Multiplexed and Reiterative Fluorescence Labeling via DNA Circuitry

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A class of reactive DNA circuits was adapted as erasable molecular imaging probes that allow fluorescent reporting complexes to be assembled and disassembled on a biological specimen. Circuit reactions are sequence-dependent and therefore facilitate multiplexed (multicolor) detection. Yet, the ability to disassemble reporting complexes also allows fluorophores to be removed and new circuit complexes to be used to label additional markers. Thus, these probes present opportunities to increase the total number of molecular targets that can be visualized on a biological sample by allowing multiple rounds of fluorescence microscopy to be performed.

INTRODUCTION

In situ imaging of molecular markers has become increasingly important to advancements in the biological and biomedical sciences (*1*−*5*). These approaches offer advantages over bulk analyses, since they allow spatially dependent expression patterns of RNA and proteins to be delineated in cells and tissues. Several antibody and nucleic acid-based fluorescent probe technologies have been developed for marker imaging, and the sensitivity of these probes has been continually improved (*5*−*8*). Yet, many contemporary cytological studies now require increasingly comprehensive molecular pathway analyses to characterize the network-level properties of cells and resolve functional relationships between cell phenotypes and their tissue distribution (*9*−*11*). In such cases, the number of markers one seeks to examine can easily exceed the number of probes that can be used simultaneously for detection due to the spectral overlap of the reporting dye molecules. Thus, various biological studies stand to benefit from methods that allow a greater number of molecular markers to be visualized.

The number of markers that can be evaluated on an individual biological sample could be increased if it were possible to remove fluorescent probes from cells such that new markers could be labeled and detected using the same fluorescent reporting molecules. However, dye molecules are typically attached covalently to probes that are engineered specifically to bind their targets with high affinity. Probe removal therefore requires the use of harsh chemical and/or physical treatments that can disrupt cell and tissue morphology and compromise subsequent marker analyses.

To overcome these limitations, we adapted a new class of DNA circuit technologies as molecular imaging probes that allow fluorophores to be sequentially coupled to, and removed from, molecular markers using exceptionally mild processing conditions. Derived from "entropically driven" circuits developed by Zhang et al. (*12*), these probes operate using principles distinct from those of fluorescence in situ hybridization (FISH) probes and molecular beacons. Whereas these latter technologies rely on more classical, sequence-dependent hybridization reaction mechanisms for target recognition, the different complexes that make up the entropically driven circuits are designed to react with one another via a process called strand-displacement: the selective exchange of individual oligonucleotides between different complexes of DNA (*13*−*16*). These reactions proceed in a sequence-dependent fashion, and hence, multiple circuits can be employed simultaneously to label different targets. Yet, the total number of matched base pairs that are formed through these reactions does not necessarily have to increase for them to proceed efficiently (i.e., reactant and product complexes can be isoenergetic). As a result, strand displacement reactions can be used to assemble, isolate, and disassemble stable intermediate-state complexes of multistep reaction cycles. Herein, we show that such capabilities can be harnessed to create erasable molecular imaging probes that can function at ambient temperatures and in mild, nondenaturing buffers (e.g., Tris-based buffers). Thus, while the circuit-based probes facilitate multiplexed (simultaneous multicolor) detection, they should also allow the same-colored dye molecules to be used reiteratively to label different markers on an individual sample of fixed cells.

EXPERIMENTAL PROCEDURES

General Methods. Oligonucleotides were purchased from Integrated DNA Technologies (IDT). The recombinant target protein GFP-Z_E, was designed with C-terminal leucine zipper (Z_E) and 6× Histidine tag (6xHis) for purification using standard cloning procedures. Artificial proteins were labeled with catalyst DNA as described in ref *17*.

DNA Circuit Design and Characterization. The DNA circuit sequences were designed as previously described (*12*). Fluorophores and quenchers were incorporated into substrate complexes as shown in Supporting Information Table S1. Strands outfitted with fluorophores or quenchers were purchased HPLC-purified. DNA complexes were formed via a thermal annealing procedure: strands were mixed together at a 1:1 stoichiometry in TAE/Mg^{2+} buffer at a final concentration of 3 μM. The temperature of this solution was then raised to 95 °C and reduced to 25 °C over 90 min. DNA complex formation was verified by 12% nondenaturing PAGE gel analyses using SYBR-Gold staining (Invitrogen).

Microarray Procedures. The DNA microarrays were printed on Vantage silyated aldehyde slides (CEL Associates) using SMP3 pins (ArrayIt) and a custom fabricated microcontact

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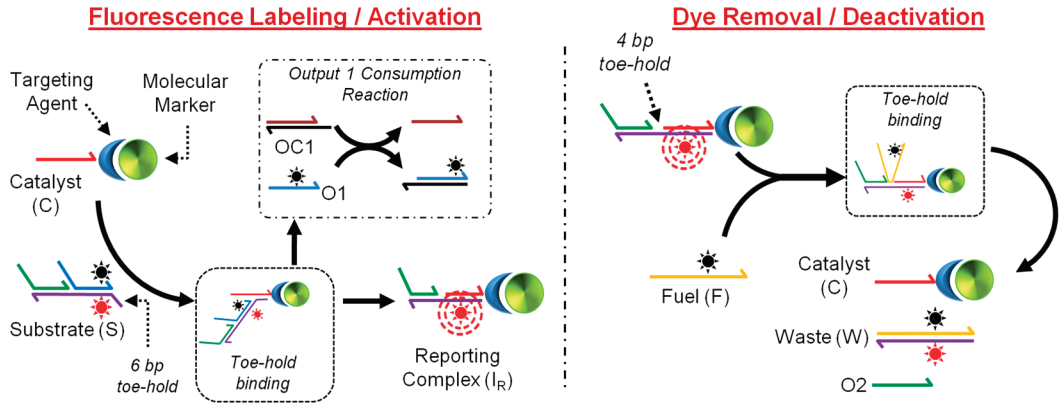
Scheme 1. DNA Circuit-Based Marker Labeling and Dye Removal Reactions



printer. Arrays were fabricated by spotting solutions of 3′-amine labeled C strands (10, 5, 1, 0.5, 0.1, and 0.05 μM stocks for gradient experiments and 2 μM stocks for all others) in PBS (pH 6.6) containing 30% glycerol. Afterward, the slides were incubated in a humidity chamber for 6 h. Free aldehyde groups on the slides were then quenched for 5 min in a sodium borohydride solution (3:1 PBS/EtOH 2.5% $NaBH_4$). Slides were blocked for 2 h in 4× SSPE buffer (600 mM NaCl, 40 mM NaH_2PO_4, and 5 mM EDTA) containing 0.1% BSA, washed 3 times with 4× SSPE buffer with 0.1% SDS, rinsed with milli-H_2O, and dried under nitrogen.

Microarray labeling/activation and dye removal/deactivation reactions were performed using a static incubation procedure or with a hybridization station (TrayMix2: ArrayIt) that provides active mixing of reagents over the slide. For static incubation, Gene frames (AbGene) were affixed over the arrays to create a reaction chamber containing 5 pmol substrate and OC1 consumption complex in TAE w/Mg^{2+}. The arrays were incubated overnight. The Gene frames were then removed and slides were washed 3 times in 4× SSPE buffer, rinsed again in milli-H_2O, and dried under a nitrogen stream. Deactivation and reiterative labeling experiments were performed similarly by affixing new Gene frames to the slides and repeating the incubation procedure. Microarray analysis was performed using *ImageJ* (http://rsbweb.nih.gov/ij/).

Cell Culture and Labeling. HeLa cells were cultured in an 8-chambered coverslips (Lab-Tek) for 24 h in DMEM supplemented with 10% FBS, 50 μg/mL penicillin, and 50 μg/mL of streptomycin. For GFP labeling experiments, the media was replaced and the cells were transfected with GFP-Z_E DNA using Fugene (Roche) transfection reagent under the manufacturer's protocol.

To label cells, coverslips were washed once with PBS and fixed with freshly prepared 4% paraformaldehyde (PFA) solution for 30 min. The cells were then washed twice for 2 min with PBS, permeabilized for 5 min with 0.2% Triton X-100, washed twice again with PBS, and stored overnight at 4 °C. Prior to circuit labeling experiments, the cells were washed again with PBS and then incubated for 2 h with a blocking solution (1% BSA, 1 mg/mL denatured Herring sperm DNA, and 0.5 μM polyT DNA in PBS). For GFP labeling experiments, the cells were also incubated with 400 nM of Z_R-ELS_6-Cat1 in PBS for 2 h. Excess polymer was then removed by washing twice with PBS prior to circuit-based labeling.

Circuit labeling reactions were carried out by incubating the cells for 1.5 h with 100 nM substrate complex and then washing twice with PBS for 2 min. Dye removal was performed similarly using 1 μM fuel. Before imaging experiments, slides were washed twice with PBS and then mounted on a glass slide using rubber cement.

Fluorescence Imaging and Analyses. All images were collected using a Zeiss Axioplan epifluorescence microscope and are contrasted identically in each figure. Correlations between GFP and Cy5-circuit signals and Cy5 signals produced during sequential labeling of cells (ON_1 and ON_2) were analyzed using a custom program written in *Matlab*.

RESULTS AND DISCUSSION

An illustration depicting our use of entropically driven circuits as imaging probes is shown in Scheme 1. Adopting the nomenclature of Zhang et al. (*12*), these circuits are composed of three main components: a single-stranded "catalyst" strand (C), a three-strand DNA complex called the "substrate" (S), and a "fuel" strand (F). In the circuit reaction cycle, the binding of C to a 6 nucleotide "toehold" domain at one end of S initiates a strand displacement reaction that releases an "output" strand (O1) and produces an intermediate-state complex (I_R) that possesses a new, internal 4 bp toehold domain. The binding of F to this toehold then initiates a second strand displacement reaction that releases C from the I_R complex and produces a "waste" product (W). To convert these complexes into imaging probes, catalyst strands can be appended to targeting agents that bind to specific molecular markers. The circuit substrates are modified by incorporating fluorescent dye (Cy3 or Cy5) and quencher (Iowa Black) molecules that are positioned such that the reaction of S with C results in an I_R complex that contains an unquenched fluorophore. The dye-bearing strand within I_R can then be removed from the marker and rendered inactive in the waste product by incubation with a modified F strand that carries a second quencher molecule. Overall, the use of quenched substrates and waste products should reduce background fluorescence resulting from potential nonspecific binding of either complex to a sample. Finally, we note that, in contrast to prior work where C truly functioned as a catalyst (*12*), marker labeling and removal in the present application is achieved by performing partial circuit reactions while using C for targeting. We therefore use the term catalyst only for continuity with previous reports.

To evaluate the efficiency of the circuit-based labeling and dye removal reactions, we first examined distributions of product complexes that were formed upon incubations of S and C, as well as S, C, and F via native PAGE-gel analyses (Figure 1a). After a partial circuit reaction of S with C, the catalyst strand is bound to the I_R complex through a total of 22 matched base pairs. This complex is therefore stable at room temperature and can be isolated on a gel. Yet, the free energy difference between S and I_R is small (ΔG ~0.4 kcal/mol). As a result, the reaction of S and C, when performed using equimolar concentrations, results in an equilibrium distribution of circuit components possessing near-equivalent concentrations of S, I_R, and free,

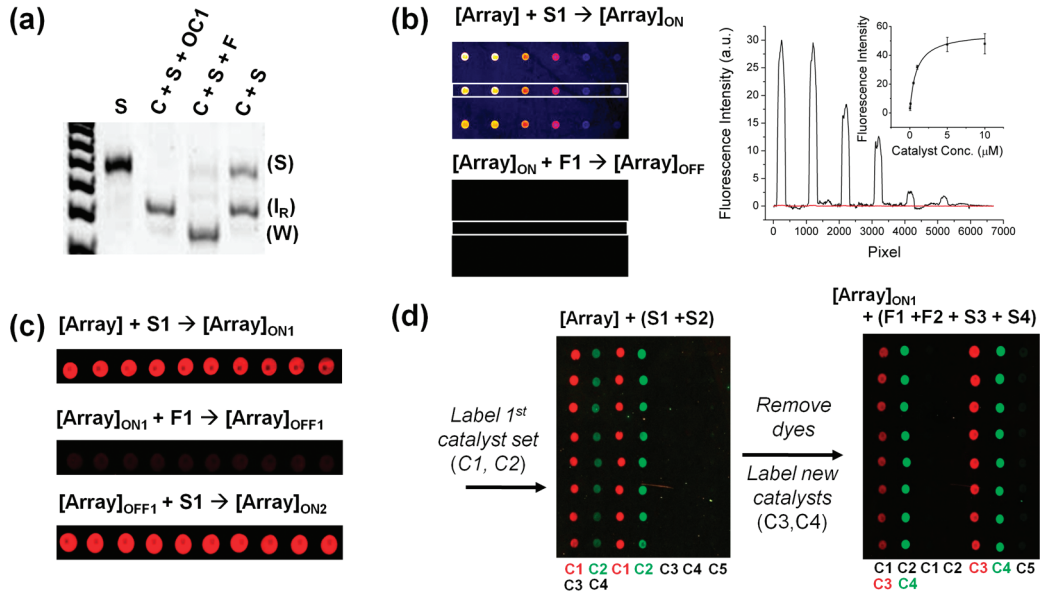


Figure 1. Multiplexed and reiterative fluorescence labeling of ssDNA catalyst. (a) Native PAGE-gel displaying DNA circuit reaction products: all components were reacted at a concentration of 200 nM. (b) A catalyst microarray possessing a gradient of spot concentrations (using 10, 5, 1, 0.5, 0.1, and 0.05 μM stock solutions) that was first labeled and then erased via the sequential addition of S (top image) and a fuel strand (bottom image). Images are rendered as heat maps. Plots of the averaged intensity profile of the boxed regions for the labeling (black line) and dye removal (red line) reactions are shown. Average spot intensities can be approximated by the Langmuir equation (inset). (c and d) Reiterative labeling of an individual (c) and multiple (d) arrayed catalysts. The reactions performed on the arrays are indicated above each image.

"unlabeled" catalyst (Figure 1a, lane 5). While this result is not optimal for marker labeling, this reaction can be driven forward by adding a second complex (OC1) that consumes (O1) once it is liberated from S (boxed reaction in Scheme 1). As demonstrated in other strand displacement systems (*18*), the sequestration of O1 shifts the equilibrium distribution of the reaction significantly toward the I_R state (Figure 1a, lane 3). Alternatively, this distribution can also be shifted using an excess of S relative to C, which should often be the case when labeling catalysts (markers) that are immobilized on a specimen. Nevertheless, the ability to drive strand displacement reactions forward through output sequestration will likely be useful for optimizing dye-labeling kinetics or when local target concentrations within a sample are high.

The removal of dyes from a sample via the reaction of I_R and F constitutes an equally important step in our marker imaging procedure. Here, the use of three-strand S and I_R complexes, as opposed to somewhat simpler two-strand complexes, allows dye-bearing strands to be displaced from the reporting I_R complex without output sequestration (Figure 1a, lane 4), since two strands (C and O2) must react simultaneously with final circuit product W for the reverse reaction W + O2 + C → I_R + F to occur. Hence, the dye removal reaction is effectively irreversible.

We next performed a series of DNA microarray experiments that were designed to evaluate the use of DNA circuit complexes as molecular imaging probes (Figure 1). In these experiments, amine-modified C strands are arrayed on the surfaces of glass slides, and the reaction of S with C produces a fluorescent I_R complex that is anchored to the slide surface and can be detected using a fluorescence microarray scanner. Analyses of arrays where a catalyst was printed at variable spot concentrations confirm that the I_R complex of the circuit can function as a reliable reporter of the levels of immobilized catalyst (Figure 1b, top). Here, spot intensity profiles can be approximated by the Langmuir adsorption equation, as is commonly found with DNA microarrays (*19, 20*). Furthermore, after the same slide is incubated with a fuel strand, each spot disappears and cannot be detected over background autofluorescence signals of the slide (Figure 1b, bottom).

Our microarray experiments also allowed us to demonstrate the use of DNA circuit probes for both multiplexed and reiterative marker labeling. We found that immobilized C strands can be labeled with dyes multiple times via sequential reactions of arrays with S, F, and then a second solution of S complexes (Figure 1c). Each labeling reaction produced arrays possessing near identical spot intensities. We also demonstrated that multiple C targets could be labeled and/or erased simultaneously using multiple S and F complexes in a single reaction step (Figure 1d). In these experiments, five different C strands were printed both as mixtures of two strands and individually on the surface of the slide. The array was then reacted with two different substrate complexes (S1-Cy5 and S2-Cy3), yielding a spot pattern that corresponded directly to the positions of the printed catalysts (C1 and C2). Subsequently, in a single incubation step, this pattern was erased with F1 and F2, and a new spot pattern was generated through a reaction with a second set of substrates (S3-Cy5 and S4-Cy3); spotted lanes where C3 and C4 were printed appear in the scanned image. Throughout this procedure, the fifth printed catalyst strand of the array (C5) remains unlabeled in both scanned images. Thus, these experiments confirm that DNA circuitry can be used for multiplexed and reiterative imaging: two fluorescent dye molecules and two spectral channels of an imaging system are used to detect four distinct markers on the same sample. Importantly, all labeling and removal reactions in these assays were performed using mild processing conditions (room temperature and Tris buffer supplemented with 12.5 mM Mg^{2+}).

We next performed a series of imaging experiments that show DNA circuit complexes can be used to selectively label molecular markers on fixed and permeablized HeLa cells. Background circuit reactivity was first tested by incubating cell samples with a quenched S complex (100 nM) for 1.5 h. The resulting images show no discernible fluorescence signals and possess signal to background ratios of 1 (Figure 2a), implying that the substrate complexes have exceptionally low background reactivity with cells. We attribute this property to the high stability of the duplexed substrate complex, the enhanced sequence specificity of strand exchange over a classical hybridization mechanism (*21*), and the ability to place dye and

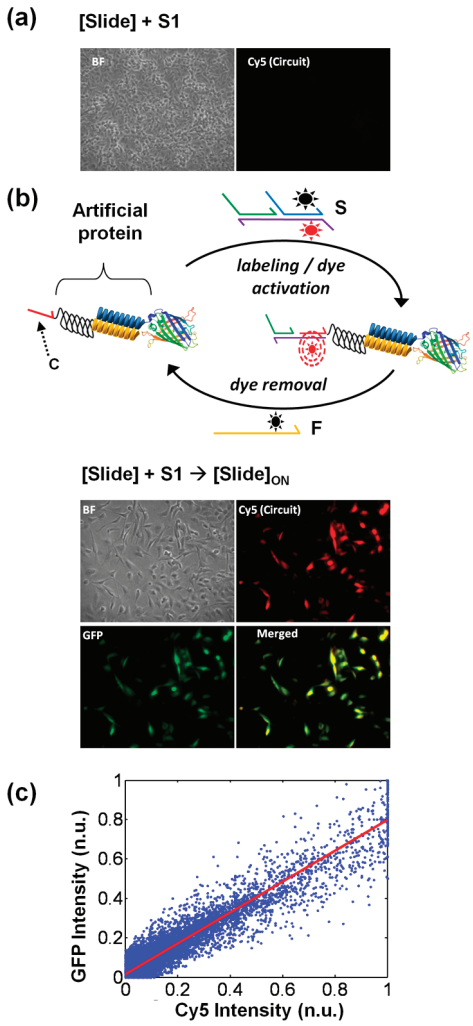


Figure 2. Selective targeting of an exogenously expressed GFP protein marker. (a) Bright field (BF) and fluorescence images of paraformaldehyde-fixed and permeablized HeLa cells reacted with a substrate complex that incorporates a Cy5 and a quencher (Iowa Black). (b) Circuit-based labeling of GFP-Z_E transfected HeLa cells. The catalyst is attached to the GFP marker via a DNA-conjugated artificial protein (Z_R-ELS_6-ssCat) that is introduced postfixation. (c) Intensity correlation analyses of GFP-Z_E and DNA circuit (Cy5) signals. The correlation coefficient, r, for the fitted line is 0.95.

quencher molecules in close proximity to one another within the substrate complex.

To label markers on cells, we chose to target the DNA circuit complexes to a transfected and expressed green fluorescent protein construct (GFP-Z_E) so that circuit labeling and dye removal efficiencies could be benchmarked directly against an internal standard (Figure 2b). The catalyst strand was coupled to the GFP using DNA-conjugated artificial-protein-based polymers (Z_R-ELS_6-ssCat) that we have previously developed for protein−DNA labeling (*17*). These polymers associate with the GFP-Z_E via a heterodimeric leucine zipper complex (Z_E/Z_R: $K_D \sim 10^{-15}$ M). Thus, after incubating GFP-Z_E transfected HeLa cells with Z_R-ELS_6-ssCat and then washing the samples to remove unbound polymer, GFP-Z_E transfected cells can be labeled by a reaction of a circuit substrate complex that carries a Cy5 dye and a quencher. As seen in Figure 2b, cells that were successfully transfected with GFP-Z_E reacted with S to produce fluorescent signals in the Cy5 channel of the microscope. While cells that were not transfected did not exhibit fluorescence, clear linear correlations are observed between GFP-Z_E and circuit labeling intensities, yielding a correlation coefficient $r = 0.95$.

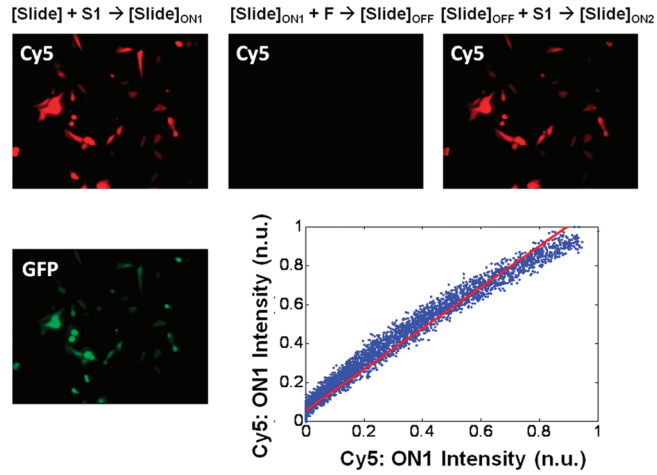


Figure 3. Reiterative circuit labeling of GFP-Z_E transfected cells. Sequential images of GFP-Z_E transfected cells where circuit complexes were used to label (ON1), erase (OFF), and relabel (ON2) the same sample of HeLa cells are shown. The pixel intensities in both Cy5 images (Cy5/ON1 and Cy5/ON2) are linearly correlated; fitted slope = 1.1, $r = 0.92$.

We also tested whether molecular markers could be labeled multiple times on a single sample of cells without loss of fluorescence signal intensities (Figure 3). After a first round of circuit labeling and imaging, fuel strands were added to remove Cy5 dyes from a sample of GFP-Z_E transfected cells. As in our microarray experiments, dye removal reactions are found to be efficient and yield signal to background ratios of 1. In addition, the transfected cells could be labeled and imaged a second time by incubating the sample with a fresh solution of substrate. Bright field imaging showed that a small portion of the cells detached from the slide surface during our manual washing and coverslip mounting procedures. Nevertheless, GFP and Cy5 signals remain highly correlated on a pixel-by-pixel basis after both rounds of fluorescence labeling ($r > 0.95$). Furthermore, strong correlations were found between cell images collected after the first and second dye labeling reactions are performed (i.e., between the ON1 and ON2 images in Figure 3 bottom). The Cy5 intensities of both images are linearly correlated and can be approximated by a line possessing a slope of 1.1. We therefore conclude that the Cy5 dyes can be coupled to the GFP-Z_E markers with near-identical efficiencies through sequential rounds of circuit-based labeling.

CONCLUSION

In summary, we have demonstrated that DNA circuit complexes can be used as erasable molecular imaging probes. Here, the sequence-dependent specificity of the DNA circuit reactions facilitates multiplexed marker detection. The use of strand displacement mechanisms also allows fluorescence reporting complexes to be disassembled, and hence, new reporting complexes can be created and used to image additional sets of molecular markers. Importantly, these reactions can be carried out at ambient temperature and in mild buffering conditions to minimize potential perturbations to a biological specimen. While such capabilities should offer opportunities to increase the number of molecular markers that one can examine on a single biological sample via fluorescence microscopy by at least a factor of 2 or 3, the next challenge will also be to develop diverse sets of molecular targeting agents (e.g., monovalent DNA-conjugated antibodies) that can facilitate efficient molecular marker recognition and react with the DNA circuitry reliably. Efforts to optimize syntheses of such agents are currently underway.

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Supporting Information Available: A listing of DNA sequences and their modifications are provided in Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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