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Visualizing glycans on single cells and tissues

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Imaging without genetically expressed probes

Glycans coating the surface of archaea, bacteria and eukaryotes have attracted significant attention of chemists and biologists in this Post-Genome Era. These biomacromolecules are not directly encoded in the genome, and the non-template driven, posttranslational modification presents grand challenges to the study of their molecular functions in native environments [1]. Development of bioorthogonal chemistry has provided a paradigm shifting solution enabling novel approaches to unravel the dynamic complexity of glycosylation. The term bioorthogonal was introduced into the published literature in 2003 by C.R. Bertozzi [2], to refer to reactions that neither interact nor interfere with the cell's biochemistry [3–5]. Installing a probe on glycans with a two-step bioorthogonal chemical reporter strategy requires the introduction of a reporter into cellular glycans and a chemical reaction that forms a stable covalent linkage between the reporter and the probe molecule. In addition to the requirement that the reaction is essentially not toxic, the reaction rate needs to be fast enough (in the biological milieu) in order to capture the kinetics of the cellular processes of interest. Several reactions have proven to be bioorthogonal [6]. Two of the earliest reactions introduced by the Bertozzi group are the Staudinger ligation [3] and 'copper-free click chemistry' which is a 1,3-dipolar cycloaddition between azides and cyclooctynes (strain-promoted azide-alkyne cycloaddition (SPAAC)) [7,8•]. More recently, it has been shown that the ligand-accelerated CuAAC (Cu(I)-catalyzed azide alkyne cycloaddition) can be exploited as a bioorthogonal reaction [6,9,10,11••].

Two broad approaches incorporate the principles of a bioorthogonal chemical reporter strategy. The metabolic oligosaccharide engineering approach, exploits the metabolic replacement of a monosaccharide by modified sugar analogues [12], while the chemoenzymatic glycan labeling (CeGL) exploits a recombinant glycosyltransferase to transfer a mono-saccharide analogue from a nucleotide sugar donor to a specific glycan acceptor [13•]. In this short review, we highlight several recent innovative applications of these two approaches to imaging glycans on single cells and tissues, rather than presenting a chronological list of the many outstanding imaging advances (e.g. [8•,14,15]). Although the focus here is the application of these approaches to imaging surface glycans, the recent tagging of intracellular carbohydrates in living cells [16] and the use of a bioorthogonal reporter strategy for Raman imaging [17•], suggests that these approaches have a bright future. The recently reported MRI imaging of glycosylated tissue in live mice using metabolic labeling and a bioorthogonal gadolinium based probe [18], suggests that we can anticipate correlated optical and MRI imaging of glycans in live animals.

Unlike super-resolution imaging with genetically expressed probes, imaging the dynamics of biological processes with bioorthogonal chemical reporter strategies is fundamentally limited by the second-order rate constants associated with the bioorthogonal reaction [6,19–21]. The Staudinger ligation (with rate constants in the range of 10^{-4} to 10^{-2} M^{-1} s^{-1}) and SPAAC (with rate constants in the range of 10^{-2} to 1 M^{-1} s^{-1}) are an order of magnitude slower than CuAAC reactions (with rate constants greater than 10^{1} to 10^{2} M^{-1} s^{-1}). Fortunately, older reactions continue to learn new tricks. For example, Wu's group has demonstrated that the introduction of an electron-donating picolyl azide combined with tris(triazolylmethyl)-amine-based ligand for Cu(I) (BTTPS) produced at least a 20-fold enhancement of CuAAC fluorescent labeling (with 1 nM concentration of metabolic precursor); this accelerated reaction enabled confirmation that the conversion rate of a monosaccharide building block into a cell-surface glycoconjugate is of order minutes [22••].

Imaging an ensemble of glycans in live cells

Studies using fluorescent recovery after photobleaching (FRAP) imaging of an ensemble of antibody labeled glycoproteins in the 1980s and early 1990s demonstrated that the extent of glycosylation and the size of the extracellular domain limit translational diffusion [23,24]. Attempts to understand and model how barriers in the cytoplasm, membrane bilayer and the external space separately restrict the translational (lateral) mobility of transmembrane proteins, showed that the diffusion of transmembrane glycoproteins was constrained as compared with the relatively free movement of glycosylphosphatidylinositols (GPI) proteins (typically

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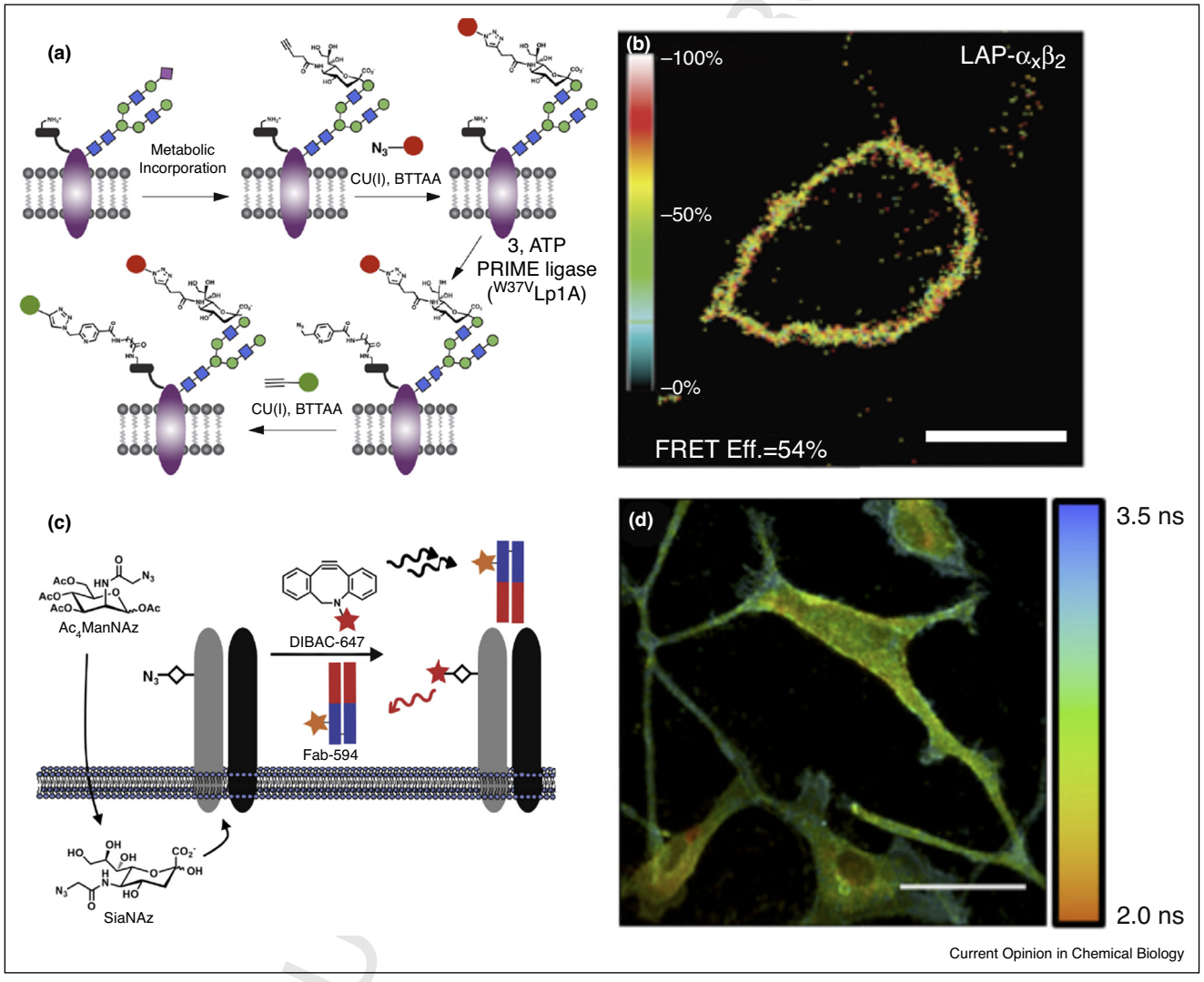
glycolipids diffuse 3 times the distance of transmembrane proteins before experiencing a barrier) [25]. FRAP measurements by Edidin's group using class I MHC molecules revealed that mutants with reduced N-linked glycans have increased lateral diffusion as compared with wild-type and that a large mobile fraction of diffusing glycoproteins enabled bleached regions to become repopulated with fluorescent molecules [24]. In contrast to these pioneering studies, contemporary FRAP imaging of the dynamics of glycolipids within the cell envelope of mycobacterial membranes exploits the power of metabolically incorporated analogues [26].

Ensemble measurements of metabolically labeled glycans, co-labeled with a site specific protein tag, enabled Lin *et al.* to apply F*o***uml;**rster resonance energy transfer (FRET) imaging to specific glycoproteins in live cells [27]. Using the enzyme-catalyzed probe ligation method, based upon lipoic acid ligase (LplA), developed by Ting's group [28], a FRET donor was installed on an extracellular terminus of a protein of interest. As shown in Figure 1a,

Figure 1



F*o***uml;**rster resonance energy transfer microscopy (FRET) of glycoproteins in live cells. **(a)** Bioorthogonal labeling in cells expressing a protein fused with LAP at the N-termini and incubated with Ac_4ManNAl. The FRET acceptor dye molecule (Alexa Fluor 647) using CuAAC assisted by BTTAA. Subsequently, LAP was conjugated with the lipoic acid-picolyl azide derivative using ^{W37V}LplA and followed by reaction with the FRET donor (Alexa Fluor 488-alkyne). Adapted from Lin *et al*. [27]. **(b)** FRET efficiency calculated using acceptor photobleaching in live HEK 293T cells expressing LAP-$\alpha_X\beta_2$ integrins. Scale bar = 10 μm. Adapted from Lin *et al*. [27]. **(c)** Fab fragment targeting moiety used to place the donor fluorophore (Fab-594) as combined with metabolic labeling with Ac_4ManNAz to introduce the acceptor cyclooctyne-fluorophore (DIBAC-647) via a bioorthogonal reaction. Adapted from Belardi *et al*. [29]. **(d)** Two-photon microscopy employing time correlated single photon counting to measure FRET with fluorescence lifetimes using a Fab fragment to install a fluorescent donor (Alexa Fluor 594) on the glycoprotein backbone and metabolic labeling with Ac_4ManNAz to place an acceptor on integrins. Scale bar = 50 μm. Adapted from Belardi *et al*. [29].

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following metabolic incorporation with an alkyne reporter (e.g. Ac_4ManNAl labeled sialic acid) into cell-surface sialylated glycans, an Alexa fluor-azide was installed as a FRET acceptor using CuAAC assisted by BTTAA. Subsequently, LplA Acceptor Peptide (LAP) was conjugated with a lipoic acid-picolyl azide derivative followed by reaction with Alexa fluor-alkyne as the FRET donor. Figure 1b shows the FRET efficiency calculated using acceptor photobleaching in live HEK 293T cells expressing LAP-$\alpha_X\beta_2$ integrins. Because this method yielded relatively high levels of FRET (≈50%), Lin *et al.* were able to apply the approach to elucidate the role of sialylation in the activation of $\alpha_X\beta_2$ integrins [27]. Following confirmation using FRET imaging that the fluorinated sialic acid analogue $3F_{ax}$-Neu5Ac effectively inhibited the sialylation of $\alpha_X\beta_2$ integrins, they showed that the removal of sialic acids impaired $\alpha_X\beta_2$ integrin activation. They also demonstrated FRET imaging of glycosylated receptors such as sialylated glycans of epidermal growth factor receptor (EGFR).

An alternative approach to implementing intensity based FRET measurements on a specific glycoprotein, Belardi *et al.* employed time correlated single photon counting with two-photon microscopy to measure fluorescence lifetimes [29]. After confirming that $\alpha_V\beta_3$ integrin in U87MG cells is sialylated with α2,3-linked residues, they used a Fab fragment to install a fluorescent donor (Alexa Fluor 594) on the glycoprotein backbone and metabolic labeling with Ac_4ManNAz to place an acceptor on integrin SiaNAz residues (Figure 1c). The measured lifetimes from FRET on $\alpha_V\beta_3$ integrins in U87MG cells cultured with Ac_4ManNAz is shown in Figure 1d. Histograms of the fluorescence lifetimes indicate that FRET reduced the lifetime of FAB-594 from 3.09 ns, *in vitro*, to an average of 2.60 ns in Ac_4ManNAz tagged cells. They also confirmed that sialidase cleavage of SiaNAz residues essentially eliminated FRET.

Single molecule tracking and super-resolution

With the explosion of single molecule tracking and super-resolution imaging of proteins (genetically encoded with photo-activatible fluorescent proteins or labeled with quantum dots or dye molecules), the extension of these approaches to glycoproteins was inevitable. Super-resolution imaging of glycans was demonstrated by two groups who published within a several month span in 2014 using stochastic optical reconstruction microscopy (STORM) on live [30,31••] and fixed cells [32••]. One of these two groups also implemented single particle tracking to follow glycans metabolically labeled with dye molecules (using biocompatible BTTPS/Cu^I catalyst) [30,31••]. Tracking of O-linked and N-linked sialylated proteins metabolically labeled with Ac_4GalNAz and Ac_4ManNAl, respectively, and tagged with dyes on cancer cells revealed constrained diffusion which was modeled as damped Brownian motion resulting from a confining harmonic potential [31••]. The slower diffusion of glycans on cells with higher metastatic potentials was conjectured to be caused by increased crowding of surface glycoproteins which could effect the formation of adhesions to the extracellular matrix [33].

An example of a 'snapshot' of the distribution of diffusing Alexa Fluor 647 dye molecules tagged to N-linked sialic acids on the surface of a live cancer cell is shown in Figure 2a (scale bar = 20 μm). Figure 2b shows a STORM image of N-linked sialic acid in HeLa cells metabolically labeled with Ac_4ManNAl and conjugated with Alexa Fluor 647 azide (scale bar = 10 μm) [31••]. Figure 2c and d show STORM images of a fixed human osteosarcoma (U2OS) cell metabolically labeled with Ac_4GalNAz and clicked with (c) CuAAC and (d) SPAAC [34] (boxed region = 2.0 μm wide). These super-resolution images highlight membrane nanotubes and adhesive filaments.

Tissue and whole-animal imaging

On a larger spatial scale, chemoenzymatic labeling protocols have demonstrated that it is possible to obtain images of tissue with glycan labeling that augments histological hematoxylin and eosin staining. In order to emphasize the capabilities of the approach, Rouhanifard, Lopez-Aguilar and Wu refer to this as: 'chemoenzymatic labeling histology method using clickable probes' (CHoMP) [35••]. Figure 3a and b shows the results of this chemoenzymatic approach with LacNAc labeling of lung tissue obtained from a fixed/frozen 10 μm mouse tissue section [35••]. This group also applied this method to other tumor tissue and to screening human tumor microarrays, where it was observed that a there was a large,13-fold decrease in LacNAc expression in grade 1 lung adenocarcinoma patient samples as compared with healthy humans.

Bioorthogonal labeling of several organ systems in living animals (e.g. heart, liver and kidney) has recently been expanded to include sialyated glycans in the brain of live mice using intravenous injection of PEGylated liposomes encapsulating 9AzSia or ManNAz that were able to cross the blood brain barrier [36••]. Because this liposome-assisted bioorthogonal reporter (LABOR) strategy can also be combined with histological staining, it is possible to relate the spatial distribution of sialyated glycans to features such as synaptic density.

Figure 3c and d shows labeling that was achieved with LABOR strategy using 9AzSia coupled with *in vivo* copper-free click chemistry. These confocal images delineate the distribution of 9AzSia-incorporated sialoglycans in the granule cell layer of dentate gyrus in the hippocampus. Using 10 μm thick sections with glycan labeling and co-immunostaining using synaptophysin and DAPI, multi-colored images highlight the biosynthesis and distribution of sialic acids on cell surfaces and synapses (as labeled

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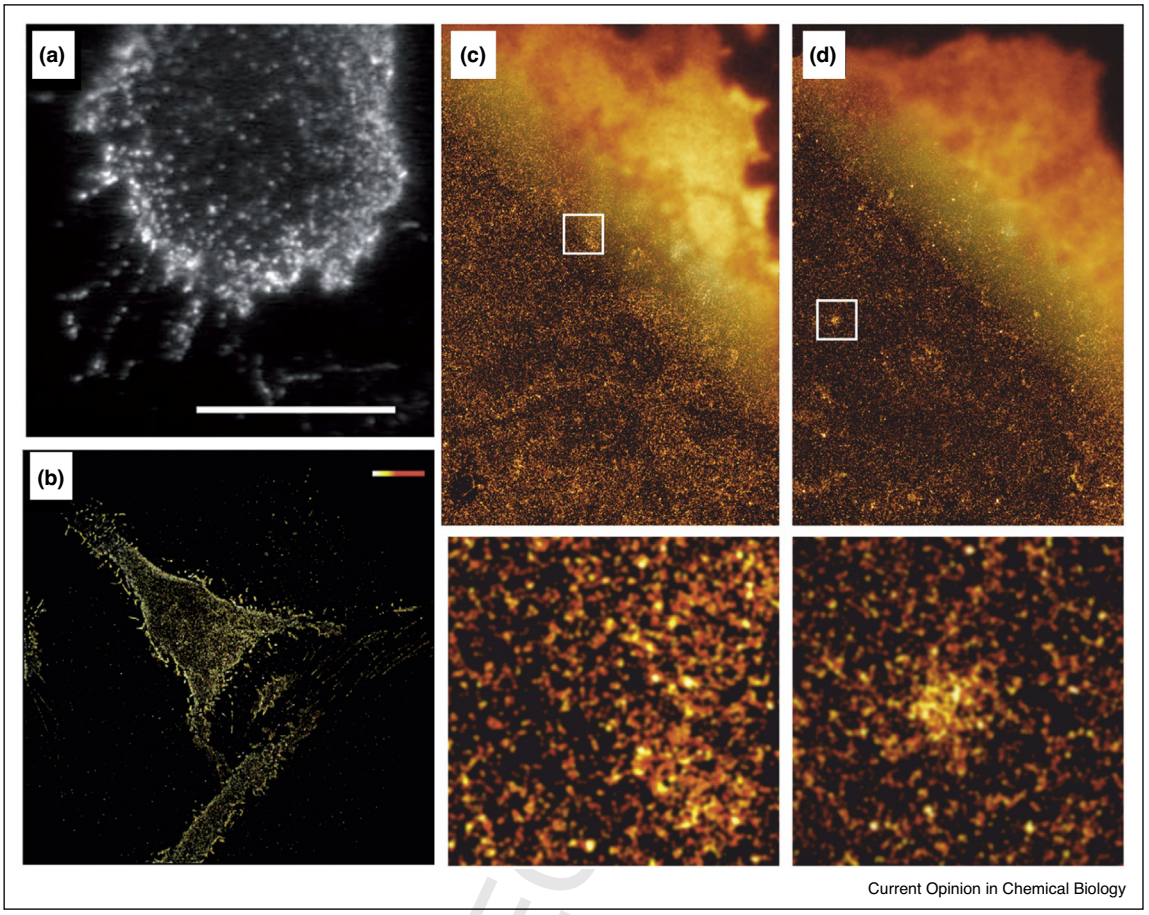
Figure 2



Single molecule tracking and STORM imaging. **(a)** Snapshot of Alexa Fluor 647 molecules on N-linked sialic acid in a live metastatic cell using TIRFM. Scale bar = 20 μm. Adapted from Jiang *et al*. [30]. **(b)** STORM imaging of sialic acid on live HeLa cells, metabolically labeled with Ac_4ManNAl and conjugated with Alexa Fluor 647 azide using BTTPS/Cu^{I} catalyst. The image was produced from 480 consecutive frames with 130021 detected deviation equal to the localization precision. The color bar represents the integrated fluorescent intensity of each molecule. Scale bar = 10 μm. Adapted from Jiang *et al*. [30]. **(c)** STORM image obtained from fixed human osteosarcoma (U2OS) cells metabolically labeled with Ac_4GalNAz and clicked with CuAAC. Adpated from Mateos-Gil *et al*. [34]. **(d)** STORM image obtained from U2OS cells metabolically labeled with Ac_4GalNAz using copper-free strain-promoted azide-alkyne cycloaddition. Adapted from Mateos-Gil *et al*. [34] (boxed region = 2.0 μm wide).

with synaptophysin) and a marker for astrocytes glial fibrillary acidic protein (GFAP).

Since the chemical reporter strategy was first applied to image surface glycans in developing zebrafish a decade ago [38], this model organism has remained the subject for advances in bioorthogonal chemistry which seek to overcome the limitations of imaging internal structures with exogenous probes. In order to prevent the high background fluorescence from unreacted probe, which can dominate glycan imaging in the transparent zebrafish, Bertozzi's group developed an alternative approach exploiting the direct injection of a cyclooctyne-functionalized sialic acid followed by subsequent injection of an turn-on tetrazine probe [37•]; using this approach, they were able to demonstrate new sialylated structures in the developing zebrafish.

Although not as efficiently incorporated as Ac_4ManNAz, microinjection of a bicyclononyne-functionalized sialic acid derivative, BCNSia, followed by injection of a fluorogenic cyclooctyne-reactive probe enabled imaging of zebrafish embryogenesis, with minimal background fluorescence. Agarwal *et al.* demonstrated that prior to the copper click chemistry reaction, the new probe produced minimal background fluorescence, but robust SiaNAl-dependent labeling of enveloping layer cells following reaction [37•]. Figure 3e shows their approach to labeling with BCNSia and **4** and images from (f and g) embryos injected with BCNSia (and fluorogenic probe CalFluor

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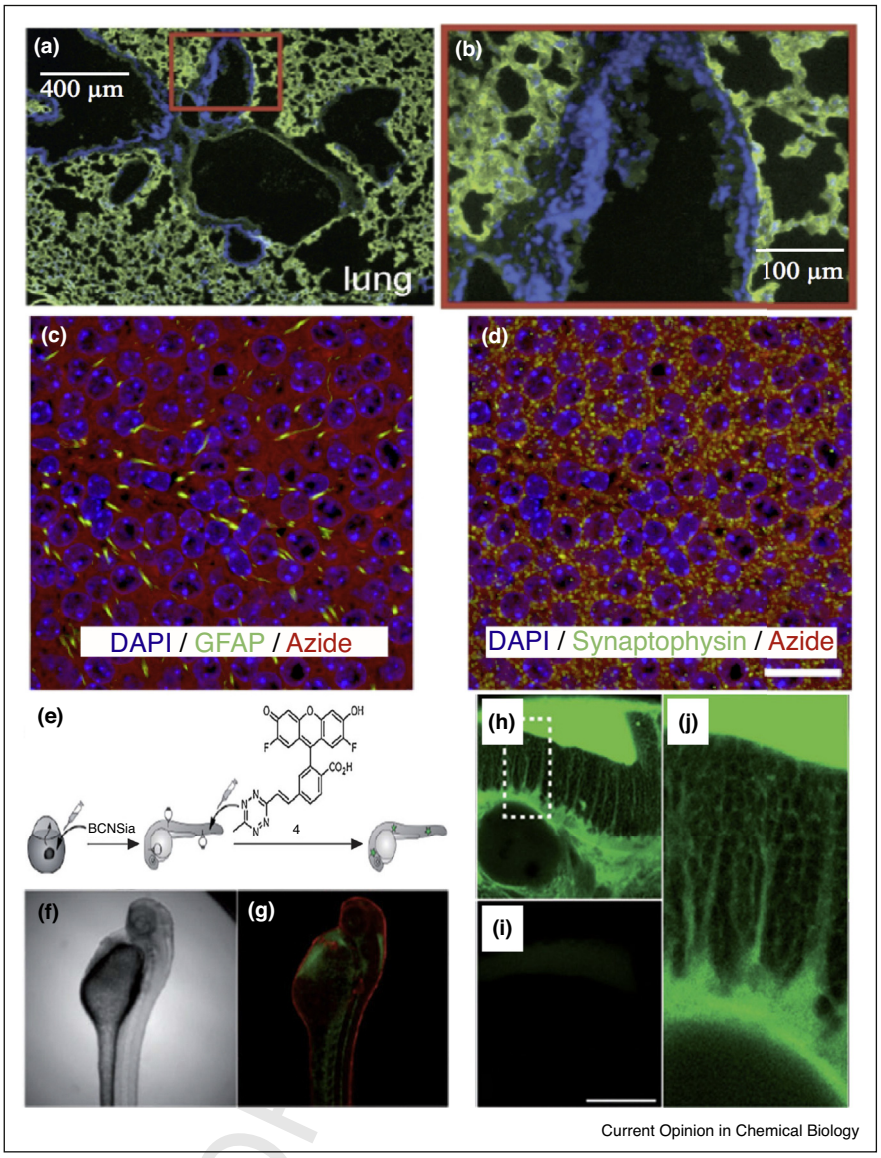
Figure 3



Tissue and whole animal imaging. **(a and b)** Chemoenzymatic labeling using clickable probes (CHoMP) applied to LacNAc labeling of lung tissue obtained from a fixed/frozen 10 μm mouse tissue section (Green: LacNAc staining; Blue: DAPI nuclear staining) Adapted from Rouhanifard *et al*. [35••]. **(c and d)** Liposome-assisted bioorthogonal reporter (LABOR) strategy used to label sialylated glycans in the dentate gyrus in mouse hippocampus with an azido sialic acid reporter molecule and copper-free click chemistry. Confocal images obtained from 10 μm thick sections with immunostaining using synaptophysin, DAPI and the marker for astrocytes, glial fibrillary acidic protein (GFAP). Adapted from Xie *et al*. [36••]. **(e)** Schema for sialylation imaging in live Zebrafish embryos with BCNSia and injection with **4**. Adapted from Agarwal *et al*. [37•]. **(f and g)** Brightfield and embryo injected with BCNSia at the 1–8 cell stage and injected with **4** and bathed in a copper click solution with CalFluor 647. Adapted from Agarwal *et al*. [37•]. **(h–j)** Zebrafish lateral view of hindbrain, (i) injections with vehicle. Scale bar = 100 μm. Adapted from Agarwal *et al*. [37•].

647 to map the vasculature). Figure 3h and j shows lateral views of labeled hindbrain and absence of labeling with injection of vehicle, Figure 3i.

Perspectives and conclusions

The applications presented in this review demonstrate that the leading microscopic methods, which have revolutionized the study of proteins in living systems, can be adapted to imaging glycans on single cells and tissues. Bioorthogonal chemical reporter strategies, using metabolic oligosaccharide engineering and CeGL, have enabled the application of FRAP, single molecule tracking and super-resolution imaging. This strategy, when combined with genetically encoded probes, has made it

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possible to visualize glycans on a specific protein via FRET and FLIM. Furthermore, it has now been demonstrated that neither the blood brain barrier nor the enveloping layer prevents *in vivo* imaging of sialylated glycans. In the not too distant future, there will be many examples of application of these new techniques to characterize glycosylation changes associated with animal models of human disease and on human samples.

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