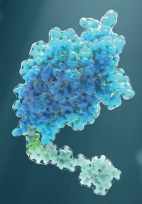
Directional conjugation of sdAbs enables their application in fluorescence technologies and advanced imaging



Protein conjugations have been around for almost a century but continue to evolve and become increasingly relevant. What started with radioactive labeling was later followed by fluorescent labeling and now extends to the labeling of a myriad of functional groups including toxins, biotin, quantum dots, DNA, and many more. These modalities, and the development of multi-color measurements, have revolutionized the detection and quantification of proteins and protein interactions.[1]

Today, functionalized antibodies also have important clinical applications in tumor imaging and treatment. Conventional antibodies are well advanced in these applications, but single-domain antibodies (sdAbs) - also called VHHs or nanobodies - offer important advantages to this[2]. These antigen-binding fragments derived from camelid antibodies (Figure 1A) consist of only one domain and are therefore very stable. They are significantly smaller (~15 kDa) than full-size antibodies (~150 kDa), which allows for better tissue penetration and access to epitopes which might not be recognized by conventional antibodies. The small size of sdAbs leads to rapid clearance from tissues, but if this is undesirable for a specific application, it can be addressed through various engineering approaches[3]. Besides, sdAbs are generally more cost-effective and easy to reformat. Various sdAb formats have already been clinically validated[4–7].

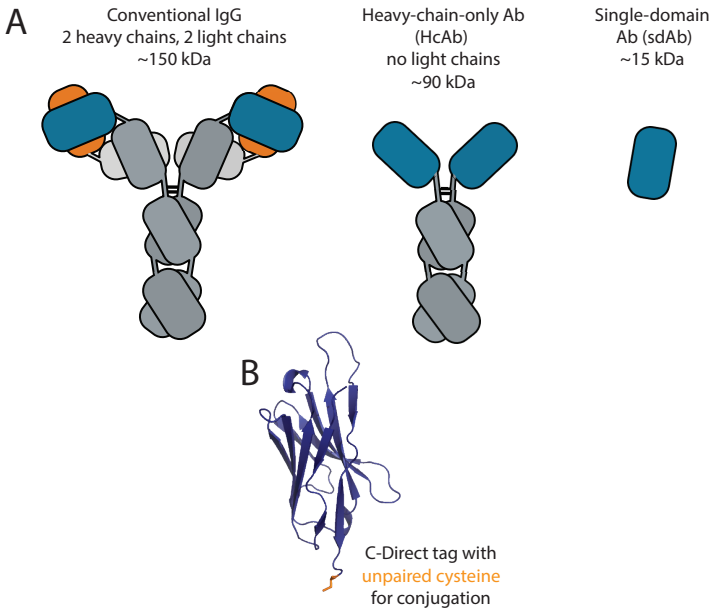


Figure 1. Structure of a sdAb. A) A conventional antibody (Ab) composed of two heavy chains and two light chains which need each other for stability (left). A heavy-chain-only antibody (HcAb) lacks light chains but remains stable (middle). A sdAb is the isolated binding domain of a heavy-chain-only Ab (right). B) Structure of a sdAb with C-Direct tag as predicted by AlphaFold2 (tag after cysteine is not shown).

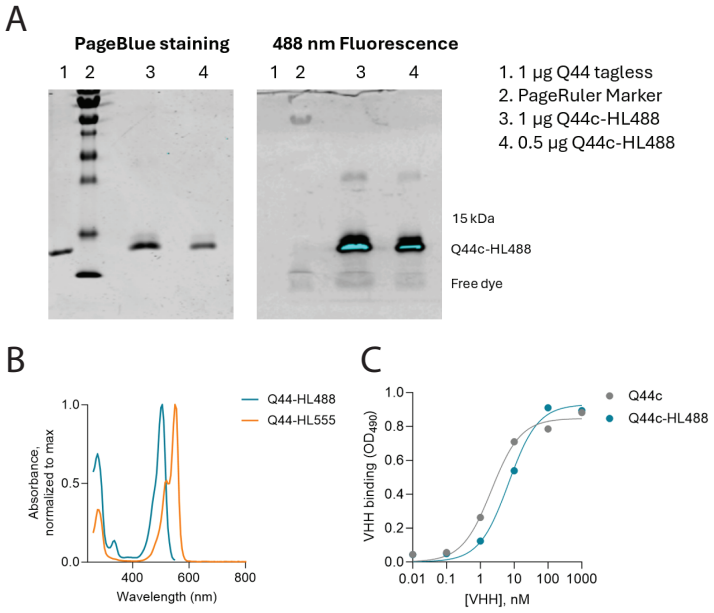


Figure 2. Quality Control of fluorescently labeled sdAb. A) SDS PAGE stained with PageBlue to assess protein integrity of fluorescently labeled Q44c sdAb (left) and scanned fluorescently to determine the amount of free dye (right). B) UV-Vis scan of fluorescently labeled Q44c sdAb to determine the degree of labeling (DOL). C) Binding affinity of conjugate determined by ELISA.

sdAbs can easily be functionalized by attaching labels. The labeling of proteins is commonly performed using NHS esters which react with random lysines of the molecule. However, this method has some drawbacks: 1) Due to multiple lysines, this labeling leads to heterogeneity, which complicates reproducibility and stability; 2) In the case of fluorescence, a high degree of labeling can result in quenching; 3) Because lysines are often found in CDRs, crucial binding sites can be compromised and loss of functionality can occur.[9] For these reasons, site-specific protein labeling of sdAbs is often preferred.

QVQ offers protein production of sdAbs fused to our C-terminal C-Direct tag. The C-Direct tag contains an unpaired cysteine which can be labeled using a maleimide-thiol reaction (Figure 2). In this way, the label will be coupled on the opposite side of the epitope-binding paratope and thereby binding affinity is maintained (Figure 1B and 2C). QVQ has ample experience with the labeling of sdAbs or target proteins via genetic fusion, random labeling and directional labeling via our C-Direct tag. The resulting imaging probes have been validated in standard lab techniques such as ELISA, flow cytometry and immunofluorescence. Moreover, directionally conjugated sdAbs have shown their advantage in various more advanced techniques. Below are several examples in which functionalized sdAbs developed by QVQ have been instrumental for advanced imaging techniques.

Super-resolution imaging with fluorescent sdAbs

Super-resolution techniques allow imaging at the level of individual proteins and deliver sharp images to study structures and stoichiometry of targeted proteins, that otherwise cannot be resolved by standard imaging methods. sdAbs are very well suited for this since their limited size allows a very small distance between the label and the epitope on the antigen. This small linkage error creates fluorescent signals that are close to the true position of the antigen and results in a higher spatial resolution as compared to the use of conventional antibodies.[10] sdAbs can be conjugated to a variety of fluorescent labels to enable their application in various super-resolution technologies such as Structured Illumination Microscopy (SIM) and direct Stochastic Optical Reconstruction Microscopy (dSTORM) (Figure 3).

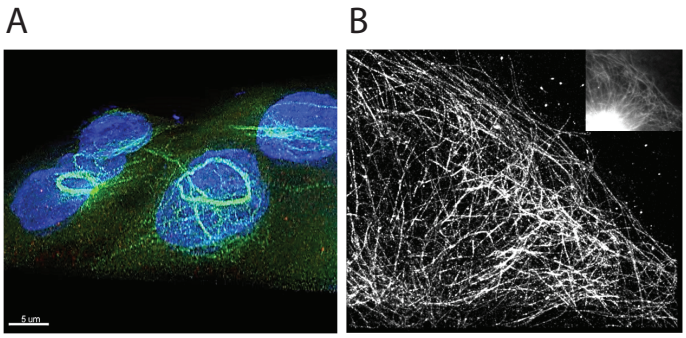


Figure 3. Fluorescently labeled sdAb used in super-resolution imaging. A) QVQ's anti-Vimentin sdAb, equipped with our C-terminal C-Direct tag (Q60c), was labeled with HiLyteFluor488 and subsequently used in SIM to reveal perinuclear vimentin rings in HeLa cells. B) Similarly, anti-tubulin sdAb11, conjugated to the blinking HiLyteFluor647 showed tubulin fibers with high resolution using dSTORM.

Target engagement of fluorescent sdAbs by bioluminescence resonance energy transfer

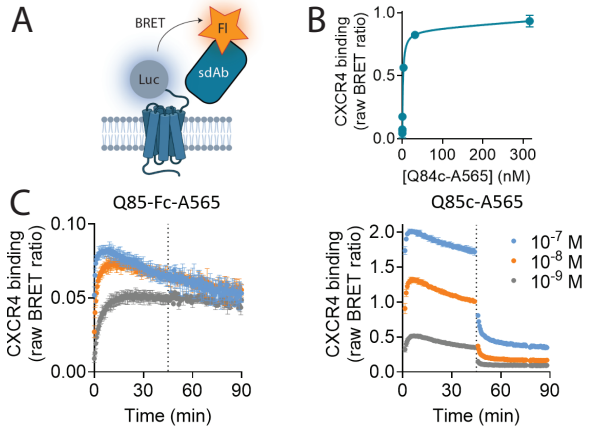


Figure 4. Study sdAb binding kinetics using BRET. A) Illustration of fluorescently labeled sdAb binding to CXCR4. B) Q84c-Atto565 binding to membrane extracts expressing luciferase (Luc) tagged CXCR4. Adapted from van der Bor et al.[13]. C) Association and dissociation of Q85-Fc (left) and Q85 (right) binding to membrane extracts containing Luc-CXCR4. Adapted from van der Bor et al.[13].

Bioluminescence resonance energy transfer (BRET) is a sensitive method to measure protein-protein interactions. This can be used to measure the binding of fluorescent sdAbs to luciferase-tagged targets (Figure 4A).[12] The energy transfer from luciferase to fluorophore only occurs when the probes are in very close proximity to each other and thus provides information on sdAbs binding, including kinetics (Figures 4B and C).

This technique can also be used to study the competition of unlabeled compounds or to sense distinct target receptor conformations.[13,14] As the energy transfer from luciferase to fluorophore depends on the proximity of both probes, the reduced linkage error of sdAbs is also advantageous here. Therefore, fluorescent monovalent sdAbs give a higher and more robust BRET signal compared to fluorescent sdAb-Fc (Figure 4C).

Photodynamic therapy using photosensitizer-conjugated sdAbs

The fast clearance, good binding affinity, excellent tissue distribution and labeling with minimal risk of reduced binding, make sdAbs highly suitable as targeting moieties of toxic substances for the treatment of e.g. solid cancers.

Immune activating or inhibiting sdAbs[19], toxic substances, or photosensitizing agents can be linked to the sdAb and thereby be delivered to its destination. For example, the CXCR4-targeting sdAb Q85c was conjugated to the very potent antimitotic agent monomethyl auristatin E to selectively kill CXCR4-overexpressing germ cell tumor cell lines.[19]

Also, light-inducible death of EGFR- and Met (HGFR)-overexpressing cells was shown by targeted photodynamic therapy using sdAbs conjugated to the photosensitizer IRDye700DX (Figures 5).[20,21]

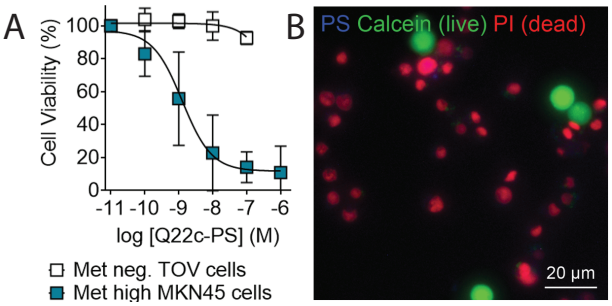


Figure 5. Targeting photodynamic therapy using sdAbs. A) Met (HGFR)-negative TOV112D or Met-overexpressing cells MKN45 cells were incubated with Met-specific sdAb Q22c, conjugated to the photosensitizer IRDye700DX (PS), and subsequently illuminated with near-infrared light to activate the photosensitizer. Cell viability was determined with resazurin. B) MKN45 cells treated as in A, after which cells were stained with CalceinAM (living cells), or propodium iodide (dead cells).[4]

Multicolor 3D tissue imaging with fluorescent sdAbs

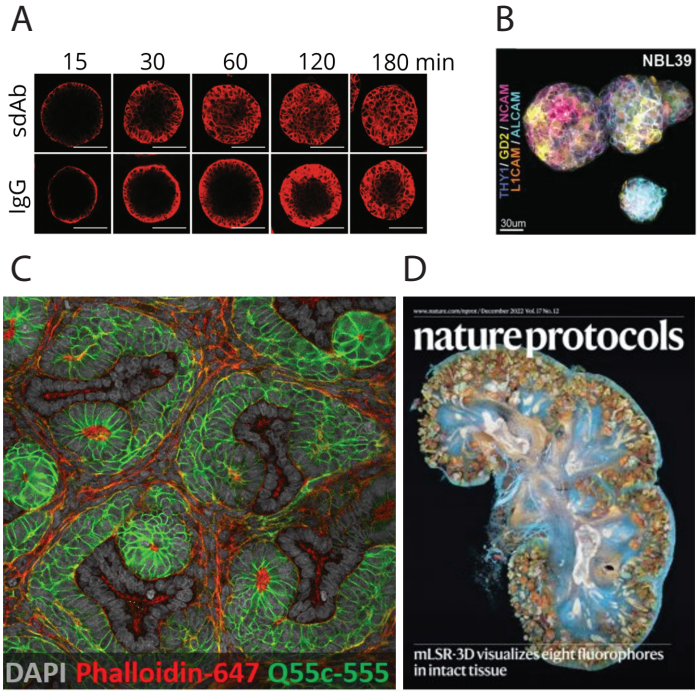


Figure 6. sdAbs allow deep tissue penetration and imaging.
A) Epidermoid carcinoma A431 spheroids imaged with labeled, EGFR-specific sdAb or conventional antibody Cetuximab by confocal imaging microscopy. The scale bar is 100 µm. Adapted from[15]. B) 3D multispectral imaging of tumor organoid. Anti-NCAM sdAb Q55c-HiLyteFluor555 is indicated in purple[16]. C) 3D imaging of human fetal kidney with Q55c-HiLyteFluor555 (green), actin-binding phalloidin-647 (red) and DAPI (grey)[17]. D) Multispectral large-scale single-cell resolution 3D (mLSR-3D) imaging of a whole human fetal kidney. Q55c-HiLyteFluor555 is colored blue[18].

3D imaging provides detailed information about the tissue's structure in all three dimensions, offering a more complete view compared to 2D imaging, which is limited to flat single-plane slices. This allows the study of biological structures and the spatial relationships between cells more extensively, improving analysis and understanding. The technique can also be combined with time-lapse imaging to observe tissue changes or movements over time, providing insight into developmental processes or disease progression.[15,16] With a size of 1/10 of a conventional antibody, sdAbs distribute more efficiently through tissues than conventional antibodies (Figure 6A).[15] Consequently, and in combination with their fast clearance, directly conjugated sdAbs are the ideal probes for imaging 3D spheroids, organoids and tissues.[15–17]

The potential of fluorescent sdAbs in 3D imaging is illustrated in Figure 6. QVQ's sdAb Q55c binding specifically to the extracellular domain of NCAM1 was conjugated to HiLyteFLuor555 and used to stain human fetal kidney (Figure 6B).[17] Q55c-HL555 was able to stain embryonic kidney tissues with excellent dept. Multispectral large-scale single-cell resolution 3D (mLSR-3D) imaging also showed whole kidney staining of NCAM with Q55c-HL555 (Figure 6C)[18]. QVQ offers high affinity sdAbs against multiple membrane-spanning proteins that can be used for 3D tissue imaging.

In vivo imaging and image-guided surgery with near-infrared-fluorophore-labeled sdAbs

The relatively small size of sdAbs not only enables efficient penetration into solid tumors, but also results in fast clearance from the bloodstream (half-life of 1-2h).[22] These features combined with good target binding affinities create probes that are very useful to image solid tumors in vivo and generate good tumor-to-background signal ratios at much earlier time points than conventional antibodies. This was shown for two different anti-EGFR sdAb by Single-Photon Emission Computed Tomography (SPECT) and near-infrared imaging.[23,24]

sdAb probes can also be applied in image-guided surgery. For example, QVQ's anti-Carbonic Anhydrase IX (CAIX) sdAb Q29c and anti-HER2 sdAb Q17c were used for dual-spectral intraoperative imaging of orthotopic breast cancer models with MCF10DCIS cells in mice.[24] This reached good tumor-to-background ratios already 2 hours after injection.

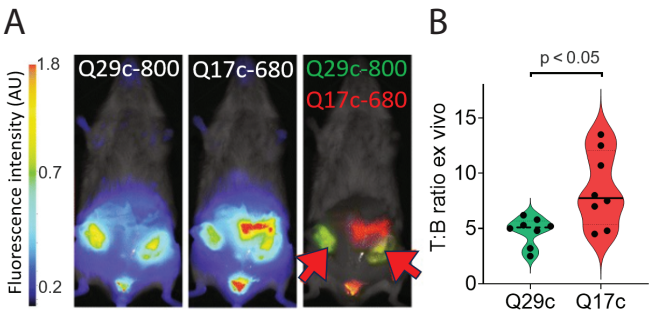


Figure 7. Dual-spectral intraoperative imaging using sdAbs.
A) Dual-spectral, intraoperative, imaging of orthotopic MCF10DCIS breast cancer tumors in mice using anti-CAIX sdAb Q29c conjugated to IRDye800CW and anti-HER2 sdAb Q17c conjugated to IRDye680RD. B) Tumor-to-background fluorescent signal ratios for Q29c-800 and Q17c-680.

QVQ
QUALITY IN ANTIBODIES

At QVQ, we specialize in custom-made sdAbs for various applications. Here we describe that labeled sdAb can be used for fluorescent techniques and advanced imaging, and how they offer advantages compared to the use of conventional antibodies.

sdAbs functionalized with fluorophores or other moieties are offered as off-the-shelf products or as follow-up of lead-development projects. QVQ offers labeling services in which sdAbs are directionally conjugated to fluorescent dyes (e.g. HiLyte Fluor dyes, IRDye), chelators (e.g. NOTA) or biotin.

QVQ offers validated sdAb products for conjugations and imaging against the following targets:

ACKR3	B7-H3	CAIX	CD80	CD163	CXCR4
DC-SIGN	EGFR	EpCAM	HER2	ITGB1	cMET
NCAM1	uPAR	PD-L2	TfR	Vimentin	VLA3

Custom modification, conjugation and genetic fusion of your sdAbs is also possible. Please do not hesitate to contact us, we are happy to think along with you.

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Key services offered by QVQ:

Lead generation	Phagemid, different tags, Immune- or non-immune libraries
sdAb libraries	Phage display panning, Customized approach
NGS & analyses	Variable number or reads. Cluster- and differential enrichment analyses
Affinity and Kinetics	ELISA, Flow cytometry, Optical fiber SPR
Epitope binning	Heterologous displacement, Optical fiber SPR
sdAb engineering	Humanization, Developability, Tm, Structural modelling, Iterative mutations
sdAb bulk production	E.coli, S.cerevisiae, HEK and CHO
Conjugates & Fusions	Biotin, PEG, Fluorophores, Luciferases, Peroxidases

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