Supporting Information

A Novel Dual-Release Scaffold For Fluorescent Labels Improves Cyclic Immunofluorescence

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Table of Contents

Experimental Procedures

Materials

Reagents and chemicals

Table S1. Reagents and chemicals used in this study.

Reagents and chemicals	Vendor
recombinant antibodies and conjugates thereof	Miltenyi Biotec BV. & Co. KG
release reagent	Miltenyi Biotec BV. & Co. KG
phosphate buffered saline pH 7.4	Miltenyi Biotec BV. & Co. KG
250/50 Aminodextran	Fina Biosolutions
EcoRIA: 5‘-C6-SS-AAT ATG GAA TTC GTC CGA GCC CGT CAA G -3‘	Sigma Aldrich
cEcoRIA-Cy5: 3’ -TTA TAC CTT AAG CAG GCT CGG GCA GTT C-Cy5 -5’	Sigma Aldrich
cEcoRIA-Biotin: 3’ -TTA TAC CTT AAG CAG GCT CGG GCA GTT C-Biotin -5’	Sigma Aldrich
S-CD4-CS: 5‘ -CGG AGA TGT GTA TAA GAG ACA GNN NNN NNN NNT TAT GAC GAT AGG CAN NNN NNN NNC CCA TAT AAG AAA-3‘	Sigma Aldrich
NR-Cy5: 5‘ -CTG TCT CTT ATA CAC ATC TCC G-Cy5- 3‘	Sigma Aldrich
1,11-bis(maleimido)triethylene glycol	Sigma Aldrich
TCEP HCl	Sigma Aldrich
THPP	Sigma Aldrich
Vio515 NHS ester	Miltenyi Biotec BV. & Co. KG
Fluorescein NHS ester	Sigma Aldrich
Sulfo-Cy5 NHS ester	Luminartis
SMCC	Sigma Aldrich
Dimethyl sulfoxide	Thermo Fisher
3.7% formaldehyde	Miltenyi Biotec BV. & Co. KG
Accutase	Sigma Aldrich

All reagents were used without further purification or analysis. 200 mM carbonate buffer pH 8.3 and 500 mM carbonate buffer pH 9.0 were prepared once by using desalted and sterile-filtered water. PEB buffer was prepared with PBS pH 7.4, 2mM EDTA and 5% bovine serum albumin.

Product and intermediate purification

Obtained intermediates were purified using NAP columns from Cytiva Lifescience pre-packed with Sephadex G-25 Grade DNA resin. For this purpose, the columns were equilibrated with PBS pH 7.4 and the sample eluted according to the procedure of the manufacturer. Product-containing fractions were then pooled in Eppendorf tubes.

Final antibody and other intermediate constructs were purified on an ÄKTA pure 25 chromatography system equipped with a Superdex 200 Increase 10/300 GL column from Cytica Lifescience. Elution was done with PBS pH 7.4 at a flow rate of 0.75 mL/min and fraction size of 0.5 mL

Antibody-oligo conjugate was purified on a ÄKTA pure 25 chromatography system equipped with a Toyopearl GigaCap® Q-650M 5 mL column from Tosoh Bioscience. Elution was done with a flow rate of 1 mL/min and fraction size of 1 mL with the following gradient:

Table S2. IEC method used for antibody-oligo conjugate aCD4-NR2.

time [min]	% buffer A (TRIS-HCl)	% buffer B (TRIS + NaCl)
0	100	0
15	80	20
28	45	55
48	0	100
58	100	0
70	end	

Concentration of intermediates and final conjugates

Protein, oligonucleotide, and fluorophore concentrations as well as the degree of labeling (DOL) were determined by absorbance at 280 nm and 260 nm with the following equations:

$$c = \frac{OD_{280}^{corr} \cdot f_d}{\varepsilon} \quad (1)$$

$$c_{oligo} = \frac{OD_{260} \cdot f_d}{\varepsilon_{260}} \quad (2)$$

$$OD_{280}^{corr} = OD_{280} - (OD_{260} \cdot f_c) \quad (3)$$

$$DOL = \frac{c_{oligo}}{c} \quad (4)$$

c: concentration of compound in mol/L

OD: absorbance at the respective wavelength

f_d: dilution factor

ε: molar extinction coefficient of protein or fluorophore in $M^{-1}cm^{-1}$ at their respective wavelength

f_c: correction factor of the oligo at 280 nm

Absorbance was measured with a GENESYS 180 UV-Vis spectrophotometer from Thermo Fisher (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 10 mm UVettes from Eppendorf (Eppendorf SE, Hamburg, Germany).

Analytical High-Performance Liquid Chromatography-Size Exclusion Chromatography (Analytical HPLC-SEC)

The purity of certain conjugates was analyzed by HPLC-SEC using the 1260 Infinity II LC System from Agilent. For SEC, the AdvancedBio SEC 2.7 µm column (4.6 x 300 mm) and Agilent AdvancedBio SEC 2.7 µM (4.6 x 50 mm) as guard column were used. PBS (pH 7.4) was used as mobile phase. The conjugates were dissolved in PBS. The parameters for the method are given below.

Analytical method:

Flow rate: 0.350 ml/min; UV-Detection: 260 nm

Oligos per dextran were determined by area under the curve (AUC) of the chromatogram at 260 nm and the molar excess n according to eq. 7.

Dex-ssDNA, Oligo/Dex = 18.6 (5.3 min)

free EcoRIA (8.3 min, dimer), free EcoRIA (9.3 min)

Dex-ssDNA, Oligo/Dex = 19.0 (5.5 min)

free EcoRIA (8.5 min, dimer), free EcoRIA (9.6 min)

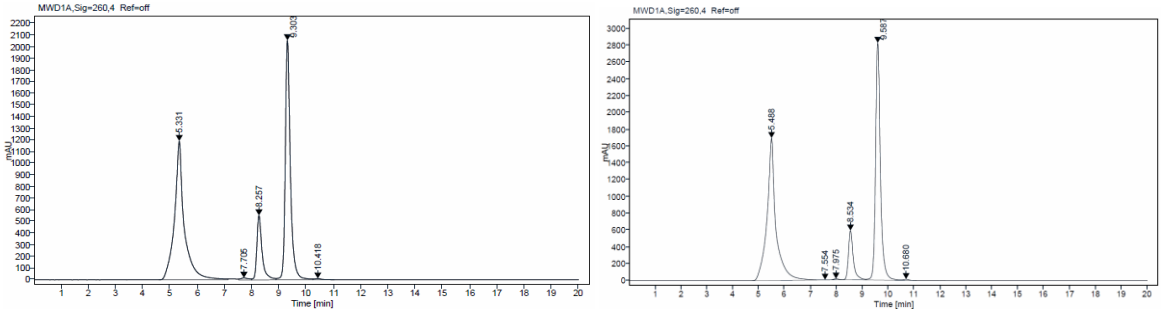


Figure S1. Analytical HPLC-SEC of Dex-ssDNA.

Table S3. Evaluation of analytical HPLC-SEC of Dex-ssDNA according to eq. 7.

Conjugate	Oligo/Dex	AUC_{bound}	AUC_{total}	n
250Dex-EcoRIA	18.6	29702	63771	40
250Dex-EcoRIA	19.0	40363	84886	40

Cell culture

Adherent AsPC-1 cells as well as suspension SUP-T1 and A431 cells were cultured in Roswell Park Memorial Institute (RPMI 1640) medium (Biowest, Nuaillé, France) supplemented with 10% fetal calf serum (FCS) (Biochrom GmbH, Berlin, Germany) and 2 mM L-glutamine (Lonza, Basel, Switzerland). They were maintained at 37°C and 5% CO2. For detachment, cells were washed with PEB buffer and subsequently covered in a thin film of Accutase. They were incubated for 5 min in the incubator and suspended in cell culture medium. For confocal imaging, cells were detached, seeded onto 24-well glass plates and left in the incubator overnight.

Staining procedure for immunofluorescence

Hybridization prior to staining: Hybridization was done according to the procedure by Rajagopalan *et al.* [1]

Suspension: Cells were fixed for 20 min in 3.7% formaldehyde at room temperature (RT). PFA was removed, cells were washed once with PBS buffer, resuspended and stored at 4 °C prior to staining. Conjugates were adjusted to 100 nM in PBS and incubated with cells for 10 min at RT in the dark resulting in final conjugate concentrations between 1 and 10 nM. Cells were centrifuged (2000 rpm, 5 min), staining solution was removed, cells were washed again and resuspended in PBS buffer. Staining was performed in Eppendorf tubes and pipetted into either 96-well plate for flow cytometry.

Adherent: Cell culture medium was removed and cells were washed with PBS once. Cells were then fixed by adding 300 µL 3.7% PFA for 20 min followed by removing the supernatant and washing 3x with PBS. Conjugates were adjusted to 100 nM in PBS and incubated with cells for 10 min at RT in the dark resulting in final conjugate concentrations between 1 and 10 nM in 300 µL total volume. Staining solution was removed, cells were washed again and covered in 300 µL PBS buffer.

Signal removal procedure

Mono-release or dual-release cocktails were freshly prepared before staining by mixing the respective reagents in the appropriate buffers:

Dextranase release: Dextranase in PBS pH 7.4

DNase I release: 900 EU/ mL DNase I in 10 mM Hepes, pH 7.4. 150mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1.8mM $CaCl_2$

For dual-release, dextranase was spiked into the DNase I cleavage cocktail. For flow cytometry analysis of signal removal, stained cells were split into two pools, whereas the first pool is measured right away to obtain the initial MFI and the second pool is resuspended in cleavage cocktail. The cell pool subjected to cleavage cocktail is again split for quenching, washing, and measurements at each given time point.

For dual-release on adherent cells in microscopy, cells are stained according to the given procedure and images recorded in the appropriate channels. Cleavage cocktail was pipetted onto the cells and time measured after addition. Focus on the cells was re-adjusted prior to recording the first image after release. After completion, cells the supernatant was discarded, cells were washed once with PBS and re-stained according to the procedure above.

Release efficiency quantification

Release efficiency describes the amount of fluorescence loss after release in percentage:

$$E_{release} = 1 - \frac{MFI_{t=x}}{MFI_{t=0}} \tag{5}$$

$MFI_{t=x}$: mean fluorescence intensity after time point x

$MFI_{t=0}$: mean fluorescence intensity before release

Standard deviation of release efficiency is calculated by Gaussian error propagation in the relative way according to the following equation:

$$\Delta E_{release} = \sqrt{\left(\frac{1}{MFI_{t=0}}\right)^2 \cdot {\sigma_{t=x}}^2 + \left(\frac{MFI_{t=x}}{{MFI_{t=0}}^2}\right)^2 \cdot {\sigma_{t=0}}^2} \tag{6}$$

$\sigma_{t=x}$: standard deviation of mean fluorescent intensity of release after time point x

$\sigma_{t=0}$: standard deviation of mean fluorescence intensity before release

Flow cytometry

Flow cytometry data was obtained with a MACSQuant X Analyzer from Miltenyi Biotec with 488 nm and 640 nm laser excitation, emission detection in B1 (FAM, Vio515), B2 (PE, PI), and R1 (APC, Cy5) channel and medium flow rate. 400,000 cells/well in a 96 well-plate were stained and 30.000 events recorded at medium flow rate. Data analysis was carried out using MACSQuantify software version 2.13.0. General gating strategy was 1) selecting main population followed by 2) exclusion of doublets/aggregates, 3) live/dead exclusion, and lastly 4) plotting the channel signal against forwards scatter signal (see Fig. S2). Fluorescence intensity was obtained as mean fluorescence intensity (MFI) value of the whole population in the respective channel.

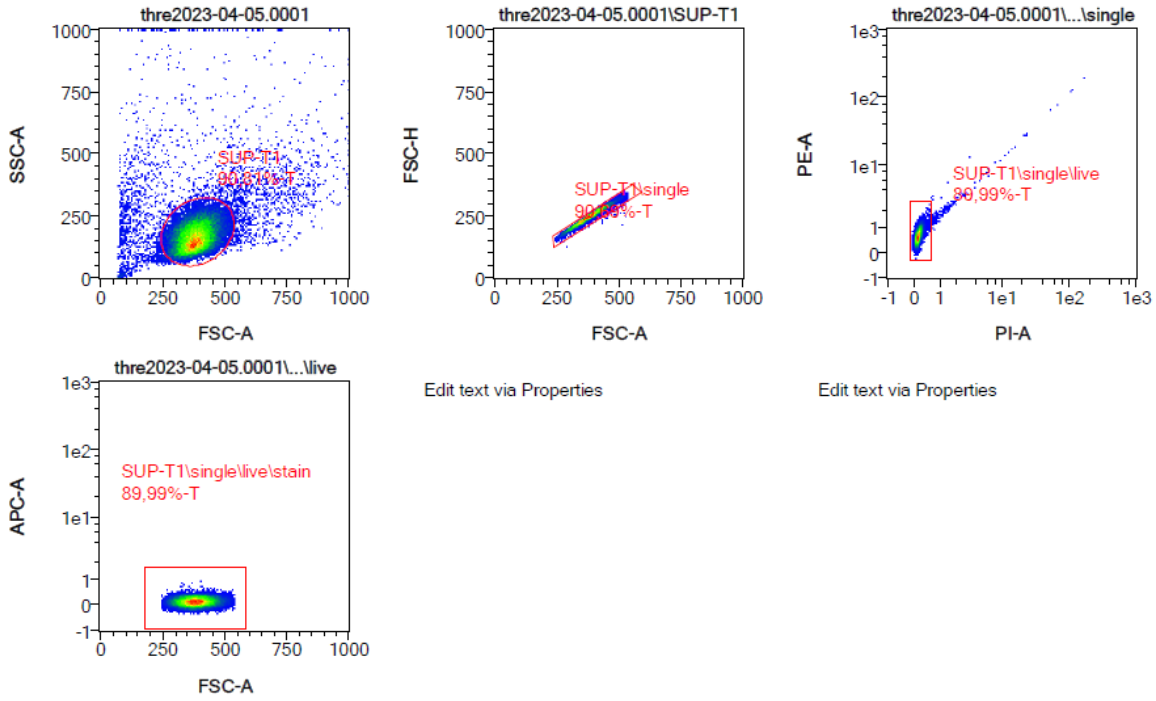


Figure S2. Gating strategy of SUP-T1 flow cytometry analysis. Gating on main population → exclusion of dublets → exclusion of dead cells → gating on CD4+ cells. The last plot shows a negative control, hence no staining intensity was observed.

Laser scanning confocal microscopy and image analysis

Stained cells were imaged on a Zeiss LSM 710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany). Conjugates were excited with the Ar-laser (488 nm) or HeNe2 laser (543 nm, 633 nm) and the images taken with a "Plan-Apochromat" 40x/0,95 Korr M27 objective.

The images were processed using Fiji software. Stained cells were segmented using the Trainable Weka Segmentation plugin with three categories: membrane, cytoplasm and background. Based on the binary mask for the membrane staining, ROI was defined and used for the quantification of mean fluorescence intensity (MFI) on the membrane of each cell in the original image. The defined ROI was used for each time point after signal erasing of the selected sample. Cells were counted with the Cell Counter plugin from Fiji.

Conjugation procedures

NHS ester antibody conjugation

For each antibody fluorophore conjugation, NHS ester of the respective dyes dissolved in DMSO was added in a ratio of 10:1 under vortexing to a solution of antibody in PBS pH 7.4 with 10 vol% carbonate buffer pH 9.0 and left on the shaker for 1 h in the dark at room temperature. The final antibody

concentration during conjugation was set to 4.5 mg/mL. To prevent protein degradation, DMSO content was kept below 5 vol%. The reaction mixture was then directly applied onto a NAP column for purification.

Conjugation of antibody-oligo

The antibody-oligo conjugate used in this study was prepared according to the procedure by Gibbs *et al.* [2] The final conjugate was purified *via* ion exchange chromatography.

Activation of aminodextran with SMCC

250 kDa aminodextran was labeled with 50-fold excess of SMCC in PBS pH 7.4 with 10 vol% carbonate buffer pH 9.0 on the shaker for 1 h with a final concentration of 20 mg/mL aminodextran. The modified aminodextran was purified *via* NAP column and concentration determined with an assumed yield of 90% as given by the manufacturer.

Conjugation of oligo to aminodextran

Capped oligos were reduced with 100 eq. of TCEP at a concentration of 2 mM in purified water for 1 h without shaking. After completion of the reaction, they were purified *via* NAP column with PBS pH 7.4 and fractions pooled depending on the absorbance at 260 nm. Next, 40-fold excess of thiolated oligos were added to SMCC-activated aminodextran in PBS at 1.75 mg/mL and 1.2 mg/mL ,respectively, and left on the shaker for 1 h. The reaction mixture was directly applied on the ÄKTA system for purification. Oligos per dextran were determined by area under the curve (AUC) of the chromatogram at 260 nm and the molar excess n:

$$Oligo/_{Dex} = \frac{AUC_{bound}}{AUC_{total}} \cdot n \tag{7}$$

Conjugation of antibodies to oligo-dextran

Antibodies were incubated with 5 eq. of DBCO NHS-activated ester for 60 min in PBS buffer pH 7.4 with 10 vol% carbonate buffer pH 9 at 4.5 mg/mL and subsequently purified by NAP column equilibrated with PBS. Simultaneously, the respective oligo-dextran intermediate was reacted with 200 eq. azidoacetic NHS ester at 0.2 mg/mL dextran concentration for 1 h in PBS pH 7.4 with 10 vol% carbonate buffer pH 9 on the shaker in the dark at RT. The reaction mixture was purified *via* Amicon filtration with 30 kDa cutoff. After concentrating of the activated intermediate and concentration determination according to equation 4. The coupling of antibody-DBCO to intermediate was carried out in PBS at room temperature for 3 h with 10-fold molar excess of antibody to azide-modified dextran conjugate at 3 mg/mL and 0.5 mg/mL antibody and dextran concentration, respectively. The final conjugate was purified by gel chromatography.

Compound information

Table S4. Synthesized conjugates and their degree of labeling (DOL) determined by absorbance.

Conjugate	DOL	Ab/Dex
aEGFR-Cy5	5.1	-
aCD4-Cy5	6.7	-
Cetuximab-FAM	8.8	-
Cetuximab-Vio515	4.5	-
aCD4-dsDNA-Cy5 (aCD4-NR2-Cy5)	2.0	-
Cetuximab-Dex-dsDNA-Biotin	18.6	1.0
Cetuximab-Dex-dsDNA-Cy5	18.6	1.0
aCD4-Dex-dsDNA-Biotin	19.0	3.0
aCD4-Dex-dsDNA-Cy5	19.0	3.0
aEGFR-Dex-dsDNA-Cy5	18.6	3.4
aEGFR-Dex-dsDNA-Biotin	18.6	3.4
aEpCAM-Dex-dsDNA-Cy5	18.6	2.9

Ion exchange chromatogram of CD4-NR2

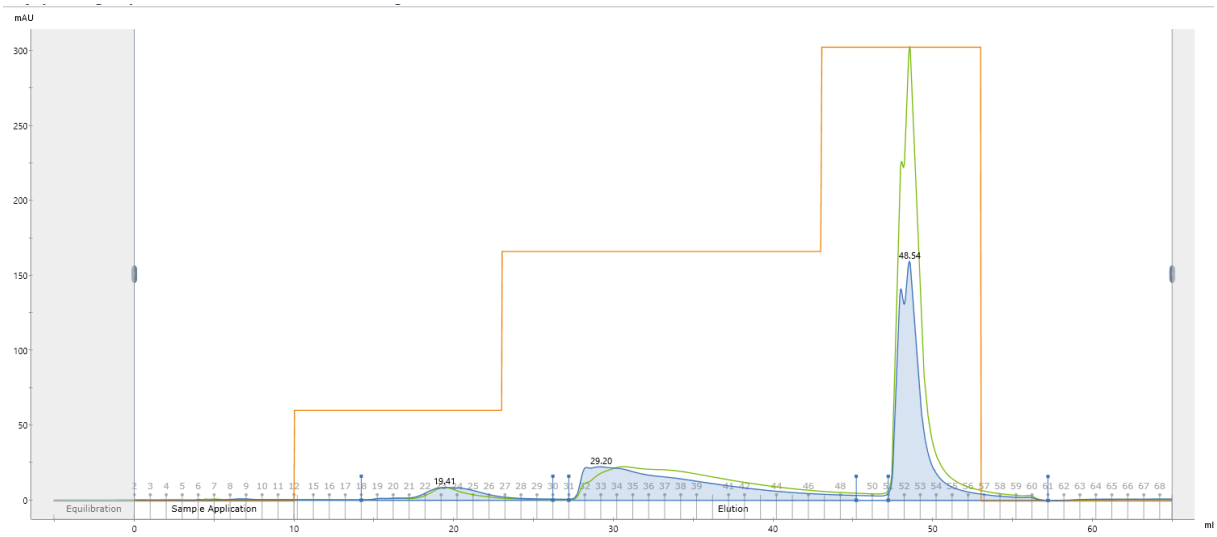
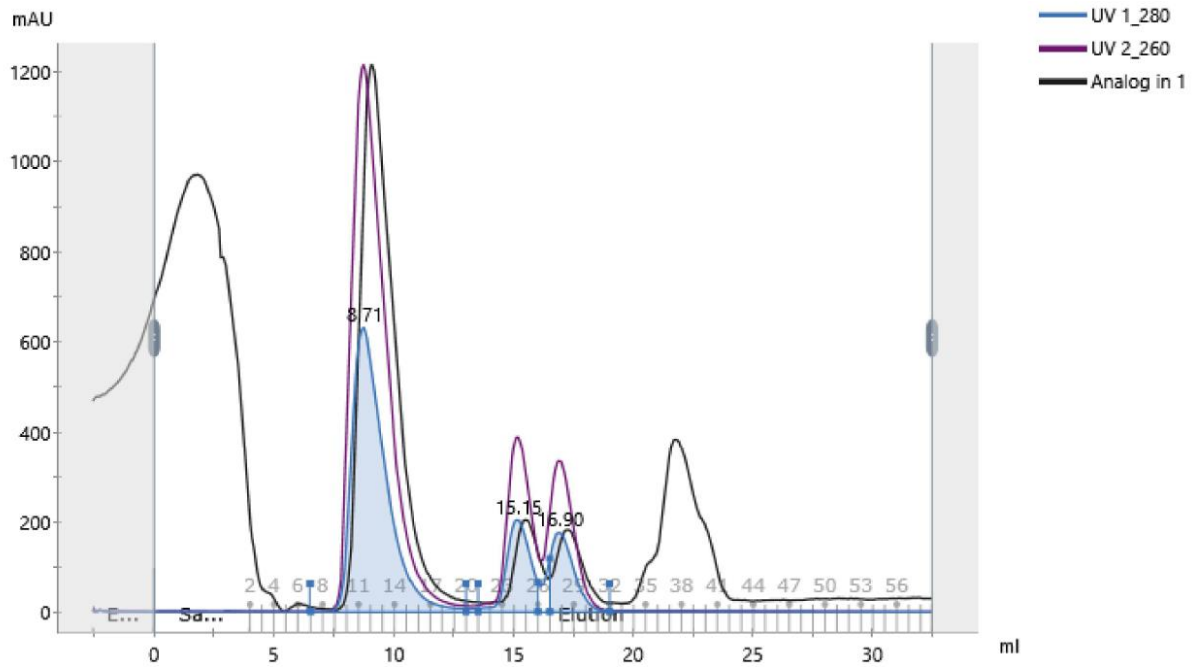


Figure S3. Ion exchange chromatogram of CD4-NR2. The compound elution starts at fraction 31 and is collected up to fraction 42. Free antibody elutes at fraction 18, while free dimeric and monomeric oligo started eluting from fraction 51. Absorbance was measured at 280 nm (blue line) and 260 nm (green line). The yellow line shows the gradient of B.

Size exclusion chromatograms of compounds

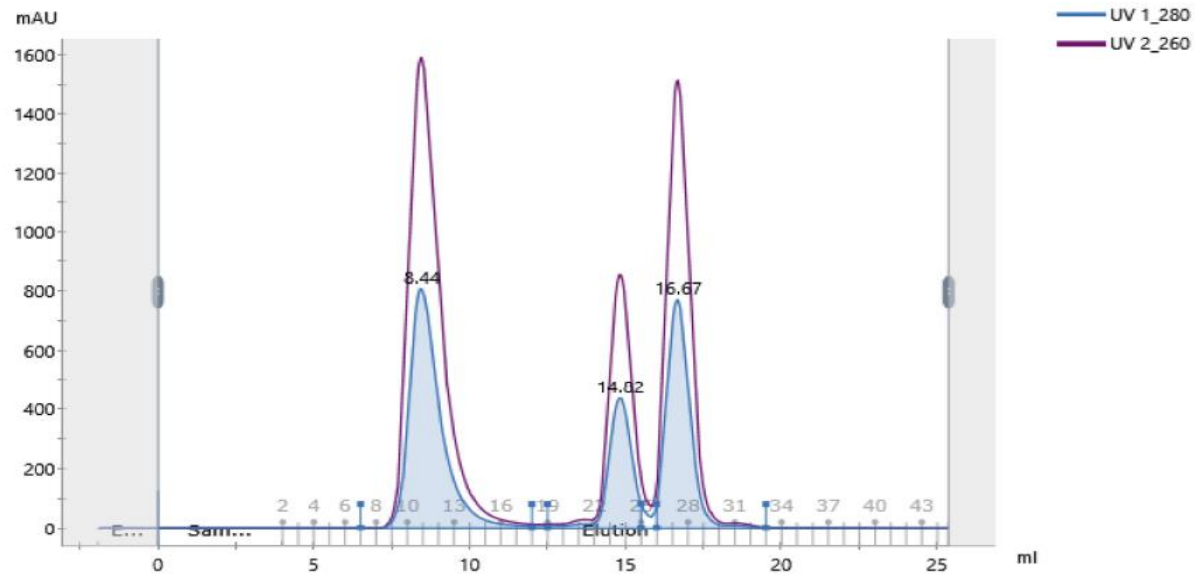
Dex-ssDNA, Oligo/Dex = 18.6



Peak A (8.7 min): Dex-ssDNA; Peak B (15.2 min): dimerized ssDNA; Peak C (16.9 min): monomeric ssDNA

Figure S4. Dex-DNA, Oligo/Dex = 18.6. The compound elution starts at fraction 9 and is collected up to fraction 17. Free dimeric and monomeric oligo started eluting from fraction 22. Absorbance was measured at 280 nm (blue line) and 260 nm (purple line). The number of oligos per dextran was determined according to equation 6.

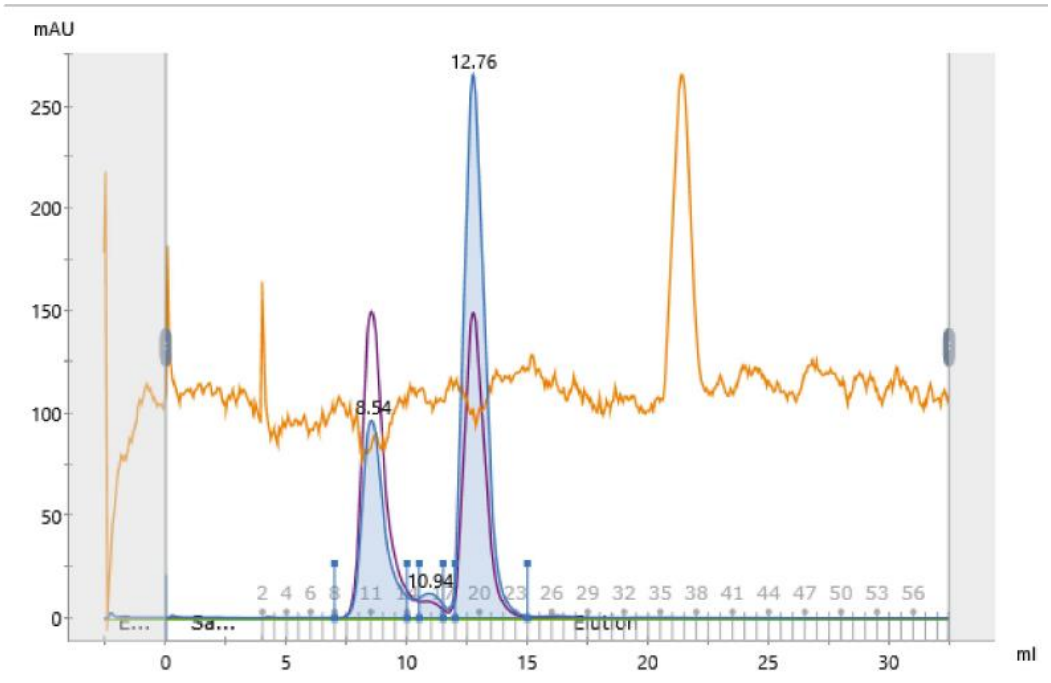
Dex-ssDNA, Oligo/dex = 19.0



Peak A (8.4 min): Dex-ssDNA; Peak B (14.0 min): dimerized ssDNA; Peak C (16.7 min): monomeric ssDNA

Figure S5. Dex-DNA, Oligo/Dex = 19.0. The compound elution starts at fraction 8 and is collected up to fraction 16. Free dimeric and monomeric oligo started eluting from fraction 22. Absorbance was measured at 280 nm (blue line) and 260 nm (purple line). The number of oligos per dextran was determined according to equation 6.

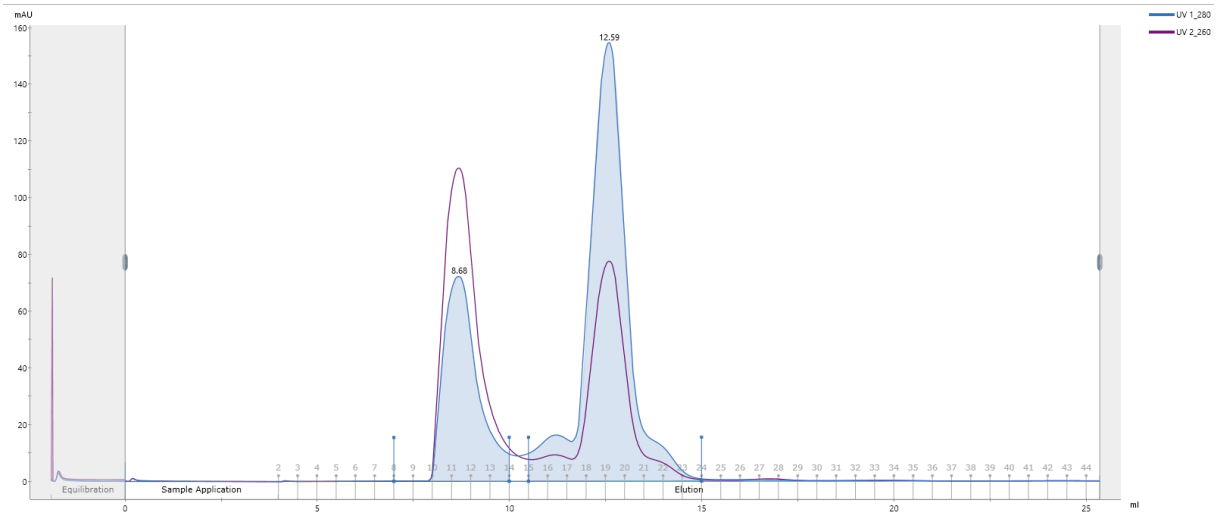
Cetuximab-Dex-ssDNA



Peak A (8.5 min): Cetuximab-Dex-ssDNA; Peak B (12.8 min): free Cetuximab

Figure S6. Cetuximab-Dex-ssDNA. The compound elution starts at fraction 8 and is collected up to fraction 13. Free antibody started eluting from fraction 17. Absorbance was measured at 280 nm (purple line) and 260 nm (blue line).

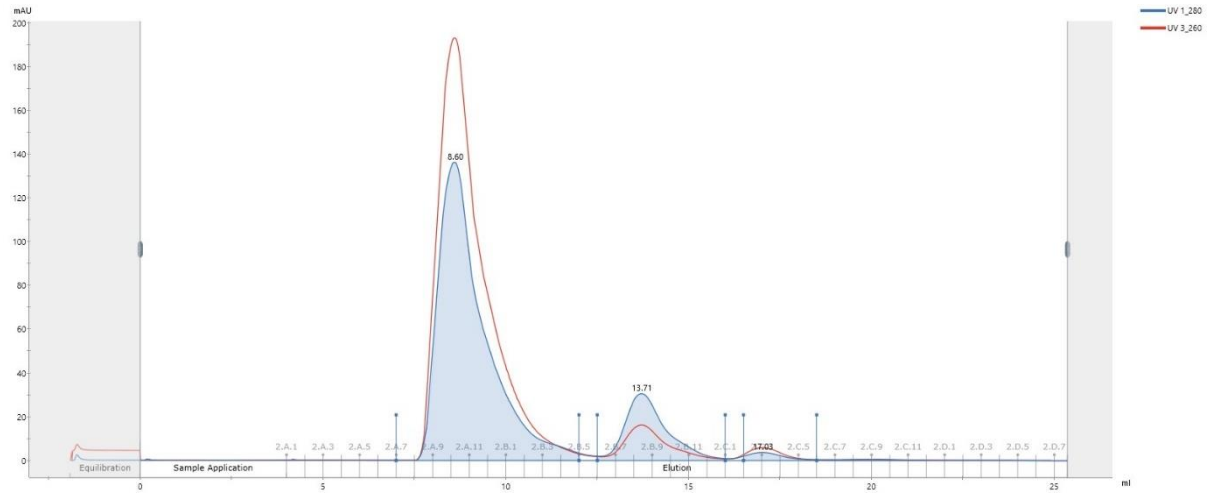
aCD4-Dex-ssDNA



Peak A (8.7 min): aCD4-Dex-ssDNA; Peak B (12.8 min): free aCD4 antibody

Figure S7. aCD4-Dex-ssDNA. The compound elution starts at fraction 9 and is collected up to fraction 12. Free antibody started eluting from fraction 17. Absorbance was measured at 280 nm (blue line) and 260 nm (purple line).

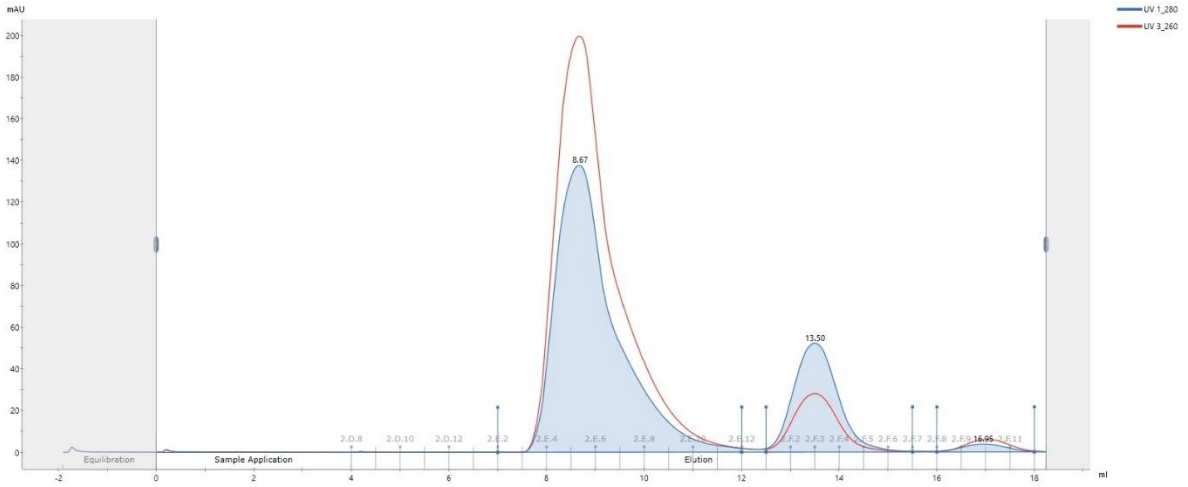
aEGFR-Dex-ssDNA



Peak A (8.6 min): aEGFR-Dex-ssDNA; Peak B (13.7 min): free aEGFR antibody

Figure S8. aEGFR-Dex-ssDNA. The compound elution starts at fraction A8 and is collected up to fraction B2. Free antibody started eluting from fraction B6. Absorbance was measured at 280 nm (blue line) and 260 nm (orange line).

aEpCAM-Dex-ssDNA



Peak A (8.7 min): aEpCAM-Dex-ssDNA; Peak B (13.5 min): free aEpCAM antibody

Figure S9. aEpCAM-Dex-ssDNA. The compound elution starts at fraction E3 and is collected up to fraction E9. Free antibody started eluting from fraction F1. Absorbance was measured at 280 nm (blue line) and 260 nm (orange line).

Complementary oligo titration

In order to test the impact of biotin and fluorophore on the staining performance, different molar ratios of complementary fluorophore- or biotin-decorated oligonucleotide to primer on the dextran were tested prior to staining of AsPC-1 cells.

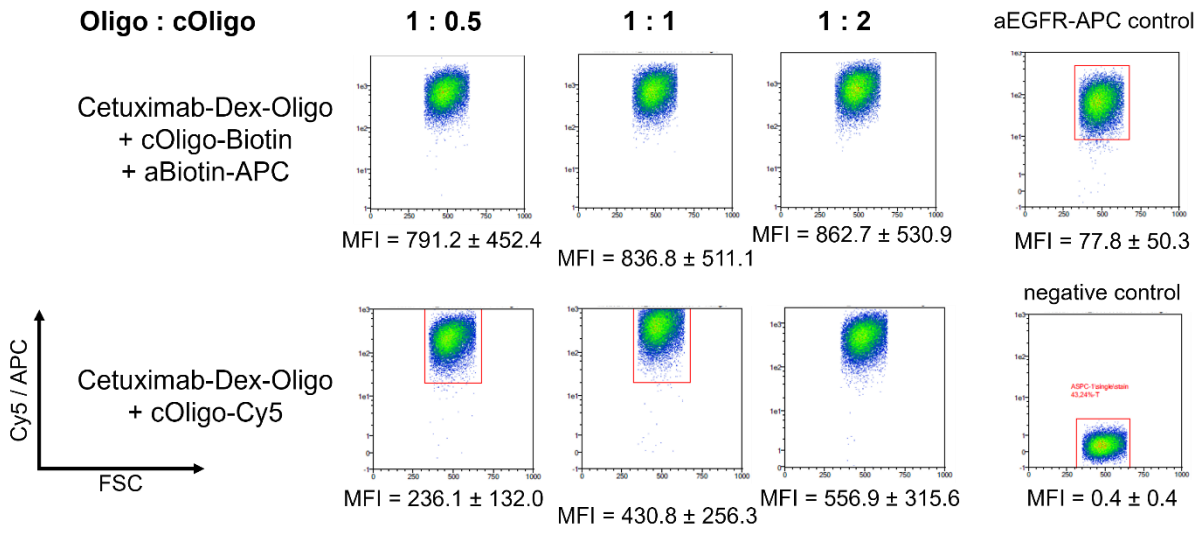


Figure S10. Titration of imaging strand/biotinylated strand to primer strand on dextran scaffold. Increase in biotin did not affect the staining intensity while increase in Cy5 imaging strand showed saturation of oligos. This indicated crowding on the dextran scaffold leading to limited amount of anti-Biotin antibodies binding to biotins. Data is given in MFI ± SD.

Anti-Biotin antibody titration

In order to test the impact of anti-biotin antibody amount on the staining performance, different molar ratios of antibody to biotin on the dextran were tested prior to staining of A431 cells.

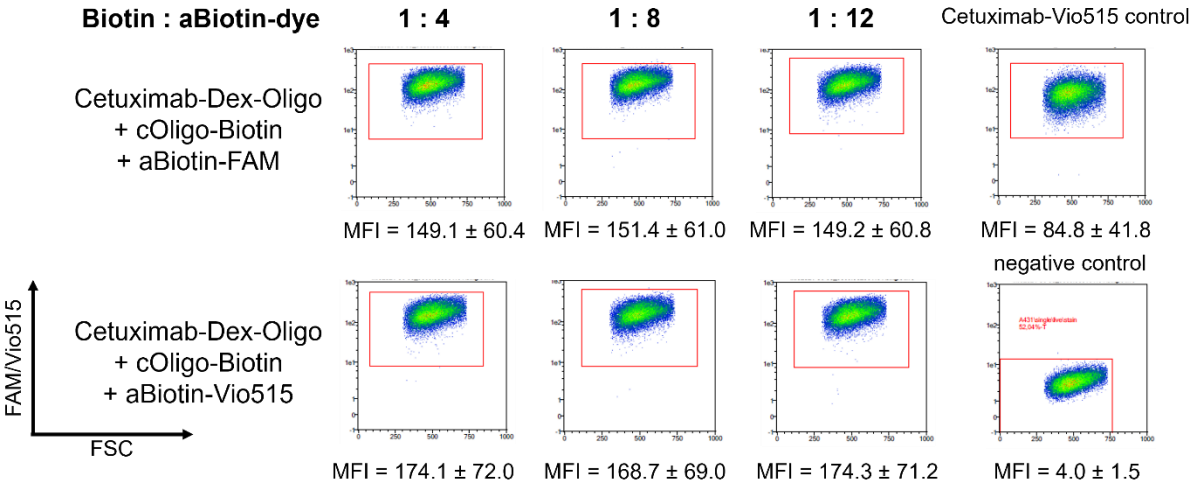


Figure S11. Titration of fluorescent anti-Biotin antibodies to biotinylated strand on dextran scaffold. Increase in antibodies did not affect the staining intensity indicating crowding on the dextran scaffold leading to limited amount of anti-Biotin antibodies binding to biotins. Data is given in MFI ± SD.

Dual release of biotin-anti-biotin conjugates in flow cytometry

In order to measure the dual release efficiency of anti-biotin antibody decorated dual-releasable conjugates, stained SUP-T1 cells were incubated for 10 min with cleavage cocktail and washed prior to flow cytometry experiment.

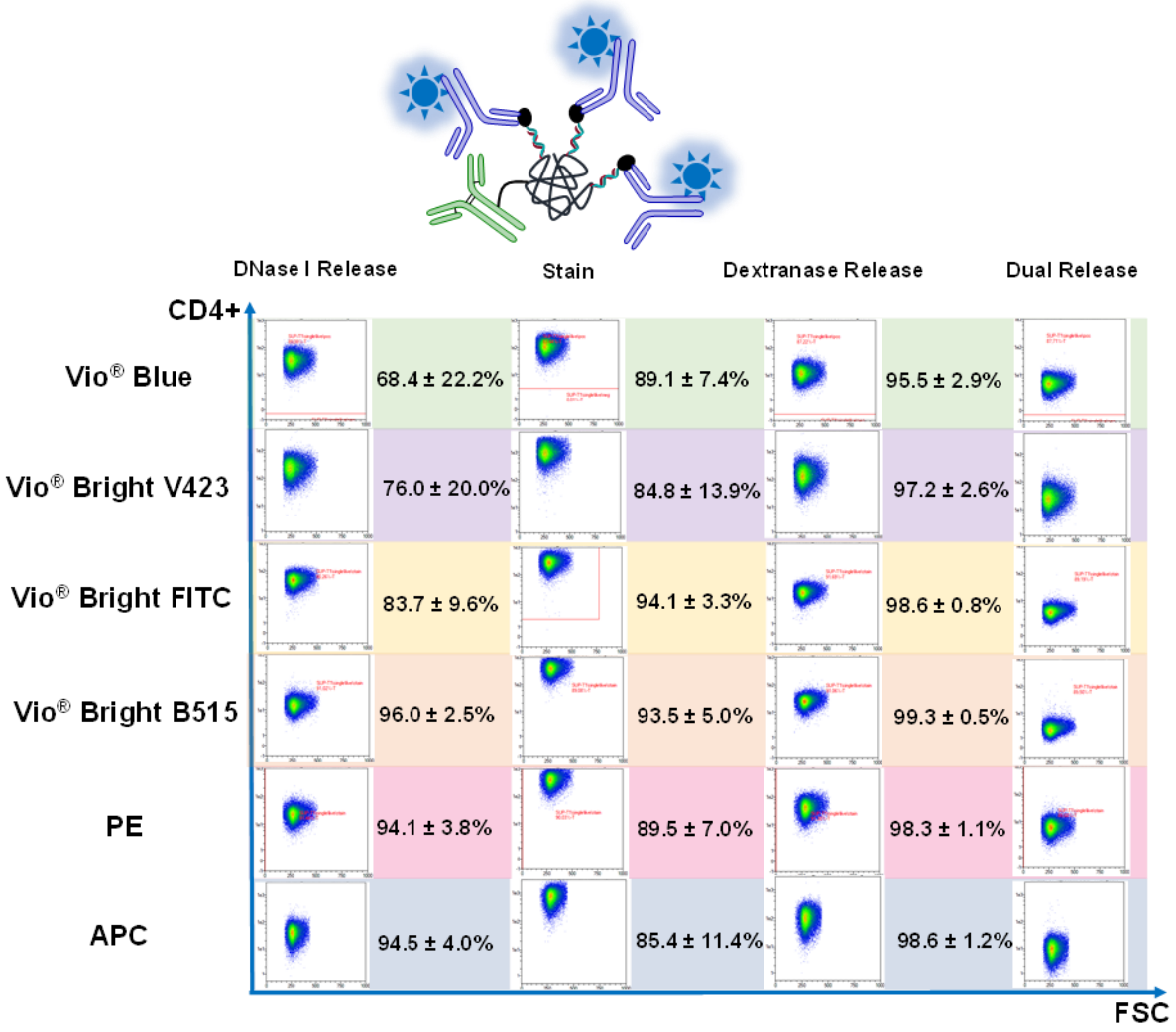


Figure S12. Dual release of aCD4-Dex-oligo decorated with fluorescent anti-Biotin antibodies in flow cytometry. Fixed SUP-T1 cells were stained at room temperature for 10 min with 5 nM conjugate. Dual release and mono-releases were performed at room temperature for 10 min. Release data is shown in percentage ± error according to eq. 6.

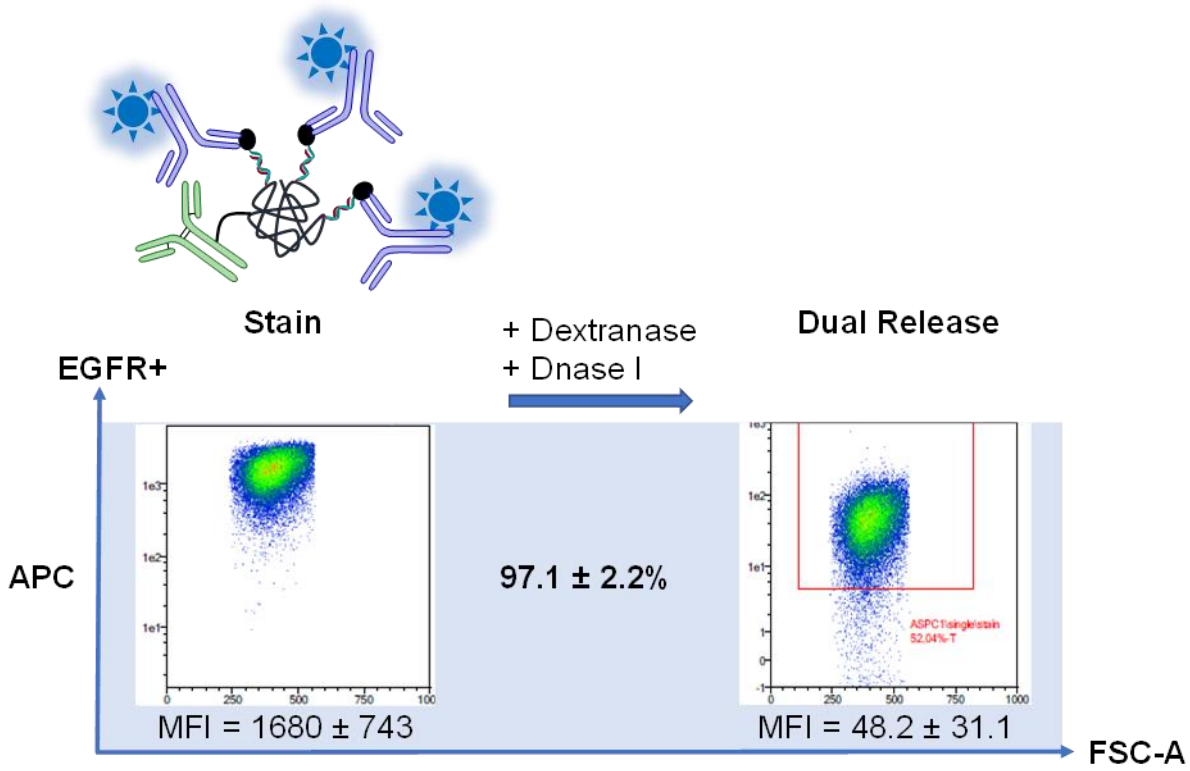


Figure S13. Dual release of Cetuximab-Dex-oligo decorated with fluorescent anti-Biotin-APC in flow cytometry. Fixed AsPC-1 cells were stained at room temperature for 10 min with 5 nM conjugate. Dual release was performed at room temperature for 10 min. Staining data is shown in MFI ± SD and release data is shown in percentage ± error according to eq. 6.

Signal removal comparison in confocal microscopy

Each staining was done at 10 nM concentration according to the procedure above for fixed adherent cells. Background was not subtracted.

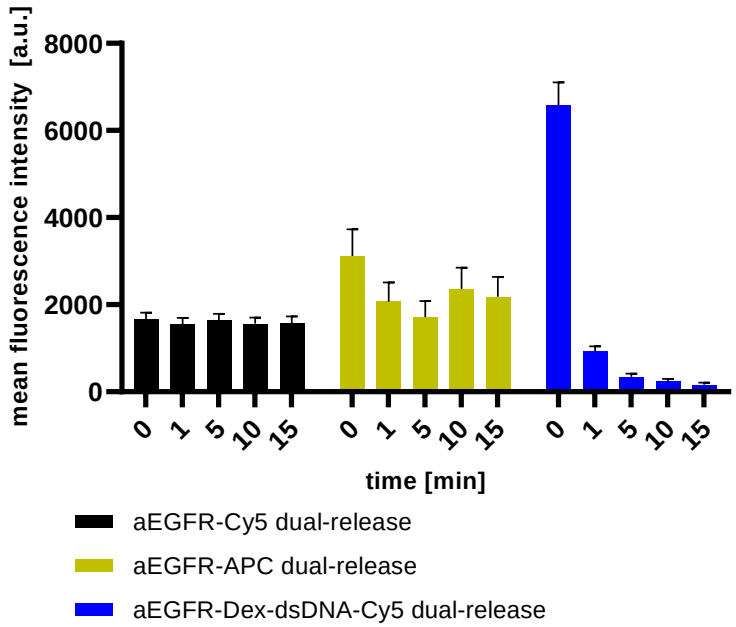


Figure S14. Quantification of signal before and after dual-release conditions for aEGFR-Dex-dsDNA-Cy5 and controls aEGFR-Cy5 and aEGFR-APC. The data represent the mean average ± SE (aEGFR -Cy5 dual release: n = 87; aEGFR-APC dual release: n = 58; aEGFR-Dex-dsDNA-Cy5 dual release: n = 67)

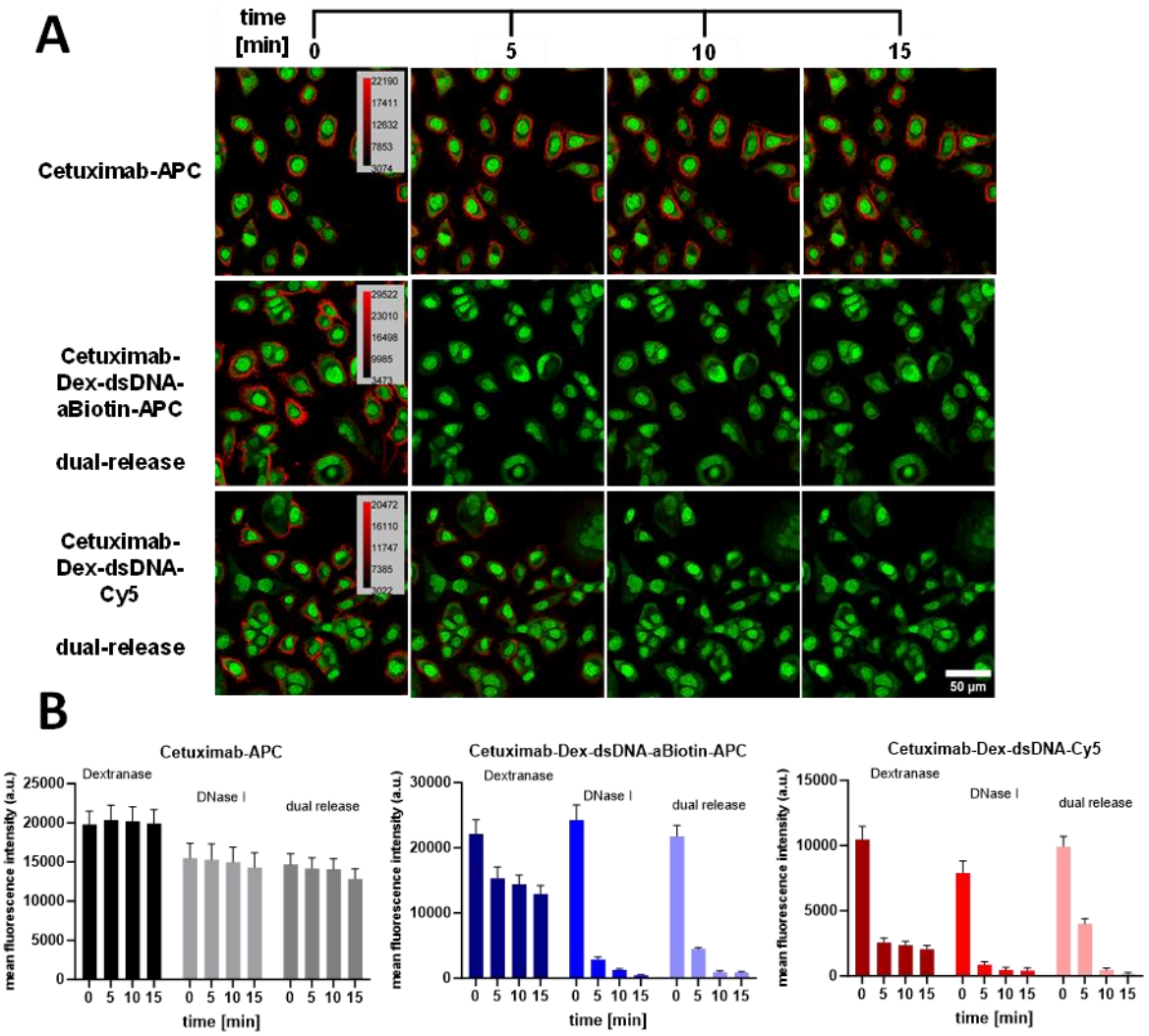


Figure S15. Confocal laser-scanning microscopy images of fixed AsPC-1 cells stained with Cetuximab-Dex-dsDNA-dye conjugates and signal quantification of initial fluorescence and during the course of release. (A) First row: dual release of Cetuximab-APC control conjugate; second row: dual release of Cetuximab-Dex-dsDNA-aBiotin-APC; third row: dual release of Cetuximab-Dex-dsDNA-Cy5. The columns indicate membrane staining after certain time points of release with t = 0 min being the initial staining before release. Cy5 and APC signal is depicted in red, nuclear staining with Sytox Orange in green. Scale bar is 50 µm and is a representation for all images. LUTs are shown on the first panels and were kept constant for each release. (B) Quantification of dual-release mode of Cetuximab conjugates. The data represent the mean average ± SE (Cetuximab-APC + Dextranase: n = 29; Cetuximab-APC + DNase I: n = 17; Cetuximab-APC dual release: n = 26; Cetuximab-Dex-dsDNA-aBiotin-APC + Dextranase: n = 26; Cetuximab-Dex-dsDNA-aBiotin-APC + DNase: n = 25; Cetuximab-Dex-dsDNA-aBiotin-APC dual release: n = 35; Cetuximab-Dex-dsDNA-Cy5 + Dextranase: n = 28; Cetuximab-Dex-dsDNA-Cy5+ DNase: n = 20; Cetuximab-Dex-dsDNA-Cy5 dual release: n = 36).

Stain-release-restain in confocal microscopy

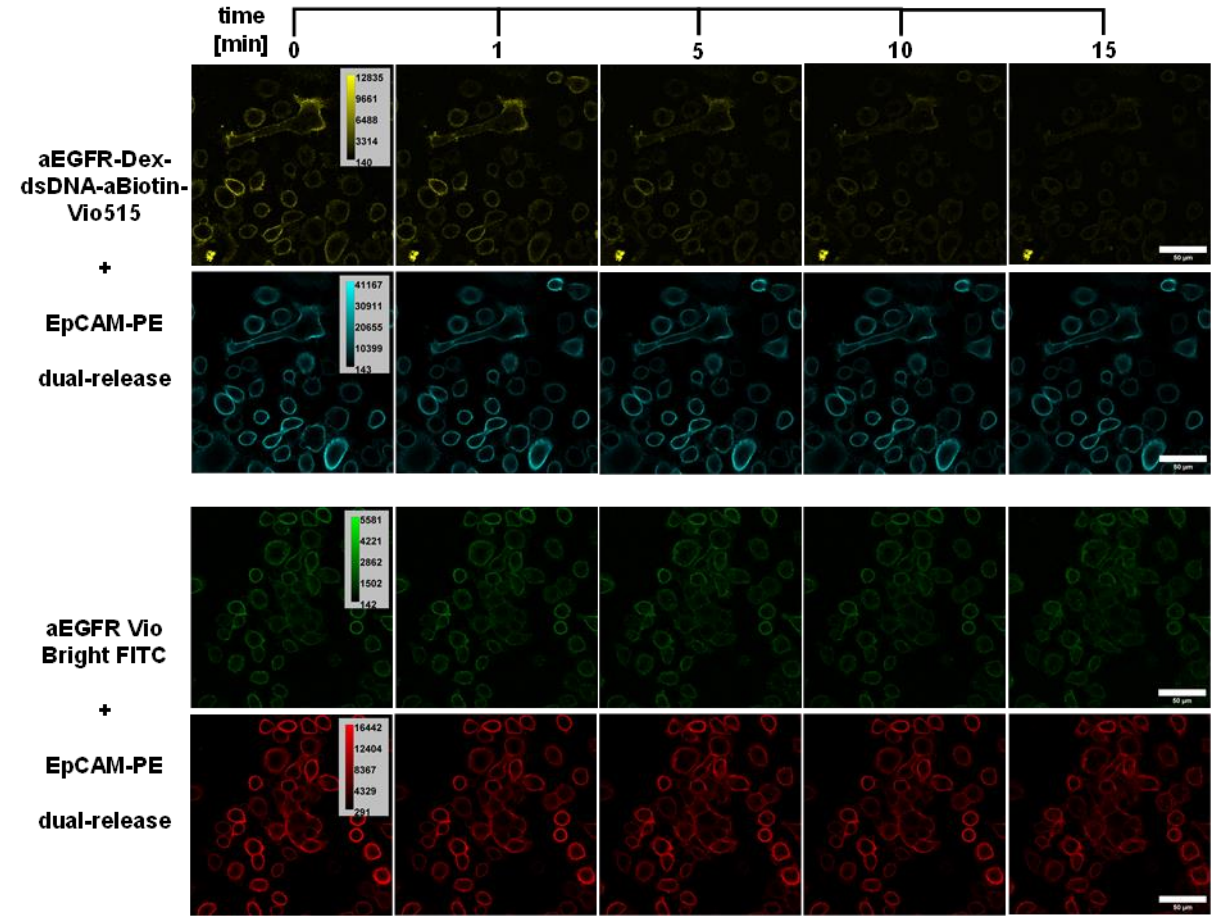


Figure S16. CLSFM of DNA-based conjugates and controls. Row 1: releasable Vio515 conjugate (yellow); row 2: aEpCAM-PE (cyan) as counterstaining; row 3-4: non-cleavable controls (green and red) that are not affected by release conditions. Scale bar is 50 µm. LUT scales show intensities for each row, which were kept constant.

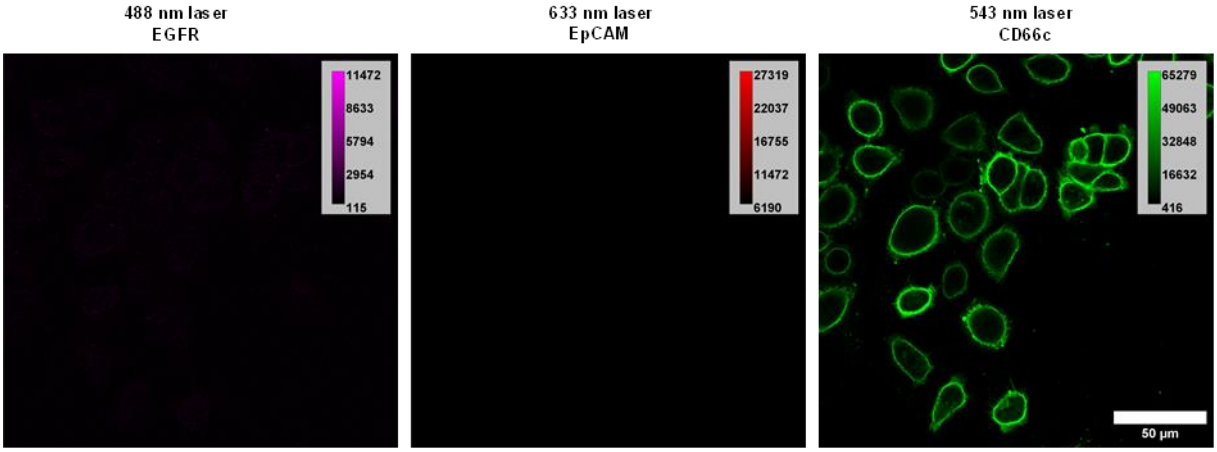


Figure S17. Images of last imaging round with non-cleavable aCD66c-PE conjugate after cleavage of aEGFR-Dex-dsDNA-aBiotin-Vio515 and aEpCAM-Dex-dsDNA-Cy5. No signal is observable within the ROI in the previously used channels indicating efficient cleavage.

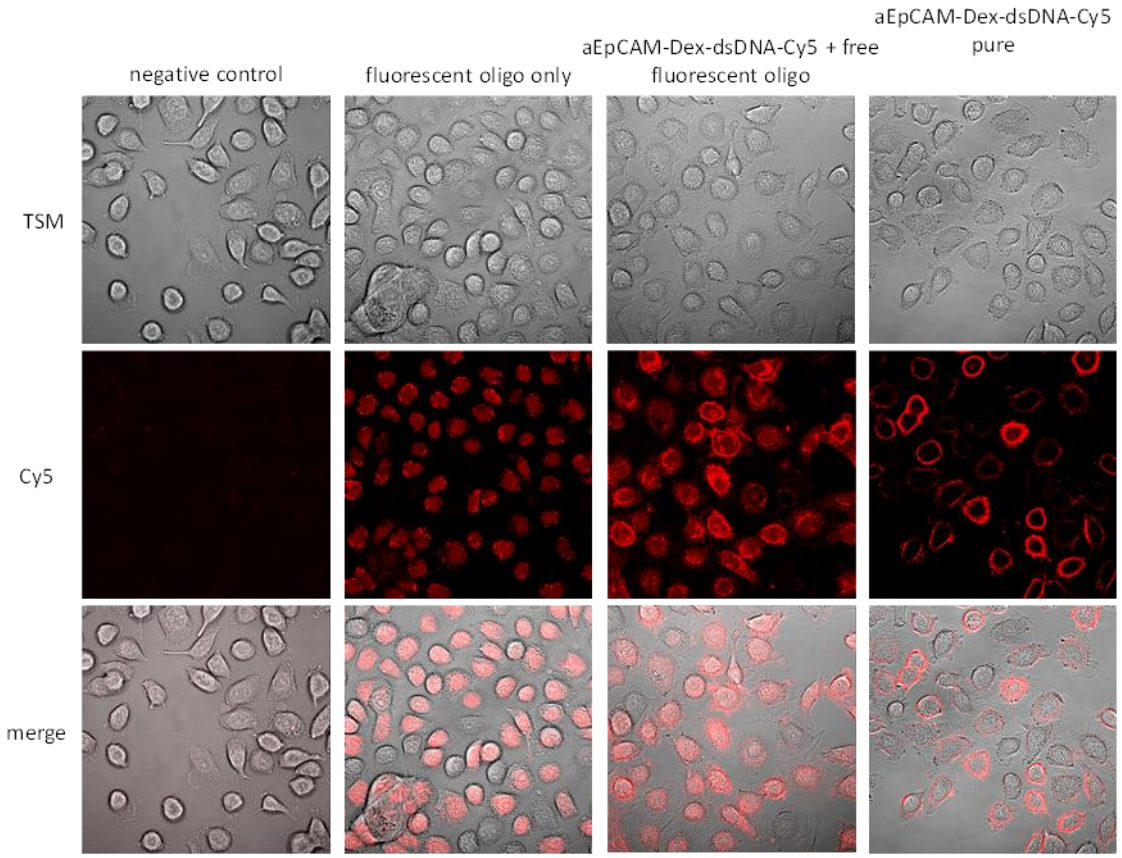


Figure S18. CLFSM of adherent AsPC-1 cells stained with anti-EpCAM conjugate and free complementary Cy5-oligo. The second column shows unspecific staining within the cells when incubated with free fluorescent oligo. The third column simulates the staining used in this study where free oligo remained in the mixture after hybridization. Here, specific membrane staining and unspecific staining within the cell is visible. Upon purification via Amicon filtration, conjugates show only specific staining on the membrane (fourth column).

References

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