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inCu-click: DNA-enhanced ligand enables live-cell, intracellular click chemistry reaction with copper catalyst

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Labeling cellular biomolecules via copper-catalyzed azide–alkyne cycloaddition (CuAAC) offers rapid reaction kinetics and uses small azide and alkyne probes that minimally disturb molecular function, making it ideal for tracking biomolecules. However, applying CuAAC inside living cells has been hindered by the high copper levels required, which compromise cell health. To overcome this barrier, here, we develop inCu-click, an intracellular CuAAC approach that employs a DNA-conjugated ligand (BTT-DNA) to localize and concentrate copper ions at the reaction site. This design permits efficient click chemistry at low intracellular copper concentrations without added copper salts and supports template-driven proximity and liposomal delivery of the ligand into cells. Here we show that inCu-click enables robust fluorescent labeling of nascent phospholipids and proteins in live cells with negligible impact on viability, establishing a platform for real-time visualization of biomolecule dynamics in complex, live cell environments.

Bioorthogonal chemistry comprises a set of widely used conjugation strategies for studying biological processes[1–4]. It demands high chemical specificity, rapid reaction kinetics, and no side reactivity with native functional groups[5]. One of the first bioorthogonal reactions--Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC)—joins a chemically inert terminal alkyne and an azide in a dipolar cycloaddition[6–10]. CuAAC proceeds in aqueous conditions[11], generates minimal byproducts[1], leverages commercially available conjugates, and uses small alkyne and azide tags that do not disturb biomolecule function[12]. However, the high copper catalyst concentration required in combination with ascorbate produces reactive oxygen species (ROS) that can damage nucleic acids[13] and harm live cells[14], limiting CuAAC's intracellular use.

Researchers have developed biocompatible ligands to lower copper toxicity and accelerate CuAAC[8,9], such as copper-chelating azides[15] and optimized reducing agents[16]. Despite these advances, intracellular labeling using CuAAC in live cells remains challenging. The copper concentration required for CuAAC (typically 50–100 μM) is toxic, intracellular uptake is low, and endogenous ligands sequester copper. A copper-chelating azide was previously shown to enhance CuAAC reactivity[17] for drug detection but requires cell fixation before fluorescence labeling. Similarly, a cell-penetrating peptide-conjugated ligand enables click detection of l-homopropargylglycine[18] but significantly reduces viability and has a low product yield in cytosolic proteins. Consequently, researchers use CuAAC mainly for extracellular labeling of surface glycans[8] and lipids[19], or fix cells before intracellular tagging[20,21].

Copper-free, strain-promoted azide-alkyne cycloaddition (SPAAC)[22] offers high selectivity[23,24] and works inside cells[25]. However, SPAAC is limited by the commercial availability of cyclooctynes[26], reagent instability, and nucleophilic addition with cellular nucleophiles[22]. The inverse electron demand Diels-Alder reaction (IEDDA) with tetrazine-dye conjugates also supports live cell labeling[27] but suffers from tetrazine instability in aqueous solutions[28] and limited activation methods[29]. These limitations make a low-toxicity, highly efficient intracellular CuAAC method highly desirable.

Here, we develop inCu-click (intracellular Cu-catalyzed click), an intracellular CuAAC approach that uses a DNA-conjugated ligand (BTT-

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DNA) to localize copper ions and drive azide-alkyne ligation without added copper salts. We show that the DNA oligonucleotide attachment localizes copper ions near the ligand, thus promoting CuAAC-mediated azide tagging with much lower copper concentrations than commercially available CuAAC ligands. The DNA oligonucleotide attachment also enables a nucleic acid template-driven proximity ligation of a fluorogenic dye. We apply this proximity ligation to detect individual RNA molecules in the cell nucleus and cytoplasm using alkyne-modified RNA FISH probes and a fluorogenic azide, assisted by our DNA oligonucleotide-conjugated CuAAC ligand in fixed cells. DNA conjugation to the ligand also provides a convenient method for intracellular delivery to encapsulate the copper within a liposome. We demonstrate that inCu-click preserves cell viability while enabling live-cell, intracellular fluorescent labeling of nascent phospholipids and proteins. By combining sensitivity, biocompatibility, and real-time intracellular activity, inCu-click advances our ability to track biomolecule dynamics in complex cellular environments.

Results

Synthesis and characterization of BTT-DNA CuAAC accelerating ligand

We adopted the strategy used to develop the BTTAA ligand[9] by ligating the precursor molecule (S1) that contains two *tert*-butyl groups bearing a single alkyne to a single-stranded DNA oligonucleotide probe bearing a single 3′ azide via CuAAC to produce BTT-DNA (Fig. 1a). We confirmed the synthesis of this probe by observing a shift in band size in a PAGE gel analysis (Supplementary Fig. 1a), then purified the BTT-DNA ligand by extensive dialysis. Interestingly, our early CuAAC studies using the BTT-DNA ligand demonstrated a ligation product without free copper (Supplementary Fig. 1b), which led us to test for the presence of copper in the purified ligand using inductively coupled plasma mass spectrometry (ICP-MS). Surprisingly, we found that the purified ligand contained copper with a ratio of Cu: BTT-DNA at 10:1 (Fig. 1a). Interestingly, this Cu remained bound after several rounds of dialysis and subsequent HPLC purification. The unmodified N_3-DNA probes were also assessed for copper and produced a ratio of Cu: DNA of 7:1, indicating that the DNA and the triazole groups are chelating the copper.

Reaction kinetics of BTT-DNA ligand

The reactivity of the CuAAC reaction in the presence of the new BTT-DNA ligand was determined using a fluorogenic plate-reader assay. We reacted a 15-mer DNA oligomer bearing a 5′ alkyne moiety (henceforth referred to as 5′ alkyne DNA) to a series of fluorogenic azide dyes[30] (3-Azido-7-hydroxycoumarin and CalFluor 488 azide) using CuAAC in the presence of BTT-DNA and BTTAA (Fig. 1b, c). This DNA oligomer sequence is not complementary to any other reaction components. Fluorescence was measured over 2 and 4 h (Fig. 1d-i). BTT-DNA consistently increased activity in accelerating the CuAAC reaction compared to BTTAA for 3-Azido-7-hydroxycoumarin in the nM range. Interestingly, the 100 nM BTT-DNA ligand outperformed the commercial ligand 1.4-fold, demonstrating that BTT-DNA is an effective ligand in the nanomolar range and in the absence of exogenous Cu(I). The difference was even more striking at 1000 nM BTT-DNA, at which the fluorescence produced was 3.0-fold higher than the commercial ligand at 2 h. At 500 nM ligand concentration, we observed that the BTT-DNA ligand outperformed the commercial ligand 4.8-fold (Fig. 1f). To ensure the completion of CuAAC reaction assisted by the BTT-DNA ligand before the calculation of the reaction rates, we performed the fluorogenic plate-reader assay using 50 μM

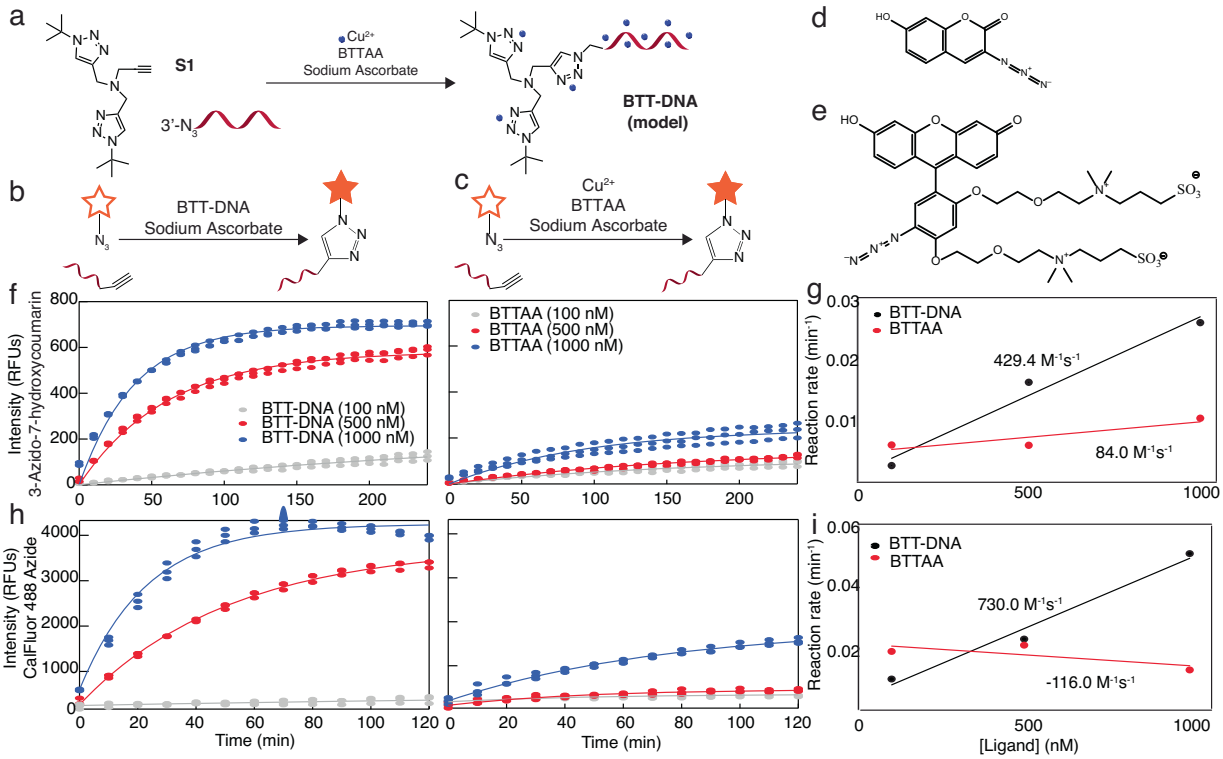

Fig. 1 | Reaction kinetics of CuAAC enhanced by the BTT-DNA ligand with two different azido fluorogenic dyes. a Strategy to synthesize BTT-DNA ligand via CuAAC using (S1). **b** Schematic showing an alkyne-labeled DNA oligonucleotide is ligated to a fluorogenic dye in the presence of BTT-DNA ligand and sodium ascorbate. **c** Schematic showing an alkyne-labeled DNA oligonucleotide is ligated to a fluorogenic dye in the presence of Cu^{2+}, BTTAA, and sodium ascorbate. **d** Structure of 3-Azido-7-hydroxycoumarin. **e** Structure of CalFluor 488 azide. **f** Reaction kinetics of CuAAC using 3-Azido-7-hydroxycoumarin. **g** Reaction rate of CuAAC using 3-Azido-7-hydroxycoumarin vs ligand concentration. **h** Reaction kinetics of CuAAC using CalFluor 488 azide. **i** Reaction rate of CuAAC using CalFluor 488 azide vs. ligand concentration. Each condition has three biological replicates.

$CuSO_4$ ([BTTAA]:[$CuSO_4$] = 2:1) as a 100% reaction yield control. After a 2-h incubation, CuAAC assisted by BTT-DNA reached the maximum yield of fluorogenic azide conversion (Supplementary Fig. 2). The rate of CuAAC reaction that was assisted by BTT-DNA ligand is 429.4 s^{-1} M^{-1}, demonstrating that the CuAAC reaction accelerated by BTT-DNA is significantly faster than the reaction accelerated by the commercial BTTAA ligand with a reaction rate of 84.0 s^{-1} M^{-1}(Fig. 1g).

Next, we performed the fluorogenic plate-reader assay using another fluorogenic dye, CalFluor 488 azide. Upon click reaction in the presence of BTT-DNA ligand, the fluorescence of the CalFluor 488 azide produced at 1000 nM BTT-DNA was 2.5-fold higher than BTTAA ligand at 2 h. The BTT-DNA ligand also outperformed the BTTAA ligand 7.5-fold at the concentration of 500 nM (Fig. 1h). The most prominent enhancement of the fluorescence was constantly achieved at 500 nM ligand concentration for both fluorogenic dyes. For the CalFluor 488 azide, the rate of CuAAC reaction that was assisted by BTT-DNA ligand is 730 s^{-1} M^{-1}, which is much higher than that of CuAAC reaction assisted by commercial BTTAA ligand (116 s^{-1} M^{-1}; Fig. 1i).

Stability of BTT-DNA ligand after cold storage

To evaluate the stability of the BTT-DNA ligand, we performed the fluorogenic plate-reader assay using 3-azido-7-hydroxycoumarin in the presence of the BTT-DNA ligand for 4 h monthly for 12 months (Supplementary Fig. 3). Using a concentration of 1000 nM BTT-DNA ligand, we observed that the reaction kinetics were unchanged. There was no significant difference between the fluorescence intensity of each sample across time points over twelve months, indicating that our BTT-DNA ligand can be stable for at least twelve months while stored at −20 °C.

Contribution of DNA sequence and BTT moiety in ligand design

We evaluated the reaction with a scrambled single-stranded DNA sequence of equal length using the fluorescent plate-reader assay to assess the DNA sequence's contribution to the BTT-DNA ligand's reactivity. We found identical activity, suggesting that the effect is not dependent on the sequence composition (Supplementary Fig. 4). Thus, the strategy to synthesize our CuAAC accelerating ligand can be applied to make a BTT-DNA ligand with any sequence. We also demonstrated the significance of the BTT moiety in ligand design by comparison the CuAAC in the presence of BTT-DNA and unmodified N_3-DNA probe (Supplementary Fig. 5). We found the CuAAC reaction in the presence of BTT-DNA showed higher fluorescence than that of unmodified N_3-DNA probe (Supplementary Fig. 5a). The presence of the BTT moiety also facilitated the nucleic acid template-driven proximity ligation which cannot be achieved without it (Supplementary Fig. 5b). We varied the length of DNA oligomer from 15 to 25 nucleotides to assess the DNA length's contribution to the BTT-DNA ligand's reactivity (Supplementary Fig. 6). We found that the longer lengths had batch variability in the quality of the modified DNA synthesis which led to variability in the performance after conversion into the BTT-DNA ligand. The 15-mer DNA oligonucleotide showed the highest and most consistent performance.

DNA and RNA template-driven proximity ligation with BTT-DNA ligand

We designed a DNA splint that is complementary to both the 5′ alkyne DNA and 3′ BTT-DNA ligand (Fig. 2a) to achieve a proximity-mediated click reaction. This design brings the alkyne close to the Cu(I) complexed with the accelerating ligand. We supplemented the reaction with spermine, a polyamine bearing multiple amino groups, and NaCl to improve hybridization (Supplementary Fig. 7). To assess the appropriate distance to position the 5′ alkyne on the DNA probe for maximum ligation to the fluorogenic azide, we performed the fluorogenic plate-reader assay for eight different 5′ alkyne DNA probes. For the reaction performed using 3-Azido-7-hydroxycoumarin, the fluorescence intensity produced from the splint in the presence of a DNA alkyne probe with a one-nucleotide distance from the BTT-DNA probe was 150.2 RFUs. For the same reaction performed using CalFluor 488 azide, the fluorescence intensity produced was 372.6 RFUs, 1.7-fold higher than the no-splint reaction. This linker distance for the DNA alkyne probe achieved the highest fluorescence enhancement and was used for all subsequent proximity experiments (Supplementary Fig. 7).

To enhance the reaction kinetics, we designed DNA splints harboring two and three repeats of the binding sequence (Fig. 2a). We assessed the fluorescence after a 6-h incubation using the fluorogenic plate-reader assay for each condition. For 3-Azido-7-hydroxycoumarin, we found that the total fluorescence produced from a single DNA splint-enhanced reaction is 301.9 RFUs, which is 1.5-fold higher than the fluorescence produced from random collisions (i.e., no splint). As expected, we also found no measurable fluorescence over background when substituting the targeted BTT-DNA strand with a scrambled BTT-DNA sequence that is not complementary to the DNA splint (Supplementary Fig. 8). We also observed that splints harboring more binding sites had higher fluorescence intensities. The fluorescence increased from 301.9 RFUs to 393.8 RFUs and 455.1 RFUs when the binding sites doubled and tripled, respectively (Fig. 2b). For the CalFluor 647 azide dye, the total fluorescence produced increased proportionally when we used the DNA splints harboring 1, 2, and 3 repeats of the binding sequence, respectively (Fig. 2b). We also found that the RNA splint enhanced reaction kinetics similarly to the DNA splint. For 3-Azido-7-hydroxycoumarin, the fluorescence produced from a single RNA splint-enhanced reaction is 282.9 RFUs, 1.4-fold higher than the fluorescence produced from random collisions. For the CalFuor 647 azide, the total fluorescence increased from 290.8 RFUs to 364.6 RFUs (Fig. 2b).

In situ proximity ligation to detect individual mRNAs in fixed cells: no wash smFISH

To test the proximity ligation in fixed cells, we used the transgenic cell line CHO-GFP-M20, which contains a transgene that includes a GFP coding region and 32 tandem copies of the probe-binding sequence (M20)[31] (Fig. 2c). We used the parental CHO cell line which does not contain the transgene as a negative control. First, we optimized the CuAAC reaction time to bind an azido dye to a 5′ alkyne DNA probe in fixed CHO-GFP-M20 cells. We optimized the CuAAC reaction time and achieved the best labeling by performing a 1-h CuAAC reaction (Supplementary Fig. 9).

After CuAAC reaction time optimization, we performed the CuAAC reaction using the BTT-DNA ligand with eight different 5′ alkyne DNA probes for targeting the M20 transgene sequence to find the best 5′ alkyne DNA probe for maximum ligation to the fluorogenic azide for transgene detection in CHO-GFP-M20 cells (Supplementary Fig. 10). Clean and robust labeling of transcription sites in the nucleus and individual RNA molecules in the nucleus and cytoplasm was observed via CuAAC in the presence of a 5′ alkyne DNA probe with a two-nucleotide distance from the BTT-DNA probe (Fig. 2c and Supplementary Fig. 10). Then, we cultivated an equal mix of CHO-GFP-M20 and parental CHO cells together. The cells were fixed and permeabilized, then reacted with CalFluor 647 azide via CuAAC in the presence of BTT-DNA ligand for 1 h. We observed specific labeling of transcription sites in the nucleus and individual RNA molecules in the nucleus and cytoplasm (Fig. 2c). The cells with bright puncta in the CalFluor 647 azide channel colocalize with GFP-positive cells, indicating that the probe binds specifically (Fig. 2c). In short, we achieved the detection of individual mRNAs specifically using a fluorogenic dye and non-exogenous copper required proximity click RNA FISH approach assisted by the BTT-DNA ligand.

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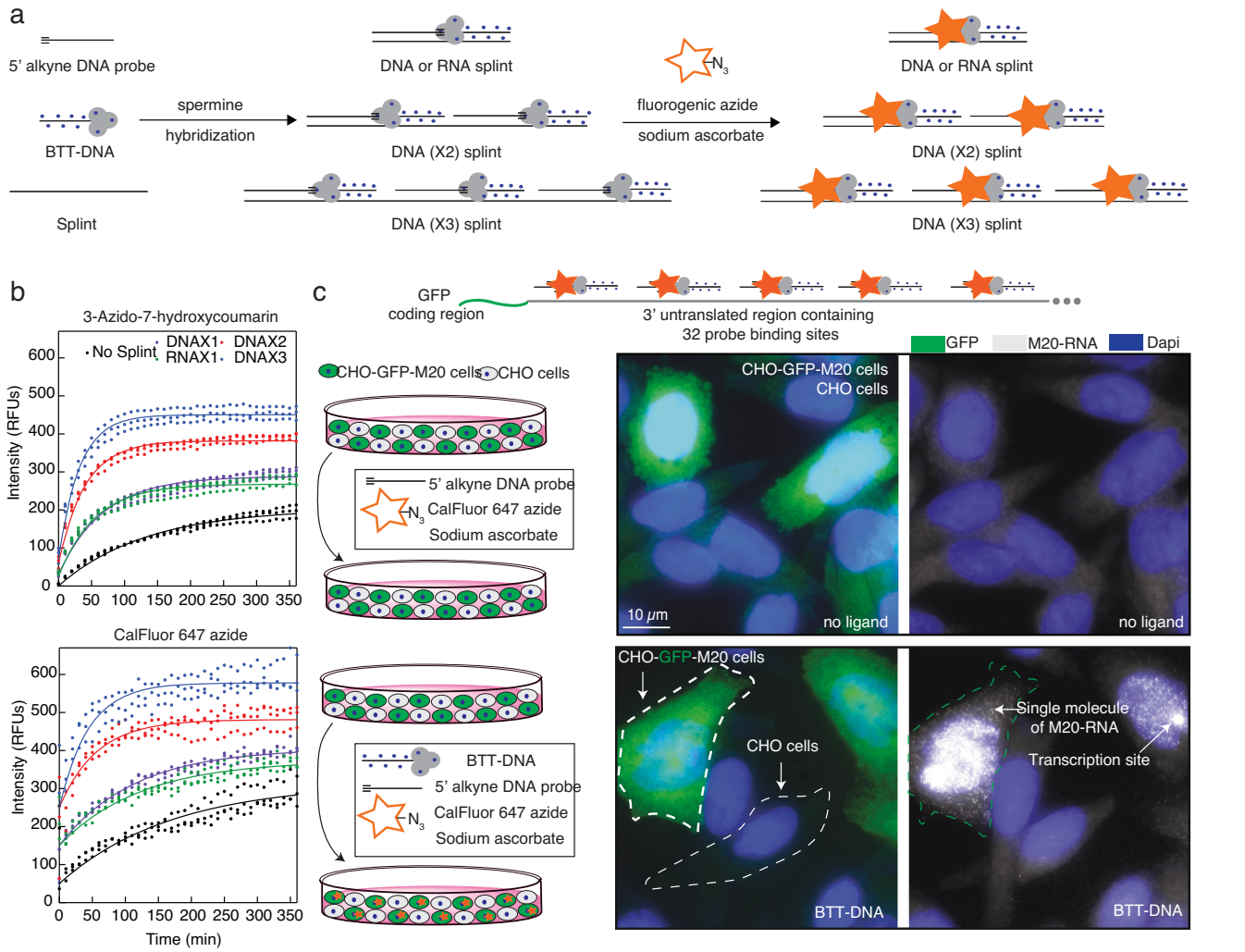

Fig. 2 | DNA and RNA template-driven proximity ligation with BTT-DNA ligand. **a** Schematic of nucleic acid template-driven proximity ligation. **b** Reaction kinetics of nucleic acid template-driven proximity ligation with DNA splints harboring different repeats of the binding sequence using 3-Azido-7-hydroxycoumarin and CalFluor 647 azide. Each condition has three biological replicates. **c** Microscopy representative (three biological replicates) of individual RNA molecule detection with nucleic acid template-driven proximity ligation using BTT-DNA. The schematic diagram depicts the proximity click FISH of M20 transgene RNA in the transgenic CHO-GFP-M20 cell line. Fixed cells are first treated with 0.2 μM alkyne-modified single-stranded DNA oligonucleotide probe (M20-left-alkyne_20_2), then treated with CuAAC in the presence of CalFluor 647 azide (10 μM), BTT-DNA ligand (20 μM), and sodium ascorbate (2.5 mM) for 1 h. (white) individual M20 RNA molecule, (green) GFP, (blue) DAPI staining of nuclei.

Fixed cell detection of biomolecules with BTT-DNA-assisted CuAAC

Next, we tested the activity of our BTT-DNA ligand in a cellular environment to detect various biomolecules in fixed cells. 5-Ethynyl-2'-deoxyuridine (EdU) is an alkyne-derivatized thymidine analog that may be metabolically incorporated into newly synthesized DNAs during active DNA synthesis in live cells[4]. Following overnight metabolic incorporation, cells were fixed and permeabilized, then reacted with CalFluor 647 azide via CuAAC. The negative control containing no ligand showed very low background fluorescence. We compared the performance of the BTT-DNA ligand to commercially available BTTAA (Supplementary Fig. 11). We observed robust labeling of nascent DNA in the nucleus using the CuAAC accelerated by our BTT-DNA ligand (Fig. 3a and Supplementary Fig. 12; 3.1-fold enhancement of signal over background), while the fluorescence of the cell nucleus using the CuAAC assisted by $CuSO_4$ and BTTAA was very low.

5-Ethynyl Uridine (EU) is an alkyne-derivatized uridine analog that may be metabolically incorporated into nascent RNAs in live cells[21]. Following a 3-h metabolic incorporation, cells were fixed and permeabilized, then reacted with CalFluor 647 Azide via CuAAC, comparing the performance of BTT-DNA ligand to commercially available BTTAA (Fig. 3b and Supplementary Fig. 11). For the cells treated with 20 μM BTT-DNA ligand, we observed strong fluorescence intensity in the cell nuclei, especially in the nucleoli[21,32](Fig. 3b). The total fluorescent signal produced in the nucleus in the presence of the BTT-DNA ligand was 2.2-fold higher than that in the no ligand control which shows low background fluorescence (Fig. 3b and Supplementary Fig. 12). In contrast, cells treated with $CuSO_4$ and BTTAA had minimal fluorescent signals.

L-homopropargylglycine (L-HPG) is a cell-permeable alkyne probe that can be metabolically incorporated into nascent proteins in methionine-starved cells[30]. We treated cells for 2 h with L-HPG, then fixed and permeabilized. We then reacted these samples with CalFluor 647 Azide via CuAAC in the presence of BTT-DNA to visualize the newly synthesized proteins (Fig. 3c and Supplementary Figs. 11, 12). We found strong fluorescence signals in the cell nucleus using the CuAAC assisted by 20 μM BTT-DNA ligand, which shows a 2.6-fold enhancement of fluorescence compared to the ligand-less ones (Fig. 3c and Supplementary Fig. 12).

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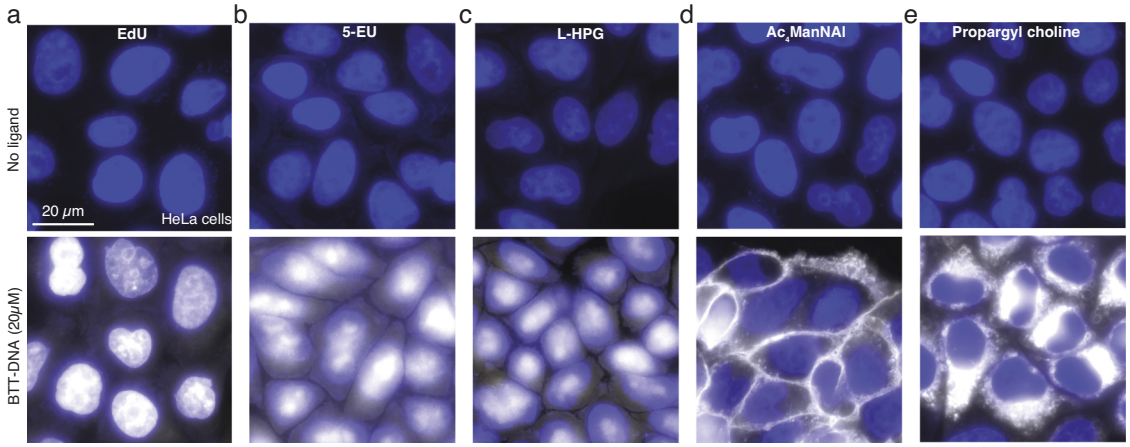

Fig. 3 | Detection and labeling of the intracellular and extracellular biomolecules using CuAAC in the presence of BTT-DNA on fixed cells. a Representative fluorescence microscopy for detection of nascent EdU-labeled DNA via CuAAC in the presence of BTT-DNA ligand on fixed cells. **b** Representative fluorescence microscopy of detection of nascent 5-EU-labeled RNA via CuAAC in the presence of BTT-DNA ligand on fixed cells. **c** Representative fluorescence microscopy of detection of nascent L-HPG-labeled protein via CuAAC in the presence of BTT-DNA ligand on fixed cells. **d** Representative fluorescence microscopy of detection of nascent Ac_4ManNAl-labeled sialic acid via CuAAC in the presence of BTT-DNA ligand on fixed cells. **e** Representative fluorescence microscopy of detection of nascent propargyl choline-labeled phospholipids via CuAAC in the presence of BTT-DNA ligand on fixed cells. On the top row, fixed cells are treated with CuAAC in the presence of 10 μM CalFluor 647 azide and 2.5 mM sodium ascorbate; on the bottom row, fixed cells are treated with CuAAC in the presence of 10 μM CalFluor 647 azide, 20 uM BTT-DNA ligand, and 2.5 mM sodium ascorbate. (white) CalFluor 647 azide labeling of nascent intracellular and extracellular biomolecules, (blue) DAPI staining of nuclei. (two biological replicates).

Further, we metabolically labeled HeLa cells with Ac_4ManNAl to introduce an alkyne tag to cell surface sialic acids[33], clicked a fluorophore to the alkynyl sugar, and imaged using fluorescence microscopy (Fig. 3d and Supplementary Figs. 11, 12). For the cells treated with 20 μM BTT-DNA ligand, a strong fluorescent signal was observed on the cell membrane where the metabolically labeled sialylated glycans are located (Fig. 3d). Compared to the control, cells treated with 20 μM BTT-DNA showed 9.1-fold enhancement of signals (Fig. 3d and Supplementary Fig. 12).

Next, we metabolically incorporated propargyl choline into newly synthesized phospholipids. Cells were incubated with propargyl choline for 24 h, then fixed and permeabilized, and then stained with CalFluor 647 Azide via CuAAC in the presence of BTT-DNA to visualize the newly synthesized phospholipids (Fig. 3e and Supplementary Figs. 11, 12). The cells showed intense staining in the cellular membrane and the intracellular structure[32] (Fig. 3e). Remarkably, the total fluorescence produced in the cytoplasm in the presence of BTT-DNA was 239.9-fold higher than that in the no ligand control, indicative of low background fluorescence in the absence of catalyst (Fig. 3e and Supplementary Fig. 12).

BTT-DNA ligand enables the detection of biomolecules on the surface of live cells

To test the activity of BTT-DNA in driving the CuAAC reaction for live cells, we metabolically labeled HeLa cells with Ac_4ManNAl (Fig. 4a and Supplementary Fig. 13), then clicked CalFluor 647 azide to the alkynyl sugar on the cell surface while the cells were incubated on ice to prevent internalization of the reagents. The BTT-DNA accelerated CuAAC produced a robust fluorescent signal on the cell membrane, showing a 7.3-fold enhancement of fluorescent signal over the no ligand background, which shows low background fluorescence (Fig. 4a, b).

Choline-containing phospholipids were also detected by metabolic incorporation of the propargyl choline to evaluate the activity of our BTT-DNA ligand for extracellular labeling in live cells (Fig. 4a and Supplementary Fig. 13). We found robust labeling of the newly synthesized phospholipids in the cell membrane (Fig. 4a, b), which is 3.7-fold higher than that of the no-ligand control.

Characterizing copper-induced cellular toxicity in the presence of BTT-DNA ligand

To evaluate the cytotoxicity of copper with the new Cu(I) accelerating ligand BTT-DNA, we compared HeLa cells that were treated with $CuSO_4$ at the copper concentration (300 μM) to HeLa cells treated with BTT-DNA ligand in the equivalent copper ion concentration (30 μM) (each molecule of ligand is complexed to ~10 copper ions; Fig. 1). Each of these was delivered under reducing conditions with sodium ascorbate, whose optimal concentration (2 mM; Supplementary Fig. 14) and treatment time (5 h; Supplementary Fig. 15) were necessary to minimize cell toxicity. We also optimized the concentration of lipofectamine RNAiMAX transfection reagents to eliminate the fluence on cell biology (0.5 μl; Supplementary Fig. 16). We observed that most cells treated with free $CuSO_4$, and sodium ascorbate were "blebbing" (i.e., cell membrane bulges out indicative of apoptosis). In contrast, the morphology of cells treated with only sodium ascorbate, or a combination of BTT-DNA and sodium ascorbate, were comparable to the negative control (Fig. 5a). This suggests that the BTT-DNA ligand can prevent cellular "blebbing" induced by Cu(I).

Next, we compared the viability of the treated cells using the ReadyProbes cell viability imaging kit. The nuclei of cells with compromised membranes are stained with the SYTOX green and measured with fluorescence microscopy (Fig. 5b and Supplementary Fig. 17). We calculated the viability for cells treated with Cu(I) to be 35.5 ± 6.8% viable. In contrast, the viability ratio for cells treated with BTT-DNA ligand is 96.0 ± 1.5% (Fig. 5b). We also stained the treated cells with CellEvent™ Caspase-3/7 Red Detection Reagent, a fluorogenic probe which fluoresces when bound to DNA after been cleaved by activated caspase-3/7 in apoptotic cells (Fig. 5c and Supplementary Fig. 17). We found that 42 ± 4.9% of cells were apoptotic following treatment by free Cu(I), while the ratio for cells treated with BTT-DNA was 3.0 ± 1.1% (Fig. 5c). These results strengthen the claim that the BTT-DNA ligand protects against Cu(I) induced toxicity.

A major concern for applying CuAAC into biological systems is the formation of reactive oxygen species (ROS), which can cause oxidative damage to proteins[34]. To compare ROS generated from the treatment of live cells with BTT-DNA ligand, we measured the amount of ROS in live cells by CellROX Green Reagent, which fluoresces after oxidation

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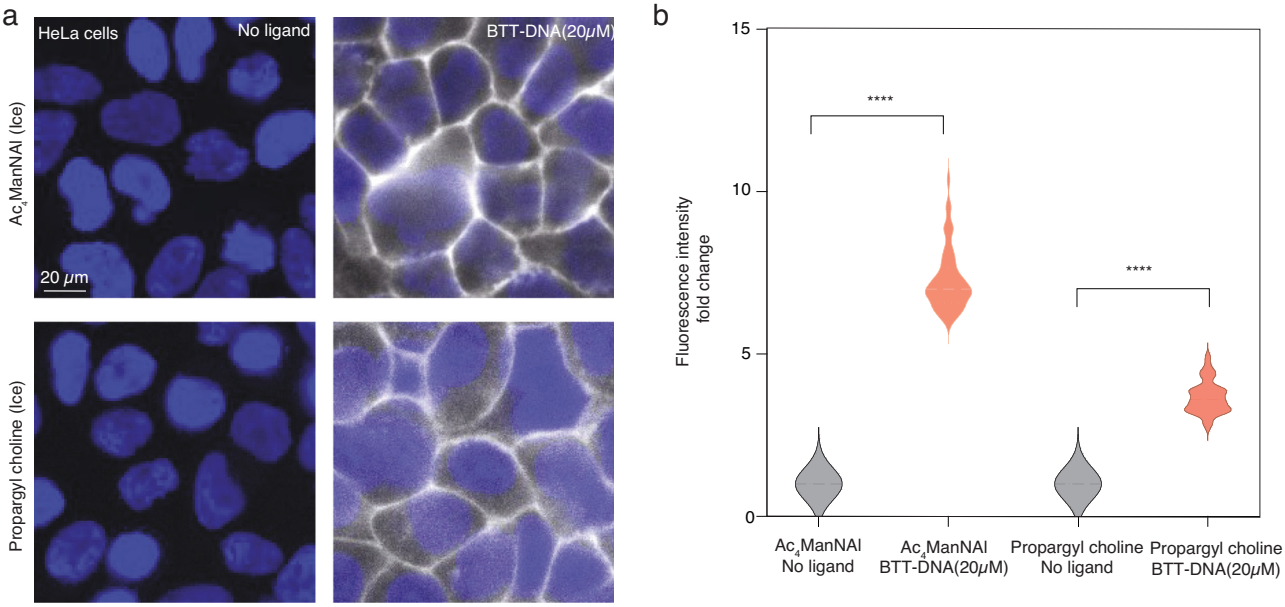

Fig. 4 | Representative fluorescence microscopy of the cell-surface biomolecule labeling and detection in the presence of BTT-DNA on live cells. a Metabolic labeling and detection of cell-surface sialic acids and choline-containing phospholipids with BTT-DNA on live cells. On the left column, live cells are treated with CuAAC in the presence of 10 μM CalFluor 647 azide and 2.5 mM sodium ascorbate; on the right column, live cells are treated with CuAAC in the presence of 10 μM CalFluor 647 azide, 20 uM BTT-DNA ligand, and 2.5 mM sodium ascorbate. (white) CalFluor 647 azide labeling of sialic acids and choline-containing phospholipids, (blue) Hoechst 33342 nuclei staining. **b** Quantifying normalized signal over background for the fluorescence microscopy assisted by BTT-DNA in panel (**a**). Statistical comparisons were performed on individual cell measurements from one representative of three independent biological replicates ($n = 60$ cells). The p value for Ac_4ManNAl is $5.389*10^{-50}$. The p value for propargyl choline is $2.768*10^{-35}$. $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, and $^{****}p \le 0.0001$. A two-sided t-test was used.

by ROS (Fig. 5d and Supplementary Fig. 17). We found that 77 ± 13.2% of cells were positive for ROS staining after free Cu(I) treatment. In contrast, the ratio for cells treated with BTT-DNA was only 3.7 ± 2.8% (Fig. 5d), indicating that BTT-DNA significantly reduces the ROS produced by Cu(I) delivery in live cells. We also used the MitoSOX green superoxide indicator. This fluorogenic dye can be readily oxidized by superoxide produced by mitochondria rather than ROS (Fig. 5e and Supplementary Fig. 17). For cells treated with Cu(I), all cells showed fluorescence in the cytoplasm, indicating superoxide production. All cells treated by BTT-DNA were negative for superoxide (Fig. 5e). Together, these results indicate that BTT-DNA has a protective effect from Cu(I) toxicity and prevents the formation of ROS and superoxide.

BTT-DNA ligand enables the detection of intracellular phospholipids on live cells

After a demonstration of the biocompatibility of our BTT-DNA ligand, we performed the intracellular biomolecule labeling via CuAAC, assisted by our BTT-DNA ligand. We started with the metabolic incorporation of propargyl choline into newly synthesized phospholipids by 24 h of propargyl choline treatment, through which the endoplasmic reticulum (ER), the Golgi, the mitochondria, and the plasma membranes were labeled with choline-containing phospholipids[32]. To ensure that our BTT-DNA ligand enters the cells to accelerate the CuAAC reaction, we used lipofectamine RNAiMAX transfection reagent, which has high transfection efficiency for siRNA. Since lipofectamine RNAiMAX encapsulates double-stranded nucleic acid, and our BTT-DNA is single-stranded, we designed a short single-stranded DNA that is complementary to the sequence of BTT-DNA and hybridized to form a duplex before transfection.

After encapsulating the BTT-DNA duplex with the liposome, 80 μM of CalFluor 647 azide was added to the mixture. This concentration of dye leads to detectable passive transport of dye into the cytoplasm while maintaining a sufficiently low dye background in live cells (Supplementary Fig. 18). We also optimized the transfection time to 4 h to transport the duplex into the cytoplasm of cells (Supplementary Fig. 19). After 4 h of transfection, we washed the cells to ensure that CuAAC occurs exclusively inside the cells and not on extracellular alkynes, followed by a 25-min sodium ascorbate incubation (Fig. 6a). We observed strong fluorescence intensity in the cytoplasm and membrane of cells treated with BTT-DNA (Fig. 6b), which is 1.7-fold higher than that of the no-ligand control (Supplementary Fig. 20).

We also conducted time-lapse imaging of intracellular labeling of phospholipids for 2 h. We observed that some cells that were non-fluorescent at 30 min began to exhibit fluorescence at 1 h and became more fluorescent at 2 h (Fig. 6c). We also showed that the fluorescence signal increased significantly over time, and the fluorescence enhancement compared to that at 30 min is 1.5 ± 0.2-fold and 2 ± 0.3-fold at 60 and 120 min, respectively (Fig. 6c, d). Cell viability measurements indicated that almost 100% of the cells were viable (Supplementary Fig. 21), indicating that the BTT-DNA ligand enables intracellular labeling of phospholipids in live cells. Additionally, to confirm the presence of free choline-containing phospholipids on the cell surface, we conducted a CuAAC reaction using Alexa Fluor 555 azide, which is a fluorescent dye with BTTAA-$CuSO_4$ complex for 5 min after intracellular phospholipids labeling. We observed colocalization of AF555 with CF647 (Fig. 6e), demonstrating the presence of the choline-containing phospholipids on the cell surface and consistent with the model in Fig. 6a. For the control sample that excludes free $CuSO_4$ and BTTAA, we observed no fluorescence signal (Fig. 6e), demonstrating that BTT-DNA is removed completely and that $CuSO_4$ and BTTAA must be added to the media for extracellular labeling. If any BTT-DNA was remaining on the surface, this was insufficient to drive the reaction on its own.

To further demonstrate that we achieved intracellular labeling of phospholipids in live cells, we treated the cells with sodium ascorbate on ice rather than at 37 °C. In this experiment, cells were treated with propargyl choline, and the BTT-DNA and CF647 were delivered via liposome. The cells were then incubated on ice, thus making the membrane rigid and not allowing Cu(II) on the inside of the cells to

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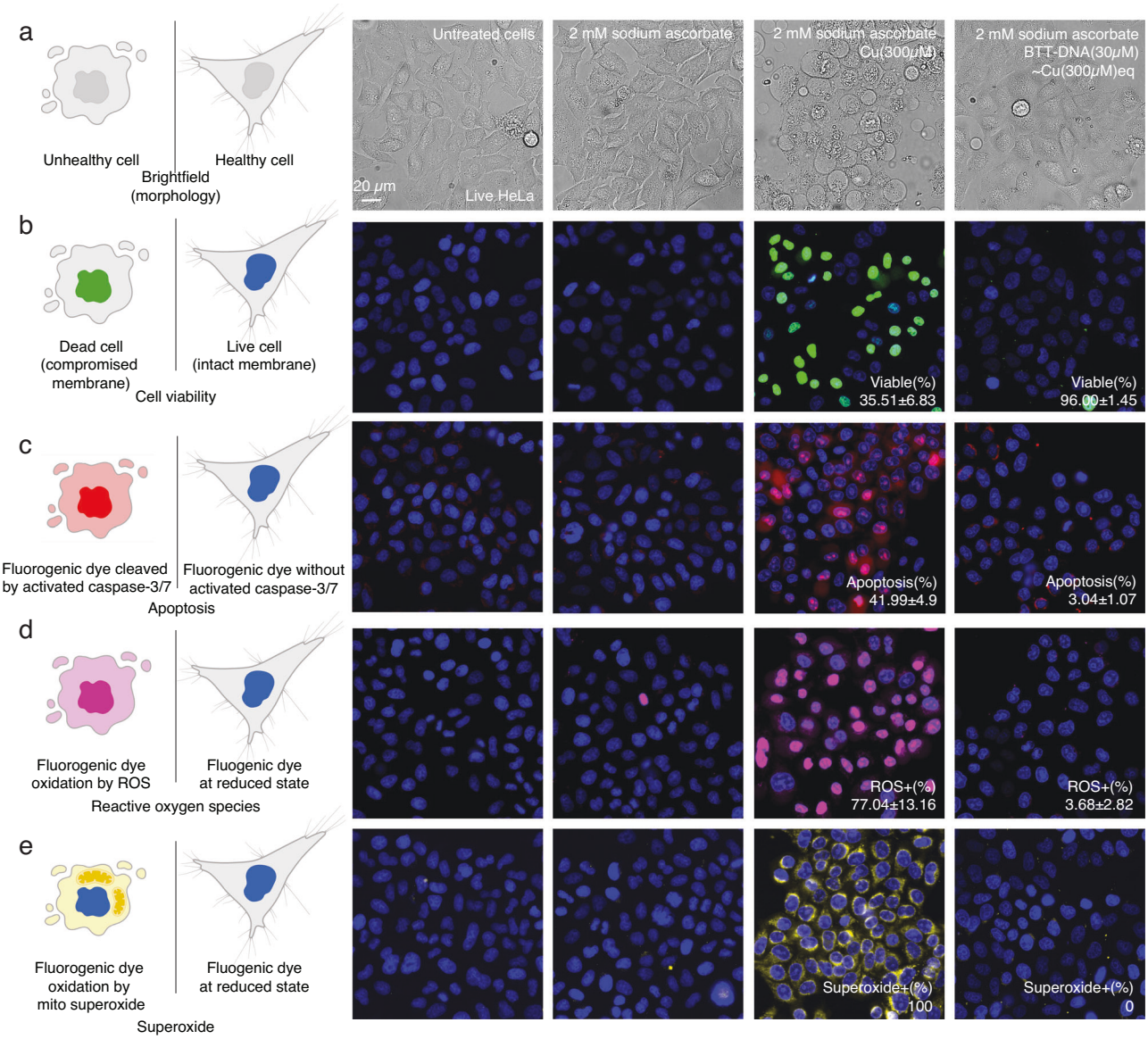

Fig. 5 | Biocompatibility of the BTT-DNA ligand. a Representative micrographs of cell morphology comparing BTT-DNA ligand (delivered via liposome) to copper on HeLa cells[41] (scale bar applies to all images). **b** Representative micrographs of cell viability assay comparing BTT-DNA ligand to copper on HeLa cells[41]. **c** Representative micrographs of cell apoptosis assay comparing BTT-DNA ligand to copper on HeLa cells[41]. **d** Representative micrographs of cell reactive oxygen species comparing BTT-DNA ligand to copper on HeLa cells[41]. **e** Representative micrographs of cell superoxide comparing BTT-DNA ligand to copper on HeLa cells[41]. (blue) Hoechst 33342 staining of nuclei of all cells, (green) SYTOX green nucleic acid staining of nuclei of dead cells, (red) CellEvent™ Caspase-3/7 Red Detection Reagent of nuclei of apoptotic cells,s(magenta) CellROX Green Reagent of reactive oxygen species of nuclei of all cells, (yellow) MitoSOX™ green indicators of mitochondrial superoxide of all cells. (two biological replicates).

convert to Cu(I), and only enabling this reduction on the cell surface (Supplementary Fig. 22). No fluorescence signal was observed after washing off the sodium ascorbate and putting the cells back at 37 °C with fresh complete medium for 2 h (Supplementary Fig. 23). These results indicate that both the BTT-DNA ligand as well as the fluorogenic dye are intracellular after delivery and washing steps.

BTT-DNA ligand enables the detection of intracellular proteins in live cells

To expand the application of BTT-DNA to label and detect other major biomolecules, we focused on the intracellular labeling and detection of nascent proteins using *O*-propargyl-puromycin (OPP), an alkyne analog of puromycin which can incorporate into newly synthesized proteins[35]. After 4 h of ligand transfection and 1 h of OPP incubation, we washed the cells to ensure that CuAAC occurs exclusively inside the cells and not on extracellular alkynes, followed by a 30-min sodium ascorbate incubation. We observed strong fluorescence intensity in the cytoplasm and nucleus of cells treated with BTT-DNA (Fig. 6f), which is 1.4-fold higher than that of the no-ligand control (Fig. 6f). The result demonstrates that BTT-DNA ligand enables the live-cell detection of intracellular proteins.

Discussion

Here, we developed inCu-click, a modified CuAAC reaction enabled by the BTT-DNA ligand that facilitates CuAAC-mediated intracellular labeling while maintaining fast reaction kinetics. Importantly, this ligand eliminates the need to add copper salt to the CuAAC reaction because it is already complexed with the ligand. With this new CuAAC accelerating ligand, the total ligand concentration necessary for the CuAAC in vitro may be decreased to the nanomolar range while

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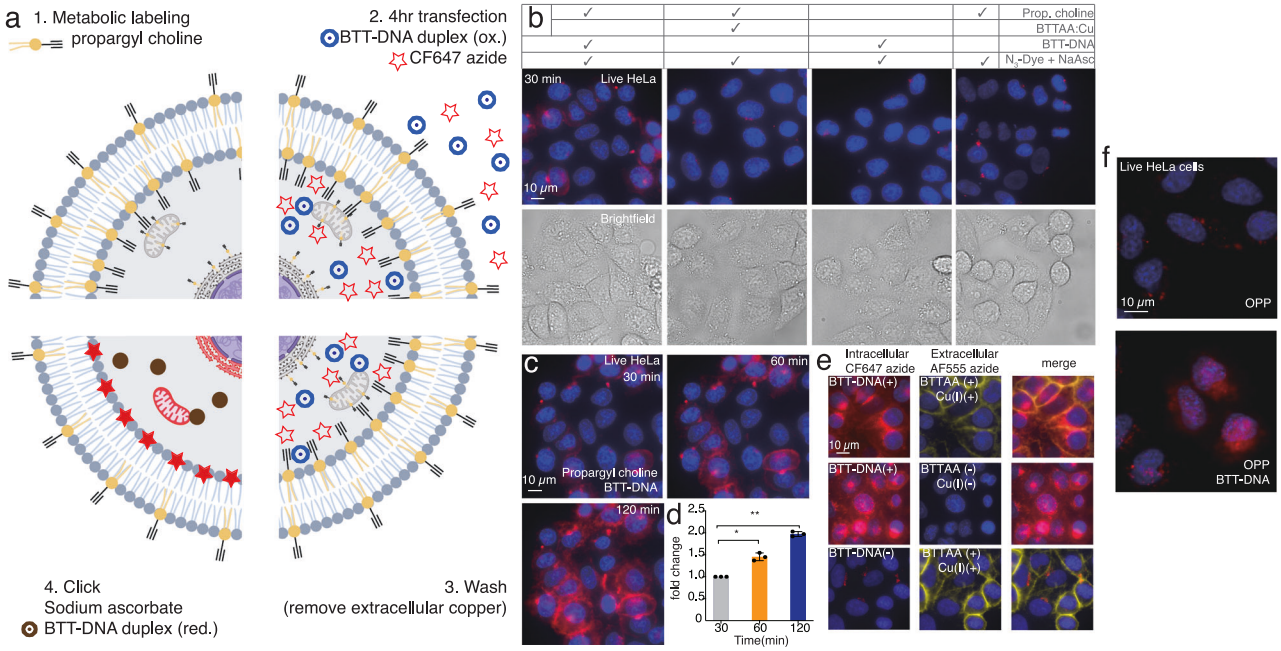

Fig. 6 | Intracellular phospholipid labeling and detection using inCu-click in live cells. a Workflow of metabolic labeling and detecting intracellular choline-containing phospholipid with BTT-DNA on live cells. (ox.) BTT-DNA duplex with oxidized copper, (red.) BTT-DNA duplex with reduced copper[41]. **b** Representative fluorescence micrographs (three biological replicates) of the intracellular phospholipids labeling and detection in the presence of BTT-DNA on live cells. Prop.-choline stands for propargyl choline. NaAsc stands for sodium ascorbate, N_3-dye stans for CalFluor 647 azide.(red) CalFluor 647 azide labeling of choline-containing phospholipid, (blue) Hoechst 33342 nuclei staining. **c** Time-lapse fluorescence microscopy (three biological replicates) of the intracellular phospholipids labeling in BTT-DNA presence for 2 h. Live cells are treated with CuAAC in the presence of propargyl choline, lipofectamine RNAiMAX transfection reagent, BTT-DNA ligand, CalFluor 647 azide, and sodium ascorbate. For reference, the 30-min time point is the same experiment as the bottom image in panel (**b**). **d** Fluorescence intensity fold-change enhancement as a function of reaction time for the BTT-DNA experiments in panel (**c**). The p value for 60 min is 0.0123. The p value for 120 min is 0.00105. Data were presented as mean ± SD. Error bars represent the standard deviation of 45 cells from three biological replicates. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, and $^{****}p \le 0.0001$. A two-sided t-test was used. **e** Representative fluorescence microscopy (two biological replicates) of the intracellular phospholipids labeling and detection in the presence of BTT-DNA and extracellular phospholipids labeling and detection in the presence of BTTAA-$CuSO_4$ complex on live cells. (red) CalFluor 647 azide labeling of choline-containing phospholipid, (yellow) Alexa Fluor 555 azide labeling of choline-containing phospholipid on the surface, (blue) Hoechst 33342 nuclei staining. **f** Representative fluorescence micrographs (two biological replicates) of the intracellular proteins labeling and detection in the presence of BTT-DNA on live cells. OPP stands for *O*-propargyl-puromycin. (red) CalFluor 647 azide labeling of puromycin-containing proteins, (blue) Hoechst 33342 nuclei staining.

remaining complexed to an average of ten copper ions per 15-mer ligand with different fluorogenic azide dyes. This new ligand also significantly drove forward the CuAAC reaction, showing more robust intracellular labeling in fixed cells and achieving live cell detection of biomolecules in the cell surface and cytoplasm. The 15-mer ligand was chosen because this length of modified oligonucleotide had high purity and consistent performance in our system. It is also the ideal length for liposome encapsulation; however, this length could change as other avenues for intracellular delivery are explored.

Previous studies have shown DNA splint-enhanced CuAAC[36–38] by combining the azide and the alkyne probes; however, these systems preclude using fluorogenic dyes, which are ideal for live-cell applications. The inCu-click reaction further drives the CuAAC reaction kinetics when hybridized to a DNA or RNA splint close to an alkyne-modified probe and clicked to a fluorogenic azide. This demonstrated that a completely non-fluorescent system may be used to drive the CuAAC reaction kinetics of a fluorogenic dye, which is highly desirable for live-cell imaging. The reaction was enhanced by adding spermine and NaCl before CuAAC. We suspect that the spermine contributes to the DNA-DNA duplex and DNA-RNA duplex stability[39], which is vital for the subsequent CuAAC reaction. Additionally, spermine can help mask the negative charge of oligonucleotides[39], which may sequester the copper away from the ligation site. We also showed that DNA splints with multiple binding sites could amplify the total fluorescence produced. Our BTT-DNA ligand could also enhance the RNA template-driven proximity ligation in situ on fixed cells.

Our experiments demonstrated that the inCu-click reaction could be used for standard CuAAC ligation assays in cellular environments to detect extracellular and intracellular biomolecules in fixed cells. For the detection of nascent DNAs in the cell nucleus using EdU labeling, we observed that the cells treated with 20 μM BTT-DNA ligand showed much stronger staining not only than cells treated without ligand but also than cells treated with 20 μM BTTAA commercial ligand, demonstrating that BTT-DNA ligand outperformed the commercial reagent[9] in detection of nascent DNAs in fixed cells. Robust labeling of nascent RNAs in the cell nuclei in the presence of BTT-DNA ligand was observed compared to the same concentration of commercial BTTAA ligand. As for the protein detection using L-HPG labeling, we found bright fluorescent signals in the nucleus and cytoplasm in the presence of BTT-DNA. For all three intracellular biomolecules, there was no significant difference in the fluorescence signal to the background using CuAAC accelerated by BTT-DNA compared to the no ligand control, indicating that the BTT-DNA ligand consistently outperforms the commercial ligand in biological environments with lower concentration. Interestingly, we achieved the highest signal-to-background ratio in phospholipid detection using propargyl choline labeling. This demonstrates that the BTT-DNA ligand can outperform the BTTAA ligand with a high yield.

We demonstrated that the inCu-click reaction significantly lowered the copper-induced cytotoxicity. When the cells were treated with free copper and sodium ascorbate for 5 h, the morphology of the cells changed, and almost all cells were “blebbing,” indicative of

pre-apoptosis. We showed that copper in the presence of sodium ascorbate could compromise the cell membrane integrity and cause cell death, and could lead to apoptosis. Interestingly, we also observed compromised cell membranes with sodium ascorbate alone when we increased the treatment time to 6 h. We found that not every blebbing or round cell showed fluorescence for SYTOX green, and cells with normal morphology could also show fluorescence for SYTOX green, which means blebbing is not the only indicator of the compromised cell membrane.

Copper-treated cells also produce oxidative stress, which can damage the biological material in cells, like ROS, which can oxidize the CellRox green reagent. This reagent is a fluorogenic probe that is non-fluorescent in the reduced state and exhibits bright green photostable fluorescence after being oxidized by the ROS and bound to DNA. We saw strong fluorescence in the nucleus, while fluorescence was dimmer in the cytoplasm. We also saw strong fluorescence in the nucleus of cells treated with 2.5 mM sodium ascorbate, indicating that a high concentration of sodium ascorbate could also cause the production of ROS in live cells. Superoxide is a form of oxidative stress responsible for damaging biomolecule structure integrity. MitoSOX green superoxide indicator measures the amount of superoxide produced only by mitochondria. We saw strong fluorescence in the cytoplasm of cells treated with copper in the presence of sodium ascorbate. The cells treated with BTT-DNA ligand appeared morphologically like the negative control for all tests. This indicates that our BTT-DNA ligand is biocompatible with living cells by lowering copper-induced cytotoxicity.

Finally, we demonstrated that the inCu-click reaction may be used for intracellular detection of propargyl choline-labeled nascent phospholipids and nascent proteins within live cells. This allowed us to track intracellular biomolecules via CuAAC, assisted by our BTT-DNA ligand for 2 h. For the lipid labeling, we cannot exclude the possibility that the reaction is occurring within the endosome rather than the cytosol, albeit still intracellular labeling. The labeling of nascent proteins using OPP is a clear demonstration of the CuAAC reaction occuring in the cytosol. Our live cell labeling scheme could enable future applications such as lipid metabolism and protein metabolism in response to drug treatment or tracking of global cellular transcription over time. Overall, the newly developed inCu-click reaction allows sensitive detection of biomolecules in fixed and live cells and holds great promise for future probing of drug or probe molecules interacting closely with biomolecules inside live cells. These studies can improve the precision of therapies in reaching diseased cells.

Methods

Synthesis and purification of BTT-DNA ligand

A 200-fold molar excess of *N,N*-bis((1-*tert*-butyl-1*H*–1,2,3- triazol-4-yl) methyl)prop-2-yn-1-amine (S1; purchased from the AECOM chemical biology core facility) was reacted with 3′ N_3-labeled 15-mer ssDNA oligo (purchased from IDT) using BTTAA ligand-assisted CuAAC ([BTTAA]:[$CuSO_4$]=2:1). The reaction mixture was prepared by adding the following reagents (Table 1): N_3-DNA (100 µM), S1-alkyne (40 mM), premixed copper sulfate (5 mM) and BTTAA (20 mM), then sodium ascorbate (100 mM). It was then shaken at 600 rpm for 30 min at 37 °C, resulting in a crude BTT-DNA ligand. The BTT-DNA ligand was purified using 3.5 kD MWCO dialysis tubing for 24 h in 3.5 L of water at room temperature (Repligen). The Invitrogen Qubit ssDNA Assay Kit was used to measure the concentration of the purified BTT-DNA ligand.

Synthesis and purification of unmodified N_3-DNA probe

A 200-fold molar excess of S1 was reacted with 3′ N_3-labeled 15-mer ssDNA oligo using BTTAA ligand-assisted CuAAC ([BTTAA]:[$CuSO_4$] = 2:1). The reaction mixture was prepared by adding the following reagents (Table 2): N_3-DNA (100 µM), S1-alkyne (40 mM), premixed copper sulfate (5 mM) and BTTAA (20 mM), then sodium ascorbate (100 mM). It was then shaken at 600 rpm for 30 min at 37 °C, resulting in the unmodified N_3-DNA probe. The unmodified N_3-DNA probe was purified using 3.5 kD MWCO dialysis tubing for 24 h in 3.5 L of water at room temperature (Repligen). The Invitrogen Qubit ssDNA Assay Kit was used to measure the concentration of the purified unmodified N_3-DNA probe.

Table 1 | Synthesis of BTT-DNA ligand

Reagent	stock	Volume added	Final concentration
N_3-DNA	100 µM	48 µl	6 µM
S1-alkyne	40 mM	24 µl	1200 µM
CuSO4	5 mM	12 µl	75 µM
BTTAA	20 mM	6 µl	150 µM
Sodium Ascorbate	100 mM	20 µl	2.5 mM
NF-H20		690 µl	
Total volume		800 µl	

Table 2 | Synthesis of unmodified N_3-DNA probe

Reagent	stock	Volume added	Final concentration
N_3-DNA	100 µM	48 µl	6 µM
CuSO4	5 mM	12 µl	75 µM
BTTAA	20 mM	6 µl	150 µM
Sodium ascorbate	100 mM	20 µl	2.5 mM
NF-H20		714 µl	
Total volume		800 µl	

Urea-PAGE gel electrophoresis

The electrophoresis of 15% TBE-Urea Gels (Thermo Fisher) was performed using the XCell SureLock® Mini-Cell (Thermo Fisher). About 0.5 ul of DNA (5 uM), 7 ul of NF-water and 7.5 ul of TBE Urea sample buffer (2X) (Thermo Fisher) was added into a 1.5 ml tube, followed by 3-min heating at 37 °C to denature the DNA sample. Then the samples were loaded into the 15% TBE Urea gel, and the wells have been flushed several times with 1xTBE running buffer to remove urea. The gel was running for 70 min at 180 voltages. After running, SYBR gold nucleic acid gel staining was used to stain the gel for 10 min in the dark. The Gel doc EZ gel documentation system (BIO-RAD) was used to make gel imaging. The ladder used in the experiment was an ultra-low-range DNA ladder (Thermo Fisher).

Inductively coupled plasma mass spectrometry (ICP-MS)

To assess the copper complexed with the BTT-DNA ligand, we prepared the ligand for ICP-MS. The sample was dissolved (0.1 ml) in 0.9 ml of concentrated nitric acid. The sample was heated for an hour at 90 °C. Then, the temperature was increased to 150 °C, and the sample was boiled. About 0.5 ml of concentrated nitric acid was added to the sample twice during the boiling period. When the sample was completely dried, we added 0.5 ml of concentrated nitric acid and diluted it with NF-H_2O to bring the final volume to 10 ml. The reagent blank was made by same preparation with only nitric acid. Samples and reagent blanks were submitted to Robertson Microlit Laboratories at Ledgewood, New Jersey, for ICP-MS.

To prepare a calibration standard, into a suitable volumetric flask, transfer appropriate amounts of certified ICP-grade standard solutions of copper to yield a concentration approximately equivalent to the expected range of copper in our sample. Dilute to volume with 1% aqueous nitric acid, and mix. Calibrate the ICP instrument using the

calibration standard. Sequentially aspirate the reagent blank, and each sample into the ICP instrument.

Plate-reader assay

The click reaction mixture was prepared by adding the following reagents (Table 3): PBS (10X), alkyne (10 μM), premixed copper sulfate (5 μM), and BTTAA (10 μM) or BTT-DNA(10 μM), azido dye (75 μM), then sodium ascorbate (25 mM). The click reaction mixture was added to the wells (20 μL/well) of the 384-well fluorescence plate, and the click reaction was carried out for 2 h at room temperature. The fluorescent alkyne-azide cyclo-adduct was detected using a Spectral Max Gemini EM plate reader with excitation/emission wavelengths at 404/477 nm for 3-azido-7-hydroxycoumarin, 500/521 nm for CalFlour 488 azide, 561/583 nm for CalFlour 555 azide, 591/609 nm for CalFlour 580 azide, and 657/674 nm for CalFlour 647 azide. For the DNA and RNA template-driven proximity ligation click reaction, 2 μl of DNA or RNA (4.5 μM) (Table 4), 2 μl of spermine (1 mM), and 2 μl of NaCl (10 M) were added after BTT-DNA. Kinetic data was analyzed using Igor Pro software (Wavemetrics).

Tissue culture conditions

HeLa cell line (ATCC, CCL-2) and CHO cell line was kindly provided by Dr. Arjun Raj were grown in Dulbecco's modified Eagle's medium (Gibco, 10566024) supplemented with 10% fetal bovine serum(Fisher Scientific, FB12999102) and 1% Penicillin-Streptomycin (Lonza,17602E). All cells were incubated in a 5% CO_2, water-saturated incubator at 37 °C.

Table 3 | CuAAC reaction components for plate-reader assay

Reagent	Stock	Volume added	Final concentration
PBS	10X	2 μl	1X
Alkyne	10 μM	2 μl	1 μM
BTT-DNA(or Cu and BTTAA premix)	10 μM	2 μl	1 μM
Azido dye	75 μM	2 μl	7.5 μM
NF-H2O		10 μl	
Sodium Ascorbate	25 mM	2 μl	2.5 mM
Total volume		20 μl	

Proximity click FISH of M20 transgene RNA on fixed cells

CHO cells and CHO-GFP-M20 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days and harvested by centrifugation (300×*g*, 3 min), resuspended in fresh media, and seeded into the eight-well chamber (Fisher Scientific). We seeded an equal concentration of CHO cells and CHO-GFP-M20 cells together, and the total cell number is 60,000 cells/well, and the total volume per well is 300 μl, and incubated for 24 h.

Cells were rinsed with 1X PBS and fixed with 4% formaldehyde for 10 min, then permeabilized with 70% EtOH in NF-H_2O at 4 °C overnight. Cells were rinsed with 1X PBS/10% formamide, followed by treatment with 100 μl smFISH hybridization buffer (10% formamide and 10% dextran sulfate in 1X PBS) containing 2 μl of M20-left-alkyne_20_2(10 μM) for 24 h at 37 °C. After hybridization, cells were washed with 300 μl 1X PBS/10% formamide for 30 min at 37 °C. Then, it was treated with 10 μM CalFluor 647 Azide, BTT-DNA ligand ([BTT-DNA] = 20 μM), 1 M NaCl, and 2.5 mM freshly prepared sodium ascorbate in 1X PBS/0.25% Triton for 1 h at 37 °C. After the click reaction, the cells were rewashed with 300 μl 1X PBS/10% formamide for 30 min twice at 37 °C and counterstained with DAPI stain diluted with 1X PBS/10% formamide for 20 min at 37 °C. Then, 1X PBS was added to each well to prepare for imaging. For negative control, we treated the cells with M20-left-alkyne_20_2 without ligand.

5-ethynyl-2′-deoxyuridine (EdU) labeling of nascent DNAs on fixed cells

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, harvested by centrifugation (300×*g*, 3 min), resuspended in fresh media, and seeded into the 18-well chamber (Cellvis). About 1.5 μl EdU (1 mM; Thermo Fisher Scientific) was added to each well, and the total volume per well was 150 μl, and incubated for 24 h.

Cells were rinsed with 1X PBS and fixed with 4% formaldehyde for 10 min, then permeabilized with 70% EtOH in NF-H_2O at 4 °C overnight. Cells were washed with 1X PBS/10% formamide for 30 min at 37 °C, followed by treatment with 10 μM CalFluor 647 Azide, premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$] = 2:1,[$CuSO_4$] = 10 μM) or BTT-DNA ligand ([BTT-DNA] = 20 μM) and 2.5 mM freshly prepared sodium ascorbate for 30 min at 37 °C. After the click reaction, the cells were rewashed with 150 μl wash buffer for 30 min twice at 37 °C and

Table 4 | List of oligonucleotides used

Oligonucleotide	Sequence
M20-right_3′azide	5′-GGTGCTCTTCGTCCA/3AzideN/
Random-M20_3′azide	5′-GCA GAG ACA CTA TTG/3AzideN/
M20-left-alkyne	5′-Hexynyl/CAAACACAACTCCTG-3′
M20-left-alkyne_20	5′-Hexynyl/CAAACACAACTCCTGGTCGA-3′
M20-left-alkyne_20_1	5′-Hexynyl/AAACACAACTCCTGGTCGAG-3′
M20-left-alkyne_20_2	5′-Hexynyl/AACACAACTCCTGGTCGAGG-3′
M20-left-alkyne_20_t	5′-Hexynyl/TCAAACACAACTCCTGGTCGA-3′
M20-left-alkyne_20_2t	5′-Hexynyl/TTCAAACACAACTCCTGGTCGA-3′
M20-left-alkyne_20_3t	5′-Hexynyl/TTTCAAACACAACTCCTGGTCGA-3′
M20-left-alkyne_20_4t	5′-Hexynyl/TTTTCAAACACAACTCCTGGTCGA-3′
DNAX1 splint	5′-CGACCTCGACCAGGAGTTGTGTTTGTGGACGAAGAGCACC-3′
DNAX2 splint	5′-CGACCTCGACCAGGAGTTGTGTTTGTGGACGAAGAGCACCAAACGACCTCGACCAGGAGTTGTGTTTGTGGACGAAGAGCACC-3′
DNAX3 splint	5′-CGACCTCGACCAGGAGTTGTGTTTGTGGACGAAGAGCACCAAACGACCTCGACCAGGAGTTGTGTTTGTGGACGAA-GAGCACCAAACGACCTCGACCAGGAGTTGTGTTTGTGGACGAAGAGCACC-3′
RNAX1 splint	5′-CGACCUCGACCAGGAGUUGUGUUUGUGGACGAAGAGCACC-3′
M20-right_3′azide-complementary	5′-TGGACGAAGAGCACC-3′
M20-right_20_3′azide	5′-TGGCTGGTGCTCTTCGTCCA/3AzideN/
M20-right_25_3′azide	5′-TCAGCTGGCTGGTGCTCTTCGTCCA/3AzideN/

counterstained with DAPI stain diluted with 1X PBS/10% formamide for 20 min at 37 °C. Then, 1X PBS was added to each well to prepare for imaging.

5-ethynyl uridine (EU) labeling of nascent RNAs on fixed cells

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, harvested by centrifugation (300×*g*,3 min), resuspended in fresh media, and seeded into the 18-well chamber (Cellvis). The total volume per well is 150 µl. After 1 day of incubation at 37 °C, 1.5 µl 5-ethynyl uridine (EU) was added to each well to a 1 mM final concentration and returned to the incubator for 3 h of incubation.

Cells were rinsed with 1X PBS and fixed with 4% formaldehyde for 10 min, then permeabilized with 70% EtOH in NF-H_2O at 4 °C overnight. Cells were washed with 1X PBS/10% formamide for 30 min at 37 °C, followed by treatment with 10 µM CalFluor 647 Azide, premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$] = 2:1,[$CuSO_4$] = 10 µM) or BTT-DNA ligand ([BTT-DNA] = 20 µM) and 2.5 mM freshly prepared sodium ascorbate for 30 min at 37 °C. After the click reaction, the cells were rewashed with 150 µl wash buffer for 30 min twice at 37 °C and counterstained with DAPI stain diluted with 1X PBS/10% formamide for 20 min at 37 °C. Then, 1X PBS was added to each well to prepare for imaging.

L-homopropargyl(L-HPG) labeling of nascent proteins on fixed cells

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, harvested by centrifugation (300 × *g*, 3 min), and resuspended in fresh media and seeded into the 18-well chamber (Cellvis). The total volume per well is 150 µl. After 1 day of incubation at 37 °C, the cell growth medium was removed and replaced with a methionine-free DMEM medium for 30 min to eliminate the methionine. Then 1.5 µl L-HPG was added to each well to a 2 mM final concentration and returned to the incubator for 2-h incubation.

Cells were rinsed with 1X PBS and fixed with 4% formaldehyde for 10 min, then permeabilized with 70% EtOH in NF-H_2O at 4 °C overnight. Cells were washed with 1X PBS/10% formamide for 30 min at 37 °C, followed by treatment with 10 µM CalFluor 647 Azide, premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$] = 2:1,[$CuSO_4$] = 10 µM) or BTT-DNA ligand ([BTT-DNA] = 20 µM) and 2.5 mM freshly prepared sodium ascorbate for 30 min at 37 °C. After the click reaction, the cells were rewashed with 150 µl wash buffer for 30 min twice at 37 °C and counterstained with DAPI stain diluted with 1X PBS/10% formamide for 20 min at 37 °C. Then, 1X PBS was added to each well to prepare for imaging.

N -(4-pentynoyl) mannosamine (Ac4MaNAl) labeling of cell-surface sialic acid on fixed cells

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, then harvested and resuspended in fresh media. About 2.5 µl of Ac_4ManNAl (5 mM; Click Chemistry Tools) was added to each well of the 18-well chamber (Cellvis) and dried for 20 min. Cells were seeded into the Ac_4ManNAl-treated 18-well chamber, and the total volume per well was 150 µl, and incubated for 24 h.

Cells were rinsed with 1X PBS and fixed with 4% formaldehyde for 10 min. Cells then were washed with 1X PBS/10% formamide for 30 min at 37 °C, followed by treatment with 10 µM CalFluor 647 Azide, premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$] = 2:1,[$CuSO_4$] = 10 µM) or BTT-DNA ligand ([BTT-DNA] = 20 µM) and 2.5 mM freshly prepared sodium ascorbate for 30 min at 37 °C. After the click reaction, the cells were rewashed with 150 µl wash buffer for 30 min twice at 37 °C and counterstained with DAPI stain diluted with 1X PBS/10% formamide for 20 min at 37 °C. Then, 1X PBS was added to each well to prepare for imaging.

Propargyl choline labeling of choline-containing phospholipids on fixed cells

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, then harvested and resuspended in fresh media. Cells were seeded into an 18-well chamber (Cellvis), 1.5 µl of propargyl choline (10 mM; Jena Bioscience) was added to each well, and the total volume per well was 150 µl, and incubated for 24 h.

Cells were rinsed with 1X PBS and fixed with 4% formaldehyde for 10 min. Cells then were washed with 1X PBS/10% formamide for 30 min at 37 °C, followed by treatment with 10 µM CalFluor 647 Azide, premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$] = 2:1,[$CuSO_4$] = 10 µM) or BTT-DNA ligand ([BTT-DNA] = 20 µM) and 2.5 mM freshly prepared sodium ascorbate for 30 min at 37 °C. After the click reaction, the cells were rewashed with 150 µl wash buffer for 30 min twice at 37 °C and counterstained with DAPI stain diluted with 1X PBS/10% formamide for 20 min at 37 °C. Then, 1X PBS was added to each well to prepare for imaging.

Click extracellular labeling of sialylated glycans on live cells

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, then harvested and resuspended in fresh media. About 2.5 µl of Ac_4ManNAl (5 mM; Click Chemistry Tools) was added to each well of the 18-well chamber (Cellvis) and dried for 20 min. Cells were seeded into the Ac_4ManNAl-treated 18-well chamber, and the total volume per well was 150 µl, and incubated for 24 h.

Cells were washed 3X with PBS/1%FBS (pH 7.4) followed by treatment with 10 µM CalFluor 647 Azide (Click Chemistry Tools), premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$] = 2:1, [$CuSO_4$] = 10 µM) or BTT-DNA ligand ([BTT-DNA] = 20 µM) and 2.5 mM freshly prepared sodium ascorbate for 30 min on ice. The cells were washed 3X with 200 µl of PBS/1%FBS (pH 7.4) to remove the unreacted CalFluor 647 Azide dye and followed by staining with 100 µl of diluted ReadyProbes™ Cell Viability Imaging Kit, Blue/Green (Thermo Fisher Scientific) for 10 min at 37 °C to stain the nucleus of the live and dead cells. The cells were then washed 3X with PBS/1%FBS (pH 7.4). Then 100 µl of PBS/1%FBS (pH 7.4) was added to each well to prepare for imaging.

Click extracellular labeling of choline-containing phospholipids on live cells

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, then harvested and resuspended in fresh media. Cells were seeded into an 18-well chamber (Cellvis), and 1.5 µl of propargyl choline (10 mM; Jena Bioscience) was added to each well, and the total volume per well was 150 µl, and incubated for 24 h.

Cells were washed 3X with PBS/1%FBS (pH 7.4) followed by treatment with 10 µM CalFluor 647 Azide (Click Chemistry Tools), premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$] = 2:1, [$CuSO_4$] = 10 µM) or BTT-DNA ligand ([BTT-DNA] = 20 µM) and 2.5 mM freshly prepared sodium ascorbate for 30 min on ice. The cells were washed 3X with 200 µl of PBS/1%FBS (pH 7.4) to remove the unreacted CalFluor 647 Azide dye and followed by staining with 100 µl of diluted ReadyProbes™ Cell Viability Imaging Kit, Blue/Green (Thermo Fisher Scientific) for 10 min at 37 °C to stain the nucleus of the live and dead cells. The cells were then washed 3X with PBS/1%FBS (pH 7.4). Then 100 µl of PBS/1%FBS (pH 7.4) was added to each well to prepare for imaging.

Click intracellular labeling of choline-containing phospholipids and puromycin-containing proteins on live cells with Lipofectamine RNAiMAX

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, then harvested and

resuspended in fresh media. Cells were seeded into an 18-well chamber (Cellvis) (2000 cells each well), 1.5 µl of propargyl choline (10 mM; Jena Bioscience) was added to each well, and the total volume per well was 150 µl, and incubated for 24 h.

About 12 µl of BTT-DNA ligand (100 µM) and 12 µl of M20-right_3'azide-complementary (100 µM) complementary to the BTT-DNA sequence were added to separate 1.5 mL eppendorf™ DNA LoBind microcentrifuge tubes (Fisher Scientific) and dried out with SpeedVac SPD1030 integrated vacuum concentrator (Thermo Scientific) at program 1 for 20 min. About 3 µl of 1X PBS was added to each tube, and we vortexed the tube for 1 min to dissolve the BTT-DNA and splint. After vortexing, we mixed and hybridized them for 1 h at 37 °C. After hybridization, we diluted the BTT-DNA ligand by adding 15.15 µl prewarmed Opti-MEM™ I reduced serum medium (Thermo Fisher Scientific) to the tube. At the same time, we diluted 0.5 µl of Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific) in 15.15 µl prewarmed Opti-MEM™ I reduced serum medium in a different 1.5 ml eppendorf™ DNA LoBind microcentrifuge tube. Then, we added all the diluted BTT-DNA ligands to the Lipofectamine™ RNAiMAX transfection reagent and incubated them at room temperature for 20 min to form the DNA-lipid complex.

After the encapsulation, 3.2 µl of CF647 azide (1 mM) was mixed into the DNA-lipid complex. Then, the mixture was applied to cells and incubated for 4 h in a 5% CO_2 incubator at 37 °C. Cells were then washed 3X with non-phenol red DMEM/10%FBS to remove extra dye and BTT-DNA ligand outside of the cells, followed by treatment with 2 mM freshly prepared sodium ascorbate for 25 min in a 5% CO_2 incubator at 37 °C. The cells were washed 3X with 100 µl of non-phenol red DMEM/10%FBS to remove extra sodium ascorbate, followed by staining with 100 µl of diluted ReadyProbes™ Cell Viability Imaging Kit, Blue/Green (Thermo Fisher Scientific) for 5 min at 37 °C to stain the nucleus of the live and dead cells. The cells were then washed 3X with non-phenol red DMEM/10%FBS. Then 100 µl of non-phenol red DMEM/10%FBS was added to each well to prepare for imaging. For the negative control, untreated cells cultured in propargyl choline and treated cells cultured without propargyl choline were included. Cells treated with BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$] = 2:1, [$CuSO_4$] = 300 µM) were also included for comparison. For intracellular labeling of puromycin-containing proteins, cells were treated with 100 µM OPP for 1 h before click reaction and the dye was delivered using streptolysin-O.

Click intracellular and extracellular labeling of choline-containing phospholipids on live cells

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, then harvested and resuspended in fresh media. Cells were seeded into an 18-well chamber (Cellvis) (2000 cells each well), 1.5 µl of propargyl choline (10 mM; Jena Bioscience) was added to each well, and the total volume per well was 150 µl, and incubated for 24 h.

About 12 µl of BTT-DNA ligand (100 µM) and 12 µl of M20-right_3'azide-complementary (100 µM) complementary to the BTT-DNA sequence were added to separate 1.5 mL eppendorf™ DNA LoBind microcentrifuge tubes (Fisher Scientific) and dried out with SpeedVac SPD1030 integrated vacuum concentrator (Thermo Scientific) at program 1 for 20 min. About 3 µl of 1X PBS was added to each tube, and we vortexed the tube for 1 min to dissolve the BTT-DNA and splint. After vortexing, we mixed and hybridized them for 1 h at 37 °C. After hybridization, we diluted the BTT-DNA ligand by adding 15.15 µl prewarmed Opti-MEM™ I reduced serum medium (Thermo Fisher Scientific) to the tube. At the same time, we diluted 0.5 µl of Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific) in 15.15 µl prewarmed Opti-MEM™ I reduced serum medium in a different 1.5 ml eppendorf™ DNA LoBind microcentrifuge tube. Then, we added all the diluted BTT-DNA ligands to the Lipofectamine™ RNAiMAX transfection reagent and incubated them at room temperature for 20 min to form the DNA-lipid complex.

After the encapsulation, 3.2 µl of CF647 azide (1 mM) was mixed into the DNA-lipid complex. Then, the mixture was applied to cells and incubated for 4 h in a 5% CO_2 incubator at 37 °C. Cells were then washed 3X with non-phenol red DMEM/10%FBS to remove extra dye and BTT-DNA ligand outside of the cells, followed by treatment with 2 mM freshly prepared sodium ascorbate for 25 min in a 5% CO_2 incubator at 37 °C. The cells were washed 3X with 100 µl of non-phenol red DMEM/10%FBS to remove extra sodium ascorbate, followed by 5-min CuAAC using 10 µM Alexa Fluot™ 555 azide (Thermo Fisher Scientific) in the presence of BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$] = 2:1, [$CuSO_4$] = 100 µM) and 2 mM sodium ascorbate in a 5% CO_2 incubator at 37 °C. The cells were washed 3X with 100 µl of non-phenol red DMEM/10%FBS to remove CuAAC components, followed by staining with 100 µl of diluted ReadyProbes™ Cell Viability Imaging Kit, Blue/Green (Thermo Fisher Scientific) for 5 min at 37 °C to stain the nucleus of the live and dead cells. The cells were then washed 3X with non-phenol red DMEM/10%FBS. Then 100 µl of non-phenol red DMEM/10%FBS was added to each well to prepare for imaging. For the negative control, BTT-DNA treated cells without BTTAA-$CuSO_4$ complex for extracellular labeling and untreated cells with BTTAA-$CuSO_4$ complex for extracellular labeling were included. BTT-DNA-treated cells with 2 mM freshly prepared sodium ascorbate for 25 min on ice was also included for comparison.

Cellular toxicity assays

Viability. Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, then harvested and resuspended in fresh media. Cells were seeded into an 18-well chamber (Cellvis) (2000 cells per well) with or without propargyl choline, the total volume per well 150 µl, and incubated for 24 h.

About 12 µl of BTT-DNA ligand (100 µM) and 12 µl of M20-right_3'azide-complementary (100 µM) complementary to BTT-DNA sequence were added to separate 1.5 mL eppendorf™ DNA LoBind microcentrifuge tubes (Fisher Scientic) and dried out with SpeedVac SPD1030 integrated vacuum concentrator (Thermo Scientific) at program 1 for 20 min. About 3 µl of 1XPBS was added to each tube, and we vortexed the tube for 1 min to dissolve the BTT-DNA and splint. After vortexing, we mixed and hybridized them for 1 h at 37 °C. After hybridization, we diluted the BTT-DNA ligand by adding 16.35 µl prewarmed Opti-MEM™ I reduced serum medium (Thermo Fisher Scientific) to the tube. At the same time, we diluted 0.5 µl of Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific) in 16.35 µl prewarmed Opti-MEM™ I reduced serum medium in a different 1.5 ml eppendorf™ DNA LoBind microcentrifuge tube. Then, we added all the diluted BTT-DNA ligands to the Lipofectamine™ RNAiMAX transfection reagent and incubated them at room temperature for 20 min to form the DNA-lipid complex.

After encapsulation, 0.8 µl of sodium ascorbate (100 mM) was mixed with the DNA-lipid complex. Then, the mixture was applied to cells and incubated for 5 h in a 5% CO_2 incubator at 37 °C. Cells were then washed 3X with non-phenol red DMEM/10%FBS to remove extra BTT-DNA ligands outside of the cells, followed by staining with 100 µl of diluted ReadyProbes™ Cell Viability Imaging Kit, Blue/Green (Thermo Fisher Scientific) for 10 min at 37 °C to stain the nucleus of the live and dead cells. The cells were then washed 3X with non-phenol red DMEM/10%FBS. Then 100 µl of non-phenol red DMEM/10%FBS was added to each well to prepare for imaging. Untreated cells and cells treated with 2 mM sodium ascorbate were included for negative control. We treated the cells with 300 µM $CuSO_4$ in 2 mM sodium ascorbate for positive toxicity control.

ROS detection. Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, then

harvested and resuspended in fresh media. Cells were seeded into an 18-well chamber (Cellvis) (2000 cells per well) with or without propargyl choline, the total volume per well 150 µl, and incubated for 24 h.

About 12 µl of BTT-DNA ligand (100 µM) and 12 µl of M20-right_3'azide-complementary (100 µM) complementary to BTT-DNA sequence were added to separate 1.5 mL eppendorf™ DNA LoBind microcentrifuge tubes (Fisher Scientic) and dried out with SpeedVac SPD1030 integrated vacuum concentrator (Thermo Scientific) at program 1 for 20 min. About 3 µl of 1XPBS was added to each tube, and we vortexed the tube for 1 min to dissolve the BTT-DNA and splint. After vortexing, we mixed and hybridized them for 1 h at 37 °C. After hybridization, we diluted the BTT-DNA ligand by adding 16.35 µl prewarmed Opti-MEM™ I reduced serum medium (Thermo Fisher Scientific) to the tube. At the same time, we diluted 0.5 µl of Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific) in 16.35 µl prewarmed Opti-MEM™ I reduced serum medium in a different 1.5 ml eppendorf™ DNA LoBind microcentrifuge tube. Then, we added all the diluted BTT-DNA ligands to the Lipofectamine™ RNAiMAX transfection reagent and incubated them at room temperature for 20 min to form the DNA-lipid complex.

After the encapsulation, 0.8 µl of sodium ascorbate (100 mM) was mixed to the DNA-lipid complex. Then, the mixture was applied to cells and incubated for 5 h in a 5% CO_2 incubator at 37 °C. Cells were then washed 3X with PBS/1%FBS (pH 7.4) to remove extra BTT-DNA ligands outside of the cells, followed by staining with 100 µl of diluted CellROX™ Green Reagent (Thermo Fisher Scientific) and NucBlue™ Live ReadyProbes™ Reagent (Thermo Fisher Scientific) for 20 min at 37 °C. The cells were then washed 3X with PBS/1%FBS (pH 7.4). Then 100 µl of PBS/1%FBS (pH 7.4) was added to each well to prepare for imaging. Untreated cells and cells treated with 2 mM sodium ascorbate were included for negative control. We treated the cells with 300 µM $CuSO_4$ in 2 mM sodium ascorbate for positive toxicity control.

Mitochondrial superoxide detection. Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, then harvested and resuspended in fresh media. Cells were seeded into an 18-well chamber (Cellvis) (2000 cells per well) with or without propargyl choline, the total volume per well 150 µl, and incubated for 24 h.

About 12 µl of BTT-DNA ligand (100 µM) and 12 µl of M20-right_3'azide-complementary (100 µM) complementary to the BTT-DNA sequence were added to separate 1.5 mL eppendorf™ DNA LoBind microcentrifuge tubes (Fisher Scientific) and dried out with SpeedVac SPD1030 integrated vacuum concentrator (Thermo Scientific) at program 1 for 20 min. About 3 µl of 1XPBS was added to each tube, and we vortexed the tube for 1 min to dissolve the BTT-DNA and splint. After vortexing, we mixed and hybridized them for 1 h at 37 °C. After hybridization, we diluted the BTT-DNA ligand by adding 16.35 µl prewarmed Opti-MEM™ I reduced serum medium (Thermo Fisher Scientific) to the tube. At the same time, we diluted 0.5 µl of Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific) in 16.35 µl prewarmed Opti-MEM™ I reduced serum medium in a different 1.5 ml eppendorf™ DNA LoBind microcentrifuge tube. Then, we added all the diluted BTT-DNA ligands to the Lipofectamine™ RNAiMAX transfection reagent and incubated them at room temperature for 20 min to form the DNA-lipid complex.

After the encapsulation, 0.8 µl of sodium ascorbate (100 mM) was mixed into the DNA-lipid complex. Then, the mixture was applied to cells and incubated for 5 h in a 5% CO_2 incubator at 37 °C. Cells were then washed 3X with PBS/1%FBS (pH 7.4) to remove extra BTT-DNA ligands outside of the cells, followed by staining with 100 µl of diluted MitoSOX Green superoxide indicators and NucBlue™ Live ReadyProbes™ Reagent (Thermo Fisher Scientific) for 30 min at 37 °C. The cells were then washed 3X with PBS/1%FBS (pH 7.4). Then 100 µl of PBS/1%FBS (pH 7.4) was added to each well to prepare for imaging. Untreated cells and cells treated with 2 mM sodium ascorbate were included for negative control. We treated the cells with 300 µM $CuSO_4$ in 2 mM sodium ascorbate for positive toxicity control.

Apoptosis detection. Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for 3 days, then harvested and resuspended in fresh media. Cells were seeded into an 18-well chamber (Cellvis) (2000 cells per well) with or without propargyl choline, the total volume per well 150 µl, and incubated for 24 h.

About 12 µl of BTT-DNA ligand (100 µM) and 12 µl of M20-right_3'azide-complementary (100 µM) complementary to the BTT-DNA sequence were added to separate 1.5 mL eppendorf™ DNA LoBind microcentrifuge tubes (Fisher Scientific) and dried out with SpeedVac SPD1030 integrated vacuum concentrator (Thermo Scientific) at program 1 for 20 min. About 3 µl of 1XPBS was added to each tube, and we vortexed the tube for 1 min to dissolve the BTT-DNA and splint. After vortexing, we mixed and hybridized them for 1 h at 37 °C. After hybridization, we diluted the BTT-DNA ligand by adding 16.35 µl prewarmed Opti-MEM™ I reduced serum medium (Thermo Fisher Scientific) to the tube. At the same time, we diluted 0.5 µl of Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific) in 16.35 µl prewarmed Opti-MEM™ I reduced serum medium in a different 1.5 ml eppendorf™ DNA LoBind microcentrifuge tube. Then, we added all the diluted BTT-DNA ligands to the Lipofectamine™ RNAiMAX transfection reagent and incubated them at room temperature for 20 min to form the DNA-lipid complex.

After the encapsulation, 0.8 µl of sodium ascorbate (100 mM) was mixed to the DNA-lipid complex. Then, the mixture was applied to cells and incubated for 5 h in a 5% CO_2 incubator at 37 °C. Cells were then washed 3X with PBS/1%FBS (pH 7.4) to remove extra BTT-DNA ligands outside of the cells, followed by staining with 100 µl of diluted CellEvent™ Caspase-3/7 Red Detection Reagent (Thermo Fisher Scientific) and NucBlue™ Live ReadyProbes™ Reagent (Thermo Fisher Scientific) for 45 min at 37 °C. The cells were then washed 3X with PBS/1%FBS (pH 7.4). Then 100 µl of PBS/1%FBS (pH 7.4) was added to each well to prepare for imaging. Untreated cells and cells treated with 2 mM sodium ascorbate were included for negative control. We treated the cells with 300 µM $CuSO_4$ in 2 mM sodium ascorbate for positive toxicity control.

Quantification methods

Quantification of labeling intensity of biomolecules with BTT-DNA-assisted CuAAC on fixed cells. Fluorescence intensities were obtained from random points in various raw fluorescence micrographs. A minimum of 175 points were acquired for analysis of EdU labeling, a minimum of 90 points were acquired for EU labeling, a minimum of 115 points were obtained for L-HPG labeling, a minimum of 85 points were acquired for Ac_4ManNAl labeling, and a minimum of 95 points were obtained for propargyl choline labeling. Intensity was normalized to the fluorescence intensity from the no ligand control.

Quantification of extracellular labeling intensity of biomolecules with BTT-DNA-assisted CuAAC on live cells. Fluorescence intensities were obtained from random cells in various raw fluorescence micrographs. Five random points were acquired from each cell membrane to obtain the average fluorescence of each cell. Sixty cells were acquired for Ac_4ManNAl labeling, and 45 cells were obtained for propargyl choline labeling. Intensity was normalized to the fluorescence intensity from the no ligand control.

Quantification of intracellular labeling of choline-containing phospholipids on live cells with Lipofectamine RNAiMAX. Fluorescence intensities were obtained from random 60 cells in three fluorescence micrographs from three biological replicates. Five random points were acquired from each cell cytoplasm to obtain the average

fluorescence of each cell. Intensity was normalized to the fluorescence intensity from the fluorescence micrographs at 30 min.

Quantification of viability. The analysis of cell viability was conducted using CellProfiler[40]. The details are in the CellProfiler project file (Viability_analysis.cpproj). Briefly, the DAPI and GFP channels of each image containing many cells were used for analysis. The DAPI channel was used to segment the nuclei by global minimum cross-entropy thresholding on the logarithm of intensity. Each nucleus was counted as one cell. The cells were classified as GFP-positive or GFP-negative based on the mean intensity of the GFP channel in the nucleus, where the threshold was set based on the intensity of the GFP channel from the untreated cells. The intensity of GFP-positive cells is higher than the threshold, and the intensity of GFP-negative cells is lower than the threshold. For the viability analysis, GFP-negative cells are viable cells with intact cell membranes, so they were accepted for further analysis. Sixteen fluorescence microscopy images from independent biological experiments were obtained for the ratio of viable cells.

Quantification of apoptosis detection. The analysis of cell viability was conducted using CellProfiler[40]. The details are in the CellProfiler project file (Apoptosis_analysis.cpproj). Briefly, the DAPI and Alexa channels of each image containing many cells were used for analysis. The DAPI channel was used to segment the nuclei by global minimum cross-entropy thresholding on the logarithm of intensity. Each nucleus was counted as one cell. The cells were classified as Alexa-positive or Alexa-negative based on the mean intensity of the Alexa channel in the nucleus, where the threshold was set based on the intensity of the Alexa channel from the untreated cells. The intensity of Alexa-positive cells is higher than the threshold, and the intensity of Alexa-negative cells is lower than the threshold. For the apoptosis analysis, Alexa-negative cells are healthy cells, so they were accepted for further analysis. Twelve fluorescence microscopy images from independent biological experiments were obtained for the ratio of viable cells.

Quantification of ROS detection. The analysis of ROS in cells was conducted using CellProfiler[40]. The details are in the CellProfiler project file (ROS_analysis.cpproj). Briefly, the DAPI and GFP channels of each image containing many cells were used for analysis. The DAPI channel was used to segment the nuclei by global minimum cross-entropy thresholding on the logarithm of intensity. Each nucleus was counted as one cell. The cells were classified as GFP-positive or GFP-negative based on the mean intensity of the GFP channel in the nucleus, where the threshold was set based on the intensity of the GFP channel from the untreated cells. The intensity of GFP-positive cells is higher than the threshold, and the intensity of GFP-negative cells is lower than the threshold. For the ROS analysis, GFP-positive cells are cells with ROS, so they were accepted for further analysis. Sixteen fluorescence microscopy images from independent biological experiments were obtained for the ratio of cells with ROS.

Quantification of mitochondrial superoxide detection. For the analysis of mitochondrial superoxide, the threshold for the intensity of mitochondria is based on the intensity of mitochondria from untreated cells because the MitoSOX Green superoxide indicators measure the superoxide produced only by mitochondria. Based on the images, all cells treated with copper in the presence of sodium ascorbate show high fluorescent intensity in the mitochondria compared to the negative controls. The cells treated by BTT-DNA ligand are negative, so we analyzed the positive cells manually rather than using CellProfiler.

Statistics and reproducibility. Quantitative analyses were performed using data pooled from three biological replicates, unless otherwise noted in the figure legends. For the analysis of fluorescence intensity fold-change in cell-surface biomolecule labeling, signals were normalized to the background from the no-ligand control. For intracellular phospholipid labeling, fluorescence intensity fold-change was normalized to the 30-min time point. Statistical comparisons were performed using two-sided *t*-tests, with *p* values reported in the figure legends.

All statistical analyses were conducted using Prism software. No data were excluded from the analyses. Experiments were not randomized, and investigators were not blinded to group allocation during experimentation or outcome assessment.

Epifluorescent microscopy of cultured cells

For HeLa cells, microscopy was performed using a Nikon inverted research microscope eclipse Ti2-E/Ti2-E/B using a Plan Apo λ 20X/0.75 objective or Plan Apo λ 60X/1.40 oil objective. The Epi-fi LED illuminator linked to the microscope assured illumination and controlled the respective brightness of four types of LEDs of different wavelengths. Images were acquired using the Neutral Density (ND16) filter for labeling with CalFluor 647 Azide. Images were acquired and processed using ImageJ and were shown as a single z-plane. Images acquired using the Neutral Density (ND16) filter are false-colored gray.

Data availability

Due to large file sizes, the raw imaging data are available from the corresponding author via email request. There are no restrictions on access, and requests will be processed within 2 weeks. Source data are provided with this paper.

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Author contributions

K.N. and S.H.R. designed the project, analyzed the data, and wrote the manuscript with input from all authors. K.N. performed all the experiments for this project. L.B. helped synthesize the BTT-DNA ligand, and Y.L. helped with the 5-ethynyl uridine (EU) labeling of nascent RNAs in fixed cells using commercial ligands. Y.Q. helped with the copper-induced toxicity assay. Z.Z. helped with the analysis of the copper-induced toxicity assay. M.W. helped with the analysis of the fluorogenic plate-reader assay.

Competing interests

The authors declare no competing interests.

Additional information

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