Supplemental information

Primed template for nanoparticle construction

Designed oligos purchased from IDT were reconstituted in dH_2O to a final concentration of 100uM. Oligo sequences were as follows:

Primer 1 - 5'-

GCC**A**GGGTGCG**A**GGGTTTGTTTC**A**TTGCTTC**A**CGCCCTT**A**CCCTCGC**A**CCCTG

GC**A**CGG

Primer 2 - 5'-TCCC**A**CGGTGGC**A**CCTCGC**A**CCT

Primer 3 - 5'-CGC**A**CGCTGCC**A**CCCTCGCTTTTGCG**A**GGGTGGC**A**GCGT

Primer 4 - 5'-GCG**A**GGTGCG**A**GGTGCC**A**CCGTGGG**A**CCGT

Extension Primer - 5'-AAGGGCGTGAAGCAATGA

Note that the underlined A's indicate sites that when amplified by rolling circle amplification will have 5-aminohexylacryomide-dUTP (Aha-dUTP, Invitrogen) incorporated.

Submix 1: Water (187.5uL), Tris-Cl (12.5uL, 1M pH 7.6) and Primer 1 oligo (50uL, 100uM) were added together. Submix 2: Water (375uL), Tris-Cl (25uL, 1M pH 7.6), Primer 2 oligo (50uL, 100uM) and Primer 4 oligo (50uL, 100uM) were added together. Submix 3: Water (187.5uL), Tris-Cl (12.5uL, 1M pH 7.6) and Primer 3 oligo (50uL, 100uM) were added together. Each submix was aliquotted into 100uL incubated at 95°C for 30 seconds, ramp temperature down to 50°C at 0.1°C/s, hold at 4°C. When incubation was completed, the three submixes were combined to obtain the final mix. Ligation of the rolling circle template then proceeded as follows. T4 ligase buffer (200uL, 10X, NEB), mixed oligos (200uL, 5uM) and water (1500uL) were added

together and mixed. T4 DNA ligase (100uL, 400,000u/mL, NEB) was added to mixture. Solution was aliquotted into a plate and incubated at 20°C for 20hrs, 65°C for 10min. Solution was pooled together and the Extension Primer was added. Mixture was then aliquotted into a plate again and incubated 70°C for 30seconds, ramp down to 40°C at 0.1°C/sec, hold at 4°C. This final anneal step was done by aliquotting into 100uL sized aliquots. This template was stored at -20°C until ready to use. (Figure Supplemental 1)

Rolling circle amplification for Nanoparticle construction

Water (969uL), phi29 reaction buffer (150uL, 10X, NEB), Dithiothreitol (DTT) (15uL, 500mM, Pierce), BSA (15uL, 100X, NEB), dGTP, dATP, dCTP mix (0.75uL, 20mM each nucleotide, NEB) and 5-aminohexylacryomide-dUTP (aha-dUTP) (0.3uL, 20mM, Invitrogen) were all added together and vortexed until mixed completely. The primed template (300uL, 0.5uM) was added and vortexed. The polymerase phi29 (50uL, 10kU/mL, NEB) was added to the pre-mix and mixed by inversion. Reaction was distributed evenly in a 96-well plate (63uL per well). Sample was incubated at 30°C for 2 hours, then 65°C for 10 min. Sample was pooled and EDTA (90uL, 250mM, Sigma) was added. Sample was centrifuged at 12,500G's for 5min at 4°C. Supernatent was recovered, and the white pellet was discarded. 20uL of sample was taken and run on a 0.8% E-gel® (Invitrogen) to verify nanoparticles were successfully made. Nanoparticle band seen was a smear between 1-3kb. The nanoparticles were then stored at -20°C until ready for use.

Sucrose purification of DNA nanoparticle

Nanoparticles from previous step were dialyzed against a low concentration of TE. The initial volume of nanoparticles determined the concentration on TE needed. For example, a 0.07X TE was used, 2X 5L using a 10,000MWCO Slide-A-Lyzer® from Pierce. Sample was removed from slide and concentrated to 800uL *in vacu.*

A sucrose gradient of 5-20% sucrose in 1X TE was performed to further purify the nanoparticles. The gradient was prepared using a Gradient Master SW50. The samples were added to the sucrose and using a Beckman Optima MAX tabletop ultracentrifuge, the samples were centrifuged for 2 hours at 4°C at 50,000G's. Fractions of 1mm were taken using a BioComp piston gradient fractionator. Fractions were analyzed using a 0.8% E-gel® and the fractions with sizes between 2-4kb were pooled together. Samples were dialyzed against water, 2X 5L using a 10,000MWCO Slide-A-Lyzer® from Pierce. The nanoparticles were concentrated *in vacu* to an approximate volume of 200uL. Concentration of nanoparticles was calculated using A260 information.

dCTP-PEG8-amine (structure 4)

This method describes the labeling of the nucleotide triphosphate dCTP (Figure Supplement 2). The remaining three nucleotide reactions were done using the same protocol.

BOC-amino PEG8 amine **(structure 1)** (2g) purchased from PolyPure (Oslo, Norway), was added to a trifluoroacetic acid/chloroform solution (1:1 v/v, 20mL total). Reaction was stirred at room temperature for 2 hours. $NaHCO_3$ (10mL, 10mM) was slowly added to the reaction. The aqueous layer was separated from the organic layer and retained. Chloroform was added and two extractions (20mL each) were performed. To the aqueous

layer, ethyl ether (20ml) was added and the extraction was performed twice. The aqueous layer was dried *in vacuo*. The final product was a clear oily residue, and weighed approximately 1 gram. Yield was estimated at less than 100% due to possible salts and water still present in sample. ^{1}H NMR, D2O: δ 3.675 (t, 4H), 3.61 (m, 24H), 3.1 (t, 4H). ESI MS [M+1] calculated 369.3. Observed [M+1] 369.2.

A total of 13.3mg dCTP **(structure 3)** from Sigma (24.8µmol, 1equiv) and 190.6mg 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) from Aldrich (992µmol, 40equiv) were added together in 500mM MES buffer pH 5.8, resulting in concentrations of 79mM and 3.2M respectively. The reaction proceeded at room temperature for 10 minutes. A PEG8-diamine **(structure 2)** solution (750mM, 186µmol, 7.5equiv) in 500mM MES buffer, pH adjusted to 6 with 5M KOH was added to the dATP/EDC (248uL). The reaction proceeded at room temperature overnight. The large excess of reagents and overnight reaction converted the entire amount of starting material to desired product. The product was first purified on a HiPrep™ DEAE 20mL column from Amersham with buffer A and B. Buffer A: 10mM phosphate+20% Acetonitrile (ACN)(Fisher Scientific) and buffer B: Buffer A in 1M NaCl. LC conditions: 0-10min 0%B, 10-15min 0-100%B, 15-20min 100%B at a flow rate of 10mL/min. The free PEG eluted from the column in void volume. The nucleotide conjugate product eluted as a broad peak at 5-10minutes. This broad peak was collected and dried *in vacu.* The product was further purified on a 30x250mm 10um Inertsil ODS-3 column from Varian , with a new buffer system A and B, where buffer A was 100mM Triethylaminoammonium acetate (TEAAc) (pH 6.6) with 4%ACN and buffer B was 100mM TEAAc (pH 6.6) with 80% acetonitrile. LC conditions: 0-5min 0-15%B, 5-

10min 15-20%B, 10-20min 20-100%B with a flow rate of 20mL/min. The product that eluted at 12minutes was collected and then dried *in vacu*. Yield averages 30% after purification steps. ^{1}H NMR d2O: δ 8.52(s,1H), 8.27 (s, 1H), 6.5 (t, 1H), 4.3 (t, 1H), 4.2 (m, 1H), 4.1 (m, 1H), 3.8(m, 29H overlaid with contaminant), 3.2 (t, 2H + contaminant), 3.1 (m, 2H+ contam.), 2.8 (m, 1H), 2.6 (m, 1H). Contaminants present in sample were TEAAc and glycerol. This particular sample was only used for NMR, no further synthesis was performed with this particular lot. ESI-MS [M+1] calculated 842.25. Observed [M+1] 842.3

Amino acid bridge attachment to modified triphosphate

FMOC-Cys(tButhio)-OPfp **(structure 5)** (Calbiochem) was dissolved in Dimethyl sulfoxide (DMSO) to a final concentration of 20mM. dCTP-PEG8-amine **(structure 4)** was dissolved in 500mM pH 8.3 phosphate buffer to a final concentration of 40mM. The nucleotide (1eq,18uL, 7.2e-4mmol) was added to the modified cysteine solution (2eq, 72uL, 1.44e-3mmol). Upon addition of the nucleotide, reaction was cloudy. Once mixed, reactants solubilized. Reaction monitored by C18 HPLC. Normal reaction times are only a few minutes (Figures Supplemental 3 and 4).

A 20% piperidine solution in DMF was added to reaction mixture (50uL). This was allowed to react for 10minutes, room temperature. A new peak was seen at an earlier retention time. Compound was isolated by C18 HPLC. Buffer A: 100mM TEAAc, 4% ACN. Buffer B: 100mM TEAAc, 80% ACN pH 6.6-6.8. Product eluted at 9 minutes with a gradient elution of 0-100%B in 15min, 3mL/min. Column used was Inertsil ODS-3 10x250 10um. Compound was dried *in vacu.* Compound was then reconstituted in

20uL pH 7, 1mM phosphate buffer and quantitated using the corresponding extinction coefficient of the nucleotide. Average yield after purification was 60%. ESI-MS [M+1] calculated = 1009.3. Observed [M+1] = 1009.4.

SYBR®101-SE conjugation with modified nucleotide triphosphate

500mM phosphate pH 8.1 buffer was added to dCTP-PEG8-cys(tButhio)-amine for a final concentration of 10mM. SYBR®101-Succinimidyl Ester (SYBR 101-SE) (Invitrogen, 1.7eq, 12.5uL, 2.125e-4mmol) was added to the nucleotide solution (1eq, 12.5uL,1.25e-4mmol) (Figures Supplemental 3 and 4). The reaction was allowed to react for 1.5hrs at room temp, in the dark. Reaction monitored and isolated by C18 HPLC. Buffer A: 100mM TEAAc, 4% ACN. Buffer B: 100mM TEAAc, 80% ACN pH 6.6-6.8. Product eluted at 13.7 minutes with a gradient elution of 0-100%B in 15min, 3mL/min. Column used was Inertsil ODS-3 10x250 10um. Compound was dried *in vacu.* Product was reconstituted in MeOH and quantitated using the extinction coefficient for SYBR®101 of 57,000 $cm^{-1}M^{-1}$. Product was dried and stored at -20°C until ready for next step. Average yield after purification was 45%. ESI-MS of deprotected material (reduced form) [M+1] calculated=1384.4. Observed [M+1] = 1384.3, [M+2] 692.8 also seen.

Derivatization of amino-nanoparticle to thiol reactive nanoparticle

The derivitization should be done immediately prior to the addition of the dye. The preparation of the dye was done concurrently with this step. N-Succinimidyl

iodoacetate (SIA) **(structure 8)** (27.1uL, 100mM, Pierce) was dissolved in DMSO and added to a sucrose purified nanoparticle solution **(structure 9)** (120uL, 80ng/uL) along with a 400mM sodium phosphate pH 8 1.5M NaCl (18uL) (Figure Supplemental 3 and 5). Solution was allowed to react at room temperature, shielded from light for 1.5hrs. For purification of the product, a phenol extraction was conducted. TE saturated phenol (Fluka) was added in a 1:1 ratio of the reaction (~170uL). The solution was mixed and centrifuged down at >10,000G's for 3min. The aqueous layer was removed and a chloroform extraction, 3X 200uL was conducted. A final ethyl ether extraction was conducted to remove any residual chloroform. The solution was cloudy upon addition of ether, but then cleared upon spinning. This solution was stored under argon until dye was added.

Covalent attachment of nucleotide-dye conjugates to nanoparticle

The dye conjugate must first be activated by reducing the disulfide linkage. A nucleotide mixture using all four conjugated nucleotides was first prepared by resuspending 10nmol of each nucleotide in 8 uL ea in a 1:1 DMSO, 50mM phosphate pH 7.4 0.9%NaCl buffer. The nucleotides were pooled together and DTT (8uL, 500mM, Pierce) was added. Reduction of compound was completed in 1 hour at room temperature, shielded from light. The sample was purified using an ethyl acetate extraction 4X100uL, then a subsequent ethyl ether extraction 1X200uL. The sample was placed under argon and flushed until the residual ether had evaporated. DMSO was added to a final volume of 40uL, yielding an approximate total nucleotide concentration of 1mM. The nucleotides were in the reduced form and ready for the coupling reaction. The derivatized

nanoparticle **(structure 10)** (166uL) was added to the activated dye conjugate solution **(structure 7)** (25.4uL) (Figure Supplemental 3 and 5). The reaction incubated at room temperature for 2hrs, shielded from light.

A mercaptoacetic acid solution was prepared and used to treat or "cap" the unreacted iodoacetyl groups on the nanoballs. A 350mM mercaptpacetic acid (1.22µL, d=1.325g/mL, Aldrich) in 50mM phosphate pH 7.4 0.9%NaCl buffer was prepared. The mercaptoacetic acid solution was added to the nucleotide conjugated nanoparticle at a final concentration of 5mM (2.8µL of 350mM mercaptoacetic acid solution) The reaction incubated at room temperature for 1 hour, shielded from light.

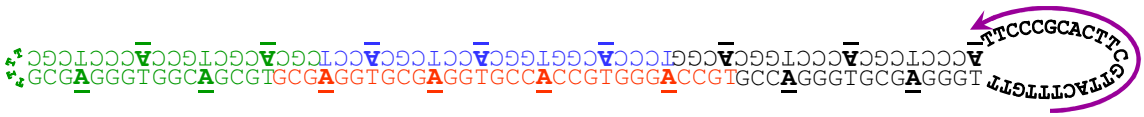
Sample was purified using a MiniElute™ PCR clean-up kit from Qiagen. Final product **(structure 11)** was eluted in 15uL of the given elution buffer. Sample was quantitated using UV data. The extinction coefficient for SYBR® is 57,000$cm^{-1}M^{-1}$ and an estimated extinction coefficient for each contributing nucleotide on the nanoparticle was 7020$cm^{-1}M^{-1}$. The design of the nanoparticle was that 1 of 8 nucleotides contained an amino-modified nucleotide. The percent labeling was calculated as followed:

(A260/7020)/8 = [possible amines for conjugation] M

A488/57000 = [SYBR-nuc] M

% labeling = ([SYBR-nuc]/[possible amines])*100

Average percent labeling seen was 30% and average percent recovery of the DNA nanoparticle was 25%.



Primer 1 – 5'-GCCAGGGTGCGAGGGTTTGTTTCATTGCTTCACGCCC
TTACCCTCGCACCCTGGCACGG

Primer 2 – 5'-TCCCACGGTGGCACCTCGCACCT

Primer 3 – 5'-CGCACGCTGCCACCCTCGCTTTTGCGAGGGTGGCAGCGT

Primer 4 – 5'-GCGAGGTGCGAGGTGCCACCGTGGGACCGT

Extension Primer -5'-AAGGGCGTGAAGCAATGA

Figure Supplemental 1: Rolling circle template construction using four primers to form the duplex DNA circle and an extension primer used to prime the reaction.

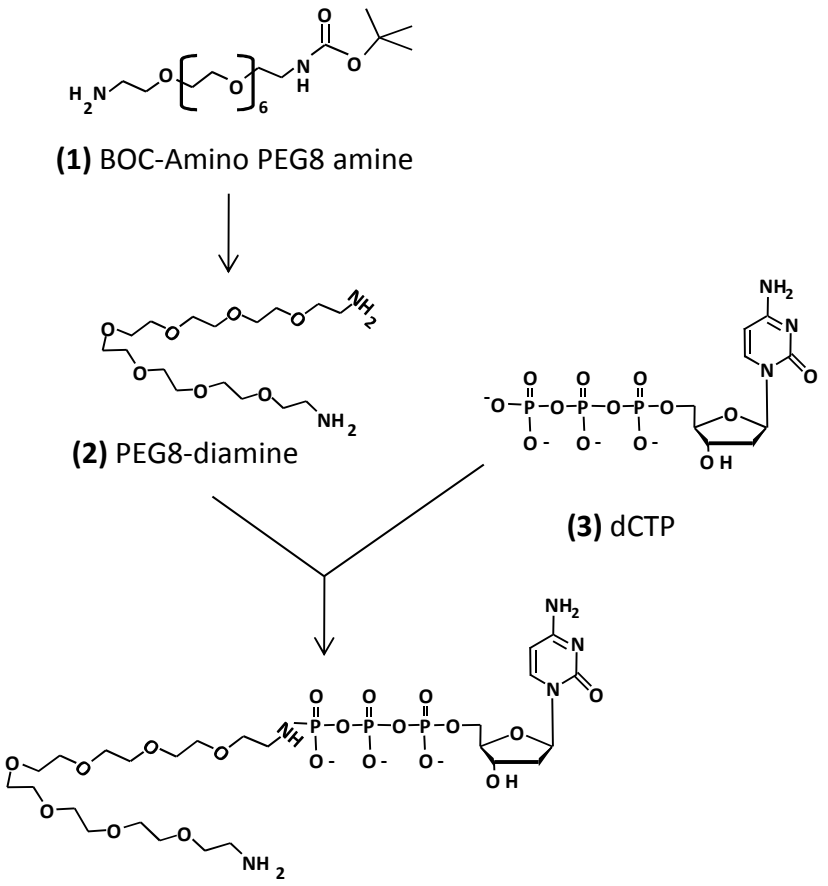


Figure Supplemental 2: Synthesis scheme for attaching a PEG8 linker to the γ-phosphate of a dNTP.

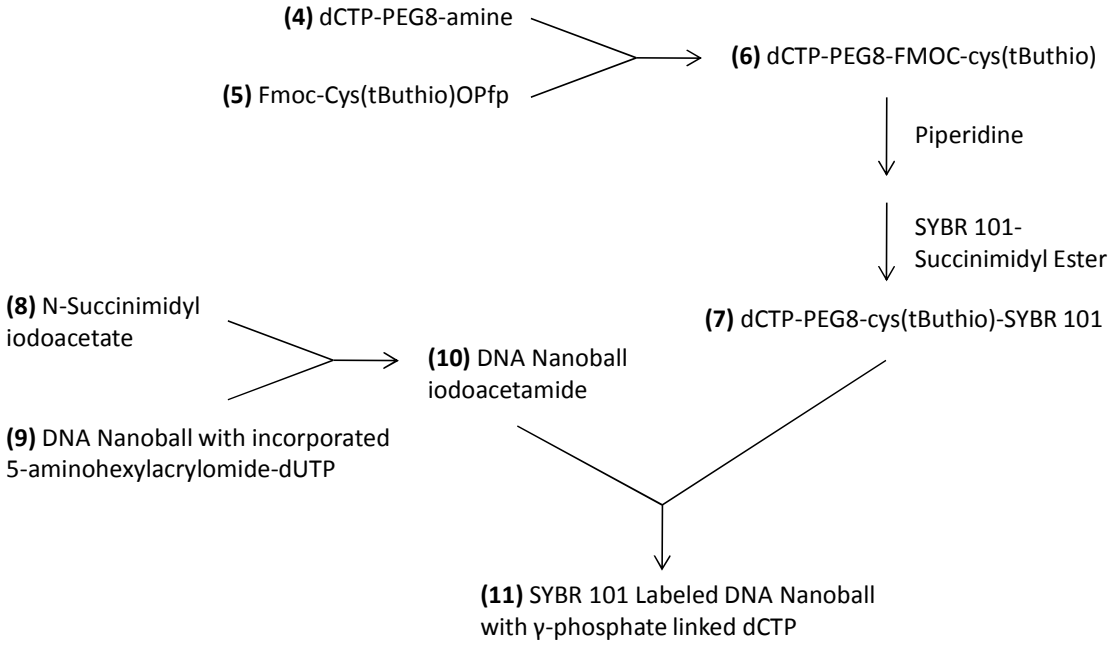
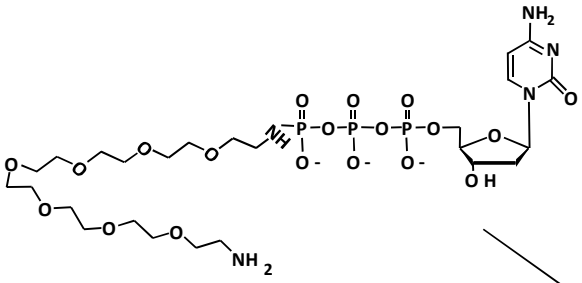
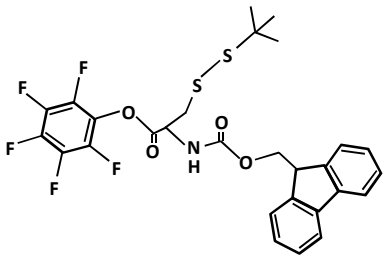


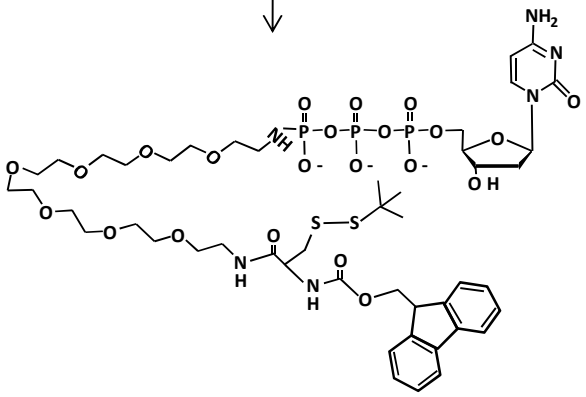
Figure Supplemental 3: Outline of nanoball synthesis.



(4) dCTP-PEG8-amine



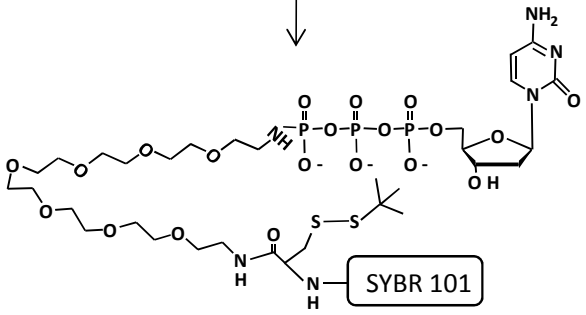
(5) Fmoc-Cys(tButhio)OPfp



(6) dCTP-PEG8-FMOC-cys(tButhio)

Piperidine

SYBR 101-Succinimidyl Ester



(7) dCTP-PEG8-cys(tButhio)-SYBR 101

Figure Supplemental 4: Synthesis of SYBR 101 attachment to a PEG8-dNTP.

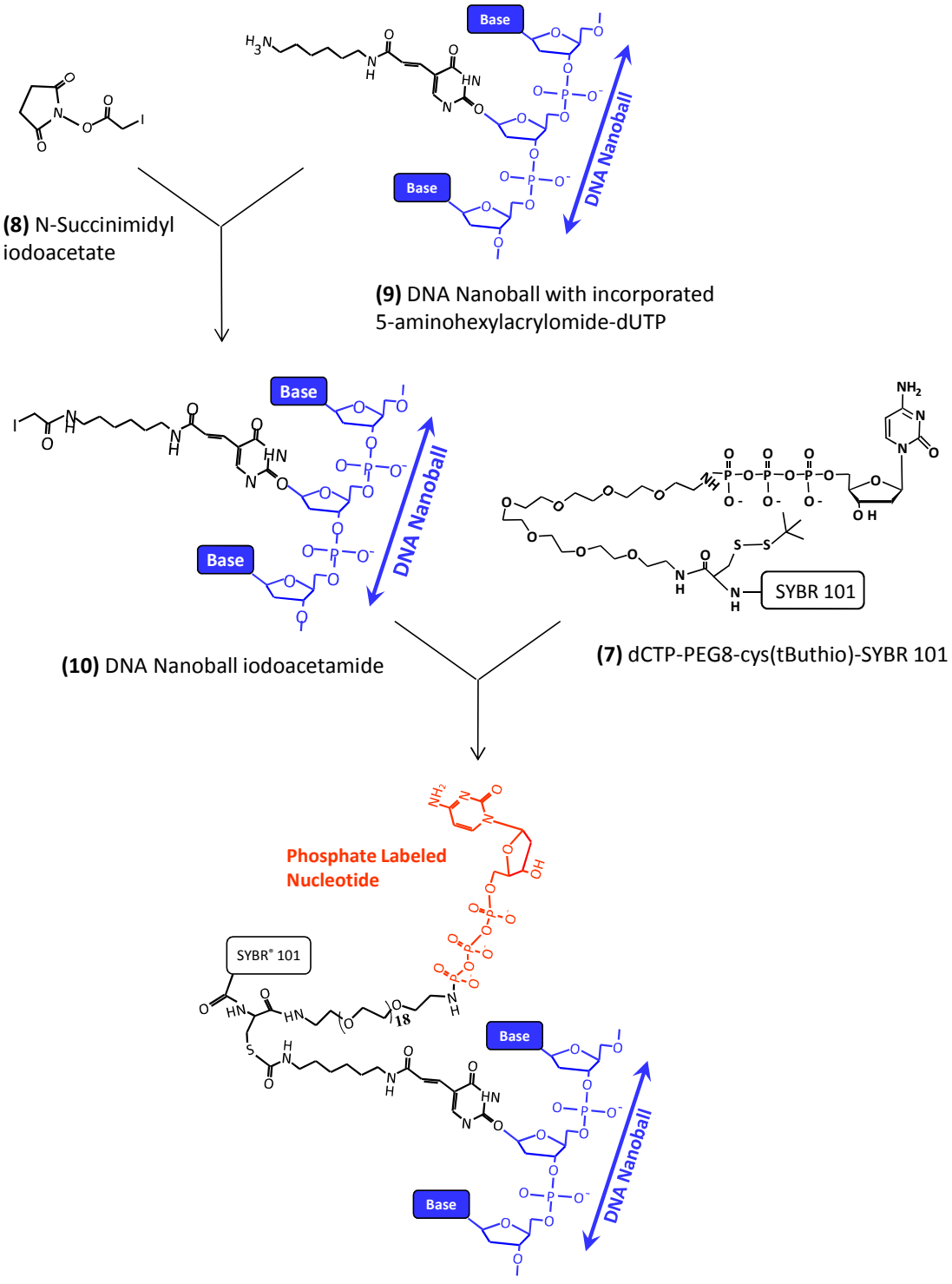


Figure Supplemental 5: Attachment of SYBR 101 labeled nucleotides to the DNA nanoballs.