Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Supplementary Information For: Dual-Sugar Cell Imaging using Isonitrile and Azido based Click Chemistries

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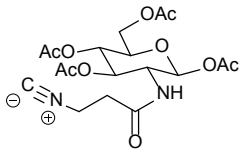
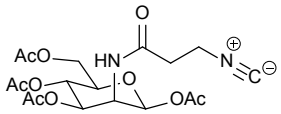
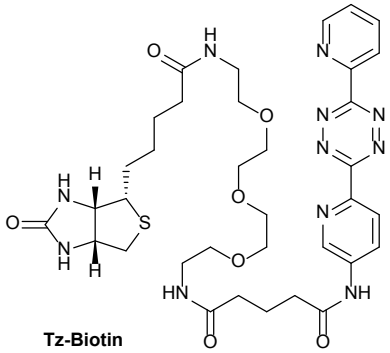
I Sugars and Probes



Ac_4GlcN-n-Iso

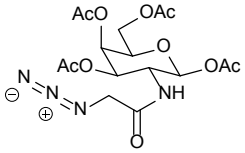


Ac_4ManN-n-Iso

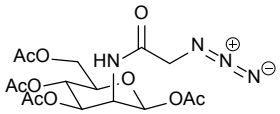


Tz-Biotin

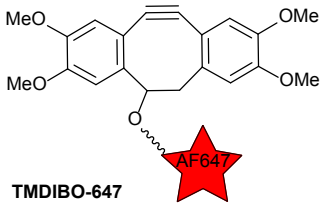
Ac_4GlcN-n-Iso, Ac_4ManN-n-Iso and Tz-Biotin were synthesized according to our recently reported syntheses.[1]



Ac_4GalN-Az



Ac_4ManN-Az



TMDIBO-647

Ac_4GalN-Az, Ac_4ManN-Az and TMDIBO-647 were synthesized according to previous work and literature procedures.[2-4]

II Biological Procedures

Cell Culture

Lewis Lung Carcinoma (LL2, from ATCC, Teddington, UK) cells were grown in a mixture of 90% DMEM (Dulbecco's Modified Eagle's Medium, Invitrogen, Paisley, UK) supplemented with 4.5 g L^{-1} glucose, 4 mM L-glutamine, 1 mM pyruvate and 10% FBS (fetal bovine serum, PAA laboratories, Yeovil, UK) and maintained in a 5% CO_2, water-saturated atmosphere at 37 °C.

Cell surface isocyanoglycan and azidoglycan labelling and detection by flow cytometry

LL2 cells were incubated for 24 hr in medium containing 50 μM of the desired azido sugar and 200 μM of the desired isonitrile sugar and vehicle (DMSO ≤ 0.25% *v/v* in buffer). Control cells were grown (i) with no sugar treatment, (ii) in the presence of the vehicle but absence of isonitrile sugar, and (iii) with single sugars only. The following procedure was followed for all the controls and sugar-pulsed cells. Cells were grown in Nunc 6-well dishes. The medium was gently removed from the wells and cells were washed with warm PBS (phosphate buffered saline; water, NaCl, KCl, Na_2HPO_4, KH_2PO_4; Fisher Scientific, Loughborough, UK). Trypsin-EDTA (0.25% trypsin and 1 mM EDTA in Hanks' Balanced Salt Solution without $CaCl_2$, $MgCl_2$ or $MgSO_4$, Invitrogen) was added to the flask (7% of the original volume) and the cells incubated at 37 °C for 4.5 min. Warm complete DMEM was then added to neutralize the trypsin. The contents of each well were transferred to eppendorf tubes, centrifuged (700 g, 4 °C, 4 min) and washed with cold FACS buffer (1% FBS in PBS).

Cells were centrifuged and resuspended in 100 μL labeling buffer A (50 μL of 200 μM Tz-Biotin in FACS buffer and 50 μL of 20 μM TMDIBO-647 in FACS buffer to get total concentrations of 100 μM and 10 μM respectively) or as a control 100 μL FACS buffer. The cells were incubated in a hot block with orbital shaking (450 rpm, 37 °C, 30 min). After 30 min the cells were washed three times with 700 μL ice cold FACS buffer. Cells were then suspended in 100 μl labeling buffer B (50 μg/ml Neutravidin-Dylight488, Invitrogen in FACS buffer) or 100 μl FACS buffer as a control. The cells were incubated in a hot block with orbital shaking (450 rpm, 37 °C, 15 min) and then the cells were washed two times with 700 μL ice cold FACS buffer. DAPI (1 μg/mL) was added to all the tubes as a cell death marker prior to filtering through a 50 μm cut-off membrane into flow cytometry tubes. Each sample was analysed by a flow cytometer (model LSRII, BD Oxford, UK) using 20,000 events. Data analysis was performed using FlowJo flow cytometry analysis software (Tree Star, Ashland, OR). The viable cell population was determined by gating cells to exclude those with high UV-near intensity in the DAPI channel (450 nm), very low cellular size (cell debris) and large cellular size (duplets or larger clusters). The far-red (for TMDIBO-647) and green (NA488) median fluorescence intensities (MFI) were recorded in the far-red and blue channel respectively. Data points were collected in triplicate.

Cell surface isocyano-glycan and azido-glycan labelling and quantification with fluorescence microscopy imaging

LL2 cells were seeded onto ibiTreat 8 well μ-slides (Ibidi GmbH, Germany) at $2\times10^4 cm^{-2}$ and allowed to adhere to the plate surface for 6 hr. After adhesion, cells were dosed with either isonitrile (200 μM) - and azido-labelled (50 μM) sugars or vehicle for 24 hr. Cells were then washed 3 times in ice cold FACS buffer before being incubated (30 min, 37 ^oC) *in situ* in 200 μL of FACS buffer containing 300 μM Tz-Biotin and 5 μM TMDIBO-647. The cells were then washed twice with ice cold FACS buffer followed by incubation with NA488 (50 μg/ml) and 300 nM DAPI (Invitrogen) in FACS buffer. After being washed as above, cells were fixed in PBS containing 4% formalin (RT, 15 min, 37 ^oC) and washed again twice in cold PBS.

Fluorescence was determined by quantitative imaging cytometry using an iCys Research Imaging Cytometer (CompuCyte, Cambridge, MA) with iNovator software (CompuCyte). A scanning protocol for quantification was configured with three channels; 405nm diode laser excitation and blue channel detection (445nm-485nm) for DAPI, 488nm argon laser excitation and green channel detection (500nm-560nm) for isonitrile sugars and HeNe 633 nm laser excitation and long red channel detection (650nm LP) for azido sugars.
High-resolution scans were acquired using the 60x objective and 0.5mm x-step size, giving a field size of 500 μm x 132 μm. A total of 350 fields were scanned for each chamber well. See Figures 3A and S3.

For analysis, watershed filters were included in the protocol to ensure separate contouring on closely spaced cells. The primary contour was set on the blue (DAPI) channel to identify events, with an integration contour of 8 pixels to include cytoplasmic staining. Fluorescence values were then determined in the green channel for the isonitrile sugars and the long red channel for the azido sugars.

Visualising of labelled cells with confocal microscopy.

Microscopy slides were prepared in an analogous fashion to above. Cells were imaged on a Leica TCS SP5 Confocal Microscope (63x objective, oil HCX PL APO, NA 1.4) followed by analysis on the Volocity 3D software (Perkin-Elmer). See Figures 3B-C and S4.

III Further Data

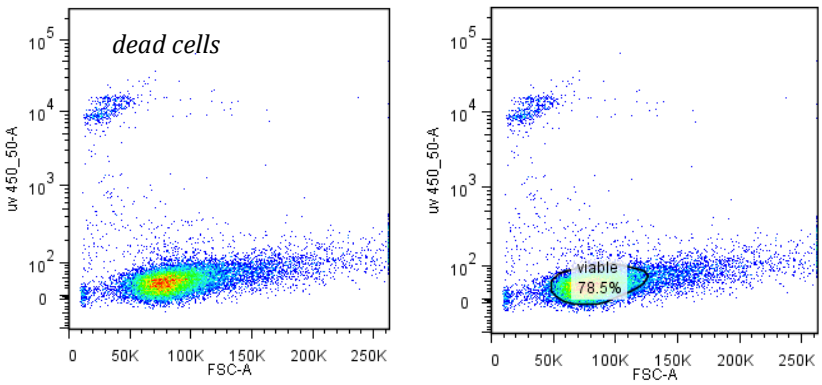


Fig. S1: Viable Cell Gating using nuclear stain DAPI (vertical axis) and Forward Scattering (FSC-A, indicating particle size). All data given are for viable cells only.

Table 1: Cell Viabilities determined by DAPI gating

	no treatment	DMSO	GalN-Az	ManN-n-Iso	GalN-Az & ManN-n-Iso
Viabilities	89.3	84.0±0.5	82.6±1.7	78.5±0.2	77±4.7

	no treatment	DMSO	GalN-Az	GlcN-n-Iso	GalN-Az & GlcN-n-Iso
Viabilities	87.9	84.4±0.8	84.1±0.3	74.5±3.2	71.8±6.1

	no treatment	DMSO	ManN-Az	GlcN-n-Iso	ManN-Az & GlcN-n-Iso
Viabilities	86.8	84.5±2.3	80.8±1.7	83.3±1.0	85.0±0.7

	no treatment	DMSO	ManN-Az	ManN-n-Iso	ManN-Az & ManN-n-Iso
Viabilities	88.7	84.2±1.5	71.2±2.4	72.2±3.3	62.5±7.3

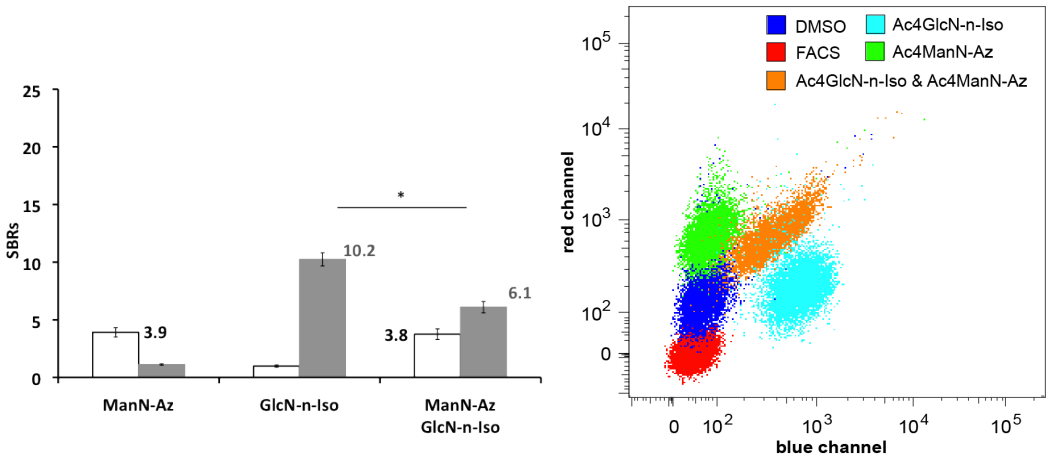


Fig. S2: Flow cytometry SBRs and Scatter Plot for cells pulsed with Ac_4ManN-Az and/or Ac_4GlcN-n-Iso.

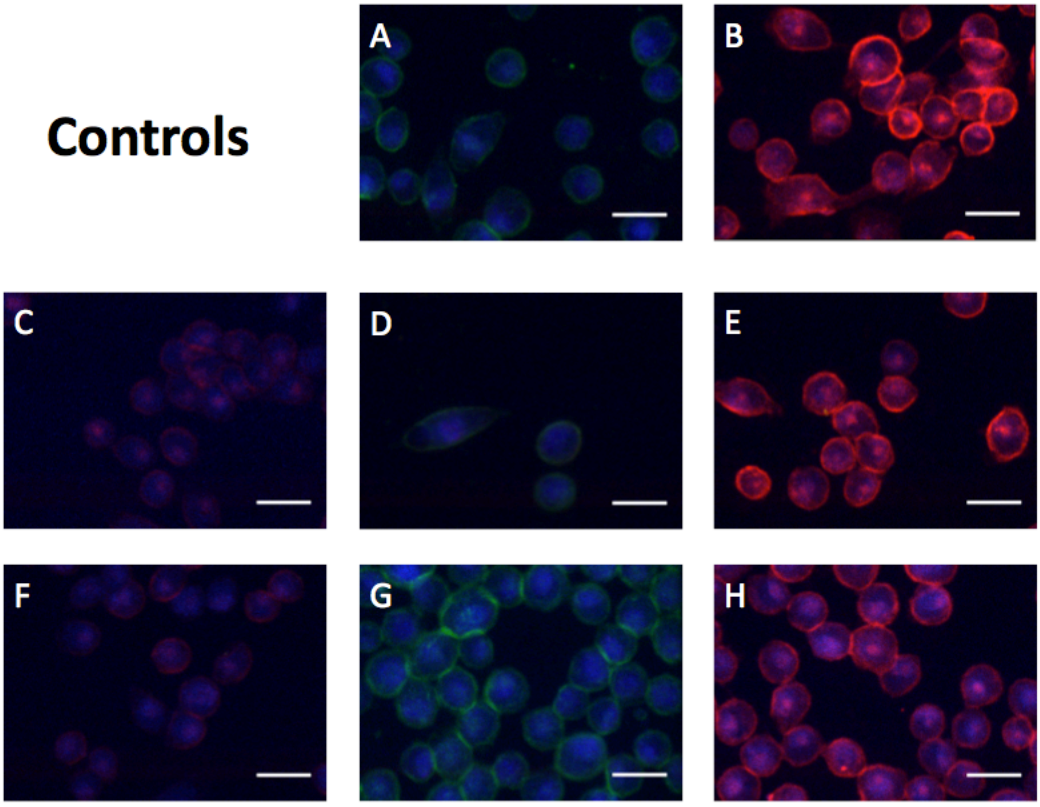


Fig. S3: Epifluorescence imaging of LL2 control cells

A= Ac_4ManN-n-Iso pulsed cells (200 μM). Cells were treated with all the probes excluding TMDIBO-647, Green Channel. **B=** Ac_4GalN-Az pulsed cells (50 μM). Cells were treated with all the probes excluding Tz-biotin, Red Channel. **C=** non-sugar pulsed cells, treated with all the probes, Red Channel. **D=** Ac_4ManN-n-Iso pulsed cells (200 μM), treated with all the probes, Green Channel. **E=** Ac_4GalN-Az pulsed cells (50 μM), treated with all the probes, Red Channel. **F=** vehicle (DMSO <0.25 *v/v*) pulsed cells, treated with all the probes, Red Channel. **G and H** Cells pulsed with Ac_4ManN-n-Iso (200 μM) and Ac_4GalN-Az (50 μM). **G=** Cells were treated with all the probes excluding TMDIBO-647. Green Channel. **H=** Cells were treated with all the probes excluding Tz-Biotin. Red Channel. Scale bars = 30 μm. All images are overlays of the specified channel with the blue channel, which shows the DAPI nuclear stain.

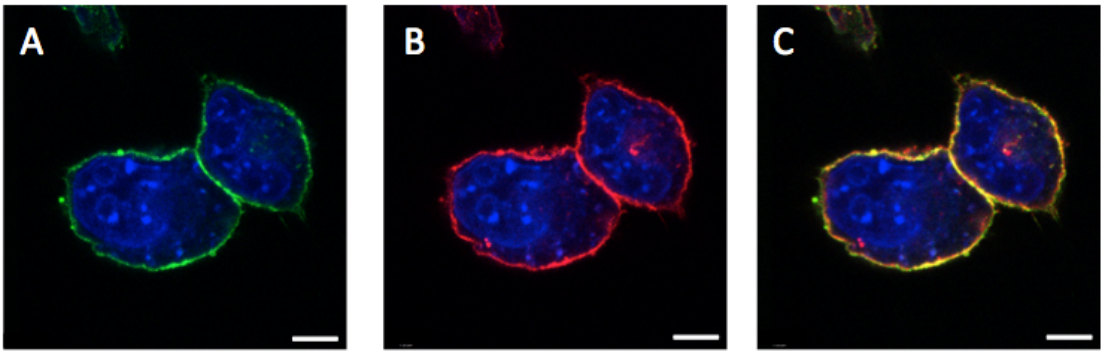


Fig. S4: Confocal microscopy of LL2 cells pulsed with both ManN-n-Iso (green channel, A) and GalN-Az (red channel, B) . Overlay: C. DAPI (blue) was used a nuclear stain.

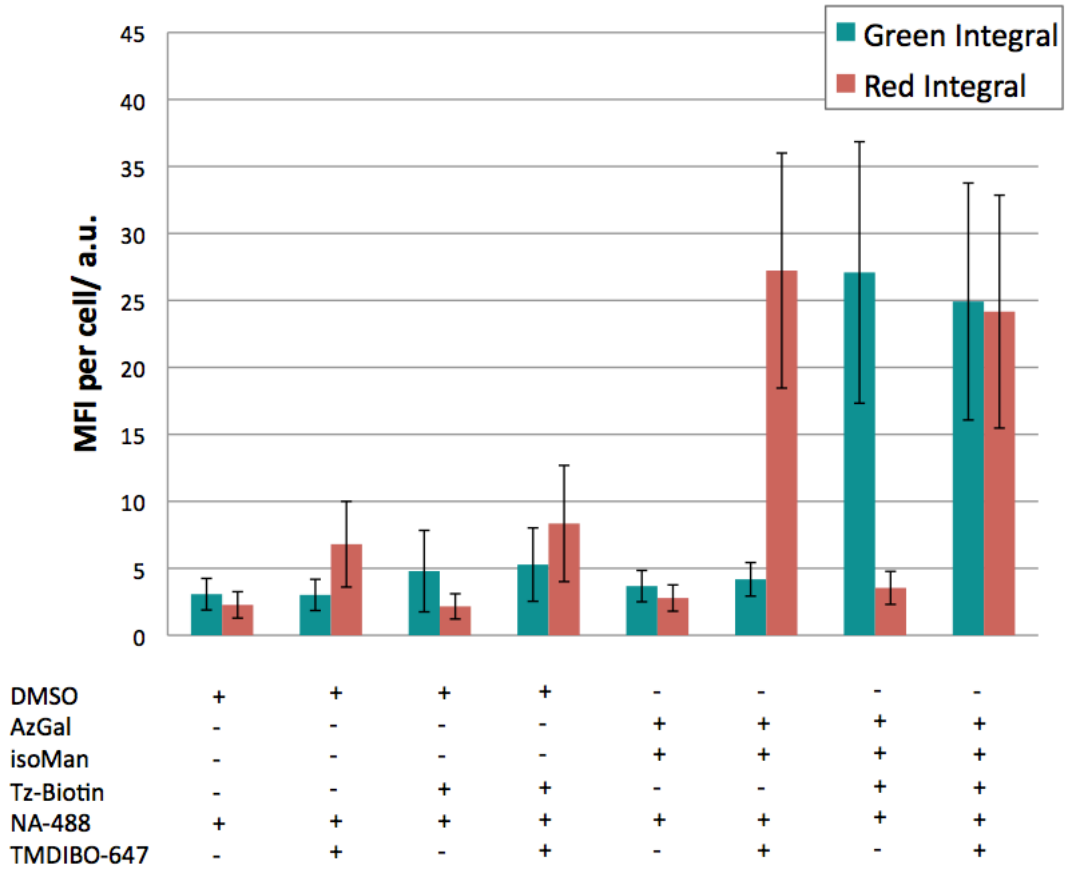


Fig. S5: Confocal microscopy of LL2 cells treated with both ManN-n-Iso and GalN-Az and then with all the probes.
A: green channel (isonitrile group). **B**: red channel (azide group). **C**: Overlay of A and B. DAPI (blue) was used a nuclear stain.

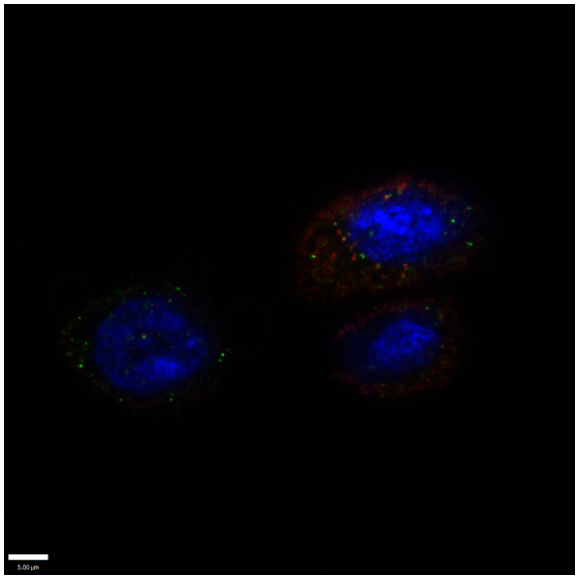


Fig. S6: Confocal microscopy of control LL2 cells treated with vehicle (DMSO) and then with all the probes. Overlay of the green and red channels. DAPI (blue) was used a nuclear stain.

IV References

1. S. Stairs, A. A. Neves, H. Stöckmann, Y. A. Wainman, H. Ireland-Zecchini, K. M. Brindle, and F. J. Leeper, *ChemBioChem*, 2013, **14**, 1063–1067.
2. H. Stöckmann, A. A. Neves, S. Stairs, H. Ireland-Zecchini, K. M. Brindle, and F. J. Leeper, *Chem. Sci.*, 2011, **2**, 932.
3. J. A. Prescher, D. H. Dube, and C. R. Bertozzi, *Nature*, 2004, **430**, 873–877.
4. D. J. Vocadlo, H. C. Hang, E.-J. Kim, J. A. Hanover, and C. R. Bertozzi, *PNAS*, 2003, **100**, 9116–9121.