Title: Quantitative super-resolution microscopy of the mammalian glycocalyx

Authors: Leonhard Möckl1,6, Kayvon Pedram1,6, Anish R. Roy1, Venkatesh Krishnan2, Anna-Karin Gustavsson1,3, Oliver Dorigo2, Carolyn R. Bertozzi1,4*, W. E. Moerner1,5*

Affiliations:
1Department of Chemistry, Stanford University, Stanford, California, USA
2Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Stanford University School of Medicine, Stanford, California, USA
3Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden
4Howard Hughes Medical Institute, Stanford, California, USA
5Lead contact
6These authors contributed equally
*Correspondence: W. E. Moerner, wmoerner@stanford.edu and Carolyn R. Bertozzi, bertzozzi@stanford.edu

Summary:
The mammalian glycocalyx is a heavily glycosylated extramembrane compartment found on nearly every cell. Despite its relevance in both health and disease, studies of the glycocalyx remain hampered by a paucity of methods to spatially classify its components. We combine metabolic labeling, bioorthogonal chemistry, and super-resolution localization microscopy to image two constituents of cell-surface glycans, N-acetylgalactosamine (GalNAc) and sialic acid, with 10-20 nm precision in 2D and 3D. This approach enables two measurements: glycocalyx height and the distribution of individual sugars distal from the membrane. These measurements show that the glycocalyx exhibits nanoscale architecture, on both cell lines and primary human tumor cells. Additionally, we observe enhanced glycocalyx height in response to epithelial-to-mesenchymal transition and to oncogenic KRAS activation. In the latter case, we trace increased height to an effector gene, GALNT7. These data highlight the power of advanced imaging methods to provide molecular and functional insights into glycocalyx biology.

Keywords:
Super-resolution microscopy, glycobiology, glycocalyx, bioorthogonal chemistry, metabolic labeling, membrane biophysics, ovarian cancer, pancreatic adenocarcinoma, KRAS, GALNT7

Introduction
Over 80% of extracellular and transmembrane proteins are predicted to be glycosylated (Steentoft et al., 2013). These glycoproteins, along with glycolipids and polysaccharides, form a peripheral compartment at the cell surface termed the glycocalyx (Figure 1A, left). The glycocalyx is present in all domains of life, and is found on nearly every cell of the human body (Varki et al., 2015). It is the first component of a cell to interact with the environment, and therefore plays an important role in cell-cell and cell-matrix interactions critical to embryonic development, immune cell trafficking, cancer progression, and many other normal and pathological processes. The glycocalyx is also known to be dynamic and undergoes shifts in
composition and physical properties in response to cues from the microenvironment. For example, human embryos at the blastocyst stage effect shedding of the uterine glycocalyx to implant (Constantinou et al., 2015), viruses deforest the glycocalyx to infect (Matrosovich et al., 2004), and cancer cells with glycocalyces that activate immune checkpoints are enriched during cancer progression by virtue of natural selection (Hudak et al., 2014).

Despite decades of observations such as these, the molecular architecture of the glycocalyx as well as its dynamics have remained difficult to elucidate. This is due to the inherent structural complexity and heterogeneity associated with the large collections of glycans that constitute the glycocalyx, and, until recently (vide infra), a limited tool kit for direct visualization of these structures using imaging methods. Also, the axial thickness of the glycocalyx is in the range of tens to hundreds of nanometers, and is therefore too small to be resolved with conventional light microscopy, which is diffraction-limited at ~200 nm. Worse, due to the heterogeneity and physical fragility of the glycocalyx, visualization of its bulk structure, e.g. by electron microscopy (EM), is difficult (Hegermann et al., 2016). There is a need for methods that allow nanometer-scale analysis of the glycocalyx while preserving its fine structure.

Localization-based super-resolution (SR) microscopy is uniquely situated to address these challenges. This flavor of SR microscopy employs single-molecule imaging and control of emitter concentration (PALM/STORM/F-PALM) (Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006). These and related methods are grouped under the general term single-molecule active control microscopy (SMACM) (Moerner, 2012). In SMACM, by actively choosing a control mechanism, only a sparse subset of the fluorophores decorating a structure are allowed to emit in any given camera frame. Then, each non-overlapping emitter, which appears on the camera’s detector as a spot, termed the point spread function (PSF), is localized. This localization achieves higher precision than the diffraction limit by taking the center of each PSF as the position of the emitter. The error in finding the center of the PSF defines the precision of the measurement, which with current methods can approach single-digit nanometers. This process is repeated over many frames, and the localizations are used in a point-by-point reconstruction of the imaged structure with information beyond the diffraction limit (Huang et al., 2016; Patterson et al., 2010). Here, we employ metabolic labeling and bioorthogonal chemistry to chemically modify specific sugar residues in the glycocalyx with small molecule fluorophores. Performing SMACM on these emitters enables visualization of single sugars in the glycocalyx at 10-20 nm precision in 2D and 3D.

This approach enables two measurements: height of the glycocalyx distal from the membrane and the distribution of sugar residues within the glycocalyx. We employ these measurements on cell lines and primary human tumor cells to show that the glycocalyx exhibits a nanoscale architecture, that glycocalyx height increases when oncogenic KRAS is expressed, and that this phenotype can be linked to a single gene, GALNT7.

Results

Bioorthogonal labeling and super-resolution imaging enable visualization of the mammalian glycocalyx at 10-20 nm precision
To introduce a small-molecule fluorophore into cell surface glycans, we used two methods rooted in bioorthogonal chemistry: (i) metabolic incorporation of N-azidoacetylgalactosamine (GalNAz) (Hang et al., 2003) in place of native N-acetylgalactosamine (GalNAc) residues, which introduces azide groups for subsequent probe conjugation; and (ii) periodate-mediated oxidation of sialic acids (Sia) to introduce aldehyde groups (Zeng et al., 2009) (Figure 1A, top and middle). Live cells bearing the corresponding modifications were labeled at 4 °C with alkyne- and aminooxy-functionalized fluorophores, respectively. This protocol selectively targets extracellular sugars as the reagents are not membrane-permeable and endocytosis is negligible at 4 °C. The cells were then fixed and subjected to confocal imaging, flow cytometry, or SR microscopy. We chose GalNAz and Sia as labeling targets based on the different positions they are thought to occupy within mammalian glycans. Whereas Sia are typically terminal residues positioned at the nonreducing end of a glycan chain, GalNAc residues tend to occupy core positions near the reducing ends of glycans and are more proximal to underlying polypeptide scaffolds (Varki et al., 2015). By labeling these sugars covalently with small-molecule fluorophores, we avoid the nonspecific labeling which often accompanies antibody- or lectin-based cell surface staining procedures (Whelan and Bell, 2015). To provide a third labeling position which visualizes cellular boundaries, we incorporated a lipid-azide (16:0 azidocaproyl phosphoethanolamine; see Figure 1B, right for structure) into the outer leaflet of the plasma membrane. This was followed by Cu-catalyzed conjugation with alkyne-AF647 (Figure 1A, bottom) (Nishimura et al., 2006). We chose a two-step method rather than direct membrane labeling with a lipid-fluorophore conjugate to ensure that the labeling reagents were capable of penetrating through the glycocalyx down to the plasma membrane.

Confocal microscopy of adherent breast cancer cells (SKBR3) revealed consistent and strong labeling across all cells for periodate-mediated labeling of Sia, metabolic incorporation of GalNAz, and lipid-azide incorporation (Figure 1B and Figure S1A). Note that by diffraction-limited confocal imaging, lipid-azide-derived labeling is indistinguishable in its membrane-localized pattern from GalNAz or Sia labeling. Since Sia labeling involves chemical modification of native functional groups on cell surfaces, we confirmed that staining was specific for sialic acids through enzymatic cleavage of Sia residues (Figure 1C and Figures S1B-C).

Optimization of the labeling workflows was needed to achieve sufficiently low intracellular and coverslip background fluorescence for SR microscopy (Methods; Figure S1D). With robust labeling protocols in place, we performed GalNAz, Sia, and lipid-azide one-color SR imaging on two morphologically different breast cancer cell lines, flat BT-20 cells and round SKBR3 cells, and one immortalized mammary epithelial cell line, MCF10A (Figure 1D; see Figure S1E for lipid-azide SR reconstructions). The quality of labeling was underscored by high numbers of localizations with low background and high photon counts (Figure S1F), enabling a localization precision of 10-20 nm across all samples. Note that the haze from widefield epifluorescence is absent from the 2D SR reconstructions. This is due to effective sectioning arising from removal of out-of-focus emitters (discussed in Figure 3A and Figures S3A-C). Inspection of 2D SR reconstructions revealed a striking feature common to all reconstructions: spiny, apparently hollow cell surface protrusions with a high density of GalNAz- and Sia-derived signal localized to their periphery (arrowheads for examples, Figure 1D). These protrusions are membrane ruffles, microvilli, or as they will be referred to here, “tubules”, which have been reported by...
several other techniques, and are commonly seen in EM images of cancer cells and tissues (Cai et al., 2017; Elgqvist et al., 2010; Kolata, 1975).

3D SR microscopy shows that glycocalyx-covered membrane tubules are an ideal target for quantitative analysis

The power of SR microscopy rests both in the revelation of previously unobserved nanoscale detail and in the extraction of quantitative parameters. Given the extreme challenge to directly measure the height of the glycocalyx distal to the membrane (Paszek et al., 2012) and given that biophysical properties of the glycocalyx such as height are of key relevance in human pathologies (Kuo et al., 2018; Pinho and Reis, 2015), we chose to explore the height of the glycocalyx as a quantitative parameter. Due to their architecture, the observed tubules provide a uniquely well-defined boundary between cytosol and glycocalyx, in contrast to less structured membrane regions (see Figure 1D). We reasoned that by leveraging the high spatial resolution at which we capture glycocalyx-covered membrane tubules and the high number of tubules found per cell, we could gain the statistical power required to extract meaningful quantitative information about the height of GalNAz and Sia distal from the membrane.

For detailed characterization of these tubules, we expanded our methodology to 3D imaging. Using a novel light sheet approach called tilted light sheet microscopy with 3D point spread functions (TILT3D, Figures S2A-B) (Gustavsson et al., 2018), we generated the first 3D SR reconstruction of the glycocalyx (sialic acid labeling, precision 10-30 nm, Figure 2A and Figures S2C-E). The tubules exhibited a roughly homogenous diameter across their length, independent of their orientation (cf. left and right insets). Projections of the 3D reconstructions onto the xy-plane using various z-slices with thicknesses of ~200 nm (Figure 2B) revealed that the apparently non-structured regions near the plasma membrane in the 2D projections likely arose from overlapping tubules in 3D and not from a true biological structure or nonspecific labeling (e.g., see intertwined tubules, Figure 2C). In addition, inspection of the boundary between the cellular cytosol and the base of the tubules confirmed that tubules were contiguous with the cell interior (Figure 2D). These measurements suggested that the observed tubules were valid targets for quantitative SR microscopy aimed at determination of glycocalyx height distal from the membrane.

To test this idea, we examined the distribution of Sia localizations along traverse slices of the tubules from the 3D reconstruction. The histograms representing these distributions yielded widths of 150-300 nm, in line with previous observations by EM (Connor et al., 2015) (Figure 2E). These “tubule widths” correspond to the sum of the following: (i) tubule size, i.e. the distance from the plasma membrane on one side to the plasma membrane on the other side, and (ii) the glycocalyx height on both sides of the tubule (Figure 2F). In the following, tubule size and glycocalyx height will be independently determined by comparing tubule widths derived from glycocalyx-labeled samples to those derived from plasma membrane-labeled samples. These comparisons form the basis for all subsequent height analysis.

Glycocalyx height can be extracted with high throughput using a 2D image analysis algorithm
3D imaging revealed that tubule widths vary widely (Figure 2E). Therefore, in order to extract meaningful information about glycocalyx height, many tubules on many cells must be analyzed. Current 3D imaging methods do not reach the throughput (sampling density, imaging speed) needed for such analysis. Furthermore, 2D methods have inherently higher resolution, which is especially important at the length scales dealt with here: given that glycan trees and the proteins that bear them are on the order of tens of nm in size (Helenius and Aebi, 2001), we expected the relevant length scales to be at the limit of what is possible to unambiguously assign, even with SR microscopy. In order to address these challenges of throughput and scale, we developed a semi-automated 2D SR imaging analysis workflow.

In single-molecule localization microscopy, 2D reconstructions represent a finite sampling of a 3D structure, which is inevitably blurred with a finite localization precision, then projected into 2D. By fitting the set of projected localizations to a model function, quantitative parameters may be extracted. To account for each of these steps (sampling, localization precision, projection, fitting), we explored how they affect the analysis of simulated “ground truth” 3D cylinders, representing glycocalyx-covered membrane tubules. Such validation is prudent when the effect sizes approach the experimental localization precision.

Single slices perpendicular to the simulated cylinders were sampled (black dots), then randomly displaced to simulate localization precision (turquoise dots), both using values corresponding to experimental datasets (Figure 3A; cf. Methods for details). The resulting yz-scatters were then projected, assuming infinitely large sectioning depth, onto the y-axis (right panels). The large sectioning depth was conservative; see Figures S3A-C for quantification of experimental sectioning depth (effectively ~120 nm in z). After projection, localizations were binned into histograms with bin width corresponding to our SR reconstructions. Agreement between the projection of the sampled localizations (gray histogram), the displaced localizations (turquoise histogram), and the ground truth total width was excellent, indicating that at length scales relevant to tubules, experimental localization precision and projection depth are sufficiently small to enable measurement of tubule width.

Note that depending on the inner and total diameter, tubules can appear in projections as non-hollow (i) or hollow (iii). We found hollow and non-hollow tubules in all cell lines examined, chiefly due to variation in tubule diameters. Therefore, a fitting function was required that captured tubule width independent of apparent hollowness. We hypothesized that a sum of two Gaussians that overlap to various extents could approximate the localization distribution across a tubule regardless of its appearance. We chose the distance between the outer inflection points over the peak-to-peak distance because the former is decoupled from the width of each single Gaussian. Simulations revealed that across tested parameter space, the maximal error for width measurements performed in this way was 0.5 nm for a tubule of 67 nm width (Figures S3D-E; see also red curves in Figure 3A). Hence, the analyses above establish a strategy to extract glycocalyx height from 2D SR reconstructions.

Figure 3B outlines the resulting analysis routine. Briefly, SR reconstructions were represented as 2D histograms of localizations (32 nm bin width, see Figure S3F for validation) and well-defined tubules were selected. A threshold was set to create a binary mask that effectively captured the morphology of the tubule and enabled its boundary to be automatically determined. Two points
corresponding to the beginning and end of the tubule were defined. Between them, 50 line profiles (LPs) oriented perpendicular to the boundary were created. Each line profile was fit to a double Gaussian, and tubule width was extracted. The average value of tubule widths derived for all line profiles in a given experimental condition was reported as the final tubule width, with accompanying standard error of the mean (conservatively taking N = number of tubules).

To benchmark the analysis routine, we chose a system where glycocalyx height could be predictably modulated. ldlD Chinese Hamster Ovary (CHO) cells are deficient in the enzyme UDP-glucose/galactose-4-epimerase (GALE), which interconverts UDP-glucose and UDP-GlcNAc to UDP-galactose and UDP-GalNAc, respectively (Figure 3C). These nucleotide sugars are donors for the glycosyltransferases that initiate and elaborate glycans, hence ldlD CHO cells display truncated glycans on their cell-surface (Kingsley et al., 1986). Fully elaborated glycans can be restored on these cells by supplementing their media with 10 µM galactose and 100 µM GalNAc. We confirmed by confocal microscopy that Sia labeling was consistent across cells and localized to the cell periphery (Fig 3D). Using the semi-automated analysis workflow, we measured Sia- and lipid-determined tubule widths of non-rescued and rescued ldlD CHO cells. As expected, the non-rescued cells had a shorter glycocalyx height, apparent from tubule widths of 119 ± 7 nm vs. 149 ± 6 nm for the rescued cells (SEM, p < 0.05, N = 42-78) (Figure 3E; see Figure S3G for SR reconstructions). The lipid-determined widths, at 115 ± 17 nm and 98 ± 14 nm for non-rescued and rescued cells, respectively, were not different from each other (Figure 3F, p > 0.1, N = 29; see Figure S3H for SR reconstructions), implying that the observed difference in Sia-determined widths originated from changes in glycocalyx height, not tubule size. Our direct measurements of glycocalyx height are in agreement with previous indirect measurements on single cells (Paszek et al., 2012). Our lipid-determined tubule widths match reported values of 60-120 nm for membrane tubules, as determined by EM (Frost et al., 2008; Guerrier et al., 2009).

As is evident from the confocal images in Figure 3D, galactose- and fully-rescued ldlD CHO cells displayed higher fluorescence intensity upon Sia labeling than their GalNAc-rescued and nonrescued counterparts, presumably due to lower sialic acid elaboration on truncated glycans. To ensure that our findings were not dependent on the extent of cellular labeling, we performed flow cytometry for each condition investigated. The labeling protocol for the flow cytometry samples was identical to the one used for SR samples. Labeling extent assessed by total fluorescence was not correlated with tubule widths (Figure 3G). The increase in height upon GalNAc addition may be due to the key role GalNAc plays in the processing and surface presentation of mucins such as MUC1, which are important structural components of the glycocalyx (Altschuler et al., 2000).

Sialic acids reside distal to GalNAc residues in the glycocalyx
For years it has been postulated that the glycocalyx exhibits an organized structure. For example, since Sia residues cap many glycan structures, they are thought to be, as a population, more peripheral than other monosaccharides (Varki and Gagneux, 2012). Peripheral localization of Sia is also consistent with their important role as ligands for viral receptors such as influenza virus hemagglutinin as well as human glycan-binding proteins such as Siglecs and selectins involved in the immune response (Varki, 2017). Any difference in height between Sia and GalNAc
populations is expected to be a fraction of total glycocalyx height. It is for this reason that while a peripheral distribution of Sia residues has been speculated, direct evidence has been lacking.

We employed tubule width measurements to examine whether Sia residues are spatially distinct from GalNAz residues (Figure 4A). For MCF10A cells, the difference was significant (202 ± 5 nm, N = 111, for Sia vs. 160 ± 6 nm, N = 52, for GalNAz, p < 0.0005). Measurements trended in the expected direction but did not achieve significance for BT-20 cells (tubule widths of 235 ± 7 nm for Sia, N = 115, vs. 212 ± 12 nm, N = 63, for GalNAz, p = 0.09) or SKBR3 cells (203 ± 7 nm, N = 61, for Sia vs. 182 ± 8 nm, N = 80, for GalNAz, p = 0.06). In all cells, the lipid-determined width was significantly less than both the GalNAz- and the Sia-determined widths (N = 19-24). With a panel of only three cell lines, conclusions were difficult to draw. Rather than screening a large number of additional cell lines, we expanded the scope of our method to include measurements on primary human cells.

Two chemotherapy-naïve patients, Patient 215 and Patient 213, underwent resective surgery for ovarian cancer (High-Grade Serous Cancer, HGSC). Primary tumor and omentum metastases were acquired and dissociated within an hour of removal and plated (cf. Methods; see Figure S4A for bright field images). Labeling, imaging, and analysis were performed as before. Cell viability was high, as indicated by robust incorporation of GalNAz after two days of incubation (Figure S4B). Sia residues were significantly distal to GalNAz for Patient 215 (193 ± 13 nm, N = 30, for Sia vs. 138 ± 11 nm, N = 29, for GalNAz, p < 0.005), Patient 213 primary tumor (235 ± 8 nm, N = 37, for Sia vs. 151 ± 10 nm, N = 34, for GalNAz, p < 0.0005), and Patient 213 omentum metastasis (186 ± 7 nm, N = 42, for Sia vs. 153 ± 8 nm, N = 25, for GalNAz, p < 0.005) (Figure 4B). As in the cell lines, all lipid-determined tubule widths were significantly smaller than GalNAz- and Sia-determined widths (Figure 4B). As seen from the SR reconstruction shown in Figure 4C, the difference in tubule width between GalNAz and Sia for Patient 213 was visually apparent. These data solidify the difference in height between Sia and GalNAz. Dissociation of freshly resected human tumors followed by metabolic incorporation and SR microscopy is currently the only way to achieve sub-20 nm precision in human tissue samples.

We next expanded the methodology to two-color labeling on single cells. Due to limitations in availability of primary human cells, we proceeded with cell lines. The approximately 20-40 nm tubule width difference between GalNAz- and Sia-determined tubule widths on BT-20, MCF10A, and SKBR3 cells would have been difficult to resolve in two-color due to lower resolution arising from requisite use of a second dye of lower quality. We therefore moved to MCF10A cells stably expressing the bulky mucin MUC1 with a C-terminus truncation to block downstream intracellular signaling (MUC1ΔCT) (Shurer et al., 2017), in the hope that a more prominent glycocalyx would result in a magnified difference between GalNAz and Sia labeling. In one-color SR imaging for GalNAz and Sia, the difference between the tubule widths was evident by visual inspection (Figure 4D). This difference in height was confirmed by quantitative analysis which yielded a difference of ~90 nm (Figure 4E, p<0.0005, N = 104 for Sia and N = 101 GalNAz). We therefore proceeded with two-color 2D SR imaging of GalNAz and Sia simultaneously. Figure 4F shows two-color binary super-resolved images of representative tubules on MCF10A MUC1ΔCT cells. Sia was distal to GalNAz across tubules. The swapped-fluorophore two-color images (Figure 4F, bottom row) confirmed that our results were not dependent on the photophysical properties of the fluorophores.
The observation that Sia extends further into the extracellular space than GalNAz can be explained by two scenarios. First, Sia and GalNAz could be stacked, i.e., the GalNAz population could reside below the Sia population in space with little overlap (Model 1, Figure 4G, left). Second, Sia and GalNAz could interpenetrate, with Sia extending further out into the extracellular space (Model 2, Figure 4G, right). To determine which scenario was best supported by our data, we selected tubules resolved as hollow and extracted the widths of each single Gaussian from the double Gaussian fit. These “wall widths” correspond to the height of a single glycocalyx on each side of the tubule. Note that this analysis can only be performed on samples that exhibit a large number of tubules appearing as hollow for both GalNAz and Sia labeling. Again, BT-20 and SKBR3 cells did not achieve significance, while in MCF10A cells Sia labeling gave a significantly larger wall width (p < 0.05, Figure 4H, N = 26-82). The observed wall widths had magnitudes between 60 and 80 nm. If doubled and summed with the lipid-determined tubule width, as required by Model 1, the wall widths yield tubule widths of at least 350 nm (cf. Figure 2F). As this value is larger than any tubule width observed across all cell lines, Sia residues as a population likely penetrate GalNAz residues and extend past them, as suggested in Model 2.

Statistical analysis of individual localizations reveals that the glycocalyx exhibits nanoscale architecture

The average widths extracted by our algorithms cannot report on the precise distribution of sugars in the glycocalyx, nor microheterogeneity along the membrane. As individual localizations correspond directly to sugar residues in the glycocalyx, we considered the possibility that analysis of raw localizations on single walls of hollow tubules could yield further insights into the shape of the sugar distributions away from the membrane. Figure 5A shows representative binned experimental localizations using a window size of 90 nm (perpendicular) x 30 nm (along membrane). The observed histograms arise from the true single emitter distribution in the glycocalyx as well a blur due to finite localization precision. Attempts to fit model functions directly to these histograms would report on the true positions of the emitters and the blur, which in this case are on the same length scale. To address this challenge, a statistical method which could account for finite localization precision was needed.

Inspection of the raw localization histograms revealed considerable heterogeneity, though all exhibited unimodality (cf. Figure 5A). Three basic patterns could account for the observed unimodal distributions: localizations could be uniform across the glycocalyx, could be concentrated at the membrane and decay outward, or could exhibit a maximum some distance away from the membrane, decaying in both directions (Figure 5B). We simulated each of these scenarios using uniform, exponential or half-Gaussian, and Gaussian distributions, respectively, as approximations of possible “ground truths”, and compared them to experimentally measured localization distributions.

We considered many 90x30 nm windows on each side of several hollow tubules for both GalNAz and Sia labeled BT-20 cells. Localizations were projected onto the axis perpendicular to the plasma membrane (x-axis, Figure 5A), yielding “experimental distributions”. Next, the four ground truth distributions ((a) through (d) in Figure 5B) were sampled with the same localization
number as in each of the experimental windows \( (n_{\text{exp}} \approx 50, \text{see Figure 5B, middle}) \), yielding “ideal distributions”. To account for two inevitable factors in localization microscopy, over-counting and localization imprecision, we duplicated 30% of the simulated localizations in the ideal distributions (estimated from merging localizations with less than 20 nm distance and one off-frame) and then perturbed each simulated localization with localization precision uncertainties sampled from the experimental data. The resulting “realistic distributions” modified the ideal models to account for unavoidable physical uncertainties.

To compare the experimental distributions to the four ground truth models (a) - (d), we employed quantile-quantile (QQ) plots (Figure S5A). In this approach, the sorted x-coordinates of the two distributions are plotted against each other, and fit to a straight line, giving a correlation coefficient, \( R^2 \) (Figure 5B, right). This procedure was repeated 2000 times, each with a newly simulated distribution, generating a set of \( R^2 \) values (Figure 5C). We performed three comparisons, progressively adding complexity. First, we determined the agreement of simulated ideal distributions compared to themselves (Figure 5C, dark gray violin plots). This estimated the effect of statistical noise due to finite sampling. Second, we compared ideal to realistic distributions (light gray). This determined the impact of the physical uncertainties on the measurement, leading to decreased mean \( R^2 \) values as expected. Finally, we compared ideal simulations to experimental data (white). The obtained sets of \( R^2 \) values contain contributions from both the physical uncertainties of the measurement and the actual positions of individual labeled sugars within the glycocalyx. To compare sets of \( R^2 \) values obtained by the ideal-realistic and ideal-experimental cases, we employed Student’s paired t-test. The resulting p-value reported on the agreement of the experimental data with the ground truth distribution in question. The Gaussian distribution exhibited the best agreement (insignificant p-values) for both GalNAz and Sia (Figure 5C), and the other three potential distributions were rejected. Note that our experimental axial sectioning depth of \(~120 \text{ nm} \) (Figure S3A-C) ensured the absence of projection artifacts in the QQ analysis (cf. Figures S5B-G for validation).

Remarkably, individual windows along one wall of a single tubule featured distributions that had a range of shapes. This local heterogeneity is shown by plotting the difference between \( R^2_{\text{ideal, exp}} \) and \( R^2_{\text{ideal, realistic}} \) along a tubule wall for two Sia-labeled tubules (Figure 5D). This analysis revealed that there were window positions along the two walls where the deviation between \( R^2_{\text{ideal, realistic}} \) and \( R^2_{\text{ideal, exp}} \) was smaller for non-Gaussian distributions (see red arrowheads), though overall agreement was still best for the Gaussian distribution, as expected. These data support the view that the glycocalyx exhibits nanoscale architecture with heterogeneity on the length scale of single-digit nanometers.

**Glycocalyx height increases upon epithelial to mesenchymal transition and oncogenic Ras activation**

Decades of genomic, transcriptomic, and proteomic studies have shown changes in the glycocalyx with malignancy (Pinho and Reis, 2015). More recently, transcript analyses have revealed upregulation of mRNA encoding “bulky” glycoproteins in primary tumors of patients with metastatic disease relative to those with confined tumors, implying that a large glycocalyx correlates with metastatic potential (Paszek et al., 2014). Those findings led to the generation of a mouse tumor model where the glycocalyx could be artificially bulked by overexpression of
signaling deficient MUC1 (MUC1ΔCT) or via surface loading of mucin-mimetic glycopolymers, which provided evidence that a bulky glyocalyx promotes metastasis (Woods et al., 2017). Despite these findings, however, direct observation of increased glyocalyx bulk as a result of transformation remained unachieved. We hypothesized that our ability to measure glyocalyx height could fill this gap and potentially uncover pro-oncogenic pathways more general than mucin upregulation that could affect glyocalyx height.

We began with two characteristic features of advanced human cancers: epithelial to mesenchymal transition (EMT) and oncogenic Ras activation. EMT is a vital component of the metastatic program (Kalluri and Weinberg, 2009). Ras is the prototypical oncogene, with mutations occurring in 9-30% of all diagnosed cancers, and is vital for a host of cellular processes that enable metastasis (Cox et al., 2014). The immortalized mammary epithelial cell line MCF10A, which we previously employed here, is a convenient system for the study of both EMT and oncogenic Ras activation. MCF10A cells can be reproducibly induced to EMT via treatment with the cytokine TGFβ (5 ng/mL) (Zhang et al., 2014). In addition, stable cell lines expressing the oncogene HRasG12V (called “MCF10AT” cells) are available, and can also be induced to EMT with TGFβ (Kim et al., 2014).

Changes in cell morphology (Figure 6A; see Figure S6A for confocal images) and Western blots of vimentin and E-cadherin confirmed that EMT occurred in TGFβ-treated cell lines and that MCF10AT cells had increased Ras signaling (Figure 6B). MCF10A cells showed a Sia-determined tubule width of 177 ± 4 nm, which increased to 201 ± 6 nm upon EMT (p < 0.005, N = 40 and 62, respectively). MCF10AT and MCF10AT + TGFβ cells had similar Sia-determined tubule widths of 217 ± 5 and 212 ± 6 nm, respectively (Figure 6C; see Figure S6C for SR reconstructions). Lipid-determined widths once again confirmed that these differences arose from the glyocalyx and not tubule size (Figure 6D and Figure S6E).

The increase in glyocalyx height upon HRasG12V expression (compare MCF10A to MCF10AT) was the largest magnitude effect (Figure 6C, p < 0.0005) and TGFβ treatment of MCF10AT cells did not increase glyocalyx height. However, this did not necessarily imply that the effects on glyocalyx height were strictly oncogenic Ras-dependent, as MCF10A and MCF10AT are different cell lines. We therefore turned to two cell lines isolated by Ying and colleagues from a doxycycline (dox)-inducible KrasG12D pancreatic ductal adenocarcinoma (PDAC) mouse model (Ying et al., 2012). Kras is mutated in 90% of human PDACs and has been implicated as a driver of disease progression (Lennerz and Stenzinger, 2015). Consistent with these previous reports, Ying et al.’s cell lines, called iKras-1 and iKras-2, are dependent on dox for tumorigenicity in the mouse. After dox removal, expression of the active KrasG12D transgene is extinguished, changing cell morphology (Figure 6E; see Figure S6B for confocal images), decreasing Kras pathway activation (Figure 6F) and slowing cellular growth without impacting viability (Ying et al., 2012). Ying and colleagues performed transcriptomic analysis on iKras-1 and -2, focusing on the effects of oncogenic Kras activation on energy metabolism. Strikingly, when we examined gene set enrichment analysis for iKras-1 and -2, we found that O-glycan and N-glycan biosynthesis gene sets are strongly downregulated upon dox removal (top third and fourth hits, respectively, when sorted by normalized enrichment score). We therefore hypothesized that removal of dox from iKras-1 and -2 cell lines might affect glyocalyx height.

10
iKras-1 and -2 cell lines were starved of dox for > 7 days until their growth slowed and morphological changes became evident. The cells were then labeled and imaged as before. We measured the Sia-determined tubule width of iKras-1 and -2 cell lines with and without dox treatment, and found a significant decrease in both cases, 180 ± 6 nm versus 130 ± 6 and 204 ± 7 nm versus 167 ± 10, respectively (p < 0.0005 and p < 0.005, respectively, N = 26-60) (Figure 6G; see Figure S6D for SR reconstructions; see Figure 6H and Figure S6F for lipid-azide stain). This represents the first experimental evidence for a direct link between a bulk property of the glycocalyx and expression of an oncogene.

**GALNT7 is upregulated in KRAS-positive human PDAC and may contribute to glycocalyx height**

*KRAS* is a master transcriptional regulator, but is not capable of direct glycosylation activity. We were curious whether the effects of oncogenic *KRAS* on glycocalyx height could be ascribed to individual effector genes under *KRAS*G12D control that directly influence glycocalyx structure. We re-analyzed Ying *et al.*’s microarray data (Ying *et al.*, 2012) to generate a list of genes enriched in the dox-on conditions. Since replicates were not available for iKras-1 and iKras-2 measurements, we took iKras-1 and -2 with dox as one group and iKras-1 and -2 without dox as a separate group for analysis (cf. Methods). A total of 47 genes were enriched in pathways that relate to glycosylation, including O- and N-glycan biosynthesis, glycosphingolipid biosynthesis, and O-linked glycosylation of mucins (see Figure 7A for heatmaps grouped according to standard pathways and Supplemental Table 1 for gene list).

We correlated mRNA transcript levels of these 47 genes to survival data in human PDAC patients (Anaya, 2016). Seven of the 47 genes gave logrank p-values less than 0.05 with higher expression predicting lower survival (top vs. bottom quartiles, Figure 7B). One of these genes, **GALNT7**, exhibited a Kaplan-Meier curve that was strikingly similar in development to the Kaplan-Meier curve for **KRAS**, especially in high expressing patients (N = 43, top vs. bottom quartiles, Figure 7C and inset). This similarity suggests that **GALNT7** could be relevant to the progression of **KRAS** positive PDAC tumors in humans.

**GALNT7** encodes polypeptide GalNAc transferase-7 (ppGalNAc-T7), which is a member of a family of Golgi-resident proteins that catalyze transfer of initiating GalNAc residues to sites of O-glycosylation on proteins. Little is known about ppGalNAc-T7’s substrate specificity, other than its preference for previously O-glycosylated substrates (Bennett et al., 1999). ppGalNAc-T7 has been linked to cancer progression, though the literature is conflicting on its role as a tumor suppressor or promoter (Gaziel-Sovran *et al.*, 2011; Peng *et al.*, 2012). Regardless, its activity has been experimentally linked to alterations in cell surface glycosylation (Gaziel-Sovran *et al.*, 2011). In PDAC, over-glycosylation on cell surfaces and in the extracellular matrix is a driver of disease progression through bulk mechanisms such as elevated interstitial pressure (Provenzano *et al.*, 2012). Therapeutic strategies targeting this bulky glycosylation currently employ intravenous injection of a glycan-degrading enzyme and are undergoing Phase III clinical trials with PDAC patients (Wong *et al.*, 2017). We hypothesized that increased **GALNT7** expression might drive increased cell surface glycosylation in iKras-1 and -2 cell lines, resulting in a larger glycocalyx.
We performed siRNA-mediated knockdown (KD) of GALNT7 in iKras-1 and -2 cell lines expressing KrasG12V (dox added) (Figure S7A). Cells were labeled and analyzed as above, yielding Sia-determined tubule widths of 179 ± 6 (N = 31) for iKras-1 transfected with an untargeted siRNA pool, 160 ± 10 (N = 19) for iKras-1 GALNT7 KD, 180 ± 11 (N = 17) for iKras-2 transfected with an untargeted siRNA pool, and 138 ± 7 (N = 24) for iKRas-2 GALNT7 KD. The decrease in tubule width upon GALNT7 KD was significant for iKras-2 (p < 0.005) and trended in the same direction for iKras-1 (p = 0.09) (Figure 7D; see Figure 7E for lipid-azide stain; see Figures S7B-D for images). These data, taken together with previous observations that glycocalyx thickness contributes to tumor survival and metastasis formation (Paszek et al., 2014; Woods et al., 2017), provide a model in which tumors can actively remodel their glycocalyx in the course of disease progression (Figure 7F). In addition, the direct link between GALNT7 protein levels and bulk on the cell surface motivates further exploration of single gene effectors of extracellular bulk, and may open doors to new therapeutic interventions for PDAC.

Discussion
Interest in single-molecule measurements for problems in glycobiology is emerging (Lakshminarayanan et al., 2018; Letschert et al., 2014). Here, we directly demonstrate that SR localization microscopy is a powerful inroad to the study of glycocalyx structure and dynamics.

An historical gap has existed in the application of advanced imaging methods to the glycocalyx despite the fact that robust and well-established methods were available for specific chemical modification of sugars with small molecule handles. In our view, this gap was due in part to a longstanding notion that the glycocalyx is a largely unstructured extracellular meshwork that, though ubiquitously present on cellular surfaces, has limited function beyond general protection and adhesion. As has been argued recently, this notion persists in the literature despite several lines of evidence to the contrary (Kuo et al., 2018). For example, mucin-domain glycoproteins present on the cell surface have been shown to act as stiff scaffolds around which receptors such as EGFR can organize (Burchell et al., 2018). Multivalent proteins such as the galectin family crosslink glycocalyx components, resulting in local lattice-like formations (Rabinovich and Conejo-García, 2016). And recently, Grinstein and colleagues provided evidence for a model in which the transmembrane hyaluronan-binding receptor CD44 forms ordered transmembrane ‘pickets’ (Freeman et al., 2018). Here, we combined metabolic labeling, bioorthogonal chemistry, and SR microscopy to show that the glycocalyx is a structured cellular compartment that is actively remodeled during oncogenesis and functionally relevant for tumor progression.

Having established and validated labeling and analysis routines, we chose as our first system of study the organization of two sugar populations, GalNAz and Sia, within the glycocalyx. Our studies of cell lines pointed to distal location of Sia relative to GalNAz in a variety of cases. To corroborate and extend the implications of these measurements, we turned to freshly dissociated tumor resections from two ovarian cancer patients. These experiments not only validated our hypothesis that sialic acids reside distal to GalNAz as a population relative to the plasma membrane, they also expanded the scope of our method to include samples with high biological relevance given the requirement for sub-20 nm precision.
The SR reconstructions that allowed glycocalyx height measurement are comprised of single-molecule localizations, which correspond to single labeled sugars in the glycocalyx. However, considerable heterogeneity is present, even in an individual tubule. As such, we employed statistical methods to elucidate patterns of localizations distal to the membrane. The key conclusion from these analyses is that sugars in the glycocalyx are not randomly distributed. The current state-of-the-art in SR microscopy does not yet enable the resolution needed to characterize these non-random distributions beyond the observation that the two sugars exhibit highest density some distance away from the plasma membrane. In the future, better precision from improved dyes may allow additional conclusions to be extracted from the highly crowded glycocalyx.

Finally, we interfaced our novel measurements with datasets generated by orthogonal methods. Our transcriptomic analysis of the iKras cell lines provided gene hits that have broad implications for glycocalyx biology. In the past, there have been few methods available to test hypotheses involving such glycocalyx-related hits, thus we suspect they have been often disfavored relative to hits relating to aspects of cellular biology with more readily available phenotypic readouts. We hope our observation that oncogenic KRAS remolds the glycocalyx will stimulate re-examination of omics datasets by others. In addition, we hope it will serve as an impetus for recognition of the glycocalyx as an organelle that is actively remodeled during oncogenesis and functionally relevant for tumor progression.

As a step in this direction, we correlated mRNA levels of glycosylation-related proteins from the iKras transcriptomics data with human PDAC survival data. This analysis revealed that GALNT7 transcripts are upregulated in cell lines expressing KRASG12D, and that siRNA-mediated KD of GALNT7 leads to a decrease in glycocalyx height. GALNT7 is therefore a potential mediator of the effect of KRAS on the glycocalyx. These data are consistent with observations that KRASG12D can decrease expression of microRNA-34a (Okada et al., 2014), a microRNA that has been reported in at least one study to decrease GALNT7 transcript levels (Li et al., 2014). Note also that the seven-member hit list we generated from the iKras transcriptomics analysis has hits other than GALNT7 which may be worth further exploration. For example, B3GNT3 and GCNT2 are involved in the synthesis of linear and branched poly-N-acetyllactosaminoglycans, large glycans which bind galectin family proteins (Shiraishi et al., 2001; Stowell et al., 2008). ST3GAL1 is a sialyltransferase, which is especially interesting given recent insights into the role surface sialylation plays in immune evasion (Xiao et al., 2016).

Broadly, we envision that future efforts will apply 2D and especially 3D advanced imaging techniques to the elucidation of additional nanoscopic parameters beyond glycocalyx height. The density, clustering, and contact sites of glycans and their interaction partners in the context of both health and disease are especially attractive as future avenues. Samples collected from patients with varying histories and disease progressions or from various sites in a single patient would be excellent targets for such analyses.
Acknowledgments: We thank Ramesh Nair (Stanford Bioinformatics Core Facility) for performing transcriptomics analysis. We thank Dr. Carl G. Ebeling (Bruker) for the use of the Bruker SRX visualization and analysis software. We thank Valerie Weaver (UCSF) for MCF10A, MCF10A + MUC1ΔCT, and MCF10AT cell lines. We thank Haoqiang Ying (MD Anderson Cancer Center) for iKras-1 and -2 cell lines. We thank Matthew Barnes (UCSF) for helpful discussions. We thank Ryan Flynn (Stanford), Elliot Woods (Stanford), and Matthew Barnes for critical reading of the manuscript.

Funding: This work was supported in part by the National Institute of General Medical Sciences (Grant No. R35GM118067) to W.E.M., by the National Institute of Biomedical Imaging and Bioengineering (Grant No. U01EB021237) to W.E.M., by a grant from the US National Institutes of Health (R01GM59907) to C.R.B., and by a US National Institutes of Health (R37GM058867) to C.R.B. K.P. was supported by a National Science Foundation Graduate Research Fellowship, a Stanford Graduate Fellowship, and the Stanford ChEM-H Chemistry/Biology Interface Predoctoral Training Program. V.K. and O.D. acknowledge support from the Mary Lake Polan Endowment. A.-K.G. acknowledges financial support from the Swedish Research Council (Grant No. 2016-00130) and from the Foundation BLANCEFLOR Boncompagni-Ludovisi, née Bildt.

Author contributions: L.M. and K.P. designed experiments; L.M., K.P., and A.R.R. carried out and analyzed experiments; V.K. and O.D. prepared human tissue samples; A.-K.G. performed TILT3D microscopy and analysis; L.M., K.P., A.R.R., C.R.B., and W.E.M. wrote the initial draft; all authors edited the manuscript; C.R.B. and W.E.M. provided conceptualization and supervision.

Competing interests: The authors do not declare any competing interest.

Data and materials availability: Raw data is available upon request.

Supplemental Materials:
Methods
Figures S1-S7
Supplemental references
References


Figures and Captions

A

Glycocalyx

Periodate

Aniline, Aminooxy-Fluor.

Cu-click with Alkyne-Fluor.

Cu-click with Alkyne-Fluor.

Glycolipids

N-glycans

O-glycans

(OM1a)

Periodate

GalNAz

Cu-click with Alkyne-Fluor.

Lipid-Azide

Glc

GlcNAc

Gal

GalNAc

Mannose

Sia

B

Sia

GalNAz

Lipid-Azide

AF647

BF

40 µm

C

Periodate

aminoxy

VC

AF647

Fluorescence (A.U.)

+ 

−

−

−

+ 

−

+ 

−

+ 

+ 

Percent of max

D

GalNAz

DL

SiA

DL

S08B3

10 µm

5 µm

BT-20

MGF10A

10 µm

5 µm

10 µm

5 µm

10 µm

5 µm

Figure 1. Labeling and SR imaging of the mammalian glycocalyx.

(A) Left. Central glycoconjugates found in the mammalian glycocalyx and important representatives of each are shown. Top row. Sialic acids (Sia) are labeled by periodate treatment, followed by aniline-catalyzed reaction of the formed aldehyde with an aminoxy-fluorophore. Middle row. Metabolic incorporation of GalNAz followed by Cu-click with an alkyne-fluorophore labels N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc). Glc is glucose; Gal is galactose; Bi-ant. refers to bi-antennary. Bottom row. Lipid-azide is docked into the outer leaflet of the plasma membrane, then labeled using Cu-click. (B) Confocal imaging of SKBR3 cells with Sia, GalNAz, and lipid labeling. Contrast settings are not equal for all fluorescent micrographs, to show all areas of cellular fluorescence. Lipid labeling is typically dimmer than GalNAz labeling, which is dimmer than Sia labeling. Structure of lipid-azide is depicted on the right. (C) Flow cytometry analysis of Sia-labeled SKBR3 cells with and without prior treatment with 30 nM Vibrio cholerae (VC) sialidase for 2 h at 37 °C. Cells were labeled using the same protocol as for SR microscopy, then lifted with trypsin, fixed, and subjected to flow cytometry. VC sialidase is specific to a subset of Sia linkages, and therefore results in partial decrease of fluorescence. (D) SR reconstructions of SKBR3, BT-20, and MCF10A cells with Sia and GalNAz labeling. Note spiny protrusions exhibiting high signal that cover the entire membrane region (arrowheads show two examples for each condition). 2D histograms shown have 32 nm bin width.
Figure 2. 3D SR imaging reveals cell-surface protrusions as glycocalyx-covered membrane tubules suitable for quantitation.
(A) 3D reconstruction of Sia on BT-20 cells. ManNAz incorporated BT-20 cells were labeled with AF647 using Cu-click and subjected to TILT3D microscopy. Left and right insets correspond to tubules that extend perpendicular to and within the xy-plane, respectively. (B)
Projection of the 3D reconstruction onto the xy-plane. The z-slices had a thickness of ~250 nm. (C) Effect of the orientation of tubules on their projections. Shown is a magnification of the tubules located at the asterisk in Figure 2A. (D) Magnification of the base of a single tubule taken from a z-slice (dagger in Figure 2B). (E) Quantitation of tubule width in 3D. A traverse section of three tubules was taken, and a histogram of single molecule localizations was plotted. (F) Schematic of the tubules as glycocalyx-covered membrane protrusions along with the two key parameters to be determined. The lipid-determined tubule width corresponds to the plasma membrane-to-plasma membrane distance. The GalNAz- and Sia-determined tubule widths correspond to lipid-determined tubule width summed with the glycocalyx height on each side of tubule.
Figure 3. Tubule width is a measure for glycocalyx height and can be extracted with high throughput from 2D SR reconstructions.

(A) Effect of projection of a 3D tubule into 2D. Three exemplary cylinders (i)-(iii) as proxies for membrane tubules are shown. The inner diameter corresponds to the membrane-to-membrane diameter of a tubule, and the difference between total and inner diameter corresponds to the glycocalyx height on the tubule. (B) Analysis routine to extract tubule widths from SR.
reconstructions. (C) *ldlD* CHO cells have a mutation in the GALE gene which leads to the absence of the key sugars UDP-Gal and UDP-GalNAc. The phenotype can be rescued by supplementation of the media with GalNAc and galactose. (D) Confocal analysis of non-rescued, partly rescued, and fully rescued Sia-labeled *ldlD* CHO cells. Contrast settings equal between all images. (E) Sia-determined tubule width of *ldlD* CHO cell rescue panel. Sugar supplementation was performed at the concentrations shown for 2 days prior to labeling and analysis. Error bars: SEM; * = p < 0.05. (F) Tubule width measurements of lipid labeled *ldlD* CHO cells. (G) Flow cytometry of starved and rescued *ldlD* CHO cells showing total AF647 fluorescence from cells labeled as in Figure 3E.
Figure 4. As a population, Sia residues likely reside distal to GalNAz residues. 

(A) Periodate-mediated Sia labeling, GalNAz incorporation, and lipid-azide incorporation were performed on BT-20, MCF10A, and SKBR3 cells. After bioorthogonal conjugation with AF647, tubule width measurements were taken using the semi-automated analysis workflow presented in Figure 3B. Error bars are SEM. * = p < 0.05, ** = p < 0.005, *** = p < 0.0005 by Student’s two-tailed t-test. (B) Tissue from Patient 213 and Patient 215 were obtained from chemotherapy-naïve patients diagnosed with ovarian cancer (High-Grade Serous Cancer, HGSC). Labeling and measurement were performed as in (A). (C) Representative SR reconstructions for GalNAz- and Sia-labeled Patient 213 cells. The visual difference between Sia- and GalNAz-labeling is striking. (D) Representative SR reconstructions of MCF10A cells expressing signaling deficient MUC1ΔCT on a doxycycline promoter. (E) MCF10A+MUC1ΔCT were labeled and measured as in (A). (F) MCF10A + MUC1ΔCT cells were sequentially Sia- and GalNAz-labeled (cf.
(G) Methods), with conjugation to AF647 or CF568 for both sugar residues, giving two-color and swapped two-color reconstructions. Binary images, which maximize contrast, of six individual magnified views are shown. Note that binarization obscures the hollowness of the tubules. (G) Two models of the glycocalyx architecture that could explain the distal position of Sia relative to GalNAz. GalNAz could either reside below Sia (Model 1), or GalNAz and Sia could interpenetrate, with Sia, as a population, extending further into the extracellular space (Model 2). (H) Wall width analysis of samples generated in (A). Individual Gaussians from the double-Gaussian fit were extracted from tubules resolved as hollow. Error bars are SEM. * = p < 0.05.
Figure 5. The glycocalyx exhibits a nanoscale architecture with sugar residues exhibiting a maximal density some distance away from the membrane.
(A) Representative localization distributions in 90x30 nm windows along a single wall of GalNAz and Sia-labeled tubules. (B) Schematic of analysis workflow for comparing possible ground truth sugar distributions with experimental localizations. (C) $R^2$ values from QQ analysis shown binned as violin plots. Each of the twelve columns represents a comparison of two distributions (see bottom). Analysis of GalNAz-labeled tubules is shown on top, and analysis of Sia-labeled tubules is shown on bottom. P-values are derived from t-tests of the means and standard deviations of the violin plots shown. Error bars: SEM; * = $p < 0.05$; *** = $p < 0.0005$. (D) Quantification of glycocalyx microheterogeneity along a single tubule wall. Arrowheads indicate example positions where the experimental data has a non-Gaussian sugar distribution.
Figure 6. Glycocalyx height increases upon transformation.

(A) Bright field images of MCF10A and MCF10AT cells with and without TGFβ treatment for >7 days. Changes in gross morphology upon transformation are visible. (B) Western blot analysis of epithelial to mesenchymal transition (EMT) in MCF10A and HRasG12V transduced MCF10A (MCF10AT) cells. pErk signaling increases in MCF10AT cells as expected from stable expression of HRasG12V. Vimentin increases and E-Cadherin decreases in TGFβ treated MCF10A cells. Vimentin increases in TGFβ treated MCF10AT cells, with E-Cadherin below detection by blot. (C) MCF10A and MCF10AT with or without TGFβ treatment were Sia-labeled and measured as in Figure 4A. (D) Lipid-determined tubule widths for MCF10A and
MCF10AT+TGFβ, the cells with the lowest and highest Sia-measured glycocalyx height, respectively. (E) Bright field images of iKras-1 and -2 cells with and without dox treatment for >7 days. Changes in gross morphology upon transformation are visible. (F) Western blot analysis of iKras lines. Dox removal decreases oncogenic Ras signaling, as evidenced by pErk both iKras-1 and iKras-2. (G) iKras-1 and iKras-2 cells with and without dox treatment were labeled for Sia and measured as in Figure 4A. (H) Lipid-determined tubule widths for iKras-1 and iKras-2 cells with and without dox treatment.
Figure 7. GALNT7 is upregulated with oncogenic Ras activation and contributes to glycocalyx bulk.

(A) Genes from the KEGG- and Reactome-O-glycosylation pathways that are significantly upregulated in dox-treated iKras-1 and iKras-2 cells compared to untreated iKras-1 and iKras-2 cells. (B) Genes from the list shown in (A) in which high mRNA transcript levels (top vs. bottom quartiles) show a significant correlation with poor patient survival in pancreatic adenocarcinoma (PDAC). Ranks in columns 2 and 3 derive from transcriptomics data of iKras-1 and -2, while p-values in column 4 are from patient data. (C) Kaplan-Meier plots for patients with PDAC tumors that show high expression (red curves) and low expression (blue curves) of Kras (left) or GALNT7 (right) (top vs. bottom quartiles, respectively). The inset shows the overlay of the
curves for high expression of Kras and GALNT7. (D) Dox-treated iKras-1 and -2 cells were subjected to siRNA-mediated knockdown (KD) of GALNT7. Control cells were transfected with a control pool of siRNA not targeting any mouse or human genes. Sia-labeling and subsequent measurement was performed as in Figure 4A. (E) Lipid-determined tubule widths for dox-treated iKras-1 and -2 cells with and without siRNA-mediated KD. (F) Proposed unified model. Upon EMT and oncogenic Ras expression, the height of the glycocalyx increases, and the resulting bulky glycocalyx contributes to tumor progression.
Methods

Cell culture

All cells were grown in T75 flasks (Thermo Fisher Scientific, Waltham, MA) and maintained at 37°C and 5% CO₂. BT-20 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. SKBR3 cells were cultured in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin. LdD CHO cells were cultured in 1:1 DMEM/F12 with 5% FBS and 1% penicillin/streptomycin. MCF10A and MCF10AT cells were cultured in phenol red free 1:1 DMEM:F12 supplemented with 5% NZ horse serum (Thermo Fisher Scientific), 20 ng/mL epidermal growth factor (Peprotech, Rock Hill, NJ), 0.5 µg/mL hydrocortisone (Sigma, St. Louis, MO), 100 ng/mL cholera toxin (Sigma), 10 µg/mL insulin (Sigma), and 1% penicillin/streptomycin. iKras-1 and iKras-2 cell lines were cultured in phenol red free RPMI supplemented with 10% Tet System Approved FBS (Takara, Mountain View, CA), 1 µg/mL doxycycline (Applichem, Darmstadt, Hessen, Germany), and 1% penicillin/streptomycin. For EMT studies, MCF10A and MCF10AT were treated with 5 ng/mL TGFβ (Cell Signaling Technology, Danvers, MA) for 1 week, then split into new flasks with complete media lacking TGFβ. For oncogenic Ras inactivation studies, iKras-1 and iKras-2 cells were lifted with trypsin, washed three times in suspension with PBS, and then seeded into fresh plates containing complete media without doxycycline. Cells were passaged in doxycycline-free media for one week prior to use for Western blot and imaging. For imaging, cells were seeded at a density of 10,000 cells/well on Lab-Tek™ II Chambered Coverglass (Thermo Fisher Scientific) coated with 0.01% poly-Lysine (Sigma). For flow cytometry, cells were seeded in 24-well plates (Thermo Fisher Scientific).

Extraction and processing of human tissue samples

Human ovarian tumor samples were obtained from the Department of Obstetrics and Gynecology under the approved IRB protocol 13939. Informed consent from patients was obtained prior to surgery for their tissue to be used in scientific research. Samples were transported and dissected within an hour of removal. Both OC213 and OC215 were obtained from chemotherapy-naïve patients diagnosed with High-Grade Serous Cancer (HGSC). The omentum-localized metastasis of Patient 213 was resected concurrently to the primary tumor and processed in parallel. Samples were washed with Dulbecco’s PBS (DPBS), and excessive connective and vascular tissue was removed. Tissues were subsequently transferred to separate 5-mL Eppendorf tubes and dissociated to single cell suspension using the Miltenyi human tumor dissociation kit (130-095-929, Miltenyi Biotec). Single cells were resuspended in Advanced DME-F12 Media (ADF) (Invitrogen), 12 mM HEPES, 1% GlutaMAX (Invitrogen), 5% FCS (Atlanta Biologicals), supplemented with 10 ng/ml human EGF (Invitrogen), 9 mM ROCK inhibitor (Y-27632, Sigma) and 1% penicillin/ streptomycin (Invitrogen), seeded in Matrigel-coated cell culture flasks and kept at 37°C, 5% CO2 in a humidified incubator. Similarly, metastatic tumor cells were collected from omental tissue and cultured as above. Epithelial cells were detached with trypsin (Gibco), centrifuged (10 min at 4 °C, 300g) and seeded in 250 µL of ADF media in pre-warmed, Matrigel-coated, 8-well Nunc Lab-Tek chamber slides (Thermo Scientific) at a density of 5,000 cells per well.

Metabolic incorporation of GalNAz and Cu-click

Two hours after seeding cells, media was supplemented with Ac4GalNAz (Life Technologies, Carlsbad, CA) at a concentration of 50 µM (from a 25 mM stock solution in DMSO). Two days after seeding, cells were moved to 4°C and washed three times with cold DPBS. DPBS with Ca²⁺ and Mg²⁺ was used at all steps during the labeling protocol to avoid cell lifting. Immediately after washing, Cu-click conjugation with AlexaFluor-647-alkyne (Life Technologies) was performed. For this, the cells were incubated with 50 µM CuSO₄ (Sigma), 250 µM BTTAA (Click Chemistry Tools, Scottsdale, AZ), 1 mM aminoguanidine (Sigma), 2.5 mM sodium ascorbate (Sigma), and 25 µM AlexaFluor-647-alkyne (Life Technologies) in DPBS for 5 min at 4°C in the dark as reported previously (Hong et al., 2010). Cells were then washed five times with cold DPBS, and fixed with 4% paraformaldehyde (Thermo Fisher Scientific) and 0.2% glutaraldehyde (Sigma) in DPBS for 30 min at room temperature. These stringent fixation procedures prevent membrane fluidity post fixation (Tanaka et al., 2010).

Periodate labeling of sialic acids

Sialic acids (Sia) were labeled as described previously (Zeng et al., 2009). Briefly, cells were moved to 4°C, then washed three times with cold DPBS + Ca²⁺ and Mg²⁺ followed by a 5 minute incubation with 1 mM sodium periodate (Sigma) in DPBS. Next, the periodate was quenched by 1 mM glycerol in cold DPBS, and washed three times with cold DPBS. Samples were stained with 25 µM AlexaFluor-647-hydroxylamine (Thermo Fisher Scientific) in the presence of 10 mM aniline in sterile filtered DPBS + 5% FBS pH 6.7 for 30 min at 4°C in the dark with gentle agitation. Cells were washed five times with cold DPBS, and fixed as above.
Two-color labeling of GalNAz and sialic acids

Two-color samples were treated with 50 µM AcGalNAz two hours after seeding, as described above. Two days later, sialic acid labeling was carried out as described above. After the incubation with AlexaFluor-647-hydroxyamine or CF658-hydroxyamine (Sigma) plus aniline, samples were washed five times with cold DPBS + Ca²⁺ and Mg²⁺, and GalNAz was labeled by Cu-catalyzed click chemistry as described above, using either CF658-alkyne (Biotium, Fremont, CA), or AlexaFluor-647-alkyne. The samples were again washed five times with cold DPBS + Ca²⁺ and Mg²⁺, then fixed as above. It is essential to add and remove wash solutions gently, to avoid cell lifting during the two-color labeling protocol.

Lipid-azide incorporation and Cu-click

Lipid-azide was incorporated into cell membranes as described previously (Nishimura et al., 2006). Briefly, a stock solution of 16:0 azidocaproyl phosphoethanolamine (Avanti Polar Lipids, Alabaster, AL) was prepared by dissolving the lipid in chloroform to a concentration of 1-4 mg/mL. For labeling a single well, 5 µL of the stock solution was dried carefully under a N₂ stream. The lipid was dissolved in 20 µL ethanol, yielding a concentration of 294 µM. 4 µL of the dissolved lipid was then added to 396 µL RPMI 1640 media with 10% FBS to obtain a final lipid concentration of 2.94 µM. Cells were incubated for 15-60 minutes at room temperature. Cells were then moved to 4°C, washed three times with cold DPBS + Ca²⁺ and Mg²⁺, and copper-Cu-catalyzed click chemistry followed by fixation was performed as described above.

Confocal microscopy

Confocal microscopy was performed on a Nikon A1R confocal microscope equipped with a Plan Fluor 60x oil immersion 1.30–numerical aperture objective. This instrument was equipped with a 561-nm green laser and a 639-nm red laser, for CF568 and AlexaFluor-647 excitation, respectively.

Flow cytometry

One-color labeling with each sugar separately was performed on cells exactly as above, with the exception that cells were fixed in suspension after trypsinization. Fixed cells were washed three times with PBS and then analyzed using a BD Accuri C6 flow cytometer.

Western blotting

For pErk, E-Cadherin, Vimentin, and tubulin blots, cells were lysed in 50 mM Tris pH 7.5, 10 mM MgCl₂, 0.5 M NaCl, and 2% Igepal (Cytoskeleton, Denver, CO) containing protease inhibitors and phosphatase inhibitors. Lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Primary antibodies were used according to manufacturer recommendations: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (20G11) (Cell Signaling Technology), E-Cadherin (4A2) (Cell Signaling Technology), Vimentin (D21H3) (Cell Signaling Technology), and α-Tubulin (clone B-5-1-2) (Sigma). Secondary antibodies (LI-COR Biosciences, Lincoln, NE) were used according to manufacturer recommendations.

For GALNT7 blots, cells were lysed in Laemmli buffer. Great than 50 µg of lysate was subjected to SDS-PAGE and transferred to nitrocellulose membrane. Anti-GALNT7 antibody (ab97645 (Abcam) was used at 1:200 in PBS containing 0.1% tween after blocking in 5% milk, overnight at 4 °C. Anti-Rabbit secondary antibody (LI-COR Biosciences, Lincoln, NE) was used according to manufacturer recommendations.

Optical setup

The core of the setup is an inverted microscope (IX71, Olympus, Tokyo, Japan). The lasers used for illumination (200 mW 561 nm, 120 mW 647 nm, CW, Coherent, Santa Clara, CA) were spectrally filtered (561 nm: ff01-554/23-25 excitation filter, 647 nm: ff01-631/36-25 excitation filter, both Semrock, Rochester, NY) and circularly polarized (LPV16B0-502 polarizers, Thorlabs, Newton, NJ, 561 nm: WPQ05M-561 quarter-wave plate, Thorlabs, 647 nm: WPQ05M-633 quarter-wave plate, Thorlabs). The beams were expanded and collimated using Keplerian telescopes. Shutters were used to toggle the lasers (VS14S2T1 with VMM-D3 three-channel driver, Vincent Associates Uniblitz, Rochester, NY). To achieve wide-field illumination, the lasers were introduced into the back port of the microscope body via a Köhler lens. The sample was mounted onto a X-Y-Z stage (Pnano XYZ Piezo Stage and High Precision XY Microscope Stage, Physik Instrument, Karlsruhe, Germany).

Emitted light from fluorophores was detected by a high NA detection objective (UPLSAP100XO, x100, NA 1.4, Olympus) and spectrally filtered (Di01-R405/488/561/635 dichroic, Semrock; for far red detection: ZET647NF notch filter, Chroma, Bellows Falls, VT, ET700/75m bandpass filter, Chroma, 3RD650LP longpass...
filter, Omega Optical, Austin, Texas; for red detection: ZET561NF notch filter, Chroma, et610/60 bandpass filter, Chroma, and FF01-593/40 bandpass filter, Semrock), and focused by the microscope tube lens. The emitted light then entered a 4f imaging system (f = 90 mm) where the first lens was positioned one focal length away from the intermediate image plane. For 3D imaging (see also section on 3D one-color SR microscopy), the desired phase pattern was implemented using transmissive dielectric phase masks (for the DH mask, Double-Helix Optics, LLC, Boulder, CO, and the Tetrapod phase mask was fabricated as described previously (Gustavsson et al., 2018) in the Fourier plane to modulate the phase and encode 3D information into the PSF. For 2D imaging, no phase mask was added. The light was focused onto the EMCCD camera (iXon3 897, Andor, Belfast, UK) by the second lens of the 4f imaging system.

For two-color two-channel imaging, the emitted light was split by a dichroic mirror (T660lpxrxt, Chroma), reflecting wavelengths shorter than 660 nm. In both paths, the emitted light passed through a 4f imaging system as described before they were merged again using a D-shaped mirror (BBD1-E02, Thorlabs).

2D one-color SR microscopy

For single-molecule localization microscopy, the DPBS in which the fixed samples were stored at 4°C in dark was replaced by a reducing, oxygen scavenging buffer (Halpern Aaron R. et al., 2015) consisting of 20 mM cysteamine, 2 µM/mL catalase, 560 µg/mL glucose oxidase (all Sigma-Aldrich), 10% (w/v) glucose (BD Difco, Franklin Lakes, NJ), and 100 mM Tris-HCl (Life Technologies). Diffraction-limited imaging was performed at low laser intensity of a few W/cm² at 647 nm excitation wavelength. Then, the laser intensity was increased to 5 kW/cm² and image acquisition was started after a short delay needed to convert the majority of the fluorophores into a dark state. Exposure time was 50 ms and the calibrated EM gain was 186. SR reconstructions were reconstructed from approx. 40000 frames using the ImageJ plugin Thunderstorm (Ovesný et al., 2014). Images were filtered with a B-spline filter of order 3 and scale 2.0. Local maxima, i.e. single-molecule signals, were detected with 8-neighborhood connectivity and a threshold of three times the standard deviation of the first wavelet level. Detected local maxima were fitted with a 2D-Gaussian using least squares. Post-processing involved drift correction by cross-correlation, followed by filtering (settings: sigma of the fitted Gaussian < 200 nm; uncertainty of localization < 20 nm). Images were reconstructed as 2D histograms with a bin size of 32 nm, corresponding to a five-time magnification (camera pixel size 160 nm). Figure S5 shows representative examples for the observed background, intensity, and sigma of the single-molecule localizations. Due to high photon counts, low background, and the large number of localizations, it was possible to set a cutoff localization precision at 20 nm to restrict the measurement to high precision.

2D two-color SR microscopy

Dual-labeled samples were stained with CF568 (Biotium) and AF647 (Life Technologies). The oxygen-scavenging buffer was identical to the buffer used for one-color imaging. First, AF647 was imaged as described. Then, the excitation was switched to 561 nm and 20000 to 30000 single-molecule frames were recorded at 30 ms exposure time with a calibrated EM gain of 186, using a laser intensity of 5 kW/cm². SR reconstructions were generated for both channels using Thunderstorm with identical settings except that CF568 molecules were detected with a threshold of twice the standard deviation of the first wavelet level. The two channels were registered by imaging a dense layer of fluorescent beads visible in both channels, followed by affine transformation using Matlab’s built-in algorithm imregtform. The found transformation matrix was then applied to the respective reconstructed image. To cross-check the transformation, the position of the beads were also super-localized using Thunderstorm, followed by approximate affine transformation and finding the nearest neighbor for each localization in one channel in the other. The control point pairs defined in this way were then used for a local weighted mean geometrical transformation. The found transformation matrix was then also applied to the respective reconstructed image. No difference was observed between the two approaches.

3D one-color SR microscopy

3D SR imaging with TILT3D was performed as described earlier (Gustavsson et al., 2018). Briefly, 647 nm light-sheet illumination with ~25 kW/cm² was used to excite AF647 molecules labeling Sia (introduced via click chemistry due to lower coverslip background when compared to periodate-based labeling) on the membrane of BT-20 cells in oxygen-scavenging buffer. With the double-helix dielectric phase mask in place, the 3D position of each single molecule was encoded in the recorded camera frames at 50 ms exposure time and a calibrated EM gain of 186. In total, 41000 frames were recorded. Every twentieth frame, a fiducial at the coverslip was imaged using 561 nm epi-illumination at ~10 W/cm². The 3D position of the fiducial bead was determined with the 6 µm axial range Tetrapod phase mask and
used for rough live axial drift correction and fine drift correction in post-processing. Custom scripts were employed to control the camera, stage, and shutters. Calibration and fitting of the Tetrapod fiducial images were performed using a modified version of the Easy-Pupil finder software (https://sourceforge.net/projects/easy-pupil-finder/). Calibration and fitting of double-helix PSF images was done using a modified version of the Easy-DHPSF software (https://sourceforge.net/projects/easy-dhpsf/). The obtained raw localizations were filtered to ensure a lobe distance of 6.1-8.0 pixels between the two lobes of the double-helix PSF. Spurious localizations were removed by means of filtering for large average distance to eight nearest neighbors. ~100000 localizations were used to reconstruct the final 3D SR reconstruction.

**Tubule width measurement**

To extract tubule width from the 2D SR reconstructions, a custom Matlab routine was used, involving three key steps for each tubule to be analyzed: (i) determination of tubule boundary; (ii) drawing of line profiles perpendicular to the tubule boundary; (iii) fitting of the obtained line profiles. After loading of the 2D SR reconstructions into Matlab, well-resolved tubules were manually selected. These defined regions of interest, each containing one tubule, were stored for individual extraction of tubule width. A two-dimensional Gaussian blur was applied to smooth the edges of the tubule. As the tubules did not exhibit rapid changes in their membrane curvature and the line profiles were drawn perpendicular to the boundary of the tubule (vide infra), this step helped to create a more accurate boundary. This was only used for determination of the boundary – the line profiles were drawn using the original SR reconstruction. The tubule to be analyzed was converted to a binary image using `im2bw`. The required threshold was set by the user. A broad range of thresholds captured the morphology of the tubule. To exactly trace the tubule boundaries, the function `bwboundaries` was used. The obtained boundary was interpolated with 10000 points using `interppolygon` (MathWorks). Then, the start and end point of the tubule were set by the user. Between these two points, 50 line profiles perpendicular to the boundary were drawn using `improfile` with bicubic interpolation. To aid later visual inspection of the line profiles, the line profiles were centered. Finally, the obtained line profiles were fitted to a double Gaussian using Matlab’s built-in `fit` function, using least squares regression. The parameter space was unrestricted except for the amplitudes; although the surrounding of tubules typically showed zero to few localizations, we wanted to ensure that no spurious localizations were picked up, potentially affecting the fit. The minimal amplitude of both Gaussians was therefore set to 10% of the maximum of each line profile. At all stages of the analysis, the user received visual feedback, e.g. an overlay of the found boundary and the SR reconstruction of the tubule, the centered line profiles, and the double Gaussian fit. Many tubules on many cells for a given sample were analyzed. The mean of the distribution was reported as tubule width, along with standard error. For these measurements, N represents number of tubules, not number of cells or line profiles.

**Quantile-quantile analysis**

To compare the experimental distributions to the four model distributions, we adapted a statistical method known as quantile-quantile (QQ) plot (Chambers, 2018). In a QQ plot, the quantiles of two data sets are plotted against each other. If the two sets have the same underlying distribution, a linear trend is observed, that is, the quantiles are maximally correlated. Thus, deviation from linearity indicates that the underlying distributions are different, hence a simple $R^2$ value can be used for comparisons.

First, we selected 11 and 10 well-resolved tubules from Sia and GalNAz labeled BT-20 cells, respectively. The x- and y-positions of the single-molecule localizations within 90x30 nm rectangular windows (N = 400 and 283, respectively) were extracted and projected onto the axis perpendicular to the wall (the x-axis), where the first localization is taken as the origin. We call these one-dimensional localization distributions the “experimental distributions”. Next, we fitted the experimental distributions to a uniform, a Gaussian, an exponential, or a half-Gaussian distribution, respectively. Note that this step only sets the parameters for simulation. The shape of the distributions is not affected, and thus, any combination of parameters will yield a linear trend in a QQ plot as long as the two compared distributions have the same underlying shape (see Figure S15).

To generate the model distributions for comparison, we generated 2000 Gaussian, uniform, exponential, and half-Gaussian distributions that were sampled using the obtained fit parameters and identical numbers of localizations as in each of the experimental windows. Each set of localizations was sampled 2000 times to rule out statistical fluctuations. We call these distributions “ideal distributions”. From the ideal distributions, we created “realistic distributions” to account for two inevitable factors in localization microscopy—overcounting and localization imprecision. The first is counting multiple frames from the same fluorophore as two different localization events, whereas the latter describes the fundamental uncertainty $\sigma$ in determining the position of the...
single emitter (in our case $\sigma = 10-20$ nm). Thus, we duplicated 30% of the values in the ideal distributions (estimated from merging localizations within a 20 nm radius, one off frame), and then perturbed each simulated localization with a $\sigma$ value chosen from the probability distribution of $\sigma$ values of the experimental data.

With this, we moved on to the QQ analysis. For each comparison, we plotted the quantiles of the two considered distributions against each other, fitted a straight line, and calculated the $R^2$ value to determine the linearity of the plot. Finally pairs of $R^2$ values were compared using Student’s two tailed t-test.

**Transcriptomics analysis**

The following two sample groups were analyzed from the GSE32277 series: GSM799693 (iKras1 +dox) and GSM799694 (iKras2 +doxilane (dox)) vs. GSM799698 (iKras1 -dox) and GSM799699 (iKras2 -dox). Affymetrix CEL files were downloaded from GEO. Background adjustment and quantile normalization followed by median polish were performed, as was the Robust Multi-array Average (RMA) algorithm for RMA expression values (RMAExpress). This was followed by gene set enrichment analysis (GSEA) using the quantile-normalized expression values from GEO for above groups with 1000 gene set permutations against canonical pathways (KEGG, Reactome, Biocarta) for the Affy Mouse430_2 chip.

**siRNA knockdown of GALNT7**

The following reagents were purchased from Dharmacon: SMARTpool: ON-TARGETplus Galnt7 siRNA (L-059912-01-0005), ON-TARGETplus Non-targeting Pool (D-001810-10-05), DharmaFECT 1 Transfection Reagent (T-2001-01), 5X siRNA Buffer (B-002000-UB-100). siRNA transfection was performed with 100 nM Galnt7 pool or 100 nM non-targeting pool, both with 1.5 $\mu$l of DharmaFECT 1 per well of a 48-well plate (seeding density of 10,000 cells/well). Cells were measured or lysed 96 h post transfection.

**Software**

Images were reconstructed and visualized in ImageJ using Fiji. 3D SR reconstructions were rendered in Vutara SRX. Data was analyzed using Matlab R2015b and organized as columnar text. Graphs were created in Origin 2016. Images were created in Adobe Illustrator CS6. Flow cytometry data was worked up with Flowjo 10. NIS-Elements AR software (Nikon Inc.) was used to acquire confocal images.
**Supplemental references**


Supplemental Figures and Captions

A

GalNAz | AF647-alkyne | Periodate | AF568-hydrazide |
--- | --- | --- | --- |
-- | + | -- | + |
-- | + | + | + |
+ | -- | -- | + |

561 exc. 20 µm

639 exc.

BF overlay

B

AF647 | BF
--- | ---
No staining | 20 µm

GalNAz | Sia | VC Sia
--- | --- | ---
-- | -- | -- |
+ | + | + |

C

GalNAz | Cu. | AF647-alkyne | VC Sia
--- | --- | --- | ---
-- | -- | -- | -- |
+ | + | + | + |

Percent of max.

AF647 Fluorescence (A.U.)

D

Lipid incorporation time (MCF10A)

Cu-Click incubation time (fdID CHO)

<table>
<thead>
<tr>
<th>No rescue</th>
<th>Full rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>5 min</td>
</tr>
<tr>
<td>30 min</td>
<td>10 min</td>
</tr>
<tr>
<td>45 min</td>
<td>5 min</td>
</tr>
<tr>
<td>60 min</td>
<td>10 min</td>
</tr>
</tbody>
</table>

E

BT-20 | MCF10A | SKBR3

2 µm

F

Counts

Intensity (photons)

Counts

Background (photons)

Counts

Uncertainty (nm)
Figure S1. Validation of labeling workflow. Related to Figure 1.

(A) Confocal images of GalNAz and sialic acid labeling on SKBR3 cells showing various controls. Dye only controls give the expected results, and labeling is consistent across cells in a given field of view. Two-color labeling gives overlaid membrane-localized signal. AF568 hydrazide (Thermo Fisher Scientific) and aminooxy-CF568 can be used interchangeably. Contrast settings are equal for each excitation channel (see lookup tables). (B) Confocal images of SKBR3 cells with and without 30 nM *Vibrio cholerae* sialidase treatment for 2 h at 37 °C (VC Sia). Labeling extent does not decrease for GalNAz labeling, in contrast to Sia labeling. Contrast settings are equal for all fluorescent micrographs. (C) Flow cytometry analysis of the effect of VC Sia treatment on GalNAz labeling. Labeling is not decreased upon exposure of GalNAz labeled SKBR3 cells to VC Sia, as expected. (D) Confocal images of lipid-azide and Cu-click labeled MCF10A MUC1∆CT (left) and *ldlD* CHO cells grown in media or media supplemented with 10 µM galactose and 100 µM GalNAc (right). For each cell line analyzed with lipid labeling, we screened 15, 30, 45, and 60 minutes incubation with lipid-azide and 5 and 10 minutes Cu-catalyzed click at 4 °C. Conditions giving the best labeling by confocal were used for further analysis. For example, for MCF10A cells (left), lipid incorporation time did not significantly alter the pattern or intensity of labeling, while in the case of *ldlD* CHO cells (right), ten minutes of Cu-catalyzed click resulted in an increase of apparent puncta, which made analysis difficult. Therefore, we chose five minutes of Cu-click labeling time for the *ldlD* CHO cells. Contrast settings are equal for all fluorescent micrographs within each cell line. (E) Representative examples for lipid-labeled tubules on BT-20, MCF10A, and SKBR3 cells. (F) Representative histograms of integrated single-molecule intensity (corresponding to numbers of detected photons with median at 3500), background, and uncertainty of single-molecule localizations.
Figure S2. Details of 3D SR microscopy method. Related to Figure 2.
(A) Single AF647 molecules were excited with a tilted light sheet at 647 nm and detected using the double-helix PSF with a 2 µm axial range. (B) Fiducial beads for live and offline drift correction were excited with epi-illumination at 561 nm and detected using the Tetrapod PSF with a 6 µm axial range. (C) 2D diffraction-limited image of the imaged cell. (D) 3D super-resolution reconstruction of the sample. (E) Histograms showing the signal photons per localization, background photons per pixel, and xy and z localization precision from the data shown in Figure 2A and in Figure S2D. The data was filtered to remove localizations with a distance between the two double-helix lobes smaller than 6.1 pixels and larger than 8.0 pixels. Spurious localizations were removed by means of filtering for large average distance to eight nearest neighbors. This resulted in ~100,000 filtered localizations with median photons per localization of 10,298, background photons per pixel of 58, and median localization precision of 8 nm and 13 nm in xy and z, respectively.
**Figure S3. Benchmarking of tubule analysis routine. Related to Figure 3.**

(A) Three example tubules taken from experimental SR reconstructions presented with various filtering settings. Top: weak threshold for point spread function (PSF) detection; middle: restrictive threshold for PSF detection; bottom: restrictive threshold and additional filtering post-reconstruction, removing localizations with a localization precision above 20 nm and PSFs with a width above 200 nm on the camera. The last, strong filtering was used for all experimental data presented in this work. By visual inspection, as the filtering becomes more restrictive, the tubules are resolved more clearly as hollow. (B) Line profiles corresponding to the three tubules shown in Figure S3A at the three filtering strengths, confirming the visual impression from Figure S3A. Thus, the ability to resolve the tubules as hollow is a result of quasi-sectioning due to rigid detection and filtering of the PSFs. This is expected as the localizations that would be projected to the middle of the tubule are the ones that are most out of focus, that is, they exhibit larger PSFs on the camera and, as a result of spreading the photons over a larger area, appear dimmer, leading to larger localization uncertainty. Hence, they are either (i) not detected in the first place due to rigid detection threshold or (ii) removed by the filter for small PSF widths or (iii) removed by the filter for small uncertainties. (C) To estimate the achieved quasi-sectioning depth, we simulated the three tubules depicted in Figure S3B as hollow cylinders using the procedure described in Figure 3A. We projected the localizations onto the xy plane assuming various sectioning depths. We binned the localizations into histograms with a bin width of 32 nm (the pixel size of the SR reconstructions) and extracted the line profile along the transverse dimension of the tubule to resemble the experimental situation. The experimental line profile using the strongest filtering conditions (light blue profile in Figure S3A) was best matched by a sectioning depth of 120 nm (compare light blue line profiles in Figure S3B and green line profiles in S3C). For comparison, the yellow line profile in Figure S3C depicts infinite sectioning depth. (D) To ensure that the double Gaussian fit is able to recover the tubule width robustly we simulated ground truth double Gaussian profiles. For this, two single Gaussian profiles were summed. The distance between their centers was changed from 0 to 250 nm in steps of 25 nm. Also, the standard deviations and amplitude ratios between the two Gaussians were varied from 25 to 87.5 nm in 12.5 nm steps and set to 1, 2, or 3, respectively. This parameter space resulted in a broad range of simulated profiles that spanned from two well-separated single-Gaussian profiles, partly overlapping profiles with shoulders, to completely overlapping profiles that were visually indistinguishable from single Gaussian profiles. (E) Each of the ground truth profiles shown in Figure S3D, with 10% noise added relative to an amplitude of one to account for random fluctuations, was fitted to a double Gaussian, the predicted tubule width was obtained, and the absolute error between the prediction and the ground truth tubule width was calculated, shown here. Evidently, the fit is able to extract the true width of the simulated profiles. None of the tested conditions exhibited an error exceeding 0.8% which corresponds to fractions of a nanometer. Hence, we are confident that the double Gaussian fit is a robust tool to determine the tubule width from the SR data, in spite of the fact that not all tubules are resolved as hollow. (F) Relation between the determined tubule width and the pixel size of the reconstruction. As we used 2D histograms to reconstruct the SR data for later tubule analysis, we wanted to ensure that the chosen bin width of 32 nm does not bias the results of the tubule and wall width measurements. Thus, we reconstructed the same set of localizations with different bin widths, analyzed identical tubules, and compared the results. The tubule widths are robustly determined with bin widths between 10-40 nm. Error bars: SEM. (G) SR reconstructions of ldlD CHO cells. (H) Representative examples for lipid-labeled tubules on ldlD CHO cells.
Figure S4. Imaging of patient-derived samples. Related to Figure 4.
(A) Representative bright-field images of Patient 215, Patient 213, and Patient 213 metastatic cells. The morphological difference between tumor cells in small colonies (white arrowheads) and spindle-like non-tumor cells (black arrowheads) is evident. Note also that non-tumor cells did not exhibit tubules when examined by SR microscopy. (B) Representative SR reconstructions for GalNAz- and Sia-labeled Patient 215 cells (left), and Patient 213 metastatic cells (right). (C) Representative examples for lipid-labeled tubules on MCF10A+MUC1ΔCT, Patient 215, Patient 213, and Patient 213 metastatic cells. Arrowheads show exemplary tubules.
Figure S5. Projection does not affect QQ analysis. Related to Figure 5.
(A) QQ plots of 20 pairwise compared Gaussian, uniform, exponential, and half-Gaussian distributions with identical (left) and different (right) parameters. A linear trend is observed in all cases. Thus, parameter values do not influence the QQ analysis. Note that the spread in the data is larger where the probability density of the underlying distribution is low, that is, where a single event has a higher impact. This effect is absent for the uniform distribution, which has a constant probability density. (B) To investigate the effect of projection on the QQ-analysis, we simulated a tubule as a hollow cylinder with an inner diameter of 70 nm and an outer diameter of 200 nm, corresponding to typical experimentally measured widths of lipid-labeled and Sia-labeled tubules. We assigned to the simulated glycocalyx a continuous density of localizations corresponding to each of the four distributions investigated (Gaussian, uniform, exponential, and half-Gaussian). (C)-(G) We projected the localization densities around the tubule using axial sectioning depths of 0 nm (C), 50 nm (D), 110 nm (E), 160 nm (F), and infinite sectioning (G). The projected localizations were binned. For visual reference, the region of the tubule corresponding to the specific sectioning depth is shown in purple next to the histograms. Inspection of the histograms confirm that sectioning depths up to 160 nm (compare histograms in (C) to (F)) do not significantly distort the shape of the four distributions, that is, the three-dimensional ground-truth density is preserved in the projection. Only for very large sectioning depths, the shape is significantly altered, and importantly, the resulting projected densities do not resemble any of the four investigated distributions (G). Thus, agreement between the experimental and any of the four model distributions cannot be explained by projection. In summary, considering our estimated sectioning depth of approximately 120 nm, we can conclude that projection does not have a strong effect on the QQ-analysis.
Figure S6. Imaging of MCF10A panel and iKras panel. Related to Figure 6.
Confocal images of the (A) MCF10A panel and (B) iKras panel. Labeling is consistent throughout a given field of view. Gross morphological changes are evident. Representative examples for SR reconstructions of cells from the (C) MCF10A panel and (D) iKras panel. Throughout all cells, a high number of well-resolved glycocalyx-covered tubules were found. Representative examples for lipid-labeled tubules on the (E) MCF10A panel and (F) iKras panel. Arrowheads show exemplary tubules.
Figure S7. Imaging and blots of iKras-1/2 knockdown lines. Related to Figure 7.

(A) Western blot analysis of dox-induced iKras-1 and -2 cell lines transfected with 100 nM siRNA pool targeting GALNT7. Arrow indicates GALNT7 band. The low affinity of the GALNT7 antibody necessitated loading of greater than 50 ng protein onto the gel, resulting in poorly resolved tubulin bands. (B) Bright field images of dox-treated iKras-1 and iKras-2 cells with and without GALNT7 knockdown reveal no visible morphological changes. (C) Representative examples for SR reconstructions of dox-treated iKras-1 and iKras-2 cells with and without GALNT7 knockdown. All cells exhibit a high number of well-resolved glycocalyx-covered tubules. (D) Representative examples for lipid-labeled tubules on dox-treated iKras-1 and iKras-2 cells with and without GALNT7 knockdown.