

A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEM **BIO** CHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

Title: Direct Intracellular Delivery of Cell Impermeable Probes of Protein Glycosylation Using Nanostraws

Authors: Alexander Minyi Xu, Derek Schawn Wang, Peyton Shieh, Yuhong Cao, and Nick Melosh

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: *ChemBioChem* 10.1002/cbic.201600689

Link to VoR: <http://dx.doi.org/10.1002/cbic.201600689>

WILEY-VCH

www.chembiochem.org

A Journal of



Direct Intracellular Delivery of Cell Impermeable Probes of Protein Glycosylation Using Nanostraws

Dr. Alexander M Xu^{[a],[b]}, Derek S Wang^[a], Dr. Peyton Shieh^[c], Yuhong Cao^[a], Prof. Nicholas A Melosh^{[a].*}

^[a]Department of Materials Science and Engineering, Stanford University, 476 Lomita Mall, Stanford, CA 94305

^[b]Current Address: Chemistry and Chemical Engineering Division, California Institute of Technology, 1200 E California Blvd, Pasadena, CA 91106

^[c]Department of Chemistry, Stanford University, 333 Campus Drive, Stanford, CA 94305

Abstract:

Bioorthogonal chemistry is an effective tool for measuring metabolic pathways and cellular activity, yet its use is currently limited due to the difficulty of introducing probes past the cell membrane and into the cytoplasm, especially as more complex probes are desired. Here we present a simple and minimally perturbative technique to deliver functional probes of glycosylation into cells using a nanostructured “nanostraw” delivery system. Nanostraws provide large scale intracellular access to cells through fluidic conduits that remain small enough to minimize cell perturbation. First, we demonstrate that our platform can deliver an unmodified azidosugar, *N*-azidoacetylmannosamine, into cells with similar effectiveness as a chemical modification strategy (peracetylation). We then show that for an azidosugar modified with a charged uridine diphosphate group (UDP) that prevents intracellular penetration, the nanostraw platform enables its direct delivery into cells, thus bypassing multiple enzymatic processing steps. By effectively removing the cell permeability requirement from the probe, the nanostraws expand the toolbox of bioorthogonal probes to study biological processes using a single, easy-to-use platform.

Keywords:

Drug delivery, Fluorescent probes, Glycosylation, Click chemistry, Nanotubes

Introduction

Metabolic labeling has become a critical tool for tracking the passage and function of biological substrates, yet has been limited by the difficulty of cellular delivery. Early experiments using radio-labeled analogs of glucose and nucleotides allowed researchers to identify their downstream biological products in metabolism and DNA replication¹⁻². More recently, metabolic labeling has been widely applied to study post-translational modifications (PTMs). PTM archetypes range from small functional group adornments such as phosphate and methyl groups to larger-scale assemblages such as ubiquitination and glycosylation³⁻⁴. By actively and reversibly modulating protein function, PTMs are essential for intracellular energy exchange, epigenetic memory, and signal transduction. As the study of PTMs has expanded, so too has the demand for observation of their localization and dynamics, driving the search for new functional metabolic analogs⁵⁻⁷.

Recently, a versatile approach has emerged, combining metabolic labeling with bioorthogonal chemistry⁸⁻⁹. With this strategy, metabolic analogs bearing a sterically-minimized bioorthogonal functional group “handle” must be delivered into cells. Once inside the cytoplasm, the handle is specifically labeled with a fluorophore by a bioorthogonal ligation reaction. This method has been especially effective for protein glycosylation studies. Natural glycosylation patterns are among the most complex and variable PTMs, composed of many unique monosaccharide subunits attached in linear and branching patterns. Their composition can vary dramatically, and these changes in composition correlate with dramatically altered phenotypes, as evidenced by the altered glycosylation status of cancer cells¹⁰. Bioorthogonal labeling provides the resolution, live-cell compatibility, and multiplexed detection necessary to map the relationship between glycosylation patterns and behavior in cells¹¹⁻¹².

The key barrier to this flexible labeling scheme is delivery of the metabolic analogs through the cell membrane and into the cytoplasm. Chemical modifications or adjuvants such as peracetylation¹³⁻¹⁴ or permeabilizing agents^{7, 15-16} can help to increase delivery effectiveness, but are not universally applicable and may engender cytotoxicity¹⁷ and lower efficiency of labeling. Moreover, the kinetics of enzymatic reactions being probed with metabolic analogs are often unknown and the intracellular levels of a metabolite may require constant upkeep over several days⁹. Unfortunately, most intracellular delivery agents are designed for single-shot delivery of oligonucleotide cargo and are too disruptive to be repeatedly applied, making them significantly less effective for consistent, extended metabolic labeling. Without more effective strategies for delivery, the full range of bioorthogonal probes remains untapped and delivery of available probes is suboptimal.

Here, we present a simple, non-perturbative technique to deliver poorly membrane permeable azido-functionalized monosaccharides into cells where they can be metabolized onto glycoproteins and labeled using bioorthogonal chemistry¹⁸. This technique uses a nanostructured platform of supported hollow tubes, called nanostraws, which deliver membrane impermeable molecules directly into the cytoplasm with minimal cell disruption¹⁹⁻²². We first show that nanostraws enable the efficient delivery of *N*-azidoacetylmannosamine (ManNAz) at comparable levels to what can be achieved by chemical modification (peracetylation). More importantly, we show that nanostraws enable the delivery of another metabolite, UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz). This molecule is an intermediate in the glycosylation pathway and is currently lacking effective strategies for delivery in cultured mammalian cells, but with nanostraw delivery it can be further exploited as a carrier for complex functional groups or tags that are incompatible with upstream biosynthetic enzymes. By directly penetrating cells to deliver cargo into the cytoplasm, nanostraws represent a powerful new approach to introduce cell-impermeable bioorthogonal probes into cellular studies. We demonstrate this using azidosugars but nanostraws are cargo-agnostic, enabling generic delivery and unlocking the study of many other biological systems.

Results and Discussion

Nanostraw membranes are polycarbonate membranes with randomly arranged hollow pores spanning the thickness of the membrane¹⁹. The pores were created using a track-etching procedure, making them highly uniform along their length with well-controlled diameters. Following deposition of aluminum oxide by atomic layer deposition and two selective etching steps, a forest of nanostraws ($\sim 3 \times 10^7 \text{ cm}^{-2}$) was formed on the membrane. The nanostraws themselves are hollow alumina tubes 100 nm in outer

diameter, 10 nm in wall thickness, and 1.5-2 μm in height. These tubes are embedded in the polycarbonate polymer substrate (Figure 1), making them stable and creating an attractive surface for cell adhesion.

Due to mechanical forces, a fraction of the nanostraws will directly penetrate the cells cultured onto them^{21, 23}, yet their small size and long leakage path largely limits cell perturbation¹⁹. Penetrant nanostraws act as conduits across the membrane, enabling molecules in solution on one side of the membrane to diffuse through the nanostraws to the other side (Figure 1B). As substrates for cell culture, nanostraws and related nanowires are robust in their materials properties and generally non-toxic, although some perturbations in cell behavior have been observed²⁴⁻²⁶. Previous applications of nanostraws have included delivery of small molecules, DNA, membrane impermeable dyes¹⁹, and ions^{20-21, 27}.

For delivery of azidosugars, the nanostraw membranes were assembled into devices consisting of a cell culture well, an adhesive layer, the nanostraw membrane, and a delivery chamber (Figure 1A, Supplemental Figure 1A). The cell culture well is a plastic tube with an approximately 8 mm inner diameter that holds 300 μL of culture media, but can be scaled up or down to accommodate lesser or greater numbers of cells (Supplemental Figure 1B). The nanostraw membrane, which is uniform over sizes up to several centimeters squared, is attached to the culture well with a ring of double-sided, biocompatible tape for the adhesive layer, providing a water-tight seal. The nanostraw membrane itself is approximately 20 μm thick and serves as the cell culture substrate. The delivery chamber is then created using a second ring of double sided tape, to store approximately 20 μl of cargo solution. This assembled device allows for cells to be cultured onto the membrane with access to the cargo chamber through the nanostraw conduits. Control experiments were conducted with flat membranes with the same density of pores but no protruding nanostraws. Reagents to be delivered were pipetted beneath the nanostraw membrane, and allowed to diffuse into the cells.

To demonstrate delivery of a bioorthogonal chemistry probe into cells using nanostraws we delivered ManNAz, which results in the introduction of azide groups onto sialylated cell surface proteins (Figure 1B). Upon incorporation onto surface glycoproteins, the azide moieties of metabolized ManNAz can be specifically labeled with fluorescent click chemistry probes (fluorophore- conjugated dibenzylcyclooctyne, DBCO). Importantly, a peracetylated derivative of ManNAz (Ac_4ManNAz), shown to be orders of magnitude more effective in biosynthetic incorporation compared to the parent compound⁹, serves as a point of comparison for the efficacy of nanostraw delivery. The cell permeable Ac_4ManNAz should label cell glycans when presented to cells in solution, while equal concentrations of the less permeable ManNAz would require a delivery method such as the nanostraws to provide the same labeling. Confirming earlier work, Chinese hamster ovary (CHO) cells incubated in control tests in standard 96-well plates with Ac_4ManNAz (100 μM for 48 hours, 10 μM Cy3 DBCO label) showed a characteristic cell-surface fluorescence profile after the bioorthogonal labeling reaction (Supplemental Figure 2A). However, cells incubated with cell-impermeant ManNAz under identical conditions showed only faint fluorescent staining (Supplemental Figure 2B).

To prepare devices for nanostraw delivery, nanostraw and flat membrane control devices were prepared by a plasma clean (<1 minute) after assembly, followed by an overnight UV light exposure and 3 hour incubation with 50 μL poly-lysine or poly-ornithine (150 μM). Following a 3x wash in PBS, 100,000 CHO

cells were resuspended in DMEM supplemented with 10% FBS and plated on the device. A 20 μ L drop of ManNAz solution was placed on parafilm and the device was placed on top, to fill the delivery chamber.

Nanostraw delivery of ManNAz was tested for two delivery time scales: a long incubation of 48 hours (Figure 2A-C) and a short incubation of 4 hours (Figure 2D-F). The ManNAz concentration in PBS was either 1 mM for the long incubation or 10 mM for the short incubation. After incubation, the media was removed from the culture chamber and the delivery chamber was washed in PBS to remove excess ManNAz solution. The culture well was incubated in PBS with 1% FBS for 5 minutes, rinsed 2x with PBS and incubated in 50 μ M Carboxyrhodamine 110 DBCO or Cy3 DBCO in phenol red-free DMEM for 15 minutes at 37 °C. Following DBCO incubation, the culture chamber was rinsed 3x with PBS and incubated in 0.25% Trypsin with EDTA for 10 minutes, and the trypsinized cells were replated onto cover slips coated with poly-lysine. After 4 hours to allow cells to adhere, slides were washed in PBS to remove excess fluorescent labels, fixed in 4% paraformaldehyde, and mounted for imaging.

Imaging of cell-impermeable ManNAz delivery after the long 48 hour incubation shows a distinct contrast between strong DBCO labeling after ManNAz delivery on nanostraw devices (Figure 2A) relative to the weaker fluorescence observed on flat membrane devices (Figure 2B). The line profile trace across cells also demonstrates a substantial increase in fluorescence intensity on nanostraw devices relative to flat membrane devices (Figure 2C). Some variations in cell-to-cell fluorescence were observed as nanostraw based delivery systems have demonstrated an inherent spread in delivery cell-to-cell²¹, and the nanostraws show markedly increased retention of cells due to improved cell adhesion to nanostraws and similar nanowires during washing steps²⁸. A small amount of ManNAz uptake is observed even on flat membrane devices due to non-specific uptake mechanisms, but non-specific uptake is unreliable and the characteristic cell-border fluorescence profile is much weaker.

Delivery using the shorter 4 hour incubation reveals that cell-surface labeling had already occurred on nanostraw devices (Figure 2D) in contrast to indistinct labeling on flat membrane devices (Figure 2E). The raw difference in intensity is lower at 4 hours compared to 48 hours (Figure 2F), which is consistent with increased labeling of accumulated azido groups over the longer time period.

These results show improved delivery efficiency of poorly permeable azidosugars with nanostraws. While a peracetylated, cell-permeable ManNAz analog was available, the true promise of nanostraws lies in facile delivery of metabolites that are difficult or infeasible to chemically modify. Within this class of metabolites are UDP-modified sugars, which bear a negatively charged diphosphate linkage that limits cell permeability. UDP-sugars are biosynthesized through multiple enzymatic steps from the free monosaccharide to be directly attached onto proteins via glycosyltransferases²⁹.

Direct delivery of UDP-sugars into the cytoplasm addresses two critical shortcomings. First, by delivering cell-impermeable secondary metabolites such as UDP-sugars and not their precursors, the activity of specific downstream enzymes within the pathway, glycosyltransferases in this instance, can be directly probed. Second, although complex functional groups such as UDP groups and fluorophores can be easily attached to free monosaccharides, the resulting modified, bulkier metabolite is often rejected by one or more of the enzymes required for biosynthetic processing and incorporation of the metabolite into its end-product. By directly delivering UDP-sugars into the cytoplasm and bypassing multiple biosynthetic

steps, the repertoire of unnatural functionalities to be incorporated onto nascent glycoproteins is freed from many enzymatic compatibility constraints.

We examined whether nanostraws are effective for a larger variety of unnatural substrates by delivering three unnatural N-acetyl galactosamine derivatives: peracetylated N-azidoacetylgalactosamine (Ac_4GalNAz), N-azidoacetylgalactosamine (GalNAz), and the uridine diphosphate modified N-azidoacetylgalactosamine (UDP-GalNAz), into GFP-labeled CHO cells (Figure 3A). These three molecules can potentially enter the N-acetylgalactosamine salvage pathway at different points³⁰. While UDP-GalNAz enters at a much later stage than GalNAz , it is significantly less cell permeable and delivery remains a critical challenge.

We studied delivery of all three forms of GalNAz sugars through both nanostraw and flat-membrane control devices. For each azidosugar, 500 μM solutions in PBS were added to the delivery chambers, incubated for an intermediate time period of 24 hours with 50,000 cells, then washed and labeled with 10 μM Cy3 DBCO for 20 minutes. Compared to the negative control condition of cells with no added azidosugars incubated with DBCO (Figure 3B, inset – GFP fluorescence), the azidosugars were delivered and labeled with varying success on nanostraws and flat membranes (Figure 3C-D, inset – GFP fluorescence). Using the nanostraws, all three sugars, including negatively charged and therefore highly impermeable UDP-GalNAz , entered the cells and were metabolized onto cell surface glycoproteins to produce the characteristic cell border fluorescence upon DBCO labeling (Figure 3C). GalNAz and Ac_4GalNAz delivery using nanostraws were both nearly 100% efficient in CHO cells and comparable to ManNAz delivery, while UDP-GalNAz delivery was nearly as effective, with only a small number of cells appearing to have weak or no fluorescence.

On flat control membranes, only Ac_4GalNAz , being cell-permeable, was seen around cells after DBCO labeling (Figure 3D). Both GalNAz and UDP-GalNAz -delivered cells showed only non-specific fluorescence when the sugar was added through a flat membrane, with a similar fluorescence profile as cells with no added sugars at all (Figure 3B). In these two negative conditions, GalNAz and UDP-GalNAz delivered through flat membranes, as well as the sugar-free condition, some fluorescence was observed, likely due to nonspecific uptake of DBCO or labeling of debris. Nanostraws appear to further promote some nonspecific labeling in the form of bright, central spots of fluorescence, but this form of labeling is accompanied by the circular, cell border labeling characteristic of bioorthogonal labeling of azido-modified glycoproteins, except in rare cases with UDP-GalNAz delivery.

These results show that physical cell penetration and delivery through nanostraws is an effective method to overcome limitations of cell-impermeant labeling molecules in metabolic labeling studies. Nanostraw delivery of membrane impermeable ManNAz , a well-characterized molecule for studying protein glycosylation, reproduced the effect of chemical modification at long and short-term delivery scales.

The nanostraws also demonstrated the capacity to deliver UDP-GalNAz , an intermediate enzymatic byproduct of GalNAz metabolism which cannot be delivered by chemical means. The principle of bypassing the cell membrane using nanostraw delivery addresses an essential theme in the application of bioorthogonal probes – their potential to be poor substrates for the endogenous biosynthetic machinery³¹. For natural metabolites that require multiple biosynthetic processing and enzymatic steps,

a metabolic analog that is incompatible with just one enzyme in the pathway will not appear in endogenous biopolymer end-products. To bypass such an enzymatic bottleneck, metabolites further downstream in the pathway can be synthesized with the desired bioorthogonal handle. However, these downstream metabolites are naturally processed to be retained in cell compartments, often with charged groups such as UDP, and are therefore poorly cell permeable, thus requiring an intracellular delivery strategy such as the nanostraws³².

Metabolic analogs that address other pathways of metabolism and post-translational modification are also excellent candidates for nanostraw delivery, including modified ATP, which can be used in conjunction with modified enzymes to discover new substrates for kinases but suffers from poor delivery options³³, or synthetic cross-linkers or dimerizing agents, which can induce novel interactions in cells to study pathways with increased specificity³⁴. Ultimately, the nanostraws represent a minimally perturbative delivery platform capable of delivering a range of freely-diffusing species, and are effective for sustained delivery for over 24 hours. Thanks to the availability of the plastic tubes used to define the cell culture area and the uniformity of the nanostraws themselves¹⁹, the platform is scalable; with delivery to cells using a larger nanostraw device, larger scale flow cytometry quantification or mass spectrometry experiments for proteomics are possible in the future. Finally, the nanostraws remove the cell permeability requirement for chemical probes to relax constraints on size and charge, allowing more diverse and effective chemical probes to be brought to bear on biological problems.

Experimental Section

Nanostraw and Device Fabrication

Nanostraws were fabricated using a track-etched membrane template (GVS). The templates were 20 μm thick polycarbonate membranes with randomly arranged pores at a density of $3 \times 10^7 \text{ cm}^{-2}$. Track-etched membrane templates are generally available in large volumes at a single prescribed density of extremely thin pores only, and are then etched to the desired pore diameter in smaller batches. The nanostraw membranes in this study were etched to 100 nm as purchased. Compared to commercially available track-etched membranes used for water filtration and other applications, the nanostraw membrane templates have relatively low porosity, with either a lower pore diameter than membranes with similar pore density or a low density than membranes with similar pore diameter.

Using as purchased membranes, nanostraws were fabricated by coating the templates with atomic layer deposition (ALD) alumina. A 10-15 nm layer of alumina is conformally applied to both sides of the membrane template as well as the inner walls of the pores using 50 cycles of ALD. Each cycle uses alternating pulses of trimethylaluminum (TMA) and water (H_2O) with a precursor pulse step of 0.015 s, an exposure step of 30 s, and a purge step of 60 s. The Savannah platform (Cambridge Nanotech) accommodates up to 4 inch wafer sized membranes, and the nanostraws are typically fabricated in smaller area batches to ensure uniformity. The nanostraws that protrude above the membrane were created by first etching one alumina coated surface of the membrane using a PlasmaQuest etcher and BCl_3 and Cl_2 plasma (40 sccm BCl_3 , 30 sccm Cl_2 , 5 sccm Ar at 300 watts, 250 s) to expose the polycarbonate beneath. The polycarbonate is then removed using an oxygen plasma etch (SPI Plasma Prep III Solid State, 200

mTorr and 100 watts, 40 min). The alumina coating the walls of the membrane pores remains to form the free-standing nanostraws.

The device materials consist of the nanostraw membranes, plastic tubing, and two rings of double-sided tape (Digi-key Electronics, 3M Acrylic Foam). The double sided tape was laser-cut to form regular rings, and the plastic tubing was polished on both ends to ensure that a water-tight seal is formed. For the lower ring of double-sided tape forming the delivery chamber, the plastic covering protecting the lower side of the tape is not removed to prevent the device from sticking to surfaces and allow access to the delivery chamber to pipette cargo solutions. Besides the nanostraws, the other materials used in the device can be easily obtained in bulk quantities and assembled.

Azidosugar Synthesis

ManNAz, Ac₄ManNAz, GalNAz, Ac₄GalNAz, and UDP-GalNAz were synthesized according to literature procedure^{29, 35}.

Cell Culture and Delivery Assays

CHO cells and GFP-expressing CHO cells were cultured in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin. Before CHO cells were added to nanostraw devices for delivery, devices were placed in oxygen plasma to sterilize, moved to the tissue culture hood, and further exposed to UV overnight to ensure sterility. A 2-3 hour incubation with 50 μ L poly-lysine or poly-ornithine promoted cell adhesion to the nanostraws. After 3x PBS wash to remove excess solution, CHO cells were trypsinized using 0.25% trypsin, resuspended in DMEM, and added to the cell culture well. At the device diameter used, the delivery chamber stores 20 μ L of cargo solution. To fill the delivery chamber, solution was placed in a droplet on parafilm, which prevents the solution from spreading. Slowly placing the device on top of the droplet ensured that air bubbles were minimized. After the delivery chamber was filled, the devices were placed in a humidified petri-dish and returned to the incubator at 37°C. For long term deliveries (>24 hr) the cargo solution may evaporate and it was necessary to replenish the chamber.

After incubation, cells are labeled using DBCO fluorophores (Click Chemistry Tools) by first washing away excess cargo solution from the delivery chamber with a PBS wash. Following a short blocking step of the cell culture chamber using 1% BSA in PBS and 2x PBS wash, DBCO fluorophores were incubated in the cell culture chamber for 15 min at 37°C. After a final 3x PBS wash, the cells were prepared for imaging. Due to the small volume of the cell culture wells as well as the fragile nature of the nanostraw membrane, thorough washing was difficult and care was taken to remove as much liquid as possible without puncturing the nanostraw membrane.

To image cells, cover slips were first prepared by placing a drop of poly-lysine on the cover slip for 15 minutes. The cover slips were then washed to remove excess solution. DBCO labeled and washed cells were resuspended by adding trypsin to the cell culture well. Cells cultured on the flat membranes were susceptible to loss during wash steps, while nanostraw-adhered cells were trypsinized for longer time periods (~5-10 minutes) to resuspend. After trypsinization, cells were resuspended in media and added to the cover slips. Following a 4 hour adherence period, the cover slips were further washed in PBS to remove excess DBCO fluorophores, a necessary step due to the washing difficulty described earlier. Cells

were then fixed with 4% paraformaldehyde, mounted on a glass slide, and imaged using a confocal microscope (Zeiss Axiovert 200 M), photometrics Cascade 512B digital camera (Roper Scientific) and MetaMorph software (Molecular Devices).

Acknowledgements

We acknowledge HFSP-RGP0048 and Bio-X Interdisciplinary Initiatives Program for support, and A.M.X. support through NSF and NDSEG graduate fellowships. We would like to acknowledge the Sarah Heilshorn lab and the Stanford Neurofab facility for assisting with microscopy and cell culture access, the David Goldhaber-Gordon and Yi Cui labs for use of equipment, and the Stanford Nanofabrication Facility and Stanford Nano Shared Facilities for fabrication and imaging.

Supporting Information

Supporting Information Available. This material is available free of charge via the Internet.

References

1. Reivich, M.; Kuhl, D.; Wolf, A.; Greenberg, J.; Phelps, M. a.; Ido, T.; Casella, V.; Fowler, J.; Hoffman, E.; Alavi, A., The [18F] fluorodeoxyglucose method for the measurement of local cerebral glucose utilization in man. *Circulation research* **1979**, *44* (1), 127-137.
2. Sanger, F.; Nicklen, S.; Coulson, A. R., DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences* **1977**, *74* (12), 5463-5467.
3. Mann, M.; Jensen, O. N., Proteomic analysis of post-translational modifications. *Nature biotechnology* **2003**, *21* (3), 255-261.
4. Deribe, Y. L.; Pawson, T.; Dikic, I., Post-translational modifications in signal integration. *Nature Structural & Molecular Biology* **2010**, *17* (6), 666-672.
5. Guo, J.; Wang, J.; Lee, J. S.; Schultz, P. G., Site-Specific Incorporation of Methyl- and Acetyl-Lysine Analogues into Recombinant Proteins. *Angewandte Chemie International Edition* **2008**, *47* (34), 6399-6401.
6. Dube, D. H.; Bertozzi, C. R., Metabolic oligosaccharide engineering as a tool for glycobiology. *Current Opinion in Chemical Biology* **2003**, *7* (5), 616-625.
7. Allen, J. J.; Li, M.; Brinkworth, C. S.; Paulson, J. L.; Wang, D.; Hubner, A.; Chou, W.-H.; Davis, R. J.; Burlingame, A. L.; Messing, R. O.; Katayama, C. D.; Hedrick, S. M.; Shokat, K. M., A semisynthetic epitope for kinase substrates. *Nat Meth* **2007**, *4* (6), 511-516.
8. Mahal, L. K.; Yarema, K. J.; Bertozzi, C. R., Engineering chemical reactivity on cell surfaces through oligosaccharide biosynthesis. *Science* **1997**, *276* (5315), 1125-1128.
9. Saxon, E.; Bertozzi, C. R., Cell surface engineering by a modified Staudinger reaction. *Science* **2000**, *287* (5460), 2007-2010.
10. Varki, A.; Kannagi, R.; Toole, B. P., Glycosylation changes in cancer. **2009**.
11. Shieh, P.; Siegrist, M. S.; Cullen, A. J.; Bertozzi, C. R., Imaging bacterial peptidoglycan with near-infrared fluorogenic azide probes. *Proceedings of the National Academy of Sciences* **2014**, *111* (15), 5456-5461.
12. Martin, B. R.; Cravatt, B. F., Large-scale profiling of protein palmitoylation in mammalian cells. *Nature methods* **2009**, *6* (2), 135-138.
13. Sarkar, A. K.; Fritz, T. A.; Taylor, W. H.; Esko, J. D., Disaccharide uptake and priming in animal cells: inhibition of sialyl Lewis X by acetylated Gal beta 1--> 4GlcNAc beta-O-naphthalenemethanol. *Proceedings of the National Academy of Sciences* **1995**, *92* (8), 3323-3327.

14. Doronina, S. O.; Mendelsohn, B. A.; Bovee, T. D.; Cervený, C. G.; Alley, S. C.; Meyer, D. L.; Oflazoglu, E.; Toki, B. E.; Sanderson, R. J.; Zabinski, R. F.; Wahl, A. F.; Senter, P. D., Enhanced Activity of Monomethylauristatin F through Monoclonal Antibody Delivery: Effects of Linker Technology on Efficacy and Toxicity. *Bioconjugate chemistry* **2006**, *17* (1), 114-124.
15. Xie, R.; Hong, S.; Feng, L.; Rong, J.; Chen, X., Cell-Selective Metabolic Glycan Labeling Based on Ligand-Targeted Liposomes. *Journal of the American Chemical Society* **2012**, *134* (24), 9914-9917.
16. Xie, R.; Dong, L.; Du, Y.; Zhu, Y.; Hua, R.; Zhang, C.; Chen, X., In vivo metabolic labeling of sialoglycans in the mouse brain by using a liposome-assisted bioorthogonal reporter strategy. *Proceedings of the National Academy of Sciences* **2016**, *113* (19), 5173-5178.
17. Haun, J. B.; Devaraj, N. K.; Marinelli, B. S.; Lee, H.; Weissleder, R., Probing intracellular biomarkers and mediators of cell activation using nanosensors and bioorthogonal chemistry. *ACS nano* **2011**, *5* (4), 3204-3213.
18. Belardi, B.; de la Zerda, A.; Spiciarich, D. R.; Maund, S. L.; Peehl, D. M.; Bertozzi, C. R., Imaging the Glycosylation State of Cell Surface Glycoproteins by Two-Photon Fluorescence Lifetime Imaging Microscopy. *Angewandte Chemie International Edition* **2013**, *52* (52), 14045-14049.
19. VanDersarl, J. J.; Xu, A. M.; Melosh, N. A., Nanostraws for Direct Fluidic Intracellular Access. *Nano Letters* **2012**, *8* (12), 3881-3886.
20. Aalipour, A.; Xu, A. M.; Leal-Ortiz, S.; Garner, C. C.; Melosh, N. A., Plasma membrane and actin cytoskeleton as synergistic barriers to nanowire cell penetration. *Langmuir* **2014**, *30* (41), 12362-12367.
21. Xu, A. M.; Aalipour, A.; Leal-Ortiz, S.; Mekhdjian, A. H.; Xie, X.; Dunn, A. R.; Garner, C. C.; Melosh, N. A., Quantification of nanowire penetration into living cells. *Nature Communications* **2014**, *5*.
22. Xie, X.; Xu, A. M.; Leal-Ortiz, S.; Cao, Y.; Garner, C. C.; Melosh, N. A., Nanostraw-Electroporation System for Highly Efficient Intracellular Delivery and Transfection. *ACS Nano* **2013**, *7* (5), 4351-4358.
23. Xie, X.; Xu, A. M.; Angle, M. R.; Tayebi, N.; Verma, P.; Melosh, N. A., Mechanical Model of Vertical Nanowire Cell Penetration. *Nano letters* **2013**, *13* (12), 6002-6008.
24. Elnathan, R.; Kwiat, M.; Patolsky, F.; Voelcker, N. H., Engineering vertically aligned semiconductor nanowire arrays for applications in the life sciences. *Nano Today* **2014**.
25. Bonde, S.; Buch-Månson, N.; Rostgaard, K. R.; Andersen, T. K.; Berthing, T.; Martinez, K. L., Exploring arrays of vertical one-dimensional nanostructures for cellular investigations. *Nanotechnology* **2014**, *25* (36), 362001.
26. Christelle, N. P., Interactions between semiconductor nanowires and living cells. *Journal of Physics: Condensed Matter* **2015**, *27* (23), 233103.
27. Xu, A. M.; Kim, S. A.; Wang, D. S.; Aalipour, A.; Melosh, N. A., Temporally resolved direct delivery of second messengers into cells using nanostraws. *Lab on a Chip* **2016**, *16* (13), 2434-2439.
28. Na, Y.-R.; Kim, S. Y.; Gaublomme, J. T.; Shalek, A. K.; Jorgolli, M.; Park, H.; Yang, E. G., Probing Enzymatic Activity inside Living Cells Using a Nanowire-Cell "Sandwich" Assay. *Nano Letters* **2013**, *13* (1), 153-158.
29. Hang, H. C.; Yu, C.; Pratt, M. R.; Bertozzi, C. R., Probing Glycosyltransferase Activities with the Staudinger Ligation. *Journal of the American Chemical Society* **2004**, *126* (1), 6-7.
30. Baskin, J. M.; Dehnert, K. W.; Laughlin, S. T.; Amacher, S. L.; Bertozzi, C. R., Visualizing enveloping layer glycans during zebrafish early embryogenesis. *Proceedings of the National Academy of Sciences* **2010**, *107* (23), 10360-10365.
31. Tanner, M. E., The enzymes of sialic acid biosynthesis. *Bioorganic Chemistry* **2005**, *33* (3), 216-228.
32. Agarwal, P.; Beahm, B. J.; Shieh, P.; Bertozzi, C. R., Systemic Fluorescence Imaging of Zebrafish Glycans with Bioorthogonal Chemistry. *Angewandte Chemie* **2015**, *127* (39), 11666-11672.
33. Banko, Max R.; Allen, Jasmina J.; Schaffer, Bethany E.; Wilker, Erik W.; Tsou, P.; White, Jamie L.; Villén, J.; Wang, B.; Kim, Sara R.; Sakamoto, K.; Gygi, Steven P.; Cantley, Lewis C.; Yaffe, Michael B.; Shokat,

Kevan M.; Brunet, A., Chemical Genetic Screen for AMPK α 2 Substrates Uncovers a Network of Proteins Involved in Mitosis. *Molecular cell* **2011**, *44* (6), 878-892.

34. Sakamoto, K. M.; Kim, K. B.; Kumagai, A.; Mercurio, F.; Crews, C. M.; Deshaies, R. J., Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, *98* (15), 8554-8559.

35. Laughlin, S. T.; Bertozzi, C. R., Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation. *Nat. Protocols* **2007**, *2* (11), 2930-2944.

Figures

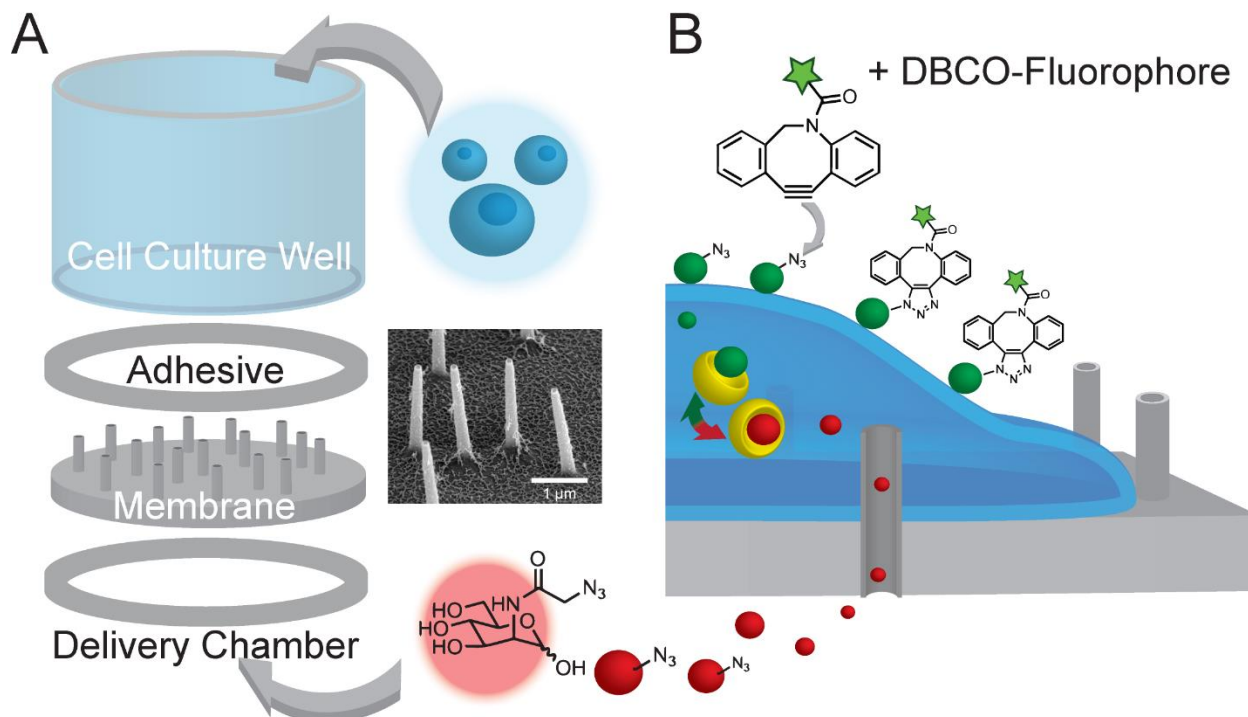


Figure 1. Nanostraw device used for azidosugar delivery. A) The device consists of four parts: a cell culture well, the adhesive layer, the nanostraw membrane, and a delivery chamber. The adhesive produces a water-tight seal between the cell culture well and the membrane, so that cargo placed in the delivery chamber below can only enter the culture well through the membrane pores. If a nanostraw membrane is used and the nanostraws have cellular access, then the cargo may pass directly into cells through penetrating nanostraws. B) Upon successful entry into the cell, an azidosugar such as ManNAz is enzymatically converted into sialic acid groups and incorporated onto cell surface glycoproteins. These groups retain the azide moiety, which can be targeted and labeled using a DBCO fluorophore.

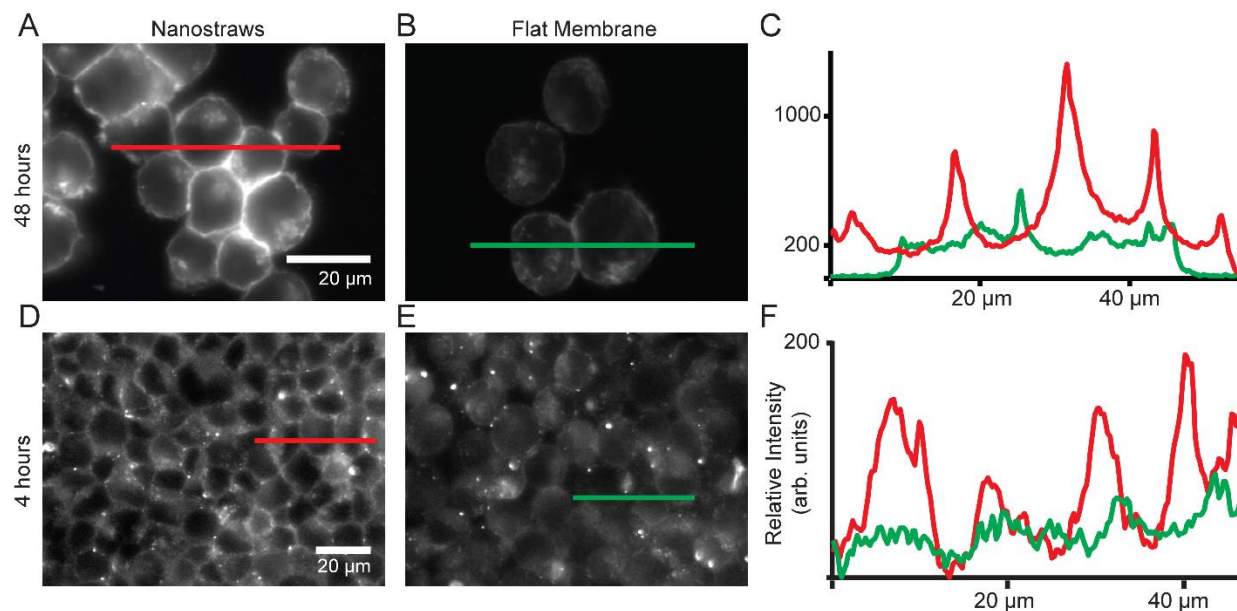


Figure 2. ManNAz delivery using nanostraws. A,B) Cells were labeled and imaged after a long term 48 hr incubation of ManNAz on nanostraw and flat membrane devices. C) A line trace shows a strong difference in fluorescent intensity between nanostraw delivery and nonspecific uptake. D,E,F) This difference was also observed at 4 hrs albeit at reduced intensity.

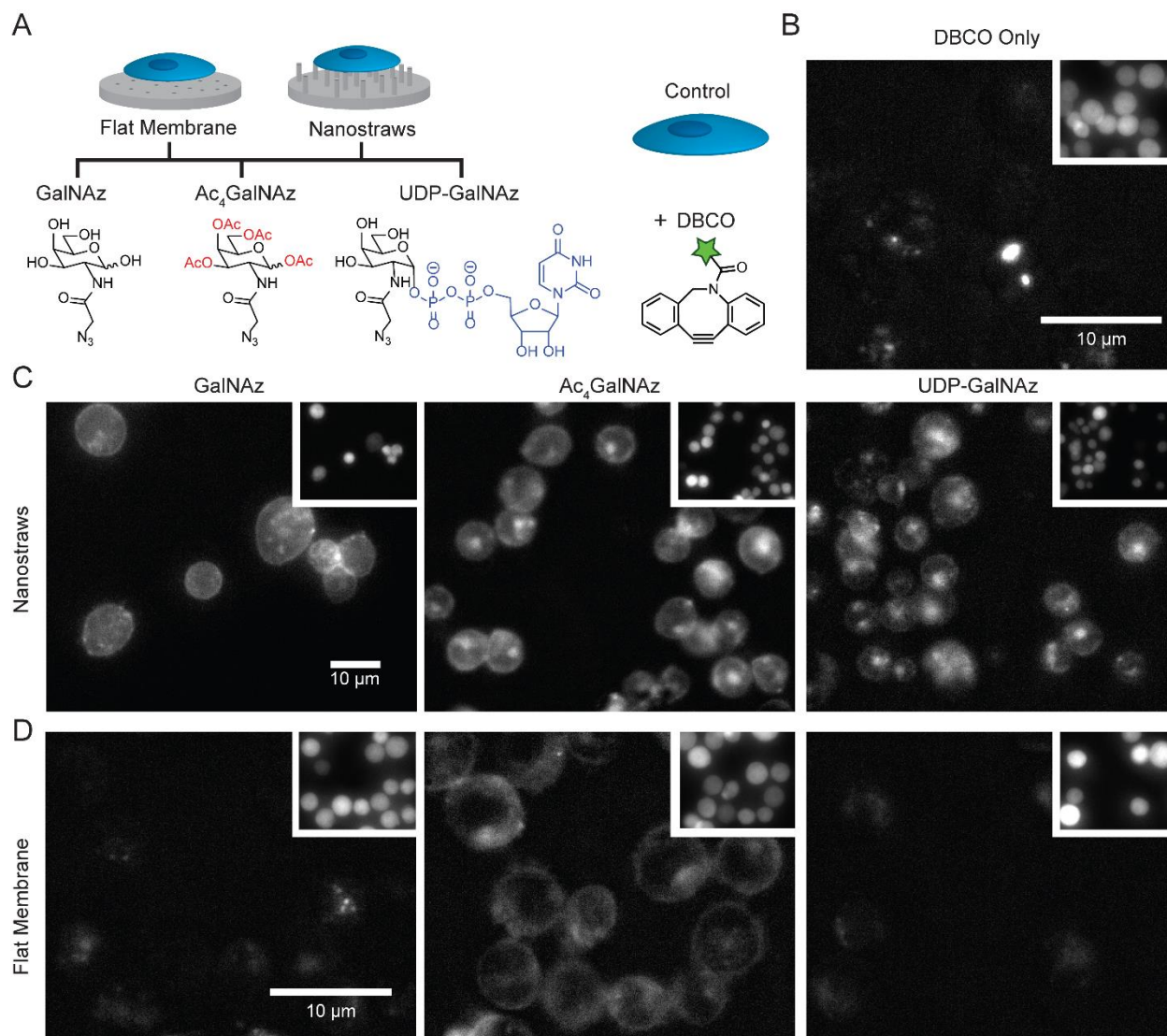


Figure 3. Delivery of modified unnatural UDP-sugars. A) Flat membrane and nanostraw delivery were performed using three sugars, GalNAz, cell-permeable Ac₄GalNAz, and negatively charged cell impermeable UDP-GalNAz. B) Cells incubated with DBCO fluorescent probes but no azidosugar showed some non-specific labeling but no characteristic cell border fluorescence (inset – GFP fluorescence). C) When nanostraws were used for delivery, all three forms of GalNAz successfully entered the cells to be incorporated onto surface glycoproteins and labeled. D) On flat, control membranes, neither GalNAz nor UDP-GalNAz was successfully delivered into cells, but the cell-permeable Ac₄GalNAz was metabolized and successfully labeled using the click chemistry reaction.