < 0.05$).

3. Results

3.1 NAD(p)H oxidation kinetics assay distinguishes human breast cancer cells based on their metabolic status

Figure 1 illustrates the main goal of this study in using the two mitochondrial parameters to characterize mitochondrial adaptation of cancer cells. As demonstrated in our recent study, we are advancing a conceptual framework where the metabolic plasticity of breast cancer cells can play a significant role besides the genetic propensity of these cells.³³ Molecular characterization of human tumor specimens has resulted in a now well-accepted classification of breast cancer into distinct sub-types based on specific receptor expression.³⁴ According to this classification, luminal A and B subtypes are positive for estrogen receptor (ER) and/or progesterone receptor (PR) but negative for HER2 receptor. HER-2 enriched subtypes are characterized by an increased HER2 copy number and/or increased HER2 receptor expression levels. These three subtypes are now currently treated using targeted therapies in addition to chemotherapy and radiation. The last subtype is usually characteristic of aggressive breast tumors that do not express any of the aforementioned three receptors (the so-called “triple negative” tumors). In Figure 2, the ER-positive MCF7 cell line belongs to luminal subtype while the other two cell lines (MDA231 and MDA436) belong to the tripe-negative subtype. Figure 2(a-d) shows representative NAD(p)H and mitochondrial membrane potential signals measured in MCF7 cells under basal conditions as well as after acute preconditioning (1 h) with either mitochondrial Complex I inhibitor, rotenone or mitochondrial uncoupler, FCCP. In cells with intact mitochondrial metabolism, it is expected that rotenone will increase the reduced (NAD(p)H) population by the virtue of decreased mitochondrial complex I activity and this increase in NAD(p)H is accompanied by a corresponding hyperpolarization of mitochondria (i.e, increase in mitochondrial membrane potential). On the other hand, FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) is an ionophore that is known to depolarize mitochondrial membrane potential (‘uncoupling’).³⁵ Figure 2e summarizes rotenone-sensitive changes in the $\{\psi_m / \text{NAD(p)H}\}$ in the three breast cancer cell lines. After stimulating with mitochondrial Complex I inhibitor (1 μ M rotenone), time-lapse measurements of NAD(p)H and mitochondrial membrane potential (ψ_m) were collected as shown for individual cell lines in Figure 2 (a-d). As can be seen, these three cell lines differ in the $\{\psi_m / \text{NAD(p)H}\}$ ratios -both in the unstimulated ($t=0$, basal) and in the metabolically preconditioned (rotenone, FCCP) situations. Even though, MDA231 and MDA436 cell lines belong to the same TNBC subtype (based on gene expression studies), these two cell lines display different time-dependent profiles of the $\{\psi_m / \text{NAD(p)H}\}$ ratio under the two metabolic perturbations. On the other hand, MDA231 and MCF7 cell lines belong to different breast cancer subtypes (TNBC and luminal, respectively) and still

displayed similar time-dependent profiles of the $\{\psi_m/\text{NAD(p)H}\}$ ratio. This points to the need for considering metabolic phenotypes along with genetic phenotypes while characterizing the various breast cancer subpopulations.

3.2 $\{\psi_m/\text{NAD(p)H}\}$ ratiometry reveals potential in sensing drug-sensitivity in breast cancer cells

Having confirmed that the $\{\psi_m/\text{NAD(p)H}\}$ ratio can discriminate the metabolic phenotypes of breast cancer cells based on their response to mitochondrial perturbation agents, we next asked if this ratio can shed light on the drug sensitivity of the breast cancer cells. Figure 3a demonstrates the genetic differences between the two breast cancer cell lines that displayed similar metabolic phenotypes (figure 2b). MCF7 cell line is less-aggressive, ER-positive luminal breast cancer cell line that displays epithelial characteristics (as marked by beta-catenin expression) while the MDA231 cell line is highly-aggressive, metastatic, triple-negative breast cancer cell line that displays strong mesenchymal characteristics (as marked by fibronectin expression). As demonstrated in earlier publications from our lab, sensitivity of mitochondrial complex I inhibitor (rotenone) is a reliable metric for the functional integrity of mitochondrial Complex I in living cells.^{13, 24} Two-photon fluorescence imaging in these two cell lines (figure 3b) shows similar rotenone-sensitive NAD(p)H kinetics. Since mitochondrial Complex I activity is a measure of NAD(p)H oxidation rate, the time-dependent NAD(p)H profiles therefore indicate that both MDA231 and MCF7 cells have similar mitochondrial Complex I function. This is in agreement with the rotenone-sensitivity as measured by flow cytometry assay (figure 3c). After confirming that these two cells have similar rotenone sensitivity, we next asked if the $\{\psi_m/\text{NAD(p)H}\}$ ratio can reliably report differential drug sensitivity between these two cell lines. Towards this, we chose a small molecule drug, erastin that has been earlier shown to cause cytotoxicity only in cancer cells with mutations in Ras-oncogene.^{36–40} MDA231 cells have been reported to display Ras-mutations while MCF7 cells are known to have wildtype Ras. As can be seen in Figure 3d, 20h treatment with 20 μ M erastin leads to a significant reduction in $\{\psi_m/\text{NAD(p)H}\}$ ratio of MDA231 while this ratio was unaltered in MCF7 cells. Figure 3e shows that erastin treatment is accompanied by a significant increase in ROS generation in MDA231 cells (and not in MCF7 cells) which concurs with earlier published studies on the mechanism of action for this small molecule drug.^{38, 39, 41} Together, this result points to the fact that $\{\psi_m/\text{NAD(p)H}\}$ ratio is a reliable indicator of drug sensitivity and in this particular example, the changes in this metric might be associated with the excessive ROS generated by erastin in MDA231 cells.

3.3 NAD(p)H cytometry reveals differential mitochondrial metabolism in breast cancer cells with varying metabolic plasticity potential

Recently we demonstrated that adaptive culture of MDA231 cells in the presence of mild doses of mitochondrial Complex I altered the metabolic plasticity regimen (Figure 1) in these cells and led to a significant improvement in mitochondrial function and further correlated with a reduction in tumorigenic potential in vivo.²⁴ Figures 4a&b show differential immunohistochemical staining of cell proliferation marker, Ki67 in tissues obtained from the animal tumors after injecting parental 231-P and adaptive 231–100R cells respectively. As described in section 2.1, adaptive culture of isogenic MDA231 cells in the

presence of rotenone and antimycin A resulted in metabolically modified 231–100R and 231–100A cells respectively. These two cell lines showed a significantly higher rotenone sensitivity than the parental 231-P cells suggesting that $\{\psi_m/\text{NAD(p)H}\}$ ratio is a reliable metric for characterizing mitochondrial enhancement in living breast cancer cells (figures 4c&d).

3.4 $\{\psi_m/\text{NAD(p)H}\}$ ratiometry can be used to obtain information on metabolic heterogeneity in tumor epithelial cells

Finally, we tested if the $\{\psi_m/\text{NAD(p)H}\}$ ratio can give reliable information on spatial variation of tumor cell metabolism within the same tumor. Towards this direction, we excised spontaneously generated adenocarcinoma tumors from the transgenic MMTV-PyMT female mice and isolated primary mammary epithelial cells from the tumor nodules (section 2.4.). Representative

$\{\psi_m/\text{NAD(p)H}\}$ plots for these three cell lines (figure 5b) and analysis of the parameters ψ_1 and ψ_2 (figures 5c&d) show that primary tumor epithelial cells isolated from region 2 differ from those from regions 1 and 3 pointing out to possible metabolic heterogeneity within the same tumor. Since these tumors have been earlier shown to harbor Ras-mutations, the primary mammary epithelial cells isolated from the three nodules (as shown) of the tumor were treated with 20 μM erastin (22 hours). Figure 5c&d summarizes the $\{\psi_m/\text{NAD(p)H}\}$ ratio profiles.

Discussion

Breast cancer is no longer viewed as a single disease but is now understood as a collection of multiple neoplasms with distinct genetic subtypes and potentially, a spectrum of metabolic phenotypes as well. Any comprehensive analysis of breast cancer specimens must therefore take into account such a multifactorial complexity and this issue becomes even more challenging when critical decisions need to be taken regarding clinical management of patient tumors. This paper is an attempt to develop a robust assay for characterizing the metabolic phenotypes in tumor cell suspensions. As demonstrated in earlier sections, $\{\psi_m/\text{NAD(p)H}\}$ ratio can clearly discriminate human breast cancer cell lines based on their metabolic phenotypes. One possible interpretation of this ratio is that this collectively represents the ability of mitochondria to generate electron donors such as NAD(p)H in the TCA cycle and to oxidize NAD(p)H by mitochondrial Complex I so as to generate mitochondrial membrane potential that is eventually used for ATP generation. Any alteration (acute and/or genetic defects) to this dynamic balance might lead to metabolic stress in the cells and we believe that $\{\psi_m/\text{NAD(p)H}\}$ ratio is a sensitive reporter of this metabolic stress at the single cell level. Since NAD(p)H signals can originate from multiple compartments and biochemical sources within the cells, we measured the rotenone-sensitive fraction so that we can interpret the $\{\psi_m/\text{NAD(p)H}\}$ ratio as specific to mitochondrial compartments. The fact that this ratio has very close relationship as measured by two different experimental configurations (two-photon imaging and flow cytometry) further add credibility to this metric. As demonstrated in Figure 2, MCF7 and MDA231 cell lines have similar mitochondrial response status despite belonging to different “genetic” subtypes. On

the other hand, MDA231 and MDA436 cell lines have quite different mitochondrial response status despite belonging to the same “genetic” subtypes. This points out to an interesting possibility where cancer cells can be thought of belonging to subtypes based not only on their genomic profiles but also on their metabolic profiles. In the current era of precision medicine, drugs developed for one type of cancer are being tested on patients with different cancer types as long as their genetic profiles have some similarity. Applying a similar reasoning, we tested if $\{\psi_m/\text{NAD(p)H}\}$ ratio can be sensitive to drug response characteristics in the two breast cancer cell lines (MCF7 and MDA231) with similar mitochondrial Complex I function phenotype. As seen from Figure 3e, this ratio clearly varies in accordance to erastin-specific modulation of mitochondrial function and the associated ROS generation. Since the human tumors display significant variability in drug response, the above finding is significant as it points out to a possibility that heterogeneous drug sensitivity in solid tumors might have their roots in the corresponding metabolic heterogeneity. Similar trends in the $\{\psi_m/\text{NAD(p)H}\}$ ratio observed in isogenic MDA231 cells (231-P, 231-100R and 231-100A) as well as in mouse tumor specimens further add value to our reasoning that $\{\psi_m/\text{NAD(p)H}\}$ ratio is a reliable and robust measure of metabolic plasticity and intratumor heterogeneity as well. We understand that there can be multiple experimental metrics that collectively define the metabolic phenotypes in living cells and it might take more than just the $\{\psi_m/\text{NAD(p)H}\}$ ratio to comprehensively characterize the metabolic phenotypes in breast cancer cells. Within the scope of this paper, we attempted to develop this metric as a high-throughput means to rapidly assess the mitochondrial Complex I activity in living cancer cells and how to explore its ramifications in metabolic phenotyping. Since normal mitochondrial metabolism entails a strong coupling between the NAD(p)H generation and mitochondrial membrane potential generation, we envision that $\{\psi_m/\text{NAD(p)H}\}$ ratio metric can be useful in mitochondrial functional screens in applications beyond the breast cancer examples described in this paper.

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Fig1: Measurement principle and perturbation schematic.

(a) Upon transformation by one or more of the oncogenic events, the breast epithelial cell undergoes multiple adaptation steps within the host environment. Depending on the nature of oncogenic potential as well as on the microenvironment, the evolution of solid tumor can have varying rates which in turn, determines the genomic stability, clonal selection and other processes that give proliferative advantages to the breast cancer cells. This genetic paradigm of cancer growth is now being revisited to include the various metabolic adaptation routes which we term as the metabolic plasticity regimen. (b) The two measurement parameters are the mitochondrial membrane potential (Ψ_m) and mitochondrial NAD(p)H oxidation kinetics. Time-lapse acquisition of these two signals from living cancer cells can be used to compute the ratio $\{\Psi_m/\text{NAD(p)H}\}$ and the two parameters Δ_1 and Δ_2 . TCA and ETC refer to the tricarboxylic acid cycle and electron transport chain respectively, within the mitochondrial matrix. In an ideal situation of well-coupled mitochondria, glycolytic output is diverted to the tricarboxylic acid cycle (TCA) within the mitochondrial matrix which in turn, is coupled to the electron transport chain (ETC) responsible for the NAD(p)H oxidation and mitochondrial membrane potential generation.

Fig2. Demonstration of metabolic phenotyping by NAD(p)H cytometry

(a-d) Representative fluorescence histograms obtained from cytometry profiles of NAD(p)H and mitochondrial membrane potential ($\Delta\Psi_m$) in MCF7 breast cancer cells. Acute preconditioning (1h before the experiment) with mitochondrial uncoupling agent (5 μ M FCCP) or with mitochondrial complex inhibitor (1 μ M rotenone) is expected to alter the ratio in a predictable way. The increase in NAD(p)H fluorescence is due to the rotenone effects in preventing the oxidation of NAD(p)H within the mitochondria while depolarization of mitochondria upon FCCP stimulus is manifested as a decrease in $\Delta\Psi_m$. (e) Mean time-lapse kinetic profiles of the ratio $\{\Delta\Psi_m / \text{NAD(p)H}\}$ obtained from three breast cancer cells as indicated. MCF7 cells and MDA231 cells show similar metabolic response trends ('metabolic phenotypes') despite belonging to different breast cancer subtype classifications. MDA436 and MDA231 cells display different metabolic response trends despite belonging to the same breast cancer subtype classifications. This is further explained in figure 3.

Fig 3: Manifestation of cancer cell drug sensitivity in NAD(p)H cytometry profiles

(a) Representative immunofluorescence images of MCF7 and MDA231 cell lines showing epithelial marker (beta catenin, green) and mesenchymal marker (fibronectin, red). Also shown are the nuclear expression levels of estrogen receptor (ER α , red). MCF7 cells display epithelial characteristics and higher ER α levels while MDA231 cells display mesenchymal characteristics and very low ER α levels. Cell nuclei are labeled with DAPI (blue). Scale bars = 20μm. (b) Mean NAD(p)H kinetics as obtained from analyzing two-photon fluorescence images. Multiple fields containing 20–30 cells per field were analyzed and the plot shows the rotenone-induced accumulation of NAD(p)H signals. (c) Rotenone-sensitive NAD(p)H changes from flow cytometry analysis in the same two cell lines were measured as depicted in figure 1. (d) Mean $\pm$ S.E of $\{ \Delta\psi_m / \text{NAD(p)H} \}$ ratios summarized for MCF7 and MDA231 cells after 20h treatment with Ras-specific cytotoxic drug, erastin (n = 3 experiments; p < 0.01). The data were normalized with respect to the untreated control values for the individual cell lines. (e) Corresponding reactive oxygen species (ROS) levels measured under the same conditions.

Fig 4: NAD(p)H cytometry profiles correlate with improved mitochondrial function in breast cancer cells displaying metabolic plasticity

(a & b) Tumor xenografts were generated by injecting 231-P (parental) and 231-100R (metabolically modified breast cancer cells) subcutaneously in athymic female mice. Ki67 immunohistochemistry images of tumor sections obtained from representative preclinical human xenografts are shown. The dark brown spots indicate the positivity of cell proliferation marker, Ki67. Tumors generated by injecting the parental 231-P cells in athymic mice show an increased Ki67 positivity as compared to those generated by injecting the mitochondrially modified 231-100R cells. Scale bars = 100 μ m. (c&d) As schematically

depicted in figure 1, the two parameters, $\Delta\psi_m$ and $\Delta\text{NAD(p)H}$ were computed from the $\{\psi_m/\text{NAD(p)H}\}$ ratio in breast cancer cells. More details on the metabolic modified cells can be found in the materials and methods section. After collecting the basal signals, time-lapse acquisition was carried out for 10 minutes after stimulating the cells with 20mM glucose, followed by 20 minutes of 1xM rotenone stimulus. $\Delta\psi_m$ is the difference in $\{\psi_m/\text{NAD(p)H}\}$ ratio between the glucose-stimulated and basal conditions while $\Delta\text{NAD(p)H}$ is the difference in $\{\psi_m/\text{NAD(p)H}\}$ ratio between the glucose stimulated (10 minute time point) and rotenone-stimulated (30 minutes time point) conditions. Preconditioning (30min-1h) the cells with mitochondrial uncoupling agent 5 μ M FCCP, is expected to enhance the mitochondrial metabolism (by increasing respiration rate) thereby increasing the values of $\Delta\psi_m$ and $\Delta\text{NAD(p)H}$. In the present case, owing to the mitochondrial modulation strategy that we adopted as well as due to the mitochondrial-specific perturbation (FCCP) used here, $\Delta\text{NAD(p)H}$ showed more significant changes than the $\Delta\psi_m$.

Fig 5: NAD(p)H cytometry in primary murine tumor epithelial cells

(a) A freshly excised mammary tumor from the transgenic MMV-PyMT female mouse displaying three tumor nodules. Primary mammary epithelial cells were isolated from these three tumor nodules and labeled as region 1, 2 and 3 respectively. (b) $\{\Delta\psi_m / \text{NAD(p)H}\}$ data were collected from the primary cell suspensions as described and the representative $\{\Delta\psi_m / \text{NAD(p)H}\}$ plots reveal that these three tumor regions contain distinct metabolic phenotypes even though they were part of the same tumor from the same animal. (c & d) Δ_1 and Δ_2 plots are shown for the primary tumor epithelial cells. Since this PyMT mouse has been shown to harbor Ras-mutations, we tested if the metabolic ratios were affected

differentially by the Ras-specific small molecule drug, erastin as demonstrated in Figure 3. No statistically significant alterations were observed with erastin treatment but the basal differences (i.e, without erastin treatment) between the three tumor regions point out to an interesting possibility for using this $\{\psi_m/\text{NAD(p)H}\}$ ratio for metabolic phenotyping of tumor heterogeneity. It is to be noted that the tumor region 3 showed lesser changes in the $\{\psi_m/\text{NAD(p)H}\}$ ratio than the other two regions which further accentuates the notion of intratumor heterogeneity.