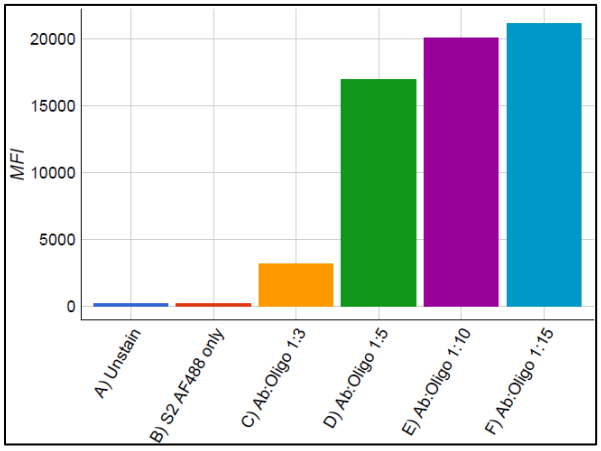
Setup and QC for ZipSeq reagents (note this only covers generation of Ab-oligo conjugates, titrating it, testing hybridization, and uncaging all read out by flow cytometry)

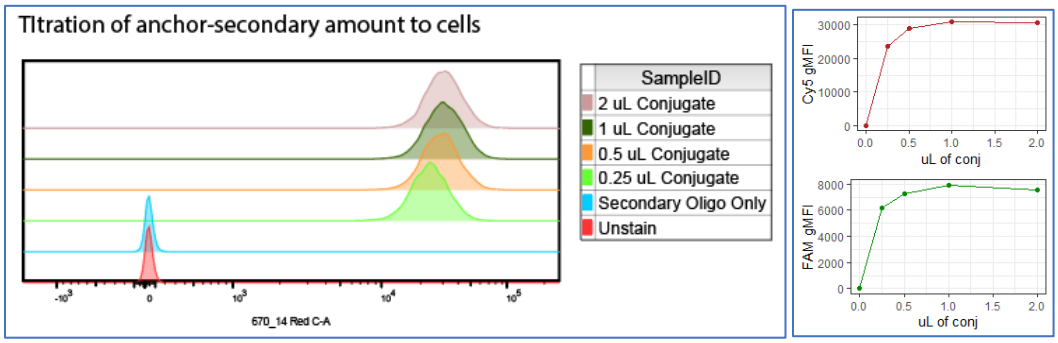
I. Generation of Anchor-Antibody Conjugates:
   a. Option A (Thunderlink PLUS from Nova) Uses free amines on both the Antibody and oligo to conjugate.
      i. Antibody must be azide free, 1 mg/mL preferable but down to 0.5 mg/mL is doable
      ii. Oligo must have either 5' or 3' amine modification, start with 100 uL of 60uM anchor strand (For example you might start with PN#1100/1101/2100)
      iii. Follow manufacturers protocol until mixing of modified Ab and oligo. I do this in the hood to maintain as sterile as possible
         1. Testing of 30F11 shows saturation of oligo binding at approx. 1:5 ratio of Ab to oligo. This may of course vary depending on antibody clone/protein you are using. Rest of the steps assume you have done 1:4 ratio using 300+80+20ul (5% BSA carrier protein) =400 ul. Let sit overnight @ 4C
            Graph showing conjugation to 30F11 with various Ab:Oligo ratios with fluorophore labeled secondary oligo hybridized (PN#1201FA)

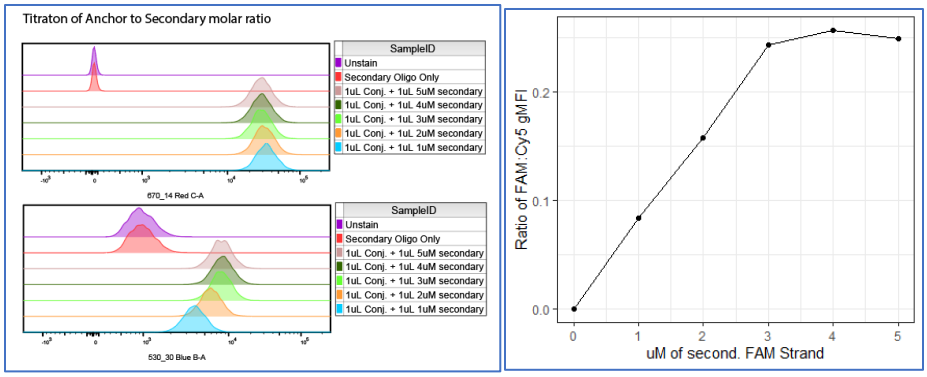


         2. Aliquot 20uL per tube and freeze @ -20C. I've found the conjugate is stable after thawing once, more cycles not guaranteed
   b. Option B: Click chemistry (tested this once, more trouble but cheaper on a per ug basis)(blank for now unless we switch to this)

II. Titration and testing of conjugate
   a. Assuming you used the volumes/amounts above, you now have at most, 60uM *100uL *80/300/400 = 4uM anchor strand in your conjugate mix .
   b. Purification of conjugate is possible as the thunderlink kit directs.
   c. We use a secondary oligo strand conjugated to a fluorophore to read out the ability to hybridize to the anchor strand. Since I often have Cy5 builtin, I use TAMRA or FAM as orthogonal fluorophores for this purpose (see PN#1201FA or 1201TA). IDT offers these modifications at wither 3 or 5' ends, in this case I recommend 5'. Add at least 2 T spacer as any nearby G can quench! In the below experiments I describe the use of **S2-5FAM** (PN#1201FA or 1201TA)(see table)

d. In the below experiment, a batch was tested on LB27.4 cells with a anti-mCD45 targeting antibody. The anchor strand contains an internal Cy5 (**S1-intCy5**)(PN#1101) while the secondary strand has a fluorescein attached. If we titrate the molar ratio of anchor strand to secondary strand, we would expect eventually the amount of FAM signal to level off as it goes into excess. You should observe the following behavior: linear increase in the FAM:Cy5 ratio which levels off. Note, it's not necessary to have the built-in Cy5, but it helps to normalize to and in downstream sort steps.





Staining was done with 200k LB27.4 cells, washed in FACS Buffer (2% FCS, 1mM EDTA). Conjugate prepared as follows:

1. 4ul of conjugate+4uL of 4uM of secondary FAM(PN# 1201) -> 10' 37C then let sit at RT for another 10'. Add 0/0.5/1/2/4 uL of this mix to cells corresponding to 0/0.25/0.5/1/2

2. 1uL of conjugate+1uL of secondary FAM(PN# 1201) from 0 to 5 uM, let sit at RT. Add 2ul to each tube.

Cells brought to 2e6/mL in 100 uL of FACS buffer, stained w/ conjugate @ 4C for 20min. Two washes following with 500uL FACS buffer, ran on flow cytometer.

III. Testing of NPOM caged oligonucleotides. Usually Biosyn does good qc, but always worth checking, especially for new designs. (below example uses **S2-4NPOM PN#1200**)

a. Resuspend to 120 uL in water, **keep protected from light**

b. Dilute to 6uM working concentration in water.

c. Hybridize with anchor strand-Antibody conjugate

d. Stain cells in compatible buffer, 4C for 20-30 minutes.

e. Wash twice w/ 5x volume of FACS Buffer
f. Illuminate cells with 365 nm light. The lamp I use requires at least 4 minutes of exposure to achieve maximal uncaging due to the greatly reduced energy density of illuminating a while tube.
g. Immediately add 1 uL of 10uM per 100 uL of the fluorescently labeled readout strand that is complement of the overhang (**S3-3Cy5 or S3-3TAMRA (PN#1301CY/TA) or full length ZC1/2/3-Cy5/TAMRA/FAM**) (PN#Z2301CY/TA/FA)
h. Incubate 5 min @ RT.
i. Wash twice w/ buffer, letting cells sit @ RT for 5min between washes.
j. Take to flow cytometer on ice.
k. Example of expected data for good binary uncaging behavior: Near 100% of uncaged (maximal signal) achieved w 365 nm light exposure and minimal <8% when uncaged. Ignore DEACM columns (other caging chemistry)

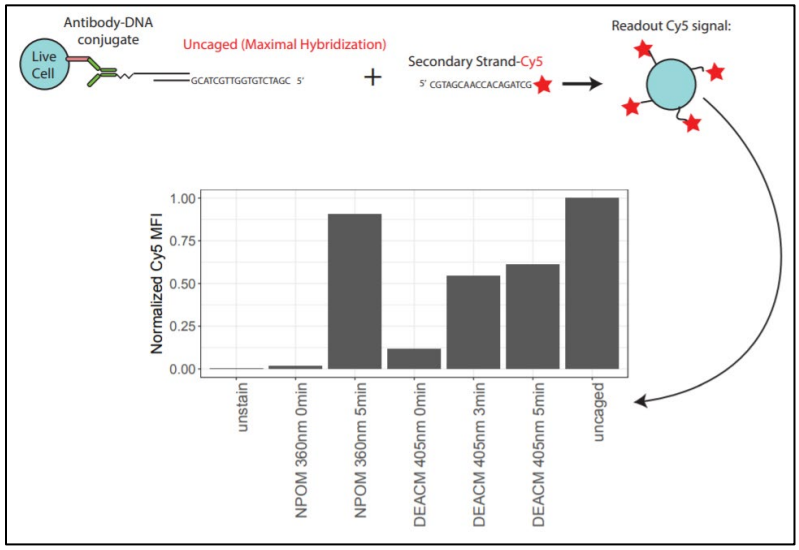


Table of oligos used for Design 1:

S1 (PN#1100): Amine modified anchor strand for conjugation to antibody: (Fig. 1, S1)
Amine-C6 linker - ATCGTTTTTTTTTTTTGATGTTGACGG
S1-intCy5 (PN #1101): Amine-modified anchor strand with internal Cy5 fluorophore for conjugation to antibody (Fig. 3,4)
Amine-C6 linker - ATCCAG\iCy5\TTTTTTTTTTTTGATGTTGACGG
S2-4NPOM (PN #1200):Caged Strand hybridized to anchor strand prior to labeling cells with overhang region O1 (NPOM modified thymidines highlighted in red): (Fig. 1, S1, 2,3,4,5)
5'-CGATCTGTGGTTGCTACCCGTCAACATCAAAAAAAAAAAA-3'
S2 Blocking Strand (PN #1210): Consists of uncaged Overhang region O1, used for sopping up excess zipcode strands (by hybridizing to their open O1' region.) Important to use between zipcoding steps as washes cannot effectively remove enough zipcode.
5'-CGATCTGTGGTTGCTAC-3'

S2-5FAM (PN #1201FA): Uncaged version of S2-4NPOM with 5' FAM modification for reading out hybridization to anchor strand
5'-FAM-TTCGATCTGTGGTTGCTACCCGTCAACATCAAAAAAAAAAAA-3'
S2-5TAMRA (PN #1201TA): Uncaged version of S2-4NPOM with 5' TAMRA modification for reading out hybridization to anchor strand
5'-TAMRA-TTCGATCTGTGGTTGCTACCCGTCAACATCAAAAAAAAAAAA-3'
S3 (PN #1300): Second layer oligonucleotide (contains overhang region O1') (Fig. 5)
GTAGCAACCACAGATCGCATGAGTCGAATCTCCCAC
S3-3Cy5 and S3-3TAMRA: Second layer oligonucleotide (contains overhang region O1') with 3' Cy5 or TAMRA modifications (PN# **1301CY** and **1301TA**)
GTAGCAACCACAGATCGCATTT-Cy5 GTAGCAACCACAGATCGCATTT-TAMRA
Second layer NPOM caged oligonucleotide with overhang region O2 (PN# 1400)(NPOM modified thymidines highlighted in red): Fig. 5
5'-CCTAGATCATGCAGTTCCGTGGGAGATTCGACTCATG-3'
Zipcode 1 w/ complementary overhang O1' (Fig. S1, 2, 3, 4, 5) (PN# Z131):
GTAGCAACCACAGATCGCACCCGAGAATTCCATGATGCAAAAAAAAAAAAAAAAAAAAAAAAAAA
Zipcode 2 w/ complementary overhang O1' (Fig. S1, 2, 3, 4, 5) (PN#Z132):
GTAGCAACCACAGATCGCACCCGAGAATTCCAAGCCATAAAAAAAAAAAAAAAAAAAAAAAAAAA
Zipcode 3 w/ complementary overhang O2' (Fig. 5) (PN# Z153)
GAACTGCATGATCTAGGCACCCGAGAATTCCATCAACGAAAAAAAAAAAAAAAAAAAAAAAAAAA
Zipcode 4 w/ complementary overhang O2' (Fig. 5) (PN# Z154)
GAACTGCATGATCTAGGCACCCGAGAATTCCATACGTGAAAAAAAAAAAAAAAAAAAAAAAAAAA
Fluorescent oligonucleotide for flow readout with complementary overhang O1':
GTAGCAACCACAGATCGTATA-Cy5
Adaptor Strands for lipid anchored oligonucleotide: (Fig. 2)
GATGCTTCACGGGATATTTTTTTTTTTTGATGTTGACGG
TATCCCGTGAAGCTTGAGTGGAATCCCTTGGCACCCGAGAATTCCA
Lignoceric acid conjugated anchor strand (Fig. 2)

Lignoceric acid - GTAACGATCCAGCTGTCACTTGGAATTCTCGGGTGCCAAGG
Co-anchor strand (Fig. 2)
AGTGACAGCTGGATCGTTAC - Palmitic acid
Design version 2 amine modified anchor strand (Fig. 5, S7) **(PN# 2100)**
Amine-C6 linker - TTTTTCACCCGAGAATTCCAC
Design version 2 universal caged strand (Fig. 5, S7) **(PN# 2200)**
CGATCTGTGGTTGCTACGTGGAATTCTCGGGTG
Design version 2 generic Zipcode strand (Fig. 5, S7) (PN# Z23NN)
GTAGCAACCACAGATCGCACCCGAGAATTCCACNNNNNNNAAAAAAAAAAAAAAAAAAAAAAAAAA
ZC1-3Cy5: Zipcode 1 strand w/complementary overhang O1 w/ terminal Cy5 (Fig. 1, 5, S7) (PN #Z2301CY)
GTAGCAACCACAGATCGCACCCGAGAATTCCACTGATGCAAAAAAAAAAAAAAAAAAAAAAAAAA-Cy5
ZC2-3TAMRA: Zipcode 2 strand w/complementary overhang O1 w/ terminal TAMRA (Fig. 1, 5, S7) (PN#Z2302TA)
GTAGCAACCACAGATCGCACCCGAGAATTCCACAGCCATAAAAAAAAAAAAAAAAAAAAAAAAAA-TAMRA
ZC3-3FAM: Zipcode 3 strand w/complementary overhang O1 w/ terminal FAM (Fig. 1, 5, S7) (PN#Z2303FA)
GTAGCAACCACAGATCGCACCCGAGAATTCCACCTCTTCAAAAAAAAAAAAAAAAAAAAAAAAAA-FAM