A Better Understanding of Bioassays

How Suitable are Primary Cell Based Assays for the Evaluation of Product Quality?

Dr. Florian Cymer, Principal Scientist – Analytics Bioassay

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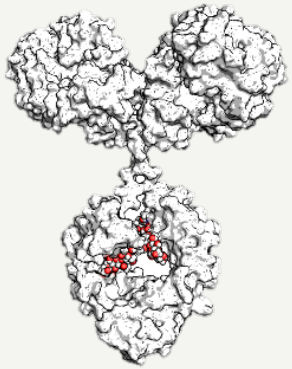
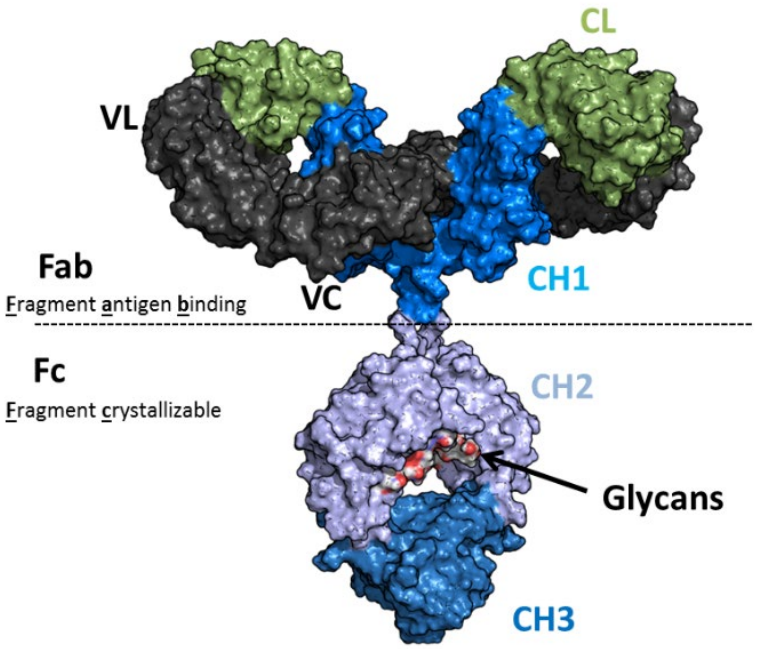
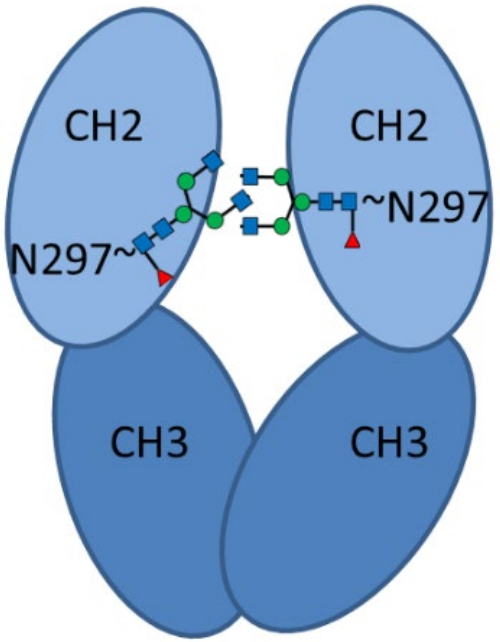
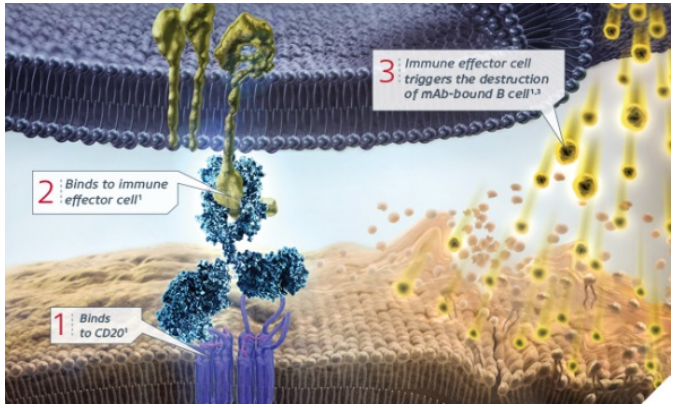
Introduction





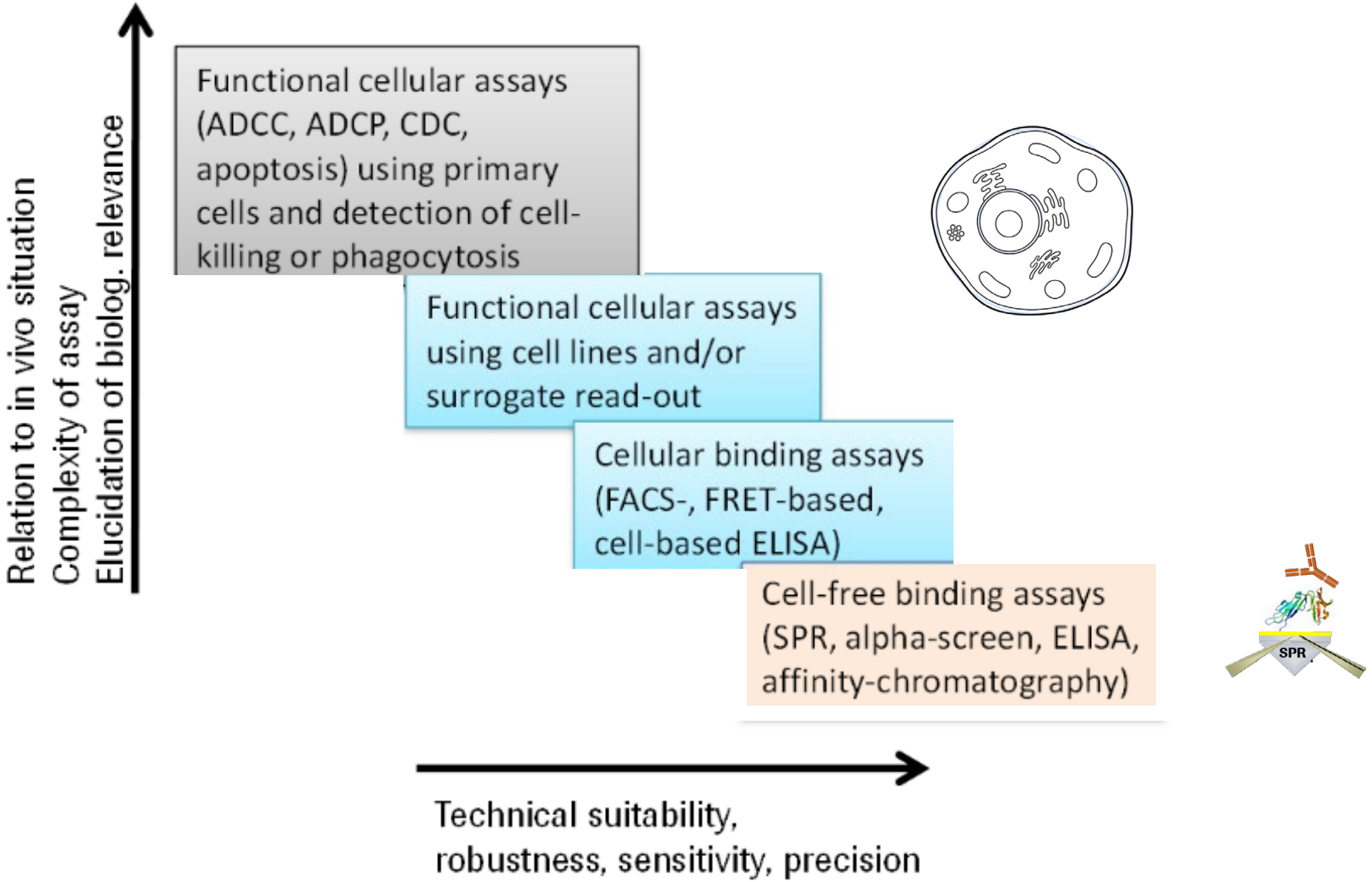


Fcγ-receptors are involved in ADCC and ADCP



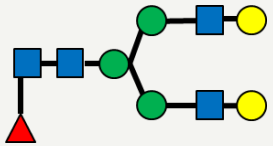
- CHO manufacturing processes result in more than 30 major glycosylation patterns and the glycan on each of the heavy chain can differ, further increasing the heterogeneiety.
- Glycans modulate the interaction with Fc-receptors on immune cells and also affect the conformational space of IgG-type antibodies.
- IgG-glycans can modulate antibody effector functions and therefore have to be controlled using functional assays to assure a safe and efficatious product.

Assay matrix



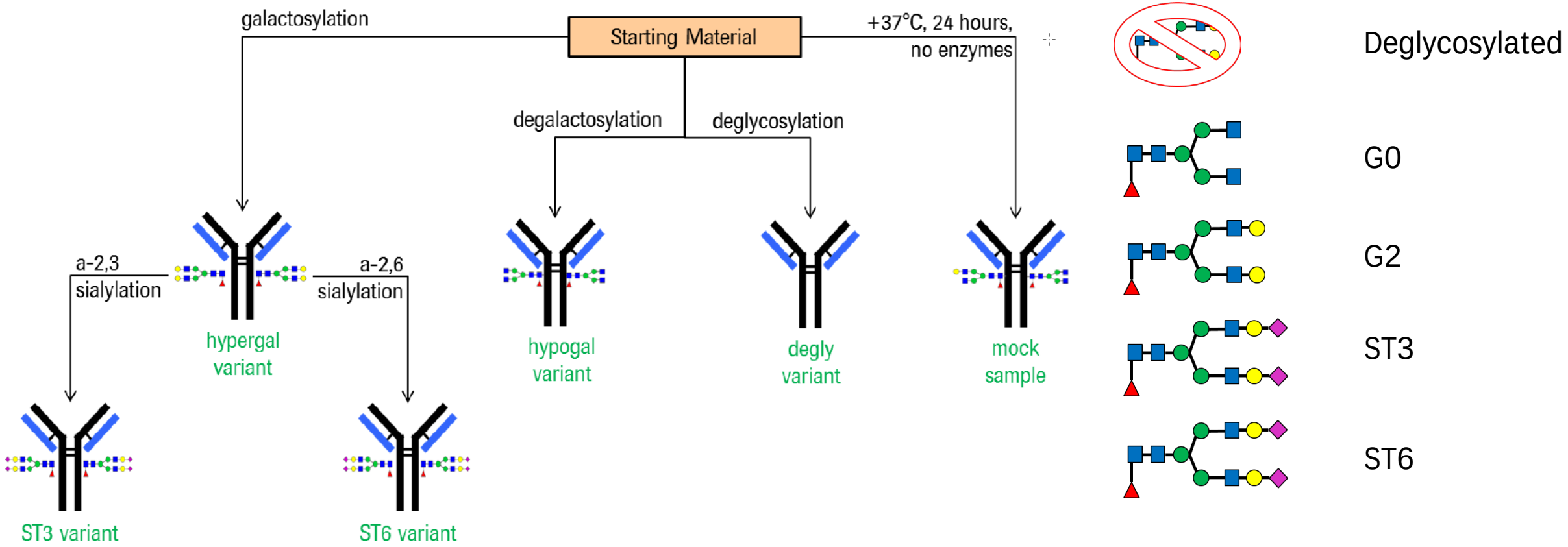
§ Cymer, F., Beck, H., Rohde, A. & Reusch, D. Therapeutic monoclonal antibody N-glycosylation - Structure, function and therapeutic potential. *Biologicals* **52**, 1-11, doi:10.1016/j.biologicals.2017.11.001 (2018).

In vitro glycoengineering of mAb samples and characterization



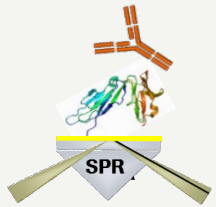
§ Thomann, M. *et al.* Effects of sialic acid linkage on antibody-fragment crystallizable receptor binding and antibody dependent cytotoxicity depend on levels of fucosylation/bisecting. *Bioanalysis* **11**, 1437-1449, doi:10.4155/bio-2019-0124 (2019).

In vitro glycoengineering of high and low afucosylated IgG1



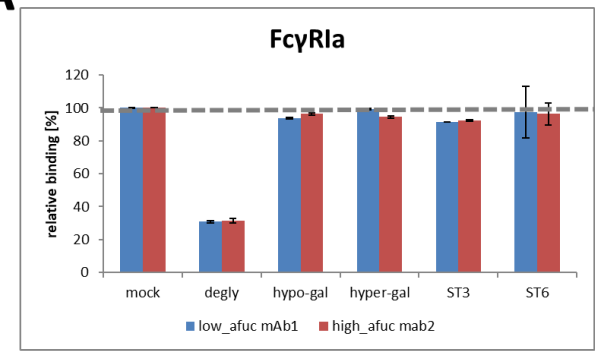
After in vitro glycoengineering, all samples were tested by SEC (to exclude aggregation or fragmentation) and LC-MS peptide mapping, to determine the level of each glycan and to check for absence of potential other modifications of each sample (side-chain oxidation, deamidation, succinimide formation, isomerization).

Binding assays and cell-line based functional assessment

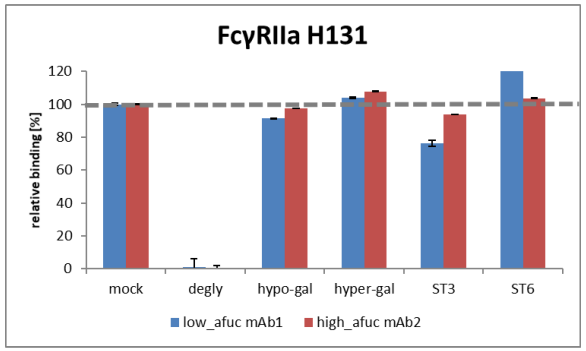


Binding experiments using SPR

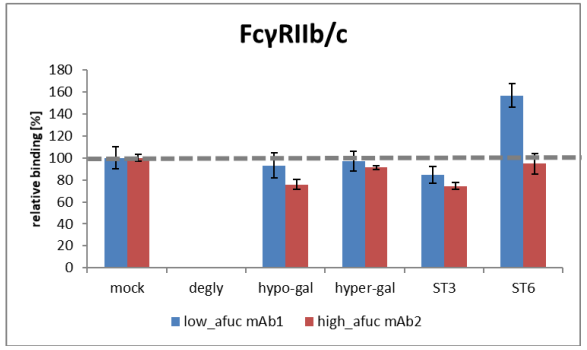
A



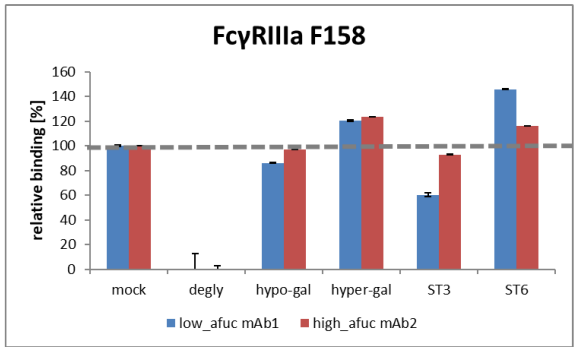
B



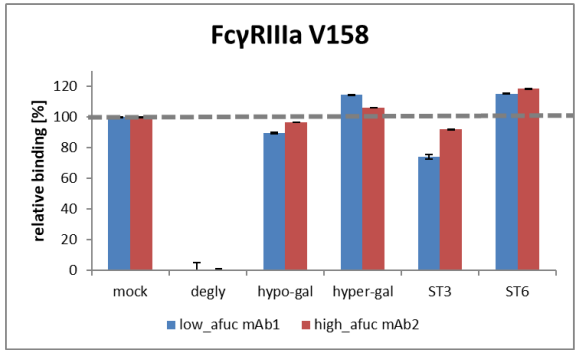
C



D

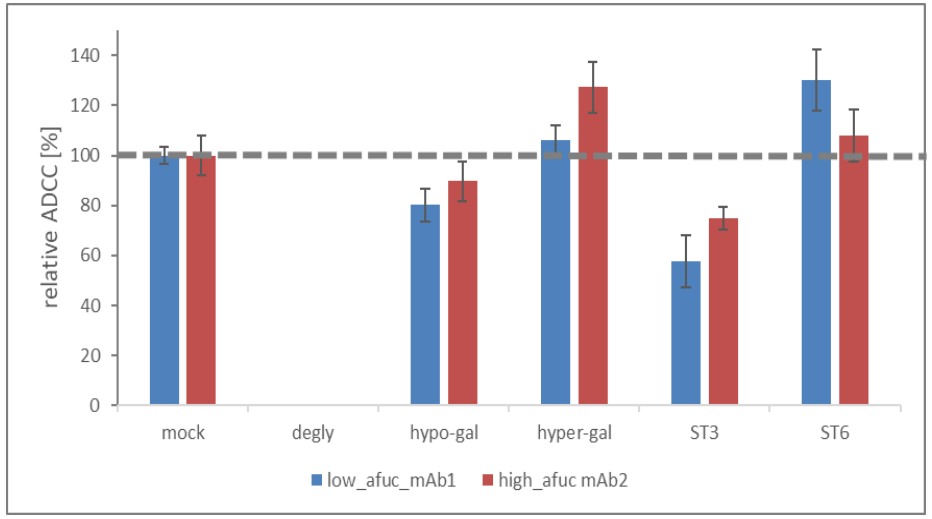


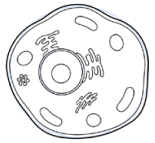
E

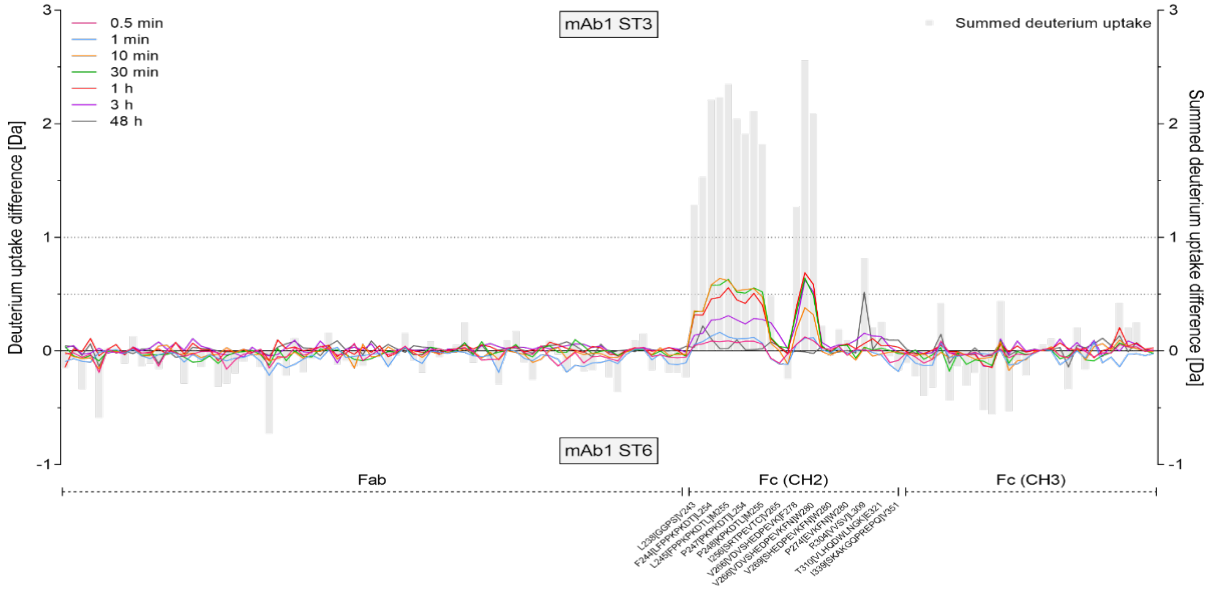


- Changes were generally more pronounced for low_afuc antibody
- Minor changes due to galactosylation but dramatic differences due to changes in sialydation. Generally a reduction in binding for α-2,3 sialydated material and increase in binding for α-2,6 sialydated material

Activity of glycoengineered material in reporter gene ADCC assays and structural changes by HDMX



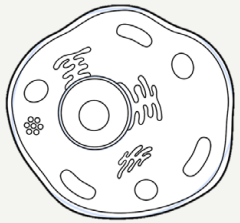




- Changes were generally more pronounced for low_afuc antibody
- Minor changes due to galactosylation but more pronounced differences due to changes in sialydation. A reduction in binding for α-2,3 sialydated material and increase in binding for α-2,6 sialydated material was observed
- Structural changes most pronounced for the α-2,3 sialydated material

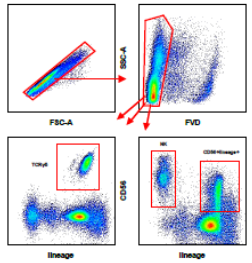
§ Kuhne, F. *et al.* The Impact of Immunoglobulin G1 Fc Sialylation on Backbone Amide H/D Exchange. *Antibodies (Basel)* **8**, doi:10.3390/antib8040049 (2019).

Characterization of primary cells and evaluation functional activities



§ Wieckowski, S., Avenal, C., Orjalo, A. V., Jr., Gygax, D. & Cymer, F. Toward a Better Understanding of Bioassays for the Development of Biopharmaceuticals by Exploring the Structure-Antibody-Dependent Cellular Cytotoxicity Relationship in Human Primary Cells. *Front Immunol* **11**, 552596, doi:10.3389/fimmu.2020.552596 (2020).

FACS marker panel



Characterization of primary cells from buffy coats

immunophenotyping of the major lymphocyte and myeloid subsets		
antigen	fluorochrome	clone
CD11c	PE-Cy5	B-ly6
CD14	BUV395	MφP9
CD38	BV421	HIT2
CD4	BV510	SK3
HLA-DR	BV605	G46-6
CD8	BV650	RPA-T8
CD25	BV711	2A3
CD16	BV786	3G8
CD3	AF488	UCHT1
CD45RA	PerCP-Cy5.5	HI100
CD127	PE	HIL-7R-M21
CD197	PE-CF594	150503
CD20	PE-Cy7	2H7
CD123	APC	7G3
TCRγ/δ-1	APC-R700	11F2
CD56	BUV373	NCAM16.2

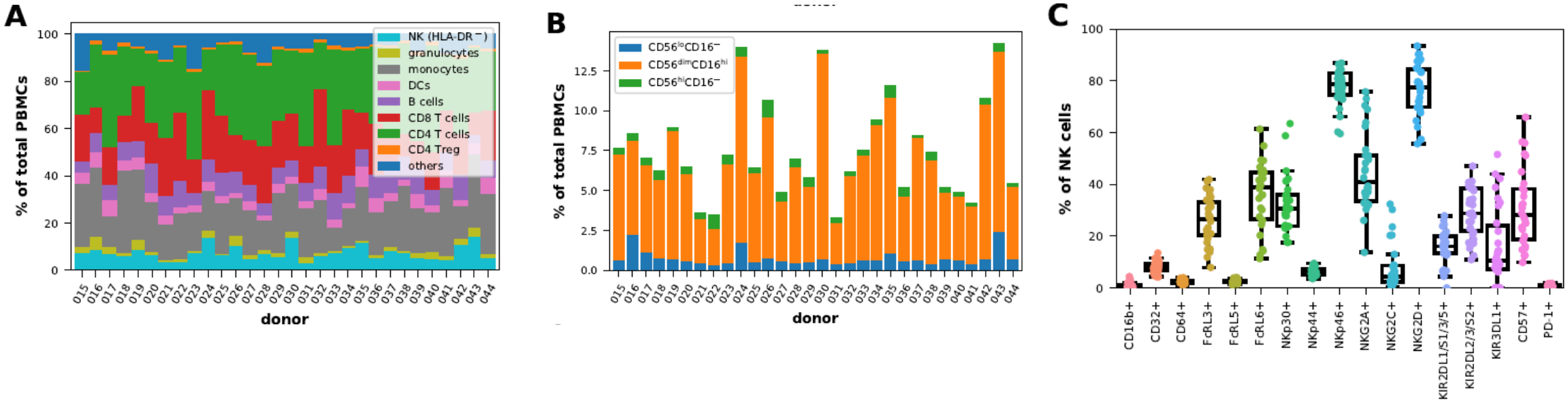
FcR and Fc receptor-like (FcRL) receptors		
antigen	fluorochrome	clone
CD56	APC	REA196
lin3	FITC	MφP9,SK7, ...
CD45	PerCP	2D1
FcRL6	BV421	2H3
CD307c	BV786	H5
FCRN	AF700	937508
CD64	BUV737	10.1
CD32	BV650	FLI8.26
CD16b	PE	REA589
CD16	BV510	3G8

markers of cytotoxicity		
antigen	fluorochrome	clone
CD159a (NKG2A)	PE	REA110
KIR-NKAT2 (KIR2DL2/S2/L3)	BUV395	DX27
CD314 (NKG2D)	BV421	1D11
CD57	BV605	QA17A04
CD335 (NKp46)	BV650	9E2/NKp46
CD336 (NKp44)	BV711	p44-8
CD337 (NKp30)	BV786	p30-15
CD158 (KIR2DL1/S1/S3/S5)	PerCP-Cy5.5	HP-MA4
CD159c (NKG2C)	PE-Vio770	REA205
CD158e (KIR3DL1)	APC	DX9
lin3	FITC	MφP9,SK7,SJ25C1,L27
CD16	BV510	3G8
CD56	BUV373	NCAM16.2

Characterization of primary cells

§ Using buffy coats from 30 healthy donors, the content of PBMCs and NK-subtypes where characterized, with high donor to donor variability. Donors were characterized wrt FcGR3a haplotype.

§ NK cells demonstrated a high diversity in expression of relevant markers.



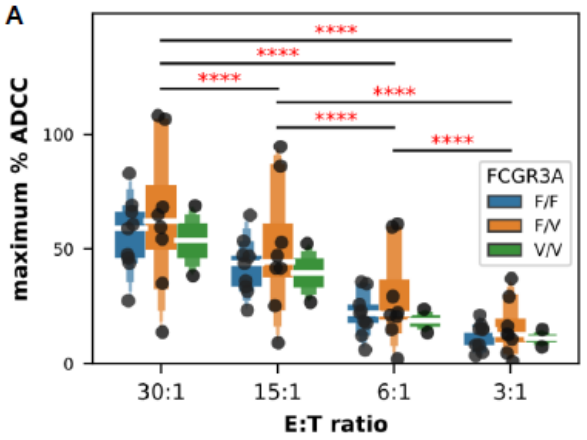
§ The distribution of immune cells was diverse and differed from donor to donor

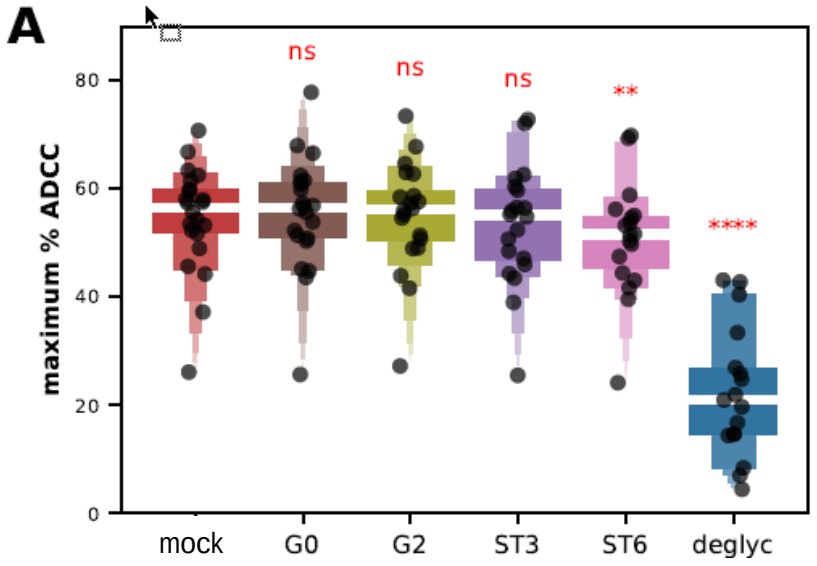
§ Among three main subsets of NK cells there was great variability in these subtypes from donor to donor

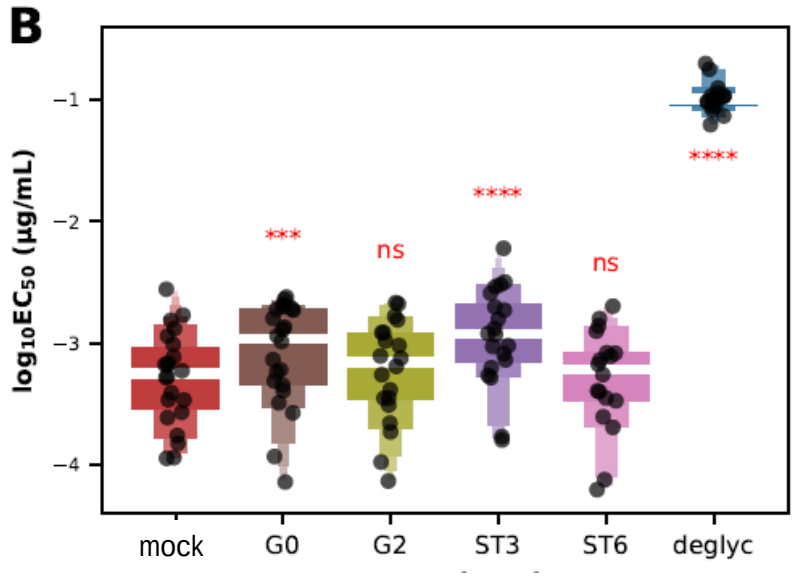
§ Cytotoxic markers on NK cells appear to be unique to each donor analyzed

Isolated primary NK cell (CD56^{+}) mediated ADCC using BT474 target cell-line and in vitro glycoengineered material

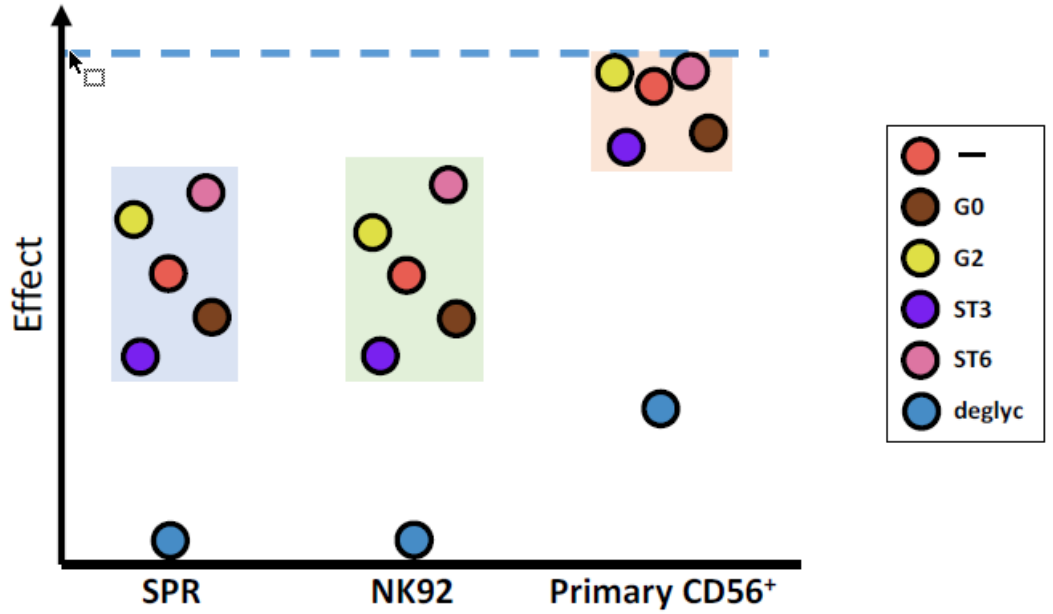
- High variations in maximum ADCC (higher asymptote) independent of FcGR3A haplotype
- No correlation of surfac marker expression and ADCC parameters
- Deglycosylated variant, ST6 (max. ADCC), ST3 (EC_{50}) as well as G0 (EC_{50}) glycoform demonstrated significant differences in ADCC





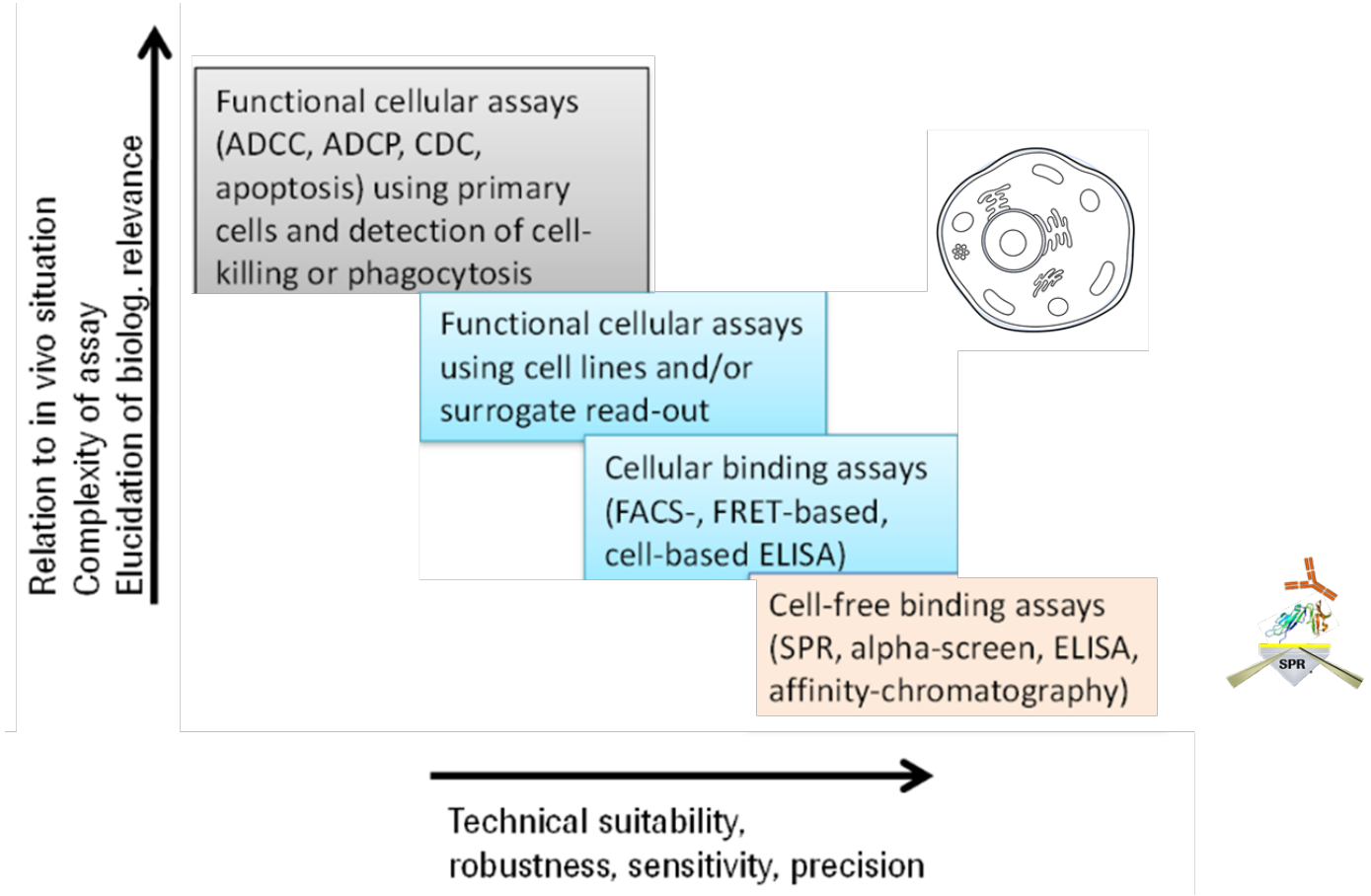


Summary



- Changes in binding and activities in cell-line based assays due to differences in glycostructures are comparable
- Differences in glycostructures could not be fully resolved in primary cell-based assays due to high variability between donors and due to an avidity plateau that marks maximum target cell lysis.

Summary



- The used primary cell-based assay does not appear to be suitable to sufficiently resolve changes in critical quality attributes such as glycosylation extremes in the used ADCC assay.

Doing now what patients need next