9-3-2018

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HHS Public Access

Author manuscript

Angew Chem Int Ed Engl. Author manuscript; available in PMC 2019 September 03.

Published in final edited form as:

Angew Chem Int Ed Engl. 2018 September 03; 57(36): 11554–11558. doi:10.1002/anie.201803034.

Surface Immobilization of Redox-Labile Fluorescent Probes Enables Single-Cell Co-Profiling of Aerobic Glycolysis and Oncogenic Protein Signaling Activities

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Abstract

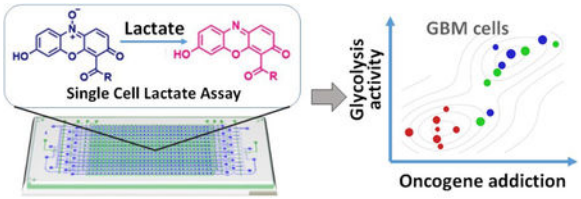
We describe an analytical method for profiling lactate production in single cells, via the use of coupled enzyme reactions on surface-grafted resazurin molecules. The immobilization of the redox-labile probes was achieved through chemical modifications on resazurin, followed by bio-orthogonal click reactions. The lactate detection scheme was demonstrated to be sensitive and specific. The method was incorporated into the single cell barcode chip platform for simultaneous quantification of aerobic glycolysis activities and oncogenic signaling phosphoproteins in cancer. We interrogated the interplay between glycolysis and oncogenic signaling activities on a glioblastoma cell line. Results revealed a drug-induced oncogenic signaling reliance accompanying shifted metabolic paradigms. A drug combination that exploits this induced reliance exhibited synergistic effects in growth inhibition.

Graphical Abstract

Conflict of Interest

Prof. J. R. Heath is affiliated with Isoplexis, which is seeking to commercialize the single cell barcode chip technology.

Supporting information for this article is given via a link at the end of the document.



Keywords

Cancer; Fluorescent probes; Metabolism; Single cell analysis; Aerobic glycolysis

The upregulation of aerobic glycolysis, known as the Warburg effect, is a hallmark of many cancers.[1] A common paradigm is that the driver oncogenic signaling confers and maintains the elevated glycolytic activities;[2] however, recent studies have revealed that metabolic processes, such as aerobic glycolysis, are also capable of actively modulating cell-signaling networks.[3] Those results highlight the complex interactions that exist between aerobic glycolysis and oncogenic signaling. Efforts in deciphering such interactions can be confounded by the intra- and inter-tumoral heterogeneity – a common feature of all cancers. Such heterogeneity facilitates tumor cells to adapt to stress through adopting new signaling and metabolic paradigms.[4]

Single cell assays hold the promise to unveil the heterogeneity as well as the interplay between metabolic and oncogenic signaling, informing the rational design of therapeutic strategies that target tumor vulnerabilities. To this end, we have recently developed integrated proteomic and metabolic single cell assays on the single cell barcode chip (SCBC) platform, where glucose influx and signaling protein activities were simultaneously quantified from individual cells with fluorescence readouts.[5] However, glucose influx provides only an incomplete view of aerobic glycolytic capabilities,[1c, 6] because the metabolic fate of the consumed glucose is not resolved. This caveat is especially prominent under drug perturbations, where subpopulations of cells may alter their glucose utilization to tolerate the drug stress.[5, 7] Thus, quantifications of both glucose influx and lactate production at the single-cell level is required for a comprehensive understanding of the aerobic glycolysis in heterogeneous tumors.

In addition, there is an ever-increasing interest in further deciphering how the interplays between the Warburg effect and the oncogenic signaling provide a metabolic advantage to cancer cells, thereby promoting tumor growth. [8] With the aim of untangling the mechanistic links among those components in heterogeneous tumors, it is necessary to perform simultaneous measurements on a panel of oncogenic signaling phosphoproteins, glucose influx and lactate production at the single cell level. To date, such task is only possible through the SCBC technology. Therefore, an orthogonal chemical approach that translates lactate quantity to fluorescence readouts so as to be incorporated into the SCBC platform is a pressing need.

Currently, quantification of lactate at the single cell level remains challenging. A major obstacle is the secretion nature of lactate, which renders those well-established uptake-based

detection schemes incompatible. Another challenge is the lack of lactate-specific antibodies, due to their extremely small size and endogenous existence. Therefore, it is impractical to develop immuno-based lactate detection methods.

Herein, we report on a chemical method for quantifying lactate production from single cells using a surface-immobilized redox-labile fluorescent probe and coupled enzyme reactions (Figure 1a). This approach was designed to be compatible with our previously reported SCBC platform (Figure 1b).[5] By integrating the quantitation of lactate production with glucose influx, as well as assays for key elements of the epidermal growth factor receptor (EGFR) signaling pathway, we were able to interrogate a glycolysis-addicted patient-derived EGFR variant III (EGFRvIII) mutant glioblastoma neurosphere model (GBM39) under different therapeutic perturbations. We resolved the aerobic glycolysis activity and its interplay with the oncogenic signaling at the single-cell level.

The lactate detection in this study was based on the surface-immobilized resazurin/resorufin as the fluorescence reporter (Figure 1a). The resazurin/resorufin pair has been widely employed in various analytical methods for redox-active metabolites due to its fast reaction kinetics and prominent fluorescence spectra changes in detecting NADH – a common mediator of enzymatic redox processes.[9] However, those studies were exclusively carried out in bulk solutions. To the best of our knowledge, there is no report of incorporating this redox pair for surface-based biosensor construction. This is probably due to the challenges in immobilizing resazurin, whose redox lability could lead to insufficient probe stability and poor assay sensitivity.

As the starting point for attempting resazurin immobilization, we first synthesized 4-carboxyresazurin (CRz) to enable further conjugation. Other modifications to resazurin, such as the alkylation and acetylation on the phenolic hydroxyl group, render the resulting resorufin counterparts non-fluorescent. A closer investigation revealed that the CRz adopted the salicylic acid structure as the preferred configuration (Figure 2, Supporting information Figure S1). Although both isomers demonstrated negative electrostatic potentials on the carboxyl groups, the salicylic form exhibited a weaker and more dispersed potential. In addition, the highest occupied molecular orbital (HOMO) for the salicylic form involved no contribution from the carboxyl group. In comparison, the carboxyl-quinone isomer had a more carboxyl-centric HOMO. All these factors contribute to the strongly compromised reactivity of the carboxyl group. As a result, direct conjugation of CRz to the surface led to negligible yield. In order to improve the CRz reactivity, we converted the aromatic carboxylic group to aliphatic reactive groups. Four types of extended CRz structures bearing amine, carboxyl, biotin and azide functional groups were successfully synthesized at appreciable quantities (Supporting information Figure S2-S5). These functional groups provided the basis for surface immobilization reactions.

In order to be adapted to the SCBC platform, the probe immobilization process should be compatible with the single-stand DNA hybridization process.[5] However, the *N*-oxide group on CRz confers strong redox lability. Consequently, the attempt of conjugating the amine-modified CRz to a single strand DNA (ssDNA) was unsuccessful. Likewise, ssDNA coupled to carboxyl-modified CRz suffered from spontaneous reduction. On the contrary, both the

biotin-modified CRz (BRz) and azide-modified CRz (APRz) demonstrated superior stability (Supporting information Figure S6, S7).

We then evaluated the BRz and APRz probes for their performance in lactate detection experiments. BRz was immobilized onto the surface through ssDNA-streptavidin conjugates. Similarly, dibenzocyclooctyne-modified ssDNA enabled surface grafting of APRz molecules (Figure 3a).[10] The resazurin-modified surfaces were exposed to a solution containing a mixture of sodium lactate, nicotinamide adenine dinucleotide (NAD^+), lactate dehydrogenase (LDH) and diaphorase. In this case, the enzyme reaction cascade shown in Figure 1a translates the lactate quantity to fluorescence readouts. As shown in Figure 3b, BRz exhibited unsatisfactory fluorescence increase. Further investigation revealed that the resorufin fluorescence was severely quenched by streptavidin (Supporting information Figure S8). On the other hand, APRz probes exhibited almost ten-fold increase of fluorescence intensity after lactate conversion (Figure 3b). More interestingly, the reduced APRz exhibited higher fluorescence intensity than unmodified resorufin (Supporting information Figure S9), possibly due to the intramolecular hydrogen bond stabilizing the structure. In addition, the APRz showed faster reaction kinetics in the diaphorase catalyzed NADH oxidation reaction than unmodified resazurin, demonstrating its superior performance (Supporting information Figure S10).

A potential concern of employing the enzymatic mechanism for lactate quantitation was the specificity of the method. The complex intracellular contents always pose strong challenges on such redox-based methods. In order to validate the detection scheme, we assessed the potential interference from common intracellular reducing agents such as glutathione and ascorbic acid, as well as glucose and glutamine. We found that those reductants at biologically-relevant levels led to insignificant fluorescence increase within the assay duration (Figure 3c, Supporting information Figure S11). In order to evaluate the combined interference from all intracellular components, we further performed validation using GBM39 cell lysates. Indeed, without the addition of extra NAD^+, the reduction of APRz was negligible (Figure 3d, Supporting information Figure S12). This result was because the endogenous NAD(P)H level was two to three magnitudes lower than that of the lactate,[11] and cells constantly produced lactate. Therefore, the intracellular NAD(P)H would contribute only negligibly to the observed fluorescence signal. Indeed, metabolic processes that produce other reducing intermediates could also interfere with the lactate detection. However, by providing excess amount of both LDH and NAD^+, the conversion of lactate would be significantly preferred.

We also found that the increase of fluorescence intensity corresponded well with the lactate concentration, both in the solution phase and on the surface (Figure 3e,f, Supporting information Figure S13). Particularly, the dynamic range of the surface APRz well covers the expected lactate concentrations at single cell level.[12] These results demonstrated that the APRz-based detection scheme was suitable for single cell lactate quantitation.

We then sought to adapt the lactate assay for single cell level quantitation. The surface-based lactate assay was incorporated onto the SCBC platform, which consisted of a two-layer elastomer microfluidics device coupled to a DNA barcoded glass slide.[5, 7] The device

contained 384 programmable microchambers where cells were loaded and lysed for analysis. Each microchamber was equipped with a set of DNA barcode stripes, which served as a scaffold for multiplex measurements (Supporting information Figure S14). We quantified glucose uptake, lactate production and a panel of signalling proteins from GBM39 single cells. GBM39 is a human glioblastoma cell line that harbours a mutated form of the epidermal growth factor receptor (EGFR), which drives cell proliferation. Cells were treated with 1 μM of erlotinib (EGFR inhibitor), 0.1 μM of oligomycin A (ATPase inhibitor) or DMSO (control) before analysis.

As shown in Figure 4a, erlotinib treatment dramatically decreased the glucose uptake capacity of GBM39 cells as well as their lactate production. Parallel measurements of oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and lactate production on bulk GBM39 cells also lead to consistent results (Supporting information Figure S15). As a commonly used inhibitor in targeted chemotherapy, erlotinib prevents EGFR binding with ATP and inhibits its phosphorylation.[13] This mechanism is consistent with our results, where significant down regulation of the phosphorylation levels on EGFR as well as downstream signaling proteins. On the other hand, under the treatment of oligomycin, GBM39 cells exhibited a slightly higher glucose uptake, but with significantly increased lactate production. These results dovetailed with the functioning mechanism of oligomycin, which inhibited ATP synthase and blocked the cellular electron transport chain, leading to elevated aerobic glycolysis activities.[14] Interestingly, oligomycin also appeared to suppress the phosphoproteins downstream of the EGFR signaling pathway, which implied that change of metabolic paradigm might also affect the oncogenic signaling network.

The single cell dataset contains rich information that can be mined through statistical analysis. To further dissect the interplay between aerobic glycolysis and phosphoprotein signalling, we employed the t-distributed stochastic neighbor embedding (t-SNE) to project the high-dimentional single cell data into a 2D space (Supporting information Figure S16). We further used PhenoGraph to partition these single data into subpopulations, using a nearest-neighbor method.[4a, 15] We resolved 17 subpopulations (clusters) across three treatment conditions (Figure 4b, Supporting information Figure S16-S18). We found that glucose uptake level and lactate production were decoupled across many subpopulations, evidenced by their distribution patterns (Figure 4b, small panels). Even within the same sample group, the two analytes correlated poorly. For instance, cluster 1 and cluster 2 exhibited similar glucose uptake level but drastically different lactate production. This result proved that glucose uptake alone could not represent aerobic glycolysis activities at single cell level.

We also found that the most proliferative cells from each treatment group (Clusters 5, 11 and 17), marked by the elevated level of Ki67 (proliferation marker), exhibited rather unique metabolic and signalling signatures. In particular, in the control and erlotinib-treated cells, the proliferation ability did not correlate well with aerobic glycolysis nor the EGFR signalling activities (cluster 5 and 11). However, cluster 17 had the highest proliferation, lactate production and EGFR signalling activities within the oligomycin-treated sample group. Further analysis also revealed that oligomycin treatment led to strengthened correlations between aerobic glycolysis and oncogenic signalling, as well as within the

phosphoprotein signalling network (Supporting information Figure S19-S21). These results indicated that by promoting the highly aerobic glycolytic phenotype, oligomycin also reinforced the cellular reliance on oncogenic EGFR signalling. In addition, cluster 17 was found to be the major contributors to this increased reliance (Supporting information Figure S22). Given time, these cells may dominate the entire cell population based on their relatively higher proliferation rate. On the other hand, the elevated oncogene addiction could render them more susceptible to EGFR inhibition.

Based on the observations above, we hypothesized that a combination of erlotinib and oligomycin would induce a synergistic growth inhibition on GBM39. Indeed, such synergistic effect across a broad dose range of erlotinib (1–10 μM) and oligomycin (0.1–1μM) was observed (Figure 4c, Supporting information Figure S23). In order to test the generality of this result, we also performed the same tests on a second EGFRvIII mutant GBM cell line (HK301) that is more sensitive to erlotinib treatment. As expected, we observed similar synergistic effects, albeit starting at lower doses of erlotinib (Supporting information Figure S24).

We then sought to test if the effect of oligomycin can be extended to other clinically relevant respiration inhibitors. We studied the therapeutic outcome of combining erlotinib with metformin or phenformin on GBM39 cells. The latter two drugs are approved for treating type-II diabetes and their reaction mechanisms are similar to that of oligomycin. Again, we observed significant synergistic effects (Supporting information Figure S25).

The described chemical approach for lactate quantification is complementary to our previously reported glucose uptake assay and enables comprehensive and true aerobic glycolysis profiling in single cells. When combined with the multiplex phosphoproteomic assays in the SCBC platform, it permits a clarifying view into how glycolytic activities relate to phosphoprotein signalling. It is worth pointing out that the analysis of the obtained single-cell data requires clear knowledge of the corresponding metabolic and/or signalling pathways. In many cases, the interpretation of such data can be confounded by the complexity of biological systems, and oversimplification may lead to incorrect conclusions.

In addition to lactate detection, the surface immobilized fluorescence probes may be adapted to detect other redox-active metabolites, through employing different enzymatic schemes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We gratefully acknowledge the following agencies and foundations for financial support. NIH Grants U54 CA199090, U01 CA217655, Eli & Edythe Broad Center of Regenerative Medicine and Stem Cell Research – Hal Gaba Director’s Fund for Cancer Stem Cell Research Award, the Ben and Catherine Ivy, the Jean Perkins, and the Phelps Family Foundations.

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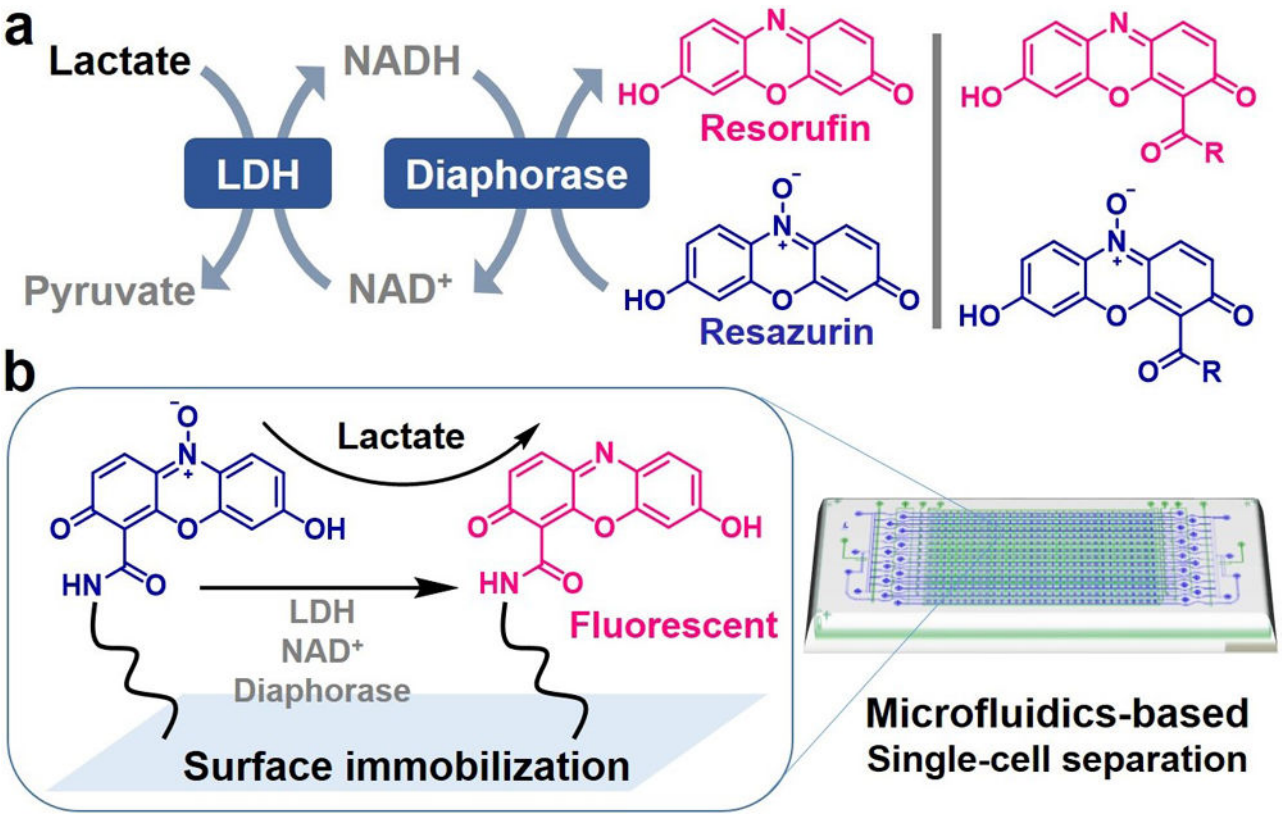

Figure 1.

a) The principle of assaying lactate through coupled enzyme reactions on resazurin substrates. The amount of lactate translates to the fluorescence intensity of resorufin. **b)** Surface immobilization of the resazurin analogs integrated with microfluidic-based technology enable the quantification of lactate production from single cells.

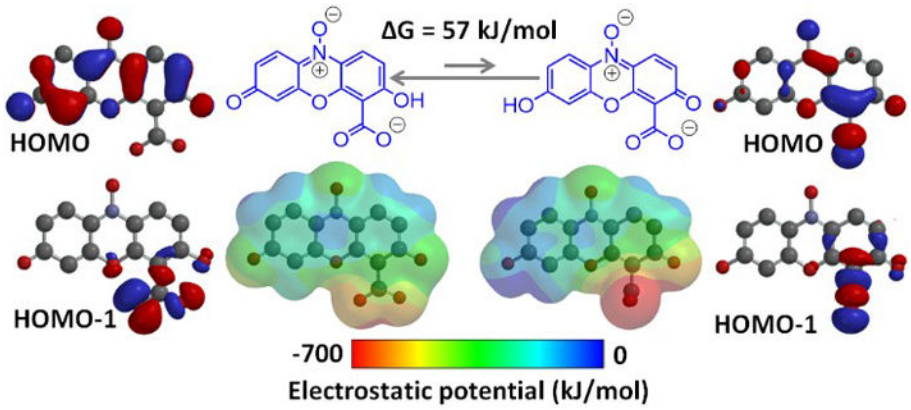

Figure 2.

Electrostatic potential and molecular orbital calculations reveal that the dominant salicylic isomer has compromised nucleophilicity and is unsuitable for further conjugations.

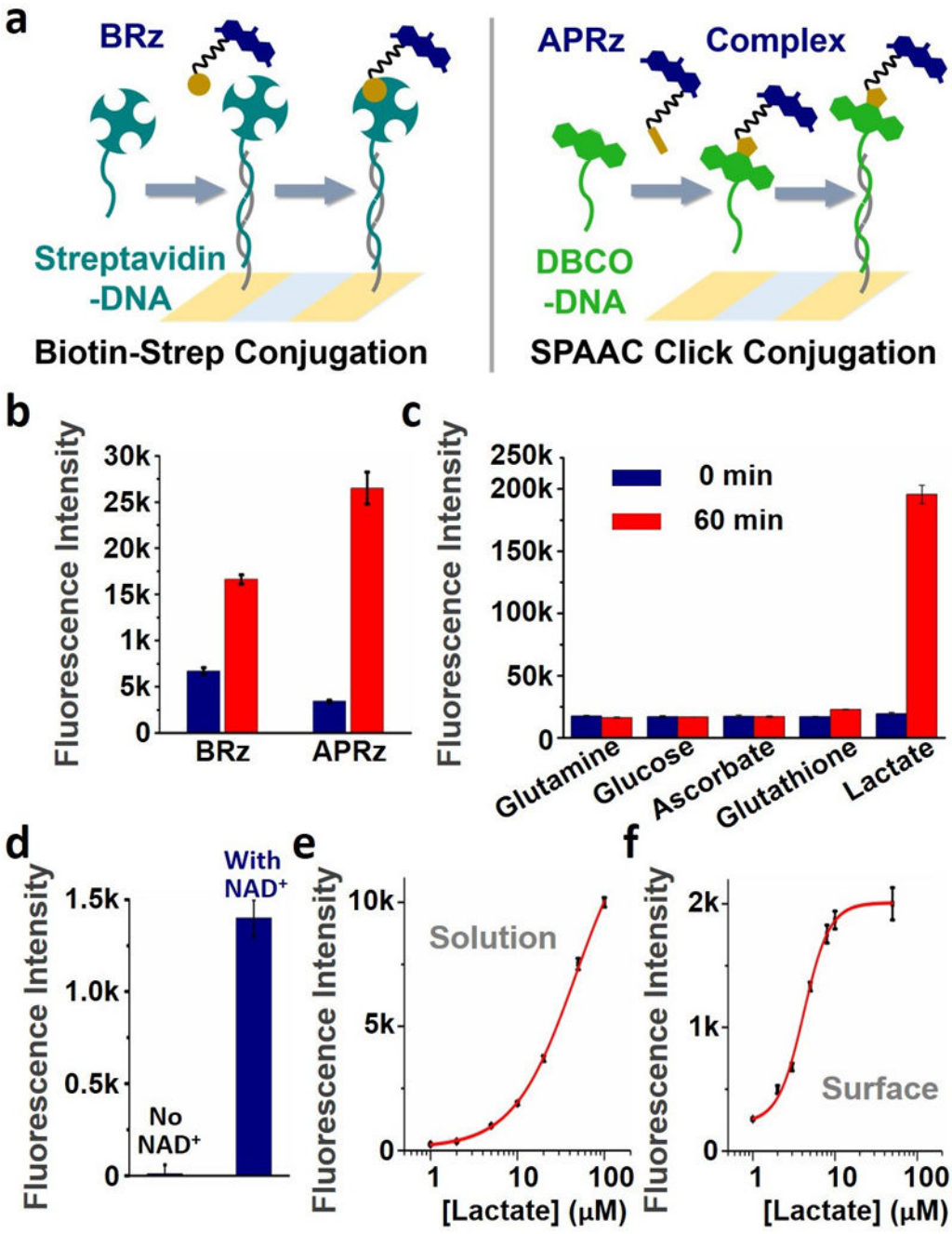

Figure 3.

a) Biotin-modified CRz (BRz) and azide-modified CRz (APRz) enable surface immobilization. **b)** Surface fluorescence intensities before (blue) and after (red) the lactate assay. Larger increase of fluorescence indicates better efficiency in lactate detection. **c)** Comparison of the fluorescence increase of APRz resulted from lactate conversion and other intracellular reducing agents. **d)** Lactate assay results using GBM39 cell lysates. APRz was mixed with diaphorase and LDH. To this mixture was added cell lysate, or cell lysate/NAD^+. Background fluorescence value obtained from a lysis buffer control was subtracted. **e,f)**

Working curve for the solution phase and surface-based lactate detection, using APRz as the substrate. The red curves were obtained through fitting with Hill functions.

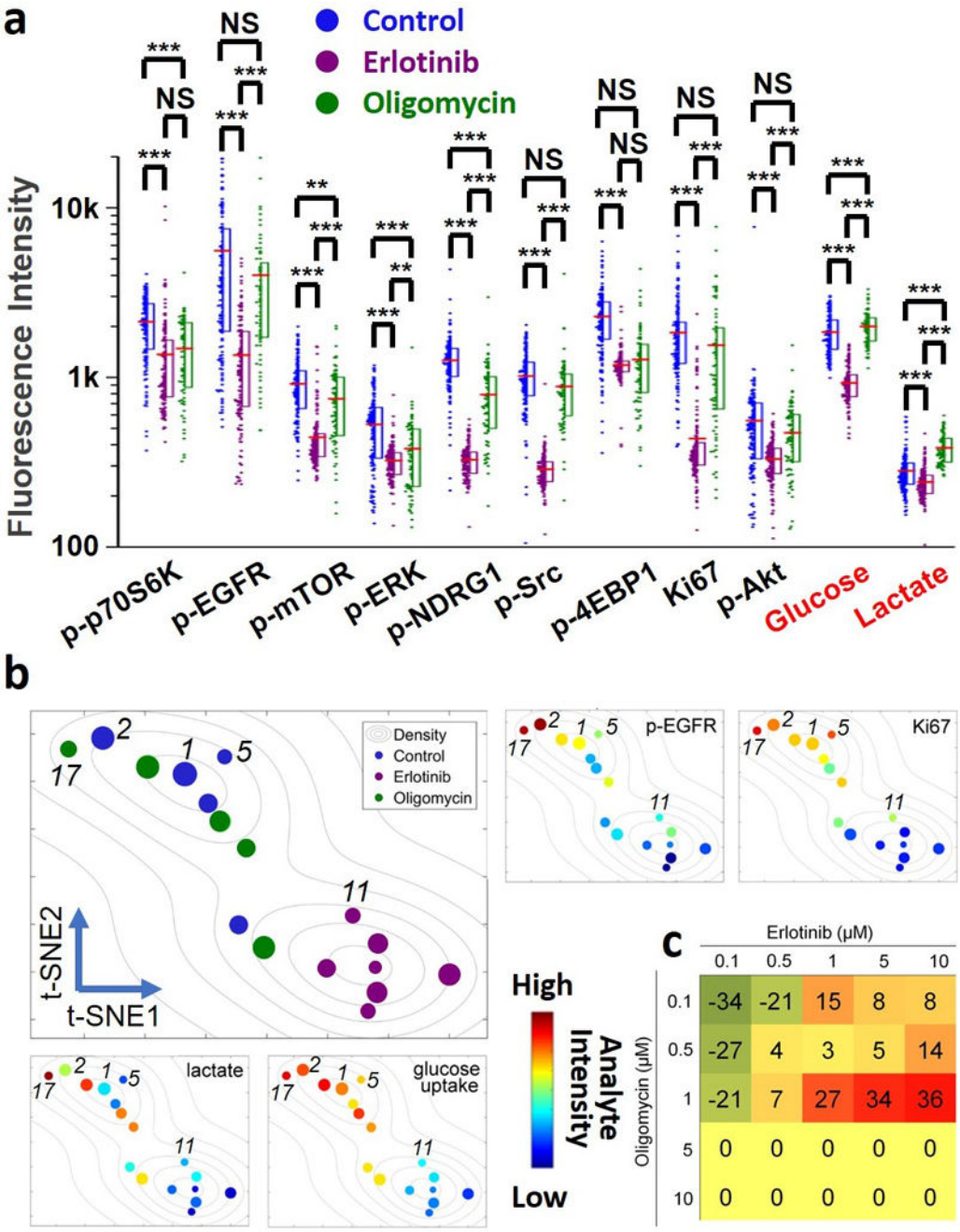

Figure 4.

a) Scatter plot of the single cell dataset. Red horizontal bars denote the average analyte levels and boxes represent the first and third quartiles of analyte distributions. Wilcoxon-Mann-Whitney significance levels: NS, $p > 0.01$; **, $p < 0.01$; ***, $p < 0.001$. **b)** t-SNE projection of the PhenoGraph clusters from GBM39 single cell dataset. Each cluster is represented by a single circle scaled to the number of cells in that cluster. The clusters are either colour coded by the treatment conditions (the larger panel) or by median expression of indicated analytes (smaller panels).The numbers next to the circles denote the cluster name. **c)**. Synergistic effects observed in erlotinib and oligomycin co-treatment experiments on

GBM39 cells. Red colour and positive numbers represent synergistic effects, and green colour with negative numbers represent antagonist effects.