Supporting Information

Bright Fluorescent Nanotags from Bottlebrush Polymers with DNA-Tipped Bristles

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SUPPORTING RESULTS

Characterization of PBiBEM-g-PMEO₃MA-N₃ (BBP-N₃)

Figure S1. Characterization of BBP-N₃. (a) Gel permeation chromatography (GPC) trace of PBiBEM-g-PMEO₃MA-N₃ in THF showing the molecular weight distribution determined using linear poly(methyl methacrylate) standards. (b) FTIR spectrum showing the azide peak of the side chains.

Absolute number average molar mass (Mn) = 8.6 × 10⁶ g/mol was determined using Multi Angle Laser Light Scattering.

Number average molar mass / Weight average molar mass (Mw/Mn) = 1.29 was determined using linear poly(methyl methacrylate) standards.
Characterization of DBBP

The signal at 2110 cm\(^{-1}\) (azide) from side chains in BBPEN (blue) disappears after the click reaction with hexynyl-A DNA. There are no detectable unreacted azide groups remaining in A-BBP (red) after the DNA conjugation.

**Figure S2.** FTIR spectra of 0.5 μM BBP-N\(_3\) and A-BBP in H\(_2\)O

**Figure S3.** FTIR spectra of 0.5 μM BBP-N\(_3\) (blue) and 0.5 μM DBBPs loaded with different compositions of DNA and PEO chains.
To determine the number of bristles on the bottlebrush polymer, we start by assuming that there are 400 azide-terminated side chains (if the grafting efficiency is 100%). When hexynyl-DNA and azide from BBP are reacted in an equimolar ratio based on this assumption, half of the hexynyl DNA was recovered from the filtrate during the product isolation from molecular weight cut off filters (30 kDa). However, when the product (DBBP) was tested using FTIR spectrometry, the azide peak had completely disappeared indicating that all the azides in BBP reacted with DNA. (Fig. S2). Therefore, we conclude that there is an average of 200 rather than 400 azide-terminated side chains (i.e. bristles) per brush.
Antibody-DBBP conjugation

Figure S4. (a) The UV traceable bisaryl hydrazone linkage between antibody and DNA oligonucleotide. (b) UV/vis spectrum showing the peak at $\lambda_{\text{max}} = 354$ nm confirming the formation of bisaryl hydrazone linkage.
Quantitation of Number of DNA Strands Attached to Antibody

Figure S5. (a) The calibration plot of $A_{260\text{ nm}}/A_{280\text{ nm}}$ vs. antibody-DNA ratio to calculate the number of DNA strands attached per antibody. (See table S3) (b) UV/vis spectrum showing the $A_{260\text{ nm}}/A_{280\text{ nm}}$ of the Ab-B' conjugate.
Labeling and Detection of c-Myc Induced or Uninduced Yeast Cells with DBBP Nanotag or Alexa 647 Tagged Antibodies.

Uninduced yeast cells and yeast cells induced to express c-Myc-tagged scFv (10^7 cells) were suspended in calcium- and magnesium-free PBS wash buffer (pH = 7.5, Na^+ = 100 mM, 1 µg/mL Pluronic® F-127). The cells were incubated with 0.5 µM final concentration of anti-c-myc mouse antibody for half an hour at 4 °C, washed three times with 500 µL of wash buffer, and resuspended in 500 µL of the same buffer. The cells were incubated with 50 nM final concentration of goat anti-mouse antibody-DBBP complex for 30 minutes at 4 °C. The cells were washed three times with 500 µL wash buffer. YOYO-1 was added to give a 50 µM final concentration in a total volume of 500 µL PBS buffer (pH = 7.5, Na^+ = 100 mM, 1 µg/mL Pluronic® F-127). Similarly, 0.5 µM final concentrations of Alexa 647-tagged goat anti-mouse IgG (H+L) (Life Technologies-Molecular Probes) was separately used as the control. (The 10x higher concentration followed the vendor’s recommendation.) A Carl Zeiss LSM 510 Meta DuoScan Inverted Spectral Confocal Microscope was used for fluorescence imaging analysis at 100x objective magnification. 20 µL of the cell suspension was placed on a 35 mm glass bottom micro well dish (Mattek, part no. P35G-1.5-14-C). The microwell plates for yeast cells were treated with concanavalin A. Enough wash buffer was added to fill the microwell before viewing under the microscope. The pin hole opening was set to the minimum setting and the tube current was set to 0.6 A for imaging of DBBP labeled samples. 4.5 A tube current and a higher pinhole setting were used for samples labeled with Alexa 647 tagged secondary antibodies. Raw images were collected using ZEN 2009 software without applying any digital enhancements. Images are shown on the next page.
**Figure S6.** Confocal microscopic images of yeast cells initially stained with primary mouse anti-c-Myc antibody, then with X-tagged secondary antibody. DIC image (grey), YOYO-1 emission (green; BP 505-550 nm) after direct excitation at 488 nm (green), Cy5 or Alexa 647 emission (red; LP 650 nm) after direct excitation at 633 nm and Cy5 sensitized emission (cyan; LP 650 nm) after direct excitation at 488 nm. **a.** Uninduced yeast cells when X = Alexa 647. **b.** c-Myc expressing yeast cells when X = Alexa 647. **c.** Uninduced yeast cells when X = DBBP. **d.** c-Myc expressing yeast cells when X = DBBP.

**Conclusion:** At the settings used for imaging, the uninduced cells do not show a measurable signal, even in the presence of the primary antibody. This indicates that the DBBP-functionalized secondary antibody does not bind nonspecifically to the cells, but rather requires the presence of both the antigen (c-Myc epitope tag on expressed scFv protein) and primary antibody in order to stain the cells.
**Staining by antibody-DBBP mixture in which DBBP lacked complementary strand**

To test whether a DBBP lacking a complementary strand for the secondary antibody would nonspecifically stain yeast cells, we prepared a mixture of DBBP functionalized only with sequence A, mixed it with secondary antibody functionalized with sequence B’, then applied the mixture to yeast cells that were induced to express the c-myc epitope-tagged scFv. As shown in the images below, the antibody-DBBP mixture in which the DBBP lacked a complementary strand failed to stain the cells (panel a). Evidently, the DBBP was eliminated during the pre-imaging washing step because it was not attached to the antibody. In contrast, the antibody-DBBP conjugate in which the DBBP hybridized to the antibody gave strong fluorescent signal in all three channels. This experiment confirms that hybridization of the DBBP to the antibody is necessary for cell surface staining. It also demonstrates the lack of nonspecific staining of yeast cells by the DBBPs.

![Confocal microscopic images](image)

**Figure S7.** Confocal microscopic images c-Myc expressing yeast cells initially stained with primary mouse anti-c-Myc antibody, then with DBBP tagged/mixed secondary antibody. DIC image-grey, YOYO-1 emission green (BP 505-550 nm) after direct excitation at 488 nm, Cy5 or Alexa 647 emission-red (LP 650 nm) after direct excitation at 633 nm and Cy5 sensitized emission-cyan (LP 650 nm) after direct excitation at 488 nm. a. c-Myc expressing yeast cells labeled with secondary antibody-DBBP mixture. b. c-Myc expressing yeast cells labeled with secondary antibody-DBBP conjugate.
Preparation of Antibody-DBBP Conjugates and Labeling of Beads

This experiment demonstrated the ability of antibody-DBBP conjugates to label polystyrene beads commonly used for fluorescence assays.

Figure S8. Labeling of polystyrene beads by an antibody-DBBP conjugate. (a) Synthesis of a DBBP with seq.A: seq.B = 199:1 via sequential click conjugation of two different DNA oligonucleotides. Sequence B is complementary to a DNA oligonucleotide conjugated to the secondary antibody. (b) Confocal microscopic images of polystyrene beads coated with biotin labeled with DBBP nanotags (Seq.A/Seq.B=199:1) synthesized from sequential conjugation method shown in (a). Beads were initially stained with primary anti-biotin antibody, then with secondary antibody-DBBP conjugate. DIC image-grey, YOYO-1 emission-green (BP 505-550 nm) after direct excitation at 488 nm, Cy5 emission-red (LP 650 nm) after direct excitation at 633 nm and Cy5 sensitized emission-cyan (LP 650 nm) after direct excitation at 488 nm.
Commercially available Alexa 647 and Quantum Dot (QD 655) tagged goat anti-mouse secondary IgG antibody probes were used as controls. Mouse anti-biotin IgG (H+L) was used as the primary antibody. The histograms and the mean fluorescence values (Supplementary Table S6) show the DBBP-tagged antibody is at least ten times brighter than the QD 655 tagged antibody (dark green histograms). At the same secondary antibody concentration to that of the DBBP nanotag (50 nM) the commercially available Alexa 647-conjugated probe gives only baseline fluorescence intensity (purple histograms). The signal from emission channel 695 significantly increases when the DBBP scaffold contains Cy5 dyes (orange histograms). DBBP nanotags show baseline fluorescence when the primary antibody is not present (blue histograms) showing labeling specificity.

**Figure S9.** Flow cytometry analysis results of biotinylated beads labeled with IgG antibodies tagged with various fluorescent labels. Fluorescence of the beads were monitored with excitation/emission at 488/530 nm (FL1-A) for YOYO-1 emission (left panel) and 633/695 nm channel (FL4-H) for Cy5, Alexa 647 and QD 655 emission (right panel).
The biotinylated beads are visible when the concentration of the secondary antibody tagged with DBBP is only 50 nM. The pinhole is set at the minimum setting and a photomultiplier tube current of 0.6 A is sufficient to get the image (Figure S10a). However, using the same instrument settings, when the fluorescent tag is Alexa 647, at the same IgG concentration, no fluorescent signal is observed (panel b). Increasing the tube current to its maximum value gives rise to a faint signal (panel c) but strong labeling is only observed when the concentration of the Alexa 647 tagged antibody is increased to 250 nM (panel d).

Figure S10. Comparison of brightness of DBBP nanotags with Alexa 647 tagged IgG antibodies. Confocal microscopic images showing the polystyrene beads coated with biotin. DIC image-grey, Cy5 emission (red; LP 650 nm) after direct excitation at 633 nm and Alexa 647 emission (white; LP 650 nm) after direct excitation at 633 nm.
Supporting Tables

Calculation of Molar Substitution Ratios (MSR) of modified antibody and DNA

**Table S1.** MSR calculated for the modified antibody (Goat antimouse IgG (H+L))

| HyNic MSR Calculator |  |
|----------------------|--|
| Name of protein      | Goat antimouse IgG (H+L) |
| Molecular weight of Protein (daltons) | 150000 |
| HyNic-modified Protein concentration (mg/mL) | 5.1 |
| Volume of HyNic-modified Protein used in MSR assay (µL) | 4 |
| Total MSR assay volume (µL) | 20 |
| A<sub>345</sub> reading (1-cm pathlength) | 0.204 |
| Molar Substitution Ration (MSR) | 1.1 |

**Table S2.** MSR calculated for the modified DNA (B')

| HyNic Oligo- MSR Calculator |  |
|-----------------------------|--|
| Name of DNA                 | NH<sub>2</sub>-B' |
| Molecular weight of DNA (daltons) | 8892 |
| HyNic-modified DNA concentration (mg/mL) | 0.753 |
| Volume of HyNic-modified DNA used in MSR assay (µL) | 4 |
| Total MSR assay volume (µL) | 20 |
| A<sub>350</sub> reading (1-cm pathlength) | 0.4 |
| Molar Substitution Ration (MSR) | 0.8 |

**Table S3.** A<sub>260 nm</sub> / A<sub>280 nm</sub> values obtained to develop the calibration plot to calculate the number of DNA strands attached to antibody.

| Ab: DNA | [Ab] (µM) | [DNA] (µM) | A<sub>260 nm</sub> / A<sub>280 nm</sub> |
|---------|-----------|------------|---------------------------------|
| 1:0     | 4         | 0          | 0.64                            |
| 1:0.5   | 4         | 2          | 0.79                            |
| 1:2     | 4         | 8          | 1.12                            |
| 1:4     | 4         | 16         | 1.51                            |
| 1:5     | 4         | 20         | 1.63                            |

Ab-B' 0.89
Mean fluorescence values from flow cytometry experiments

Table S4. Mean fluorescence (530 nm emission channel) values of c-myc tagged yeast cells labeled with IgG antibodies with X-tags.

| Sample name     | Subset Name | Count | Mean fluorescence 530 nm emission channel |
|-----------------|-------------|-------|--------------------------------------------|
| X = Alexa 647   | cells       | 9903  | 2097                                       |
| X = QD 655      | cells       | 9906  | 2035                                       |
| X = DDBP(-)Cy5  | cells       | 9919  | 173475                                     |
| X = DDBP(+)Cy5  | cells       | 9911  | 96366                                      |
| X = DDBP(+)Cy5 (-)1⁰ Ab | cells | 9912  | 2120                                       |
| Yeast only      | cells       | 9908  | 2083                                       |

Table S5. Mean fluorescence (695 nm emission channel) values of c-myc tagged yeast cells labeled with IgG antibodies with X-tags.

| Sample name     | Subset Name | Count | Mean fluorescence 695 nm emission channel |
|-----------------|-------------|-------|--------------------------------------------|
| X = Alexa 647   | cells       | 9903  | 215                                        |
| X = QD 655      | cells       | 9906  | 2171                                       |
| X = DDBP(-)Cy5  | cells       | 9919  | 254                                        |
| X = DDBP(+)Cy5  | cells       | 9911  | 25057                                      |
| X = DDBP(+)Cy5 (-)1⁰ Ab | cells | 9912  | 334                                        |
| Yeast only      | cells       | 9908  | 218                                        |
*Table S6.* Mean fluorescence (530 nm emission channel) values of biotin coated polystyrene beads labeled with IgG antibodies with X-tags.

| Sample name         | Subset Name     | Count | Mean 530 nm emission channel |
|---------------------|-----------------|-------|-------------------------------|
| X = Alexa 647       | Biotin beads    | 8495  | 3450                          |
| X = QD 655          | Biotin beads    | 8501  | 2958                          |
| X = DDBP(-)Cy5      | Biotin beads    | 8499  | 198925                        |
| X = DDBP(+)Cy5      | Biotin beads    | 8503  | 84852                         |
| X = DDBP(+)Cy5 (-)1⁰ Ab | Biotin beads | 8492  | 2859                          |
| Biotin beads only   | Biotin beads    | 8505  | 2785                          |

*Table S7.* Mean fluorescence (695 nm emission channel) values of biotin coated polystyrene beads labeled with IgG antibodies with X-tags.

| Sample name         | Subset Name     | Count | Mean 695 nm emission channel |
|---------------------|-----------------|-------|-------------------------------|
| X = Alexa 647       | Biotin beads    | 8495  | 240                           |
| X = QD 655          | Biotin beads    | 8501  | 4908                          |
| X = DDBP(-)Cy5      | Biotin beads    | 8499  | 270                           |
| X = DDBP(+)Cy5      | Biotin beads    | 8503  | 50254                         |
| X = DDBP(+)Cy5 (-)1⁰ Ab | Biotin beads | 8492  | 298                           |
| X = Biotin beads only | Biotin beads   | 8505  | 206                           |
METHODS

General Experimental Methods

Synthesis of Azide Functionalized BBP [Poly(2-(2-Bromoisoctyl)oxy)ethyl Methacrylate)-graft-Poly(2-(2-(2-Methoxyethoxy)ethoxy)ethyl Methacrylate) (PBiBEM-g-PMEO₃MA-N₃)]

Figure S11. Synthesis of PBiBEM-g-PMEO₃MA-N₃ (N₃-BBP) by atom transfer radical polymerization (ATRP)

A poly[2-(2-bromoisoctyl)oxy]ethyl methacrylate] (PBiBEM) macroinitiator with a degree of polymerization in the backbone of 400 = m (Figure S10) was synthesized by following a previously reported procedure. 2-(2-methoxyethoxy)ethyl methacrylate) (MEO₃MA), used as monomer, was polymerized from PBiBEM macroinitiator by ATRP via a grafting-from method using Cu(I)Br, Cu(II)Br₂, and 4,4'-diononyl-2,2'-dipyridyl(dNbpy). The initial ratio of reagents in the grafting-from reaction was [MEO₃MA]/[BiBEM]/[Cu(I)Br]/[Cu(II)Br₂]/[dNbpy] = 500/1.0/0.9/0.1/2.0. MEO₃MA (4.0 g, 17 mmol), PBiBEM (0.0096 g, 0.034 mmol of bromine initiating groups (BiBEM)), Cu(II)Br₂ (0.00077 g, 0.0034 mmol), dNbpy (0.028 g, 0.069 mmol), and 40 % (v/v) anisole were added to a 25 mL Schlenk flask equipped with a stir bar. The mixture was degassed via three freeze-pump-thaw cycles, and then Cu(I)Br (0.0044 g, 0.031 mmol) was added when the solution was frozen during the final cycle. The flask was sealed and placed in an oil bath at 25 °C. Conversion was analyzed by gas chromatography (GC) using anisole as the internal standard. The polymerization was stopped after 6 h and the flask was opened to air. The solution was passed through a column of neutral alumina, and then precipitated into hexanes three times. Finally the resulting polymer was dialyzed against three changes of THF for 2 days. The polymer was dried under vacuum at room temperature for 24 h providing PBiBEM400-g-PMEO₃MA180 with a degree of polymerization in the side chain of MEO₃MA = 180 estimated by GC. A Schlenk flask was charged with PBiBEM400-g-PMEO₃MA180 (1.0 g, 0.088 × 10⁻³ mmol), sodium azide (0.023 g, 0.35
mmol), and 10 mL DMF. The reaction mixture was stirred at room temperature for 48 h. The solution was dialyzed against four changes of DMF for 3 days to remove excess sodium azide, and then the DMF solvent in dialysis was replaced with THF. The solution was evaporated and dried under vacuum at room temperature for 24 h. The polymer was analyzed by $^1$H-NMR spectroscopy in CDCl$_3$, gel permeation chromatography (GPC) in THF, and FTIR spectroscopy providing PBiBEM400-g-PMEO$_3$MA180-N$_3$: $\text{Mn} = 8.6 \times 10^6$, $\text{Mw/Mn}= 1.29$, and 2110 cm$^{-1}$ ($\text{-N=N+=N-}$).

**Oligonucleotides**

DNA sequences with 5' terminal hexynyl and 5' Cy5 modifications were synthesized using solid phase oligonucleotide synthesis on a MerMade-4 synthesizer (Bio-automation, Plano, TX, USA). Commercially available starting materials were used without further purification. Phosphoramidites (dA, dC, dG and T) with labile PAC protecting groups and appropriate reagents were purchased from ChemGenes (Wilmington, MA USA) and Glen Research (Sterling, VA USA). Synthesis and de-protection of the oligonucleotides were conducted under standard protocols for PAC-protected amidites, as recommended by the manufacturers. The DNA synthesis columns were purchased from Biosearch Technologies, Inc (Novato, CA USA). 5'-amino-modified DNA and any unmodified DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. and obtained as lyophilized powders.
Oligonucleotide Sequences (Underline indicates the complementary regions.)

**Hexynyl-A** - 5’-Hexynyl- GCA CT GCA GTT GGA TCC CAT AGC-3’ (23 mer)

\[ \text{Hexynyl-B} - 5’-\text{Hexynyl-} \underline{\text{GCT AT CCA TCA GAA TTC GCG ACG}} -3’ \text{ (23 mer)} \] - completely complementary to Seq.B' 

\[ \underline{\text{A}_{\text{comp}}} - 5’-\text{ATC GA GCT ATG GGA TCC AAC TGC} -3’ \text{ (23 mer) (IDT)} \]

\[ \underline{\text{Cy5-A}_{\text{comp}}} - 5’-\text{Cy5- ATC GA GCT ATG GGA TCC AAC TGC} -3’ \text{ (23 mer)} \]
**NH₂-A'** - 5'-NH₂-GCT ATG GGA TCC AAC TGC AGT GC -3' (23 mer) (IDT) - completely complementary to Seq. A

![NH₂-A'](image)

**NH₂-B'** - 5'-NH₂-ATCGA CGT CGC GAA TTC TGA TGG ATA GC-3' (IDT) - completely complementary to Seq. B

![NH₂-B'](image)
Click Conjugation of Hexynyl-A to BBP-N₃

BBP-N₃ (4 mg) was dissolved in 100 µL of deionized (18.0 MΩ) H₂O to give a 1 mM azide stock solution. A lyophilized powder of hexynyl-A was dissolved in H₂O to obtain a 500 µM hexynyl stock solution. The BBP-N₃ (10 µL, 1 mM azide) and hexynyl-A (20 µL, 500 µM) solutions were mixed and degassed several times by blowing with argon, to remove any dissolved oxygen. Acetonitrile (1.5 µL, 20% ACN in H₂O V/V) and a freshly prepared solution of sodium ascorbate prepared in deionized H₂O (8 µL, 100 mM) were mixed and degassed in a separate vial. The two solutions were added to 4.5 µL of degassed deionized H₂O and the final pH and salt concentration were brought to pH = 7.5 and 100 mM Na⁺ with 5 µL of 10 X PBS buffer. The solution was mixed thoroughly and degassed. A degassed solution of CuSO₄ (1 µL, 100 mM) was added to initiate the reaction and was allowed to run for 3 hours at room temperature with gentle shaking. The DBBP product was purified with a 30 kDa molecular weight cutoff filter (Millipore) and dissolved in PBS buffer (pH = 7.5, Na⁺ = 100 mM). The DNA concentration was calculated using the UV absorbance at 260 nm using a NanoDrop UV/Vis spectrometer. The final DBBP product was stored in a -14 °C freezer to be used later. For solution phase FTIR experiments the isolated DBBP from the molecular weight cutoff filter was dissolved in deionized water instead of PBS buffer.
Click Conjugation to Achieve Varied Loading of SeqA and PEO Loading on BBP

PEO-alkyne

![PEO-alkyne](image)

**Figure S12.** Structure of PEO-alkyne and synthesis of DBBPs with low loading of DNA

**Synthesis of A-BBP with 40% Saturation of Azide Terminating Side Chains**
The general procedure for the conjugation of hexynyl-DNA to BBP-N₃ described on page S21 was followed by using 40% as much hexynyl-A DNA (8 µL, 500 µM stock).

**Synthesis of PEO/A-BBP with 40% Hexynyl-A and 60% PEO-alkyne by Sequential Click Conjugation**
An alkyneterminated poly(ethylene oxide)(PEO-alkyne) synthesized using a previously reported procedure² was used for the click reaction. PEO-alkyne (2.08 mg) was dissolved in 5 mL of deionized water to make a 200 µM stock of alkyne. Azide from BBP-N₃ (10 µL, 1 mM) and hexynyl-A (8 µL, 500 µM) were mixed and degassed several times to remove any dissolved oxygen. Acetonitrile (1.5 µL, 20%
ACN in H$_2$O V/V) and a freshly prepared solution of sodium ascorbate prepared in deionized H$_2$O (8 µL, 100 mM) were mixed and degassed in a separate vial. The two solutions were added to 1.5 µL of degassed deionized H$_2$O and the final pH and salt concentration were brought to pH = 7.5 and 100 mM Na$^+$ with 5 µL of 10 X PBS buffer. The solution was mixed thoroughly and degassed. A degassed solution of CuSO$_4$ (1 µL, 100 mM) was added to initiate the reaction and was allowed to run for 1 hour at room temperature with gentle shaking. After 1 hour a degassed solution of PEO-alkyne (15 µL, 200 µM) was added and the reaction was allowed to run for two more hours. The DBBP product was purified using molecular weight cutoff filters.

**Synthesis of A/B-BBP with 99.5% Hexynyl-A and 0.5% Hexynyl-B**

![Figure S13](image)

**Figure S13.** Sequential click conjugation method to synthesize DBBPs with Seq.A: Seq.B in 199:1 ratio.

The sequential click conjugation method (Figure S13) was followed using BBP-N$_3$ (10 µL, 1 mM azide), hexynyl-B (5 µL, 10 µM) and hexynyl-A (19.9 µL, 500 µM).

**Hybridization of DNA Duplexes and YOYO-1 Intercalation**

![Figure S14](image)

**Figure S14.** Hybridization of an equimolar mixture of complementary DNA to give double stranded DNA on BBP followed by intercalation of YOYO-1
An equimolar mixture of Seq.A from DBBP and Cy5-A_{comp} were mixed together in buffered aqueous solution (PBS buffer, pH = 7.5, 100 mM Na^{+}) to give a 100 nM final concentration of duplex DNA. Nonbrush DNA duplexes were prepared by mixing seq. A and Cy5-A_{comp} in an equimolar ratio in buffered solution to give the same final concentration of duplex DNA. The same step was followed to prepare brush and nonbrush duplex DNA without Cy5 dye by using A_{comp} in place of Cy5-A_{comp}. The vials were incubated in 90 °C water bath for 2 minutes and quickly transferred to a water bath at 65 °C. After incubating for 15 minutes at 65 °C the samples are cooled down to 25 °C within a period of 1 hour. The DBBP samples with 40% loading of seq. A were mixed with equimolar ratio of Cy5-A_{comp} and A_{comp} separately in buffered solutions to give double stranded DBBPs with low loading of DNA.

YOYO-1 (Life Technologies-Molecular Probes) was added to 500 nM final concentration to 100 nM pre-annealed DNA duplex and mixed for one minute before taking fluorescence measurements.

**Preparation of Antibody-DNA Conjugates**

Antibody-DNA conjugation (Figure S15) was performed using the Solulink protein-oligo conjugation kit (catalog S-9011-1, Solulink) which uses bisaryl hydrazone conjugation chemistry. Antibody and DNA modification reactions were optimized to obtain a single DNA strand conjugated per antibody. Prior to functionalization, affinity-purified, unlabeled goat-antimouse IgG antibody (Jackson Immunoresearch) was desalted using MicroSpin G-50 columns (GE Healthcare) and buffer exchanged (modification buffer, 100 mM phosphate, 150 mM NaCl, pH 8) using molecular weight cutoff filters (50 kDa, Millipore Corporation) to bring the final protein concentration to 2.9 mg/mL. 1.1 mg of SANH (succinimidyl 6-hydrazinonicotinamide acetone hydrazine) reagent provided in the kit was dissolved in 150 µL of N,N-dimethylformamide (DMF). The SANH solution and the desalted, buffer-exchanged antibody was mixed in a 5:1 (SANH:antibody) molar ratio. Separately 1.7 mg of Hydralink SFB (Succinimidyl 4-formylbenzoate) reagent provided in the kit was dissolved in 80 µL of DMF. 5’-aminated DNA (NH2-B’, Integrated DNA Technologies, Inc.) was dissolved in modification buffer to make a 1 mM stock solution. The SFB reagent and the NH2-B’ was mixed in a 5:1 (SFB:DNA) molar ratio. Both antibody and DNA coupling reactions were performed at room temperature for 2 hours on a gently rotating shaker. Excess SANH and SFB were
removed using MicroSpin G-50 and G-25 columns, respectively. The modified antibody and the DNA were dissolved in conjugation buffer (100 mM phosphate, 150 mM NaCl, pH 6). Molar substitution ratio (MSR) assays were performed using the standard protocols provided by the vendor to determine the number of modifications per antibody and DNA strand (Supplementary Tables S1 and S2). SFB-derivatized DNA was then combined with the SANH-derivatized antibody in 1:5 ratio and allowed to react overnight at room temperature on a gently rotating shaker. Unreacted DNA was removed using 50 kDa filters. Fractions from each filtration round were tested for presence of unbound free DNA (A$_{260}$ using NanoDrop spectrophotometer). The conjugation of antibody-DNA was verified spectrophotometrically, by the presence of the bisaryl hydrazone linkage ($\lambda_{\text{max}} = 354$ nm) formed between SANH and SFB (Figure S4).

![Diagram](image1)

**Figure S15.** Synthesis of Ab-B'. (a) Functionalization of antibody with succinimidyl-6-hydrazinonicotinate acetone hydrazine (SANH) (b) Functionalization of NH$_2$-B' with succimidyl-4-formylbenzamide (SFB). (c) Formation of UV traceable bisaryl hydrazone linkage between antibody and B'. (d) UV trace showing the peak at $\lambda_{\text{max}} = 354$ nm confirming the formation of bisaryl hydrazone link.
Determination of Antibody:DNA Ratio
To quantitatively determine the number of DNA strands conjugated per antibody, a calibration experiment was conducted. 20 µL solutions with various ratios of antibody to DNA were mixed as follows: 1:0 (4 µM and 0 µM, respectively), 1:0.5 (4 µM and 2 µM, respectively), 1:2 (4 and 8 µM, respectively), 1:4 (4 and 16 µM, respectively), and 1:5 (4 and 20 µM, respectively).(Supplementary Table S3) Using the NanoDrop spectrophotometer, the $A_{260\text{ nm}}/A_{280\text{ nm}}$ absorbance ratios were determined and recorded for each solution. The obtained calibration curve was then used to determine the number of DNA strands per antibody (Supplementary Figure S5). This procedure accounts for the fact that the absorbance of the DNA (260 nm) overlaps with the absorbance of the antibody (280 nm). For the Ab-B′ conjugates reported here, an average of one DNA strand was attached per antibody.

Antibody-DBBP Hybridization
DBBP with Seq. B: Seq A in a 1:199 ratio was first hybridized with Cy5-A_comp by mixing equimolar amount of Cy5-A_comp and Seq. A on DBBP in PBS buffer (100 mM Na+, pH 7.5) and annealing. The resulting DBBP should have, on average, one unhybridized Seq.B strand available for hybridization to the antibody functionalized with its complement (Ab-B′). Antibody and DBBP (10 µM each final concentration) were mixed and allowed to hybridize at 25 °C overnight on a gently rotating shaker. The antibody-conjugated DBBP (10 µM) was stored at 4 °C until further use.

Labeling and Detection of c-myc Targets of Yeast Cells with Antibody-DBBP Nanotag
Yeast cells expressing c-myc-tagged scFv (10^7 cells) were suspended in calcium- and magnesium-free PBS wash buffer (pH =7.5, Na^+ = 100 mM, 1µg/mL Pluronic® F-127). The cells were incubated with 0.5 µM final concentration of anti-c-myc mouse antibody for half an hour at 4 °C, washed three times with 500 µL of wash buffer, and resuspended in 500 µL of the same buffer. The cells were incubated with 50 nM final concentration of goat anti-mouse antibody-DBBP complex for 30 minutes at 4 °C. The cells were washed three times with 500 µL wash buffer. YOYO-1 was added to give a 50 µM final concentration in a total volume of 500 µL PBS buffer (pH = 7.5, Na^+ = 100 mM, 1 µg/mL Pluronic® F-127). Similarly, 50 nM final concentrations of Alexa 647-tagged goat anti-mouse IgG (H+L) (Life Technologies-Molecular Probes) and Qdot® 655 tagged goat anti-mouse IgG (H+L) (Life Technologies) were separately used as controls. Labeled cells were analyzed by fluorescence activated cell sorting (FACS) using a Coulter Epix Elite flow cytometer (Beckman-Coulter, Fullerton, CA). The following
dichroic lenses (DL)/band-pass (BP) filters were used: 550DL/530BP for YOYO-1-donor fluorescence channel, and 720DL/695 BP for Cy5-acceptor fluorescence channel. Further, a Carl Zeiss LSM 510 Meta DuoScan Inverted Spectral Confocal Microscope was used for fluorescence imaging analysis at 100x objective magnification. Yeast cell samples were prepared according to the yeast cell fluorescent staining protocol outlined above, except the final volume of each sample was 60 µL of PBS wash buffer (pH =7.5, Na⁺ = 100 mM, 1 µg/mL Pluronic® F-127). 20 µL of the cell suspension was placed on a 35 mm glass bottom micro well dish (Mattek, part no. P35G-1.5-14-C). The micro well plates for yeast cells were treated with concanavalin A. Enough wash buffer was added to fill the micro well before viewing under the microscope. The pin hole opening was set to the minimum setting and the tube current was set to 0.6 A for imaging. Raw images were collected using ZEN 2009 software without applying any digital enhancements.

Labeling and Detection of Biotin Targets on Polystyrene Beads by Antibody-DBBP Nanotag

Biotin-coated polystyrene beads (10⁶ beads) were suspended in 60 µL total volume of calcium- and magnesium-free PBS (pH = 7.5, Na⁺ = 100 mM, 0.02% Triton X-100) buffer. The beads were incubated with 0.5 µM final concentration of anti-biotin mouse IgG(H+L) (Jackson Immunoresearch) for half an hour at 25 °C, washed three times with 500 µL of wash buffer, and resuspended in 500 µL of the same buffer. The beads were incubated with 50 nM final concentration of goat anti-mouse antibody-DBBP complex for 30 minutes at 25 °C. The beads were washed three times with 500 µL wash buffer. YOYO-1 was added to give a 50 µM final concentration in a total volume of 500 µL PBS buffer (pH =7.5, Na⁺ = 100 mM, 1µg/mL Triton X-100). Similarly, 50 nM final concentrations of Alexa 647-tagged goat anti-mouse IgG (H+L) (Life technologies-Molecular Probes) and Qdot® 655 tagged goat anti-mouse IgG (H+L) (Life technologies) were separately used as controls. Samples were allowed to incubate for 30 minutes at 25 °C and washed with 150 µL PBS. Samples were resuspended in 500µL PBS. Labeled beads were analyzed by fluorescence activated cell sorting (FACS) using a Coulter Epix Elite flow cytometer (Beckman-Coulter, Fullerton, CA). The following dichroic lenses (DL)/band-pass (BP) filters were used: 550DL/530BP for YOYO-1 donor fluorescence channel, and 720DL/695 BP for Cy5 acceptor fluorescence channel. Further, a Carl Zeiss LSM 510 Meta DuoScan Inverted Spectral Confocal Microscope was used for fluorescence imaging analysis at 100x objective magnification. Bead samples were prepared according to the microbead fluorescent staining protocol outlined above, except the final volume of each sample was 60 µL of PBS wash buffer (pH = 7.5, Na⁺ = 100 mM, 1 µg/mL Triton X-100). 20 µL of the bead suspension was placed on a 35 mm glass bottom microwell dish (Mattek, part no. P35G-1.5-14-C). Enough wash buffer was added to fill the microwell before viewing under the microscope. The pin hole opening was set to the minimum setting and the tube current was set to 0.6 A.
for imaging. Raw images were collected using ZEN 2009 software without applying any digital enhancements.

**Dot Blot Experiments**

Maltose-binding protein was blotted on a pure nitrocellulose transfer and immobilization paper (pore size 0.45 µm, Perkin Elmer) in increasing amounts. (spots of 0.5 ng to 25 ng in duplicate to get two rows) and was allowed to dry for 30 mins at 25 °C. Non-specific binding was inhibited with blocking buffer (5% milk) for 1 hour at 25 °C. The 5% milk was prepared in Tris-buffered saline and Tween (TBST) buffer (pH = 8.5). The membrane was washed three times using the TBST buffer to get rid of any unbound protein and excess milk. 1 µL of the anti-MBP monoclonal antibody-HRP conjugate (New England Biolabs) was added to 1 mL of 5% milk (1:1000 dilution) and the membrane was allowed to be covered in this solution containing the primary antibody overnight at 4°C with gentle shaking. The membrane was washed in 25 mL TBST for 5 minutes with gentle shaking, 3 times for 2 minutes each. A 1:1000 dilution was made with goat anti-mouse IgG-Alexa 647 conjugate or the goat anti-mouse IgG-DBBP-nanotag with 1 ml TBST to get similar concentrations of the secondary antibody (final concentration = 2 µg/mL = ca. 10 nM). The membrane was incubated in the solution for 3 hours. The washing steps were performed in triplicate with TBST and TBS buffers. The membrane was dried and was imaged using the Typhoon FLA 9000 scanner. YOYO-1 was excited using the excitation wavelength at 473 nm at 250 V and the direct excitation of Cy5 and Alexa 647 was performed at 650 nm at 250 V. The blots were scanned to get the emission at 695 nm.

**References**

1. Beers, K. L.; Gaynor, S. G.; Matyjaszewski, K.; Sheiko, S. S.; Moeller, M., *Macromolecules*, **1998**, 31, 9413–9415
2. Gao, H., Min, K.; Matyjaszewski, K., *Macromol. Chem. Phys.*, **2007**, 208, 1370–1378
3. Protein-Oligonucleotide Conjugation Kit, Technical Manual, Catalog S-9011-1,
   [http://www.solulink.com/library](http://www.solulink.com/library)