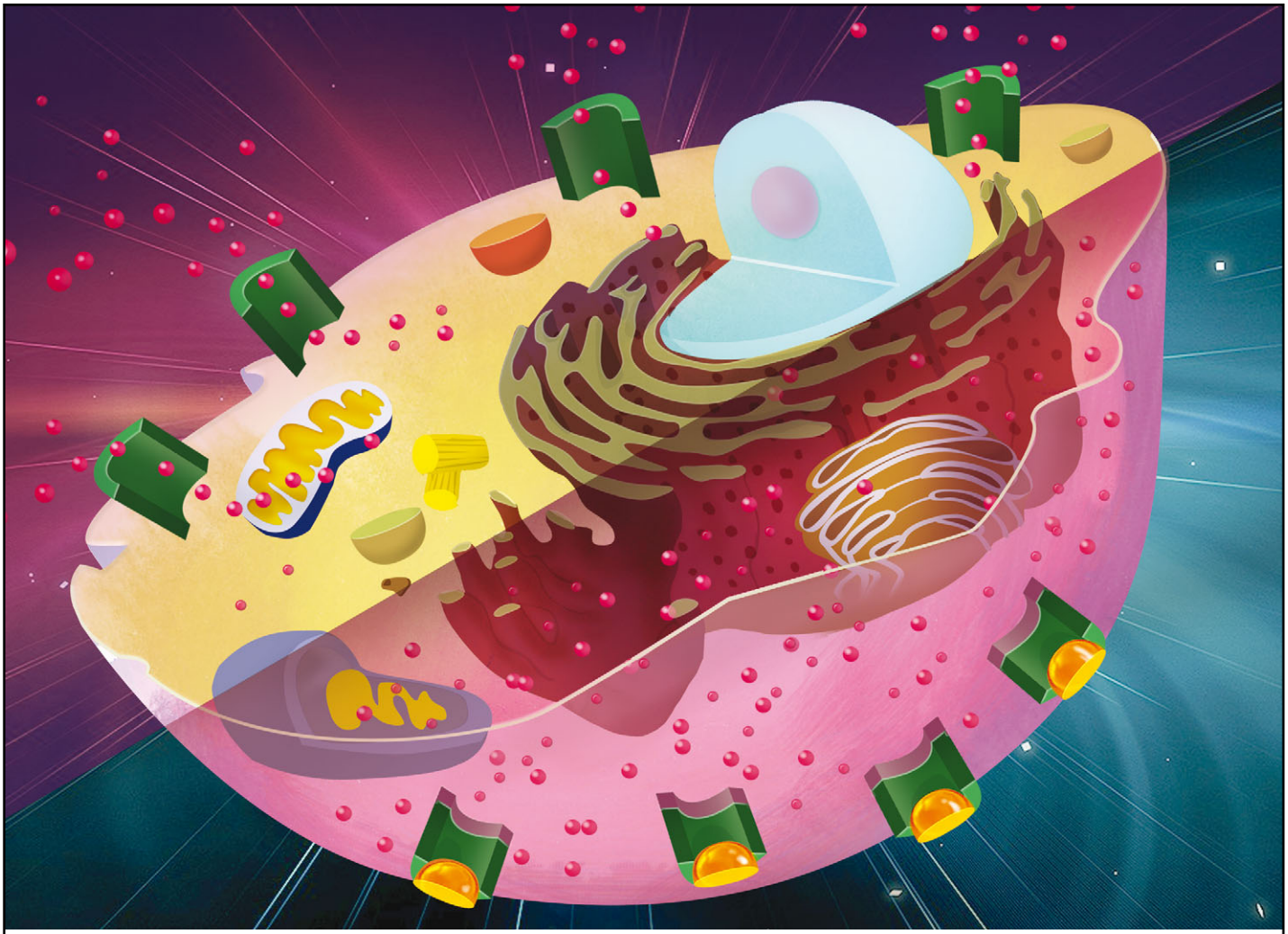

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**Exploiting the mechanism of cellular glucose uptake to develop an image-based high-throughput screening system in living cells**

A novel image-based assay system using a fluorescent glucose bioprobe, GB2, was developed to monitor the perturbation of cellular glucose uptake in a high-throughput manner. Based on our biophysical studies, we effectively suppressed the efflux of GB2 with phloretin, a transient and selective GLUT inhibitor. With this breakthrough, we developed the first image-based HTS system to monitor the glucose uptake in living cells with robustness, reproducibility, and accuracy.

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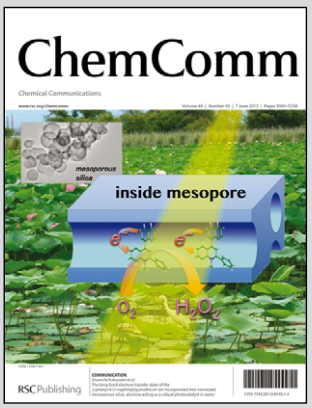


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# Exploiting the mechanism of cellular glucose uptake to develop an image-based high-throughput screening system in living cells†

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**Biophysical understanding of cellular glucose uptake led us to the development of an image-based high-throughput screening system by using a fluorescent glucose bioprobe, GB2. The accuracy, robustness, and practicality of our image-based HTS system were demonstrated through the pilot screening and the subsequent *in vitro* confirmation.**

Glucose is the main energy source in organisms, and its cellular uptake is the key phenomenon that indicates metabolic activity. Glucose homeostasis is extremely important in energy balance and is tightly controlled through the interplay between glucose uptake and utilization, which is highly associated with the physiological states of organisms and is regulated by insulin-dependent and insulin-independent pathways.[1] Thus, an imbalance in glucose homeostasis is one of the principal markers for various diseases.[2] Therefore, the measurement of cellular glucose uptake has been of prime interest for the development of early diagnostic tools and for the discovery of novel therapeutic agents to treat cancer, diabetes, and obesity.[3]

A radioactive glucose analogue, [$^{18}$F]-2-fluoro-2-deoxy-D-glucose ($^{18}$FDG), has been extensively used for imaging tumors and metastatic diseases in the field of clinical oncology.[4] Even though it has huge impact in clinical diagnosis, $^{18}$FDG and other radioactive glucose analogues are not practically applicable in laboratory settings, especially for large-scale screening systems in the drug discovery process, because of their high costs, inconvenience in use, and the hazards associated with their radioactive wastes. These limitations have led researchers to the development of fluorescent glucose bioprobes. For example, 2 fluorescent glucose analogues, 2-NBDG[5] and 6-NBDG,[6] were developed and their cellular uptake mediated *via* glucose transporters (GLUTs) was demonstrated. However, both NBDGs have several limitations, including a weak fluorescence intensity, high treatment dosage, and non-compatibility in the physiological environment.[7] To overcome these limitations, we have reported the development of novel glucose bioprobes, Cy3-glc-α[8] and GB2 (ref. 9) (Fig. 1a), and showed their application in real-time and quantitative monitoring of glucose uptake in metabolically active cells by using fluorescence microscopy and flow cytometry.[9] Further to our previous contribution to the field of glucose bioprobes, we envisioned the development of a novel, image-based high-throughput screening (HTS) system to identify new bioactive compounds for the treatment of metabolic diseases. In fact, there is a growing demand for

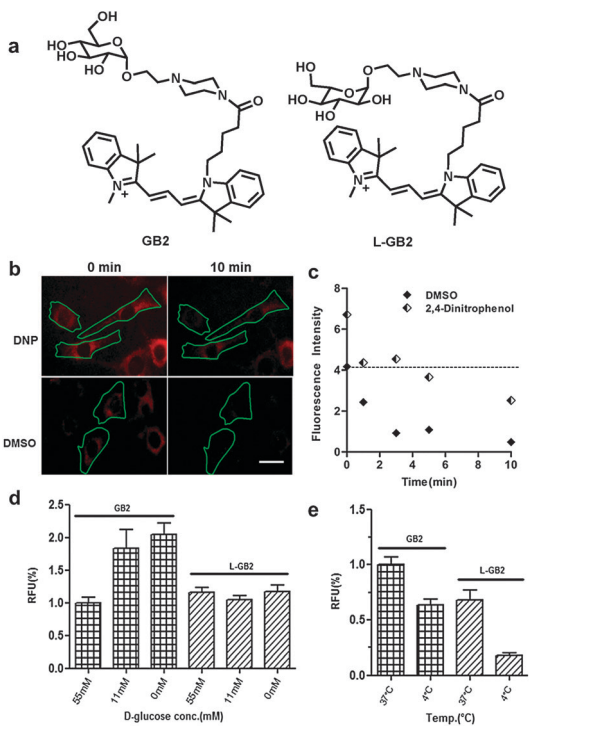


**Fig. 1** (a) Chemical structures of GB2 and L-GB2. (b) Time-dependent signal loss of GB2 within the cytoplasm of C2C12 myoblasts. The scale bar represents 20 μm. (c) Continuous monitoring of the time-dependent signal in living C2C12 myoblasts upon treatment with DNP. Fluorescence intensity was determined from the region of interest (ROI) in C2C12 cells. (d) D-Glucose competition assay of GB2 and L-GB2. (e) Suppression of endocytosis and its influence on GB2/L-GB2 cellular uptake. Relative fluorescence units (RFU) were determined from the ROI, and the data are the average values obtained in measurements performed on 40–50 cells. Error bars indicate SEM.

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image-based high-content screening (HCS) as a key approach for the discovery of novel first-in-class therapeutic agents.[10] In the case of small-molecule modulator screening for cellular glucose uptake, several HTS systems using either $^{14}$C-labeled 2DG or 6-NBDG have been pursued with limited practicality and applicability to HCS.[11]

Unlike fluorescent bioimaging of individual samples, the image-based HTS system requires a higher level of robustness and fidelity. In the context of the fundamental role of glucose as a cellular energy source, the phosphorylation of glucose is an essential step to generate energy currency (ATP) and other cellular elements *via* the pentose-phosphate pathway once D-glucose enters the cytoplasm through GLUTs along its concentration gradient (Fig. S1, ESI†).[12] $^{18}$FDG and 2-NBDG are known to be phosphorylated by hexokinases as glucose and thereby $^{18}$FDG and 2-NBDG can be trapped within cells for an extended period of time, although the phosphorylation of 2-NBDG leads to rapid degradation into non-fluorescent products.[13] However, GB2 is not a substrate for hexokinases (Fig. S2, ESI†). Therefore, a conventional bioimaging procedure will lead to time-dependent loss of fluorescent signals, caused by the efflux of non-phosphorylated GB2 from the cells through GLUTs due to its reversed concentration gradient after the washing step. When C2C12 myoblasts were treated with 2,4-dinitrophenol (DNP)—a reported small-molecule enhancer of cellular glucose uptake through shuffling protons across the mitochondrial membrane,[14] we observed high intensity of GB2 fluorescence signals in the cytoplasm. However, this fluorescence signal deteriorated down to that of the DMSO control within 5 min, as observed by continuous monitoring of fluorescence intensity (Fig. 1b and c). This phenomenon is a crucial challenge for the development of a HTS assay with GB2 because the signal read-out will be significantly affected by the inevitable difference in its detection time in the HTS format.

To address this challenge, we first investigated the detailed uptake/release mechanism of GB2 in the cellular system. We previously confirmed that GB2 enters the cytoplasm through GLUTs in an energy-independent and competitive manner with D-glucose,[9] but it is still possible for GB2 to enter the cytoplasm partly *via* either energy-dependent endocytosis or passive diffusion. In addition, endogenous GLUTs can differentiate the cellular uptake of D-glucose from that of L-glucose.[15] Therefore, we hypothesized that the chiral environment in GLUTs can selectively allow the entrance of GB2 into the cytoplasm through GLUTs. To test this hypothesis, we synthesized L-GB2, an enantiomeric pair of GB2 (Fig. 1a and see ESI†) and performed a glucose competition assay with GB2 and L-GB2 in C2C12 myoblasts to monitor physiological behavior of GB2 and L-GB2. As shown in Fig. 1d, D-glucose successfully competed out the cellular uptake of GB2 in a concentration-dependent manner, indicating that the translocation of GB2 is mainly mediated by GLUTs, but there was no difference between L-GB2 cellular uptake at the highest concentration of D-glucose (55 mM) and that in the D-glucose-depleted medium. This result confirmed that there are distinctly different cellular uptake mechanisms between GB2 and L-GB2 owing to their difference in stereochemistry (D- and L-glucose), and GB2 can mimic the plasma membrane translocation of D-glucose in cellular systems because of its GLUT specificity. In contrast, the basal level of cellular uptake of L-GB2 might be caused by either nonspecific endocytosis or passive diffusion. When we inhibited the endocytotic process by lowering the temperature during the incubation,[16] a drastic reduction in L-GB2 uptake (75%) was observed, while the GB2 cellular uptake was marginally affected under the identical conditions (Fig. 1e).

Based on these observations, we were confident that the cellular uptake of GB2 was mainly associated with cellular GLUT systems and thereby the image-based monitoring of GB2 fluorescence signal can serve as a measure for the cellular uptake of glucose, interactively regulated by cellular translocations or the expression levels of GLUTs. Since glucose utilization in skeletal muscles is significantly impaired in type 2 diabetic patients,[3] a GB2-based phenotypic HTS assay can be an excellent platform to identify anti-diabetic agents that perturb the cellular glucose uptake after addressing the GLUT-mediated excretion of GB2. In this context, we aimed to suppress the GB2 release after the washing step from the cytoplasm by using specific GLUT inhibitors to complete the GB2-based phenotypic HTS assay platform. Among the reported specific GLUT inhibitors, we selected phloretin—a natural dihydrochalcone known to be a transient and competitive inhibitor of GLUTs.[17] The treatment of phloretin was expected to inhibit the GLUT-based escape of GB2 and to retain the fluorescence signal within the cytoplasm (Fig. 2a). As shown in Fig. 2b and c, the initial fluorescence intensity of GB2 in the cytoplasm was significantly decreased within 30 min, but the treatment with phloretin preserved the fluorescence intensity of GB2 over 60 min (Fig. S4, ESI†). This result indicated that the major loss of fluorescence signals in the cytoplasm was caused by GLUT-mediated excretion of GB2, which can be effectively blocked by treatment with phloretin.

As aforementioned, we aimed to develop an image-based HTS assay with our novel fluorescence glucose bioprobe, GB2. The treatment with phloretin during the imaging step suppressed the substantial efflux-based decrease in fluorescence signals over the extended measurement time (at least 30 min) which ensures the high level of robustness and fidelity for the phenotypic screening (Fig. S6, ESI†). On the basis of this breakthrough in the bioimaging step, we established a novel screening protocol by high-content

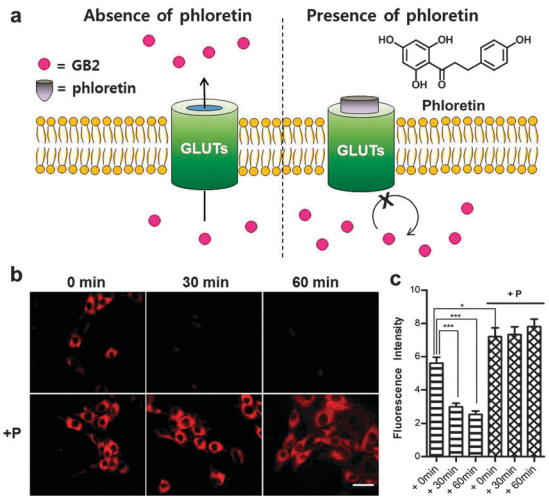


**Fig. 2** (a) Schematic of the physiological behavior of GB2 in the presence or absence of phloretin, with its chemical structure. (b) Fluorescence microscopic images of cells obtained after washing with cold PBS in the presence and absence of phloretin (20 μM). The scale bar represents 20 μm. (c) Fluorescence intensities were determined from the ROI and the data are the average values obtained in measurements performed on 40–50 cells. Error bars indicate SEM; ***$p < 0.0001$, **$p < 0.005$, and *$p < 0.01$.

image analysis of living C2C12 myoblasts with GB2 in a 96-well plate for the identification of small-molecule modulators as potential anti-diabetic agents (Fig. S7 and S8, ESI†). Unlike 2-NBDG and 6-NBDG, the brightness and minimal photobleaching of Cy3 in GB2 ensures efficient detection of the fluorescence intensity in a short exposure time, which is essential for screening a large number of compounds in a high-throughput manner (Fig. S9, ESI†). These results indicate that our phenotype-based screening system meets the criteria of a practical HTS assay for drug discovery owing to its robustness, reproducibility, and accuracy.

Finally, we performed our image-based HTS assay against 264 compounds from the pDOS library[18] to identify bioactive small molecules that perturb the cellular glucose uptake. As shown in Fig. 3a and b, we successfully identified a series of small molecules that increased the cellular glucose uptake as observed by monitoring the fluorescence intensity of GB2 in the cytoplasm. By the virtue of image-based phenotypic screening, we were able to examine the assay results to eliminate false positives; some compounds enhanced the fluorescence intensity in the cytoplasm, but the cell morphology and staining pattern showed whether they affected the cell viability or other cellular processes (Fig. 3b, P33H11 and see ESI†). To confirm the reliability of our image-based HTS assay, 4 selected hit compounds were subjected to 2 orthogonal glucose uptake measurements using $^{14}$C-labeled 2-deoxyglucose ($^{14}$C-2-DG) and enzyme-based uptake measurement assay of non-radiolabeled 2-deoxyglucose (2-DG). As shown in Fig. 3c–e, the 4 hit compounds identified from our GB2-based HTS assay consistently showed enhanced cellular glucose uptake in both commercial methods, although these methods are not practically applicable in HTS systems. Therefore, we have clearly demonstrated the reliability of our image-based HTS system to identify novel candidates for treatment of glucose homeostasis-related diseases.

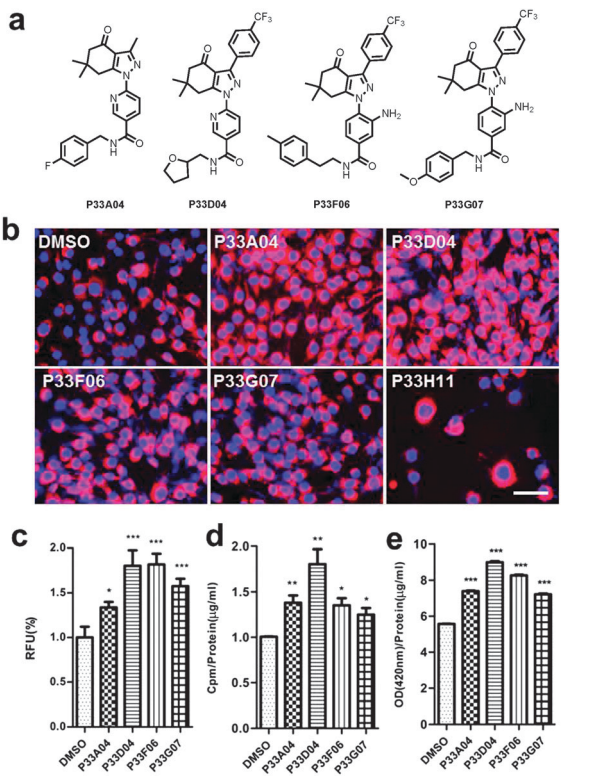


**Fig. 3** (a) Chemical structure of primary hit compounds. (b) Fluorescence images of C2C12 myoblasts. The scale bar represents 40 μm. (c) RFU was determined from the ROI. C2C12 myoblasts were treated with DMSO and 4 of the primary hits, P33A04, P33D04, P33F06 and P33G07 (10 μM), for 24 h. (d) Radioisotope-based measurement of $^{14}$C-labeled 2-DG uptake. Data are representative of three independent experiments. (e) Commercial enzyme-based measurement of cellular glucose uptake using non-radiolabeled 2-DG. Data are representative of three independent experiments. Error bars indicate SEM; ***$p < 0.0001$, **$p < 0.005$, and *$p < 0.01$.

In conclusion, we have developed a novel image-based screening system to monitor the perturbation of cellular glucose uptake in a high-throughput manner. Our mechanistic studies confirmed that the fluorescent glucose bioprobe GB2 enters the cells mainly *via* GLUT-mediated translocation and the gradual decrease in its fluorescent signal inside the cytoplasm is caused by the efflux of non-phosphorylated GB2. Based on our biophysical studies, we used phloretin, a transient and selective GLUT inhibitor, to effectively suppress the efflux of GB2 for bioimaging. With this breakthrough, we developed the first image-based HTS system to monitor the glucose uptake in living cells with robustness, reproducibility, and accuracy. Furthermore, we anticipate the application of our image-based phenotypic assay in HCS systems in conjunction with other orthogonal assays, such as intracellular trafficking, signaling processes, and glucose utilization in living cells. Considering the high demand for the discovery of therapeutic agents for the treatment of diabetes, obesity, and cancer, our image-based HTS assay provides a powerful tool to identify novel hit compounds with a new mechanism of action.

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