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Supramolecular Probes for Assessing Glutamine Uptake Enable Semi-Quantitative Metabolic Models in Single Cells

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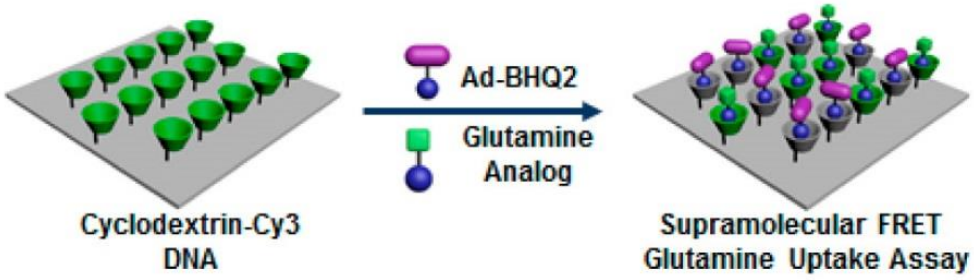
DOI: 10.1021/jacs.5b12187
J. Am. Chem. Soc. 2016, 138, 3085−3093



Azhar
14-01-2017

Introduction:

Cancer may be considered a metabolic disease, where multiple deregulated metabolic pathways contribute to disease progression. The prototypical metabolic alternation in cancer is the Warburg effect, where cells exhibit an elevated glycolysis in aerobic or anaerobic environments.

Upregulated glutamine metabolism has recently been recognized as another unique feature of many tumors. In those cases, glutamine can participate in the tricarboxylic acid (TCA) cycle through conversion to α-ketoglutarate and provide an alternative energy source to glucose.

Certain cases of drug resistance in cancer are also accompanied by heightened glutamine metabolism. Methods for the analysis of cellular glutamine uptake have provided powerful biological insights, but they are largely limited to isotopic labeling followed by radioactivity or mass spectrometric analysis of bulk samples.

Background

Absolute quantitation of intracellular metabolite concentrations by an isotope ratio-based approach

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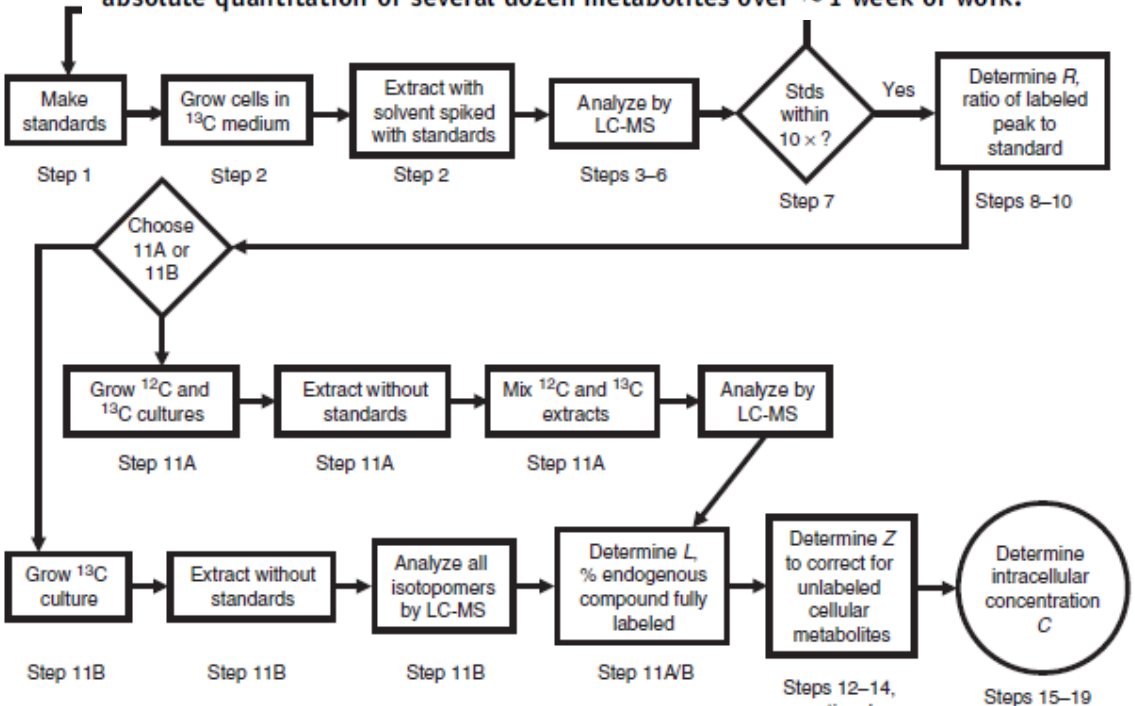
Published online 17 July 2008; doi:10.1038/nprot.2008.107

This protocol provides a method for quantitating the intracellular concentrations of endogenous metabolites in cultured cells. The cells are grown in stable isotope-labeled media to near-complete isotopic enrichment and then extracted in organic solvent containing unlabeled internal standards in known concentrations. The ratio of endogenous metabolite to internal standard in the extract is determined using mass spectrometry (MS). The product of this ratio and the unlabeled standard amount equals the amount of endogenous metabolite present in the cells. The cellular concentration of the metabolite can then be calculated on the basis of intracellular volume of the extracted cells. The protocol is exemplified using *Escherichia coli* and primary human fibroblasts fed uniformly with ^{13}C-labeled carbon sources, with detection of ^{13}C-assimilation by liquid chromatography–tandem MS. It enables absolute quantitation of several dozen metabolites over ~1 week of work.



Nat Protoc. 2008, 8, 1299-1311
doi: 10.1038/nprot.2008.107

Glutamine Uptake and Metabolism Are Coordinately Regulated by ERK/MAPK during T Lymphocyte Activation

Erikka L. Carr, Alina Kelman, Glendon S. Wu, Ravindra Gopaul, Emilee Senkevitch, Anahit Aghvanyan, Achmed M. Turay, and Kenneth A. Frauwirth

Activation of a naive T cell is a highly energetic event, which requires a substantial increase in nutrient metabolism. Upon stimulation, T cells increase in size, rapidly proliferate, and differentiate, all of which lead to a high demand for energetic and biosynthetic precursors. Although amino acids are the basic building blocks of protein biosynthesis and contribute to many other metabolic processes, the role of amino acid metabolism in T cell activation has not been well characterized. We have found that glutamine in particular is required for T cell function. Depletion of glutamine blocks proliferation and cytokine production, and this cannot be rescued by supplying biosynthetic precursors of glutamine. Correlating with the absolute requirement for glutamine, T cell activation induces a large increase in glutamine import, but not glutamate import, and this increase is CD28-dependent. Activation coordinately enhances expression of glutamine transporters and activities of enzymes required to allow the use of glutamine as a Krebs cycle substrate in T cells. The induction of glutamine uptake and metabolism requires ERK function, providing a link to TCR signaling. Together, these data indicate that regulation of glutamine use is an important component of T cell activation. Thus, a better understanding of glutamine sensing and use in T cells may reveal novel targets for immunomodulation. *The Journal of Immunology*, **2010, 185: 1037–1044.**

J Immunol 2010; 185:1037-1044; Prepublished online 16 June 2010;
doi: 10.4049/jimmunol.0903586

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Communication

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Chemical Methods for the Simultaneous Quantitation of Metabolites and Proteins from Single Cells

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ABSTRACT: We describe chemical approaches for integrated metabolic and proteomic assays from single cells. Quantitative assays for intracellular metabolites, including glucose uptake and three other species, are designed as surface-competitive binding assays with fluorescence readouts. This enables integration into a microarray format with functional protein immunoassays, all of which are incorporated into the microchambers of a single-cell barcode chip (SCBC). By using the SCBC, we interrogate the response of human-derived glioblastoma cancer cells to epidermal growth factor receptor inhibition. We report, for the first time, on both the intercellular metabolic heterogeneity as well as the baseline and drug-induced changes in the metabolite−phosphoprotein correlation network.

In this paper…

They have extended glutamine uptake assays to the single cell level to help resolve the heterogeneous nature of bulk tissues or tissue cultures that can mask deeper meanings.

They combined those measurements with assays for a panel of 15 other metabolites and proteins to help resolve relationships that are hard to establish when the analytes are measured independently.

In their approach, the glutamine uptake assay is based upon a **novel supramolecular chemistry approach.**

They have described how the addition of the glutamine uptake assay to a combined metabolite/protein panel that includes glucose uptake and assays for the levels of glutathione (GSH), cAMP, and cGMP, plus a number of associated enzymes permits the construction of a **semiquantitative metabolic model** for understanding the dose-dependent response of model glioblastoma multiforme cancer cells to receptor tyrosine kinase inhibition.

Results and discussion:

Surface Patterned Supramolecular FRET Pair

Screening of Glutamine Analogs

Glutamine Uptake Measurements on an Integrated Metabolic/Proteomic SCBC

A Simplified Model for Analyzing Integrated Metabolic/Proteomic Data

Surface Patterned Supramolecular FRET Pair

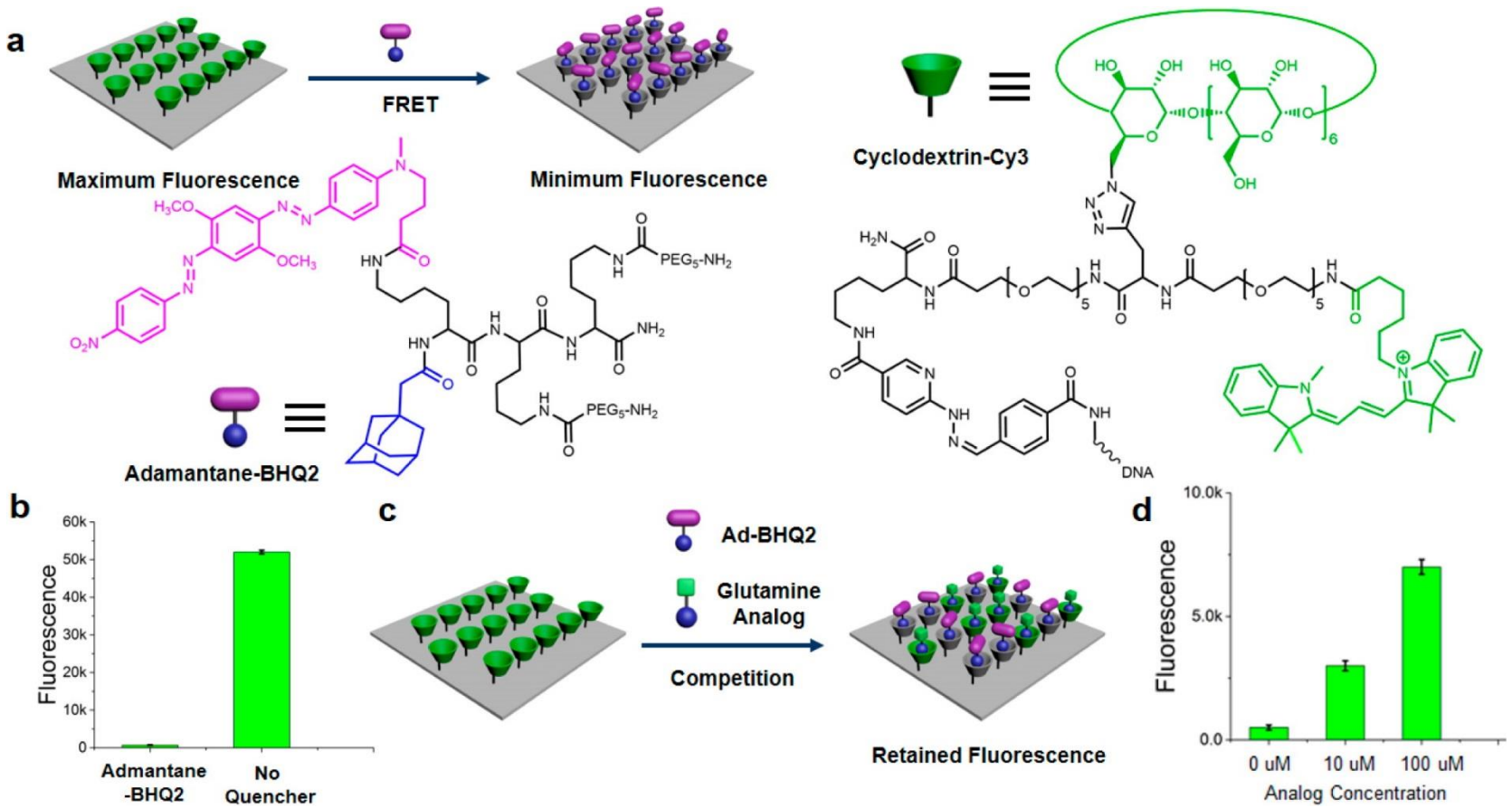

Figure 1. Illustration of the supramolecular glutamine assay. (a) The chemical structures and the functioning mechanism of the supramolecular FRET pair. The cyclodextrin-Cy3 was conjugated to a single strain DNA and then immobilized onto the glass slide through DNA hybridization. The Cy3 groups serve as FRET donor and the cyclodextrins as supramolecular host. A dark quencher group, BHQ2, was conjugated to an adamantane to form the FRET acceptor/supramolecular guest. The binding between adamantane and cyclodextrin brings BHQ2 to the vicinity of Cy3 and quenches the fluorescence. (b) Fluorescence intensities of the surface Cy3 with and without 100 nM of adamantane-BHQ2. (c) An adamantane labeled glutamine analog competes with the adamantane-BHQ2 for the binding site of surface cyclodextrin, thus inhibiting the quenching process and retaining Cy3 fluorescence. (d) Fluorescence intensities of the surface Cy3 with 100 nM of adamantane-BHQ2 and different concentrations of the glutamine analog.

Screening of Glutamine Analogs

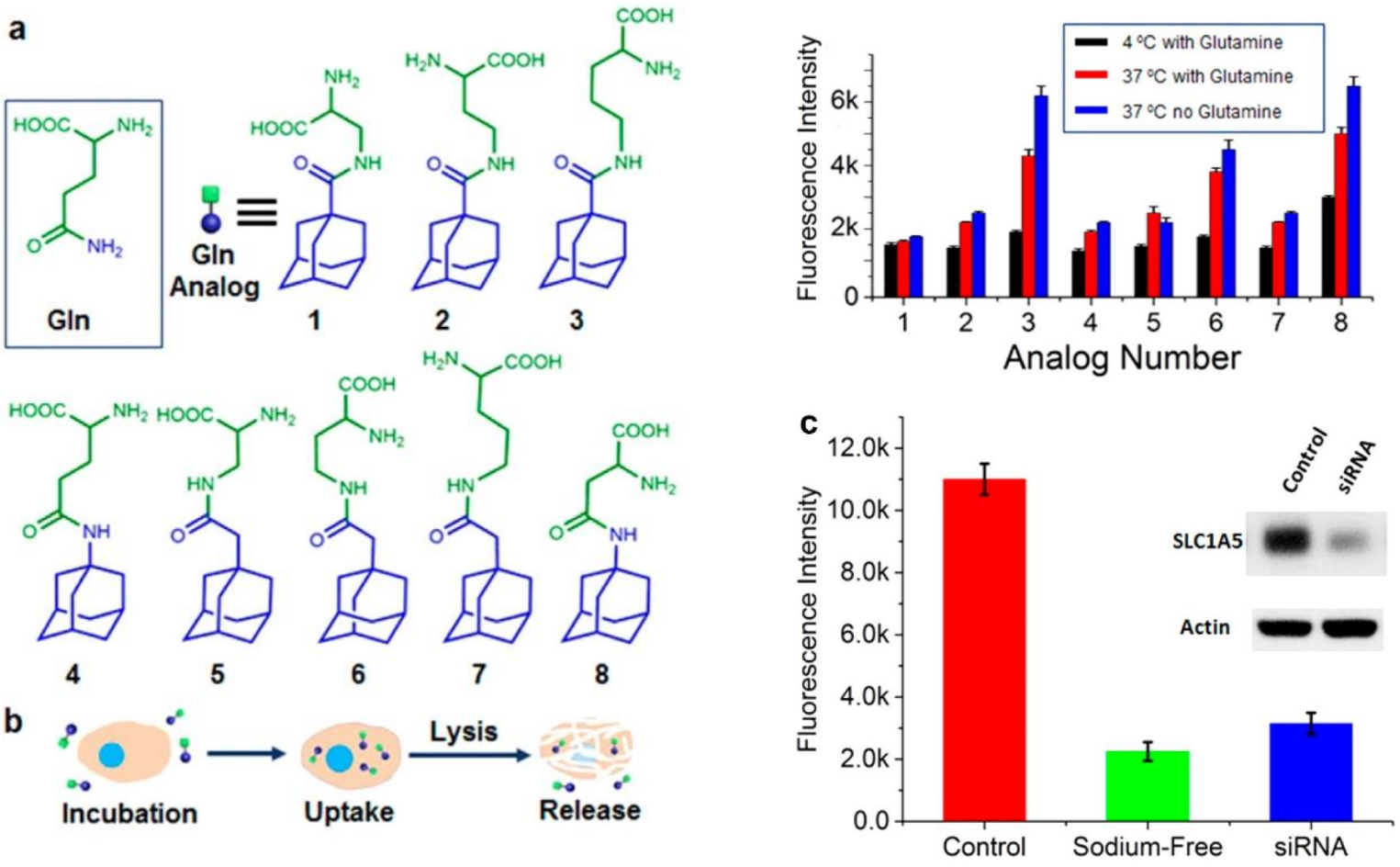

Figure 2. Screening of the glutamine analogs. (a) The structures of glutamine and the library of glutamine analogs. (b) The illustration and the results of the cell uptake experiments. Compounds 3 and 8 exhibit expected temperature dependency and glutamine dependency. Compound 3 was selected as the glutamine analog due to the better response. (c) The uptake of compound 3 in control, sodium-free media, and SLC1A5-knock down U87 cells.

Glutamine Uptake Measurements on an Integrated Metabolic/Proteomic SCBC

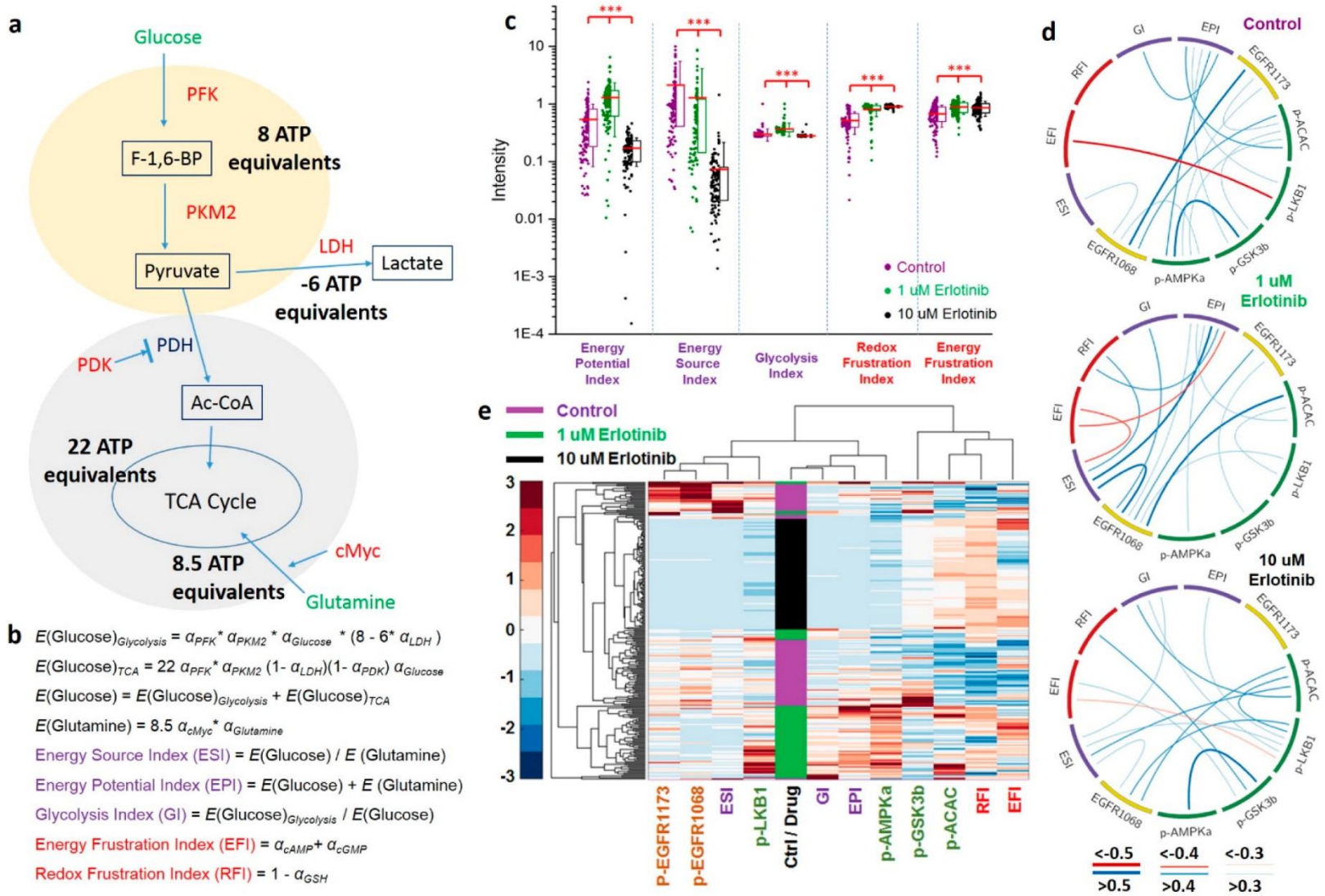

Figure 3. SCBC experiments on the U87EGFRvIII cells. (a) Illustration of the SCBC platform. (b) SCBC results of U87EGFRvIII cells treated with 1 and 10 µM of erlotinib for 24 h. Compound 3 was used as the glutamine analog. The red horizontal lines denote the mean fluorescence value of the measured analyte, the boxes cover the second and the third sample quartiles, and the whiskers label the standard deviation. (*, $p < 0.1$; ***, $p < 0.001$).

A Simplified Model for Analyzing Integrated Metabolic/Proteomic Data

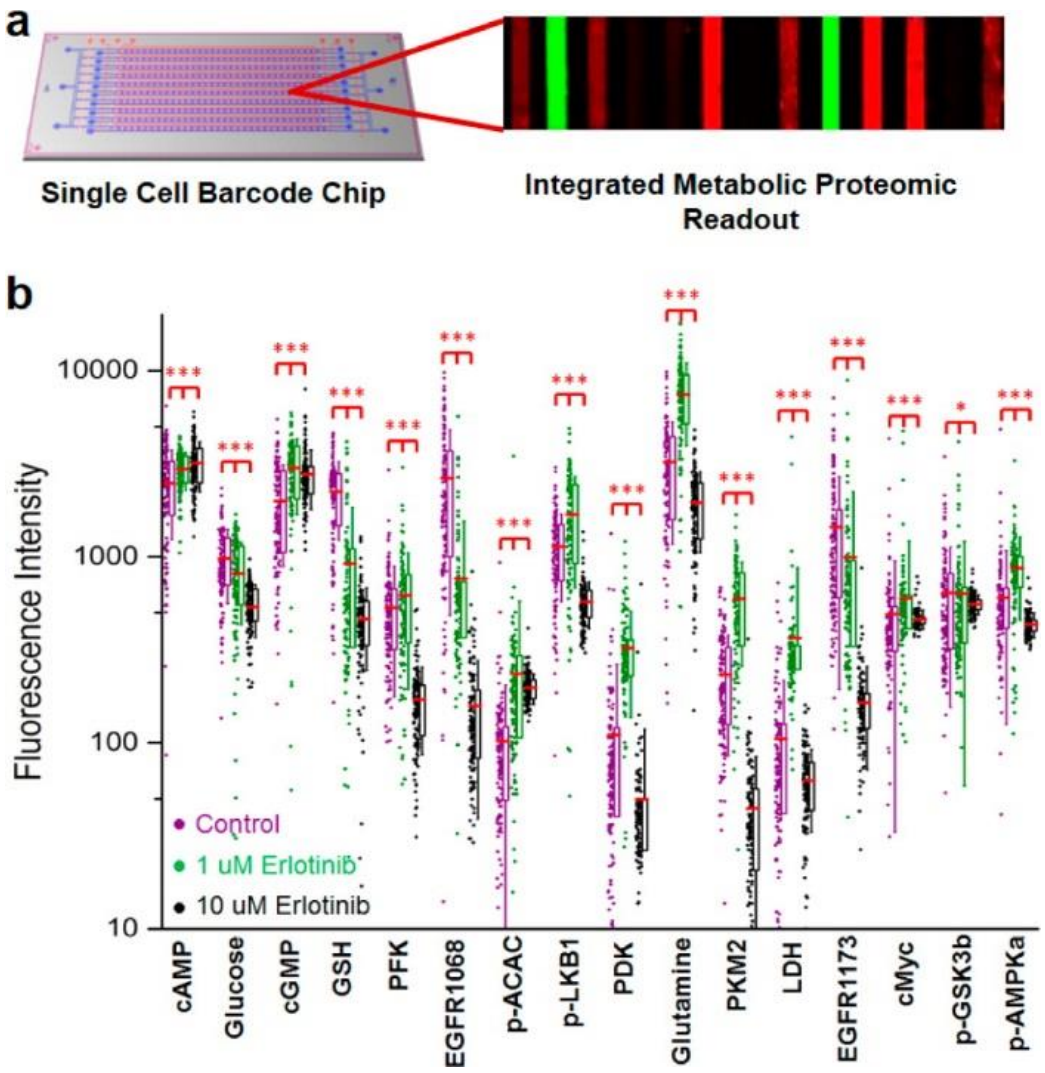

Figure 5. Data analysis based on the metabolic model. (a) The simplified metabolic model. The production number of various high-energy molecules (ATP, NADH, FADH2, etc.) is converted to the production of ATP equivalents. (b) The definition of the energy indices. (c) Scatter plots of the indices. (d) The correlations networks for different samples. (e) The clustering result of the combined SCBC data set.

Conclusion:

- They have described a supramolecular surface competition assay for quantifying glutamine uptake from single cells.
- They immobilized Cy3-labeled cyclodextrins on a glass surface as a supramolecular host/FRET donor, and employed adamantane-BHQ2 conjugates as the guest/quencher. Adamantane-labeled glutamine analog was selected through screening a library of compounds and validated by cell uptake experiments.
- When integrated onto a single cell barcode chip with a multiplex panel of 15 other metabolites, associated metabolic enzymes, and phosphoproteins, the resultant data provided input for a steady-state model that describes energy potential in single cells and correlates that potential with receptor tyrosine kinase signaling.
- They utilized this integrated assay to interrogate a dose-dependent response of model brain cancer cells to EGFR inhibition and found that low-dose (1 μM erlotinib) drugging increases cellular energy potential even as glucose uptake and phosphoprotein signaling is repressed. They identified new interactions between phosphoprotein signaling and cellular energy processes that may help explain the facile resistance exhibited by certain cancer patients to EGFR inhibitors.

Future plan:

As in this paper, other suitable chemical techniques can be employed so that other metabolites can be estimated by integrating with single cell barcode chip.

Immobilasation of cyclodextrin for localized surface plasmon resonance spectroscopy and sensing of metal based nanosystems.

Glutamine plays an important role in cellular metabolism and has special significance in cancer cell maintenance and survival. Although the ability to quantify glutamine uptake at a single cell level is meaningful, the value of that assay is significantly amplified when it is integrated into a multiplex panel of related metabolites, metabolic enzymes, and signaling proteins. Such an integrated analysis was enabled by the compatibility of the supramolecular-based glutamine uptake assay with the core DNA barcoding method that is used in SCBCs. The design of the DNA-cyclodextrin-Cy3 donor was engineered for competitive binding and required a balance between high-surface coverage of cyclodextrin and steric access to enable the DNA hybridization-driven surface assembly of the donor. Thus, there is a trade-off on the amount of cyclodextrins per DNA oligomer, and this trade-off was experimentally optimized. Because of the high density of cyclodextrin groups on the surface and the confined space in the microfluidic device, the effective cyclodextrin/adamantane binding is much stronger than that in solution. For the quencher molecule, both BHQ2 and adamantane are hydrophobic, and so PEG moieties are appended to improve the aqueous solubility. Additionally, the p-nitroazobenzene group on BHQ2 can also bind with cyclodextrin, further increasing the binding affinity between cyclodextrin and adamantane-BHQ2. This enhances the quenching efficiency and improves assay sensitivity. The design of the small library of glutamine analogs (Figure 2a) was based on the substrate specificity of the human glutamine transporter (SLC1A family), that is, a neutral amino acid side chain with hydrogen-bonding capabilities. Because the binding mechanism and the exact binding pocket structure of SLC1A are unknown, it is difficult to interpret the differing performances for each analog. In addition, because there are other potential carriers for glutamine transport in different types of cells, these analogs may not serve well as universal glutamine uptake surrogates.

Multiplex single cell measurements of functional analytes metabolites and enzymes yield several classes of information, in addition to the average value of the analyte (which is also obtained from bulk assays). First, the distribution of each analyte level provides insight into the sample heterogeneity. Second, analyte−analyte correlations can provide information regarding signaling interactions that are activated or repressed between different experimental conditions. Finally, clustering analysis can provide a more global view for the statistical analysis of sets of analytes or permit comparisons across different experimental conditions. In our case, the inclusion of metabolites with intrinsically correlated metabolic proteins within the panel permits the use of a semiquantitative and simplified physical chemistry model (Figure 5a) for guiding the interpretation of the single cell data. Although the model excludes many other metabolic pathways that may be active, it should have some meaning within the context of global cellular energy demand. The metabolic model, combined with insights from bulk metabolic assays, yields energy indices that help guide the interpretation of the dose-dependent response of the GBM cells to erlotinib (Figure 5c). For example, the EPI increases upon low-dose drug treatment. This is in seeming contrast to the decreased glucose uptake, but the EPI provides a more global view of energy flux. The increased level of metabolic enzymes and the heightened glutamine uptake point to elevated metabolic activities of the cells under low-dosage (but clinically relevant) drug stress. At the higher dose, the metabolic activity of the cells is repressed, by all measures reported here. The lowdosage results are further validated through intracellular ATP concentration measurements and extracellular oxygen consumption assays (Figure 4). This result may have clinical relevance. At low dose, erlotinib successfully engages its target (decreased p-EGFR level) and inhibits glucose uptake (corresponding to a decreased 18F-FDG PET signal in clinical molecular imaging).

However, the cells are still very metabolically active, and the cell growth is not inhibited in vitro, thus anticipating a poor patient response. The value of the EPI index as a gauge of drug effectiveness, relative to protein assays and/or glucose uptake assays alone, is seen in the clustering analysis of Figure 5e. Here, the EPI index readily differentiates control/low-dose drug/high-dose drug populations and correlates well with cell survival. The single cell resolved metabolic model also provides deeper biological information. For instance, a linkage between receptor tyrosine kinase signaling and GBM cell metabolism is resolved. The ESI is highly correlated with the p-EGFR level of the cells (Figure 5e), while no single metabolite or metabolic protein exhibited such a strong correlation. This suggests that EGFR signaling may regulate the cellular glutamine dependence. Further, the pEGFR1068 and the pEGFR1173 sites are decoupled via drugging, and we can infer different functional consequences of these two phosphorylation sites, with the pEGFR1068 possibly playing a role in regulating cellular glutamine dependency, and thus cell survival upon erlotinib treatment. This information may be useful in terms of understanding the rapid development of resistance to erlotinib that is seen in GBM patients. The metabolic protein panel and the metabolic model were established with the motivation for establishing a picture of the metabolic demand that can be resolved at the single cell level, using metabolites that are especially important for cellular energy consumption. It is straightforward to assay more proteins on the SCBC platform.30 It should also be possible to develop additional chemical methods to expand the panel of metabolites that are simultaneously assayed. For instance, quantifying ATP/ADP ratio would complement the assays reported here. Further, a more comprehensive panel with metabolic intermediates and products, such as lactate, pyruvate, α-ketoglutarate, etc., would significantly help resolve a more sophisticated and quantitative picture of cellular energy flux, thus removing some of the assumptions that went into the semiquantitative model provided here.