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A Proximity-Dependent Biosensor System for Visualizing Cell-Cell Interactions Induced by Therapeutic Antibodies

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Abstract

Despite the promise of therapeutic antibodies in engaging the immune system to eliminate malignant cells, many aspects of the complex interplay between immune cells and cancer cells induced by antibody therapy remain incompletely understood. This study aimed to develop a biosensor system that can evaluate direct cell-cell physical contact and interactions between immune effector and target cells induced by therapeutic antibodies in physiologically relevant environments. The system uses two structural complementary luciferase units (SmBit and LgBit) expressed on the respective membranes of effector and target cells. Upon cell-cell contact, the two subunits form active NanoLuc, generating a luminescent signal, allowing for real-time monitoring of cell-cell interactions and quantitatively assessing the pharmacological effects of therapeutic antibodies. We optimized the system to ensure selectivity by adjusting the spacer lengths between two luciferase units to minimize interference from nonspecific intercellular contact. The system was applied to quantitatively monitor cell-cell interactions between NK and target cells induced by rituximab and between T and target cells induced by blinatumomab in a 3D cell culture system. The biosensor system has the potential to characterize antibody pharmacology through a deeper understanding of antibody-mediated cell-cell interactions.

Introduction

Therapeutic antibodies can engage innate and adaptive immune cells to eliminate pathogenic or malignant cells¹. Activating and attracting immune cell to find and engage with infected

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Author Contributions

Conceptualizations: Y.T., and Y.C.; methodology: Y.T., and Y.C.; formal analysis: Y.T., and Y.C.; investigation: J Y.T., and Y.C.; writing-original draft: Y.T., and Y.C.; writing-reviewing and editing: Y.T., and Y.C.; supervision: Y.C.

Conflict of Interest

All the authors declare no competing interests.

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or malignant cells are the fundamental pharmacological actions to many antibody-based therapeutic approaches, including but not limited to checkpoint blockades, circulating cytokine neutralizers, bispecific T cell engagers, and virus neutralizers^{2,3}. Characterizing antibody-induced intercellular interactions between immune effector and target cells is therefore crucial to understanding antibody pharmacological effect and pharmacodynamics (PD), provide insights into the mechanisms of immune evasion and treatment resistance, and inform strategies for optimizing antibody therapeutics in various therapeutic areas^{4,5}.

Despite the fact that understanding the dynamics of cell-cell interactions and the formation of immunological synapse upon cell-cell interactions is critical for optimizing antibody-based therapeutics, to date, investigations in this area have focused primarily on molecular-scale interactions^{6,7}, leaving gaps in our understanding of the intercellular steps at the cell population level involved in effective immune cell activation and target cell killing. Cell population-level interactions are complex and involve dynamic and coordinated responses of multiple cells within a population⁸. These interactions can be influenced by various factors⁹, such as cell density, spatial arrangement, and environmental cues, which can impact the overall effectiveness of immune cell activation and target cell killing mediated by therapeutic antibodies.

Numerous technical methods have been developed to detect cell–cell interactions involved in various physiological processes, spanning developmental biology, neural networks, and immune responses. These intercellular interactions exhibit significant variability in their duration and complexity, often involving multiple cell types^{4,8,10,11}. In broad terms, these methods can be proximity-dependent or –independent, depending on their purpose for intercellular imaging or labeling. Our research group has also contributed to this field by developing an intercellular labeling approach for assessing interactions between immune and tumor cells in both in vitro and in vivo studies^{12–14}. In pathological environments, such as tumor microenvironments, environmental factors can restrict the functions of effector cells, such as impairing their ability to find and engage target cells¹⁵. Inside tumors, many factors work together to create a niche that restricts effector cell infiltration, motility, adhesion, and effector functionality^{16,17}. Thus, characterizing cell-cell interactions at the population level in physiologically relevant environments can provide valuable insights into the mechanisms of immune evasion and treatment resistance, and inform strategies for optimizing antibody-based therapeutics.

Here we constructed a proximity-based biosensor system to detect stable intercellular contact and interaction. The biosensor system described is designed to detect stable intercellular contact and interaction between immune effector and target cells using two structurally complementary luciferase subunits. Briefly, two structurally complementary luciferase subunits in the NanoBiT[®] system¹⁸, Large_BiT and Small_BiT, upon transfection, were respectively expressed on the surfaces of immune effector and tumor cells. Upon cell-cell contact and interaction, the proximity between tumor and immune cells allows the binding of Large_BiT and Small_BiT, forming active luciferases that emit strong luminescence upon substrate stimulus¹⁸. The system was further applied to detect NK-tumor cell interactions induced by an anti-CD20 antibody rituximab and T-tumor cell interactions induced by a bispecific T cell engaging antibody blinatumomab in a three-dimensional

(3D) cell culture system. The biosensor system offers a promising approach to monitor and evaluate cell-cell interactions in relevant physiological conditions and to understand the factors that impede effective intercellular interactions induced by therapeutic antibodies.

Materials and Methods

Cell Culture and Reagents

Three cell lines were employed in this study: HeLa cells, Phoenix-AMPHO cells, and Human Natural Killer cells. HeLa cells were cultured in DMEM with 10% FBS (Invitrogen), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco). Phoenix-AMPHO cells (ATCC® CRL-3212™) were cultured in RPMI 1640 medium supplemented with 10% FBS (Invitrogen), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco). Human Natural Killer cell line (NK-CD16; ATCC® PTA-6967) was cultured in the Alpha-MEM medium. Cells were maintained in a humidified incubator at 37 °C.

Plasmid Construction and Cell Transduction

We first constructed a plasmid for the expression and detection of SmBiT and LgBiT on cell membranes. Figure 1A depicts the schematic representation of the pDisplay vector and its key components. From the N-terminus to C-terminus of the inserted gene, the pDisplay vector contains a murine Ig k chain leader sequence (Signal peptide), a HA epitope, SmBiT or LgBiT, spacers, a myc epitope, and a platelet-derived growth factor receptor transmembrane domain (PDGFR-TM). The Signal peptide and the PDGFR-TM help direct and anchor the fusion protein to the cell membrane. The HA and myc epitopes enable the detection of fusion peptides¹⁹.

The sequences of SmBiT and LgBiT were kindly provided by Promega. Spacers were added to the C terminus of LgBiT or SmBiT to yield different distances between split luciferases and cell membranes. The LgBiT-(GGGGS)_n or SmBiT-(GGGGS)_n sequences were inserted into the pDisplay plasmid at XmaI and SaLI restriction sites.

The CMV-LgBiT-(GGGGS)_n-PDGFR-TM gene was then cloned into PBMN-I-eGFP to produce recombinant retrovirus (Figure 1B). PBMN-I-GFP was a gift from Garry Nolan (Addgene plasmid # 1736).

SmBiT or LgBiT fusion genes expressed from pDisplay™ were introduced to HeLa cells via Lipofectamine 3000 (Invitrogen). For the NK-CD16 cells, transduction was carried out using a previously established protocol²⁰. This process involved transfecting the Phoenix-AMPHO cells with the engineered vector (PBMN-IRES-EGFP-SmBiT) via Lipofectamine 3000 to produce recombinant retrovirus. This retrovirus was subsequently collected from the supernatant of transfected Phoenix-AMPHO cells for NK-CD16 transduction.

Selection of NK-CD16 Cells with Positive SmBiT Expression

NK-CD16 cells with positive SmBiT expression were selected via cell sorting by probing HA tag, probing CD16, and probing eGFP simultaneously. Anti-HA antibody (Invitrogen #26183, 1:500), anti-mouse secondary antibody Alexa Fluor 647 (Invitrogen #A-21235,

1:200), anti-CD16 antibody (Abcam #246222, 1:600), and anti-rabbit secondary antibody Cy3 (Abcam #6939, 1:200) were used for the selection.

SmBiT and LgBiT Expressions in Hela and NK cells

Transfected HeLa cells were harvested at 96-hour post-transfection, suspended in Opti-MEM, and transferred to a 96-well plate (1×10^5 cells per well). HiBiT control protein, a 11-amino acid peptide with a higher affinity to LgBiT than SmBiT (0.7 nM vs. 190 μ M), was used. NanoLuc substrate (furimazine) solution (1:50 diluted) containing 40 nM HiBiT control protein was added to the 96-well plate (20 μ L per well). The luminescence was measured at 460 nm by Cytation 3 equipped with 460/40 nm bandpass filter. To evaluate SmBiT expression, LgBiT protein extracted from membrane and cytosol was used. The membranous and cytosolic LgBiT proteins were extracted from LgBiT-positive, SmBiT-positive, and HeLa WT cells using Mem-PER Plus membrane protein extraction kit. LgBiT-containing (1:100 diluted) furimazine solution was added to 96-well plates to detect SmBiT proteins.

SmBiT expression on NK-CD16-SmBiT cells was assessed. NK-CD16-SmBiT cells were transferred to a 96-well plate (1×10^5 cells per well). HeLa-SmBiT cells (SmBiT-0L, 1L, 3L, and 12L) were used as positive controls. HeLa WT and NK-CD16 WT cells were used as negative controls. LgBiT-containing (1:100 diluted) furimazine solution was added to SmBiT⁺ cells to detect SmBiT expression levels. The S/N ratios were calculated as described in Equation 1.

$$S/N \text{ ratio} = \frac{NK_{CD16}^{SmBiT}}{NK_{CD16}^{WT}} \text{ or } \frac{HeLa_{CD16}^{SmBiT}}{HeLa_{CD16}^{WT}}$$

Eq. 1

Cetuximab-induced NK and HeLa cell interactions

To evaluate the binding of the engineered SmBiT and LgBiT proteins in the absence of cellular spatial limitations, HeLa cells were transfected by pDisplay-SmBiT and pDisplay-LgBiT genes via Lipofectamine 3000 (Invitrogen) either collectively or separately. Two types of separately transfected HeLa cells were co-incubated and then briefly centrifuged at 200 g before adding the furimazine solutions.

The ability of the biosensor system to detect antibody-induced cell clustering was then validated in HeLa-EGFR: cetuximab: NK system. NK-CD16-SmBiT-12L cells were mixed with pDisplay-LgBiT-12L-HeLa cells at E:T ratios of 10 or 5 with or without 10 nM cetuximab (MedChemExpress). For E:T ratio of 10, 1×10^5 NK cells and 1×10^4 HeLa cells were co-incubated in 100 μ L medium per well. For E:T ratio of 5, 5×10^4 NK cells and 1×10^4 HeLa cells were co-incubated in 100 μ L medium per well. As a control, NK-CD16 WT cells were mixed with pDisplay-LgBiT-12L-HeLa cells at the same conditions.

Imaging cell-cell interaction in a 3D cell culture system

At 96 hour post-transfection, CD20⁺LgBiT⁺ HeLa cells were harvested suspended in a 1:1 mixture of Matrigel (BD Bioscience). Matrigel-encapsulated cells were seeded in 24-well plates (20 μ L in each well) and solidified at 37 °C for 30 min. In the 3D cell culture imaging studies, NK-CD16 cells with or without SmBiT expression were added to solidify 3D cell cultures with or without rituximab. The images were acquired at 30-min after substrate supplement (furimazine, 1:100 dilution) using an IVIS optical imaging system (Caliper Life Sciences) with an electron multiplying charge-coupled device camera. Acquired images were processed and quantified using Living image 4.5.2 (Caliper Life Sciences).

To compare the sensitivities in detecting rituximab-induced cell clustering between LgBiT-3L+SmBiT-12L and LgBiT-12L+SmBiT-12L, corrected bioluminescent signals were calculated as described in Equation 2.

$$\text{Corrected BLI signal} = (\text{NK}_{\text{CD16}}^{\text{SmBiT}}[\text{CTX}] - \text{NK}_{\text{CD16}}^{\text{SmBiT}}[\text{control}]) - (\text{NK}_{\text{CD16}}^{\text{WT}}[\text{CTX}] - \text{NK}_{\text{CD16}}^{\text{WT}}[\text{control}])$$

Eq. 2

The peak corrected signals of each spacer combinations were calculated and normalized by control group signals, as described in Equation 3. Multiple spacer combinations were investigated: LgBiT-0L+SmBiT-0L, LgBiT-1L+SmBiT-1L, LgBiT-3L+SmBiT-3L, LgBiT-0L+SmBiT-12L, LgBiT-1L+SmBiT-12L, LgBiT-3L+SmBiT-12L, and LgBiT-12L+SmBiT-12L.

$$\text{Normalized signal} = \frac{\text{Corrected BLI signal}}{\text{NK}_{\text{CD16}}^{\text{SmBiT}}[\text{control}]}$$

Eq. 3

Results

The proximity-based biosensor system

Upon the LgBiT⁺ and SmBiT⁺ cell-cell contact, the two complementary parts on opposing cell surfaces come together and emit bright luminescent signals upon the addition of NanoLuc substrate. The molecular bonds between LgBiT and SmBiT did not noticeably interfere with the natural intercellular interaction between effector and target cells, due to their low binding affinity (190 μ M). To optimize the biosensor system, the spacer length between LgBiT and SmBiT was adjusted to ensure high specificity and sensitivity in detecting stable intercellular contact and immunological synapse formation (Figure 2A). Spacer-fused SmBiT biosensor components were constructed in plasmid DNA and viral vector forms. Literature evidence shows that the theoretical distances between opposing cell membranes in a synapse typically fall within the range of 15 – 40 nm²¹, a spacer length around this range might be optimal. Therefore, we tested the biosensor system with a wide range of combined spacer lengths from 0 – 48 nm (Table 1), and the optimized spacer lengths were selected for given pair of effector and target cells with the highest S/N ratios

(Figure 2B, upper panel). When combined spacer lengths were too short, the sensitivity of detection by the biosensor system decreased (Figure 2B, middle panel). When combined spacer lengths were too long, the biosensor system would show high noise with reduced selectivity for detecting stable cell-cell interaction (Figure 2B, lower panel).

Engineered the biosensor system with high sensitivity

We first characterized the expressions and functions of the biosensor system in HeLa cells. As Figure 3A shows, the LgBiT and SmBiT expressions in HeLa cells were significant ($p < 0.0001$). Both LgBiT and SmBiT were detectable in cytosols (Figure 3B), but their expressions on the cell membrane were much higher than in cytosols ($p = 0.0006$). The high membranous expression of the biosensor system suggests its potential for use in detecting intercellular interactions (Figure 3B).

The biosensor system was then characterized using different cell systems to determine the interaction probability between LgBiT and SmBiT. When SmBiT and LgBiT expressed on the same cells (without spatial restriction), strong bioluminescence signals were detected (Figure 3C). However, when SmBiT and LgBiT were expressed on separate cell membranes (SmBiT⁺ and LgBiT⁺ HeLa cells), the bioluminescent signal was low when both cell types were in suspension (Figure 3D). The intercellular interaction probability was increased by centrifugation, which resulted in 5–10 times higher bioluminescent signals in centrifuged LgBiT:SmBiT cell mixtures, indicating that the bioluminescent signal is contact-dependent (Figure 3D). Centrifugation enforced cell-cell contact and triggered the complimentary interactions between LgBiT and SmBiT (Figure 3D).

The biosensor system was then applied to investigate antibody-induced cell-cell interactions. Specifically, intercellular interaction between NK-CD16 and HeLa cells induced by an anti-EGFR antibody cetuximab was first evaluated. SmBiT⁺ NK-CD16 cells were incubated with LgBiT⁺ HeLa cells at different E:T ratios (5 or 10), with or without cetuximab (Figure 3E). Without cetuximab, no significantly different bioluminescent signals were detected in the effector-target cell mixtures regardless of E:T ratio, suggesting limited intercellular interactions without cetuximab. An increased bioluminescent signal was observed in the presence of cetuximab, suggesting that the cetuximab promotes the interaction between NK-CD16 and HeLa cells, consistent with its pharmacological actions. The bioluminescent signals were significantly higher in the 10 E:T ratio group than the group with 5 E:T ratio ($p = 0.02$), indicating cell density dependency.

Interactions between NK and target cells induced by rituximab in a 3D system

NK-CD16 cells transfected with SmBiT with different linker lengths (SmBiT-0L, SmBiT-1L, SmBiT-3L, and SmBiT-12L cells) had comparable CD16 and HA expression levels (Figure 4A). All four SmBiT⁺NK-CD16 cell lines (SmBiT-0L, SmBiT-1L, SmBiT-3L, and SmBiT-12L) had substantial SmBiT expressions (Figure 4B). No significant difference in SmBiT expressions among the four SmBiT⁺NK-CD16 cell lines was observed ($p = 0.1$, one-way ANOVA). CD20 expression levels were largely comparable among CD20⁺LgBiT⁺ HeLa cell lines (LgBiT-0L, LgBiT-1L, LgBiT-3L, and LgBiT-12L cells) (Figure 4C). HeLa

cells had relatively lower expressions of LgBiT-0L and LgBiT-1L and the expressions of LgBiT-3L and LgBiT-12L were comparable (Figure 4C).

The rituximab-induced NK-CD16 and CD20⁺HeLa interactions were assessed using LgBiT-12L: SmBiT-12L pair in the 3D cell culture system for 48 hours. Figure 4D shows the study design. Figure 4E and 4F show the real-time NK-CD16 and CD20⁺HeLa interactions. The interactions were relatively low at the beginning of the imaging study (< 0.5 hr), then increased and peaked around the 2.5 hours, suggesting increasing frequency of effector and target cell interactions over time. Interestingly, the control group without rituximab also exhibited moderate bioluminescent signals, indicating that NK-CD16 cells may naturally interact with target cells without antibody stimulation, higher than the natural interactions between NK-CD16 and CD20⁻ Hela cells (Figure 3D). The interactions mediated by rituximab gradually decreased over time from 2.5 to 9 hour, and were no longer detected at around 48 hours, which is consistent with the typical duration of antibody-dependent cell-mediated cytotoxicity (ADCC). At earlier time points (< 0.5 hour) the bioluminescent signals were relatively higher in the periphery than the central region, indicating that the interactions started in the periphery of the spheroids (Figure 4E and 4F).

The optimized biosensor system with improved sensitivity

The biosensor pair was further tested with varying combined spacer lengths. The rituximab-specific signals were normalized by the peak signals from the control group. The biosensor pair with the shortest combined spacer length (LgBiT-0L+ SmBiT-0L) had the lowest rituximab-specific signal, while the pair with the longest theoretical combined spacer length (LgBiT-12L: SmBiT-12L) had the highest rituximab-specific signal (Figure 5A). The longitudinal profiles and images are shown in Figure 5B and Figure 5C. However, the combined spacer length was not the only factor influencing the rituximab-specific signals selectivity, as the LgBiT-0L: SmBiT-12L pair had lower rituximab-specific signals than the LgBiT-3L: SmBiT-3L pair ($p = 0.005$) (Figure 5A), suggesting lower selectivity. Biosensor pairs with different combined spacer lengths, e.g., LgBiT-3L: SmBiT-3L, LgBiT-1L: SmBiT-12L, and LgBiT-3L: SmBiT-12L, had comparable rituximab-specific signals ($p = 0.9$). Based on these results, LgBiT-12L: SmBiT-12L biosensor pair was selected for subsequent dose-dependent study.

The intercellular interaction was investigated at two rituximab concentrations (1 and 100 $\mu\text{g/mL}$). It was observed that the signal peaked at 2 hours in both dose groups, similar to previous observations. However, the high dose group (100 $\mu\text{g/mL}$) showed significantly higher cell-cell interaction throughout the imaging window compared to the low dose group (Figure 5D and 5E), suggesting an antibody concentration-dependent effect on the cell-cell interactions.

Interactions between T and tumor cells induced by blinatumomab

The intercellular interactions induced by the bispecific T cell engaging antibody blinatumomab were also assessed using the system (Figure 6A). The system sensitivity and selectivity were tested by comparing the signals across a range of linker lengths. Interestingly, the optimal linker pair was found to be LgBiT-3L: SmBiT-3L, which is shorter

than the optimal length for NK and target cell interactions induced by rituximab, indicating cell line-specific optimal linkers. This finding is consistent with previous observations that natural killer¹⁸ cells are capable of exerting their lytic effect at a relatively broader cell-cell distance compared to cytotoxic T cells²². Figure 6B shows that the blinatumomab-induced T and target cell interactions peaked at 6 hrs, which is slower than the rituximab-induced NK and target cell interactions, suggesting different patterns

Discussion

In this study, we developed a biosensor system that provides a potential tool to enable the visualization and quantification of intercellular interactions between immune cells and target cells mediated by therapeutic antibodies. Specifically, it allows for the real-time monitoring of interactions between immune cells and target cells induced with good temporal resolution. The biosensor system can be used to evaluate the interactions induced by different therapeutic antibodies, involving interactions between NK and tumor cells induced by rituximab and T and tumor cells induced by bispecific T cell engager. Overall, the biosensor system represents a promising platform for studying antibody-induced cell-cell interaction, which has potential applications in understanding the pharmacological actions of therapeutic antibodies.

By integrating the split luciferases (LgBiT and SmBiT) with the spacer $-(GGGS)_n-$, the biosensor system was fine-tuned by adjusting the spacer lengths, allowing for improved sensitivity in detecting intercellular interactions. The weak binding (190 μ M) between the luciferase subunits in the biosensor system is not expected to interfere the natural intercellular interaction, and two luciferase subunits can be easily interrupted by cellular separation. LgBiT-12L: SmBiT-12L pair provided the highest sensitivity in detecting NK-Tumor cell conjugations, possibly due to the proper flexibility on both sides. Yielding approximately 48 nm theoretical combined spacer lengths (LgBiT-12L and SmBiT-12L), which are close to the theoretical distances between opposing cell membranes in a NK-Tumor immunological synapse (15 – 40 nm)²¹. The select biosensor pairs showed highest bioluminescent signals between NK and Hela cells induced by rituximab.

The biosensor system allowed for real-time monitoring of NK and target cell interactions and clustering dynamics, providing temporal resolution. ADCC, mediated by NK cells, is a crucial mechanism of pharmacologic action for many therapeutic antibodies, such as tranzusumab, rituximab, cetuximab, and other antibodies that trigger strong effector functions²³. The process of ADCC is potentially influenced by multiple factors, such as the concentration and distribution of the antibody, the density of effector cells, the tumor microenvironment, and the expression of Fc receptors on the effector cells^{24–28}. However, the mechanisms and dynamics of ADCC in vivo and the involvement of ADCC to antibody therapeutic effect have not been entirely clear; most measurements of ADCC consist of bulk assays or discontinuous methods, obscuring the spatial and temporal resolutions of antibody-mediated cell-cell interactions and often lacking the physiological context. The biosensor system we developed provides a complementary tool for noninvasively assessing ADCC in a 3D culture system, enabling the investigation of the mechanisms and dynamics of ADCC at the population levels with sufficient resolutions.

The longitudinal monitoring of the intercellular interactions could provide insights into antibody pharmacological actions. The peak of intercellular interaction between NK and tumor cells induced by rituximab was around 2 hours, which was consistent with previous studies regarding the cell lysis during ADCC, which was around 80 minutes for most NK cells in a previous study²⁹. The ADCC effect can persist for a duration of up to 20 hours³⁰. The decrease in the signal of intercellular interaction after 2 hours could result from either tumor cell lysis or exhaustion of NK cells during the incubation period. It remains unclear if the installation of these biosensor units genetically would influence the functions of effector cells. We find it unlikely that this decline is linked to the dissociation of the antibody from its target, given the high binding affinity of the evaluated antibody. To further clarify, it is essential to perform a cell viability assay or assess the functional status of the effector cells, neither of which was included in the present study.

Interestingly, blinatumomab-induced T and target cell interactions peaked at 6 hours, which is much slower than the peak observed in the rituximab-induced NK and target cell interactions. The slower peak observed in the blinatumomab-induced T and target cell interactions may be due to the need for T cells to be extensively activated before engaging with tumor cells, leading to a slower onset of target cell killing compared to the more rapid and innate killing ability of NK cells. It is important to note that the specific mechanisms of action and experimental conditions for each drug may also play a role in the observed differences in cell-cell interaction kinetics.

The method has two major limitations. First, the bioluminescent signal for intercellular interactions isn't as intense as fluorescence. Consequently, the biosensor system lacks necessary sensitivity for single-cell imaging and is better suited for imaging at the cell population or tissue levels. Second, the bioluminescent signal arises from the interaction of two complementary luciferase units upon cell-cell contact, without providing insights into molecular interactions on cell membranes. It remains uncertain whether the cell-cell interactions detected by the system are associated with specific molecular interactions or the formation of immunological synapses. Further studies are warranted to explore the connection between the imaging signals and particular molecular interactions.

Overall, the proximity-based biosensor system described in the study offers a promising tool for noninvasive and continuous monitoring of cell clustering dynamics induced by therapeutic antibodies. Further evaluation of the system across various experimental systems is required, as it may emerge as a valuable tool for assessing the pharmacological effects of therapeutic antibodies.

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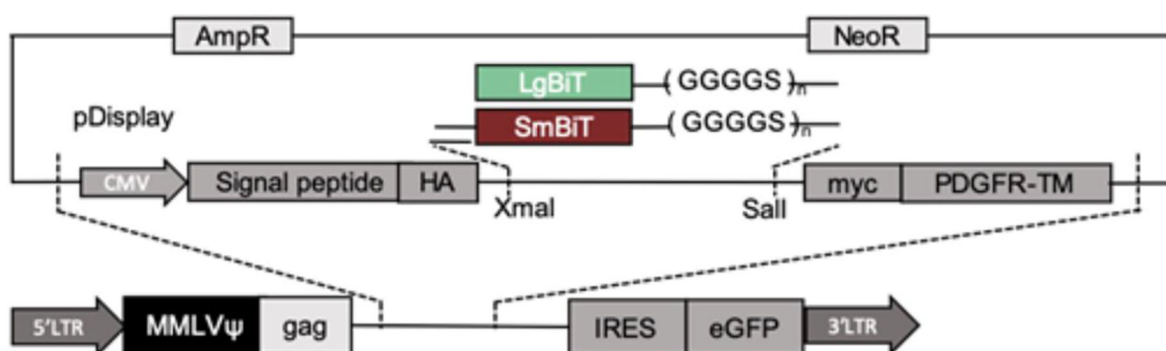


Figure 1. Construction of the biosensor system plasmid.

(A) LgBiT or SmBiT constructed in pDisplay vector. (B) The CMV-LgBiT-(GGGGS)_n-PDGFR-TM gene was cloned into PBMN-I-eGFP.

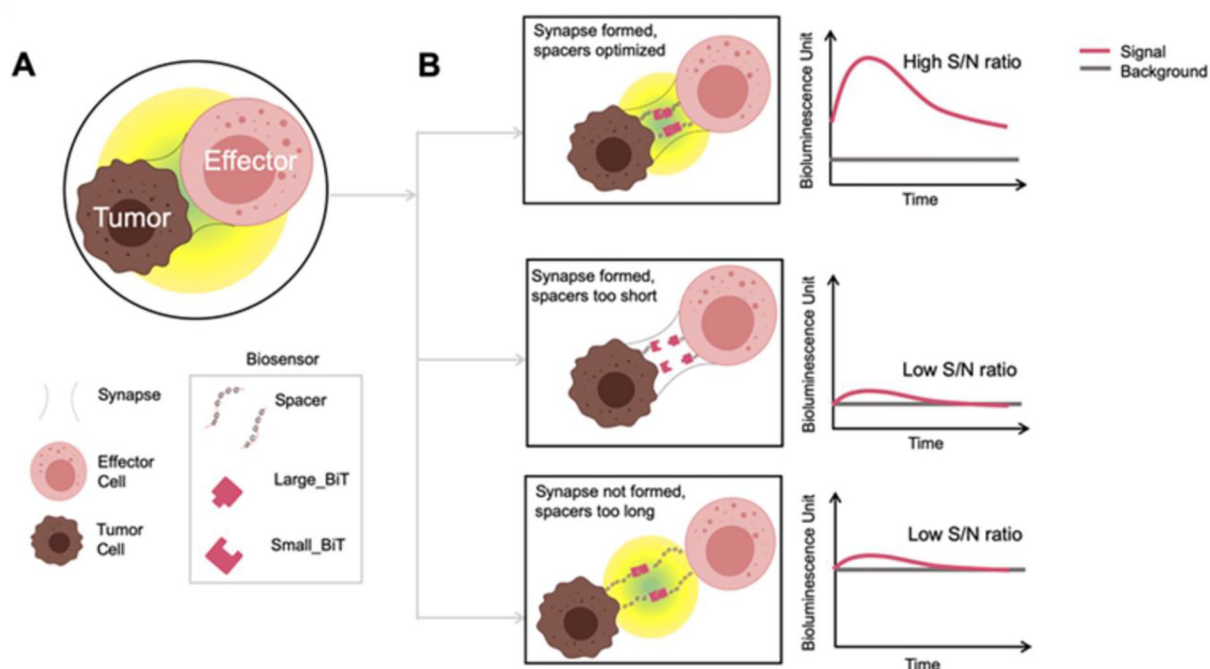


Figure 2. The design of the proximity-based luminescent biosensor system for detecting proximity-dependent intercellular interactions.

(A) The schematic of the biosensor system. Briefly, two structurally complementary luciferase subunits in the NanoBiT[®] system, Large_BiT and Small_BiT, are separately expressed to the surfaces of immune and tumor cells with varying length of the spacers. Upon cellular interaction, the proximity enables complementary interactions of Large_BiT and Small_BiT, forming active luciferases that emit strong luminescence upon stimulus. The spacer used in constructing the biosensor system is (GGGS)_n. (B) The concept of the optimized sensitivity and selectivity of the biosensor system by adjusting the combined spacer lengths.

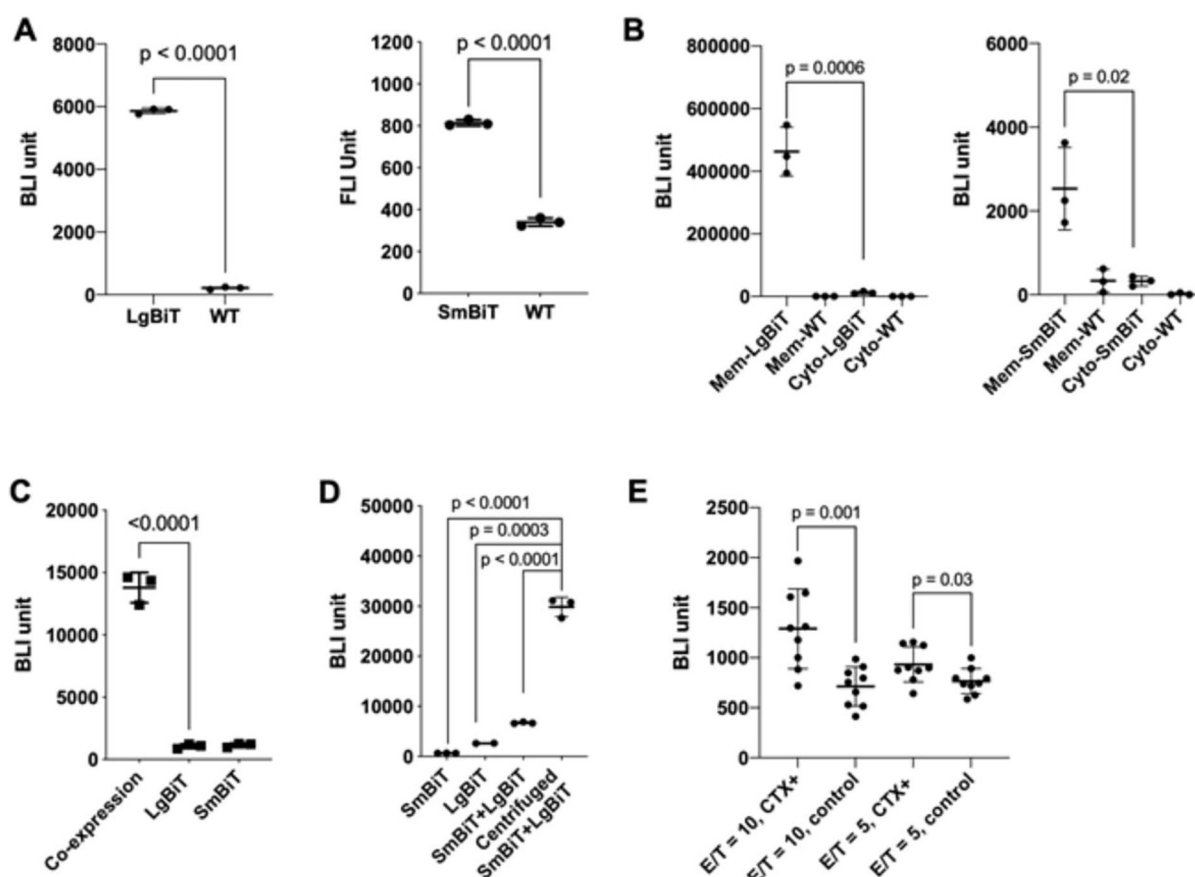


Figure 3. The biosensor system detected cell-cell interactions in a proximity-dependent manner. (A) LgBiT and SmBiT were significantly expressed in HeLa cells as indicated by HiBiT probing (Left panel) or HA tag probing (Right panel). (B) LgBiT and SmBiT were majorly expressed on cell membranes. LgBiT expression (left panel) and SmBiT expression (right panel). (C) Co-expressed LgBiT and SmBiT emitted strongest bioluminescent signals compared to LgBiT- or SmBiT-positive cells alone. (D) Biosensor system detected cell contacts upon centrifugation. (E) Hela: NK-CD16 interactions were increased by anti-EGFR antibody cetuximab. The bioluminescent signals were measured after 30-min incubation of Hela and NK-CD16 in both E/T= 10 and E/T= 5 groups. Cetuximab-specific signals were higher in E/T= 10 group than E/T= 5 group. MEM = membranous; cyto = cytosolic; E/T = effector/target cell ratio; WT = wild type.

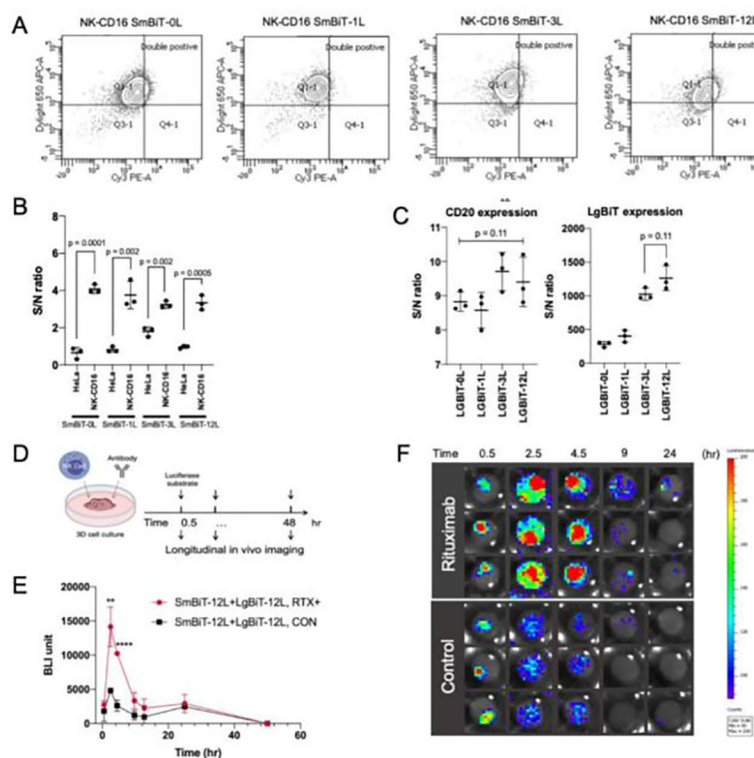


Figure 4. The biosensor system detected intercellular interaction between NK-CD16 and CD20⁺HeLa cells.

(A) NK-CD16 cells stably expressing SmBiT (SmBiT-0L, SmBiT-1L, SmBiT-3L, and SmBiT-12L) were sorted by HA tag (Dylight 650) and CD16 (Cy3). (B) SmBiT expression with different linkers SmBiT-0L, SmBiT-1L, SmBiT-3L, and SmBiT-12L were significantly higher than positive controls. (C) CD20 expressions were comparable between CD20⁺LgBiT⁺ HeLa cell lines (LgBiT-0L, LgBiT-1L, LgBiT-3L, and LgBiT-12L cells, $p = 0.11$, one-way ANOVA) ($n = 3$). (D) Study design and bioluminescent images of NK-CD16 and CD20⁺HeLa interactions at 0.5, 2.5, 4.5, 9, and 24 hour. RTX = rituximab; CON = control. (E) (F) Bioluminescent image revealing direct interaction between NK-CD16 and CD20⁺HeLa cells. LgBiT-12L: SmBiT-12L pair was used.

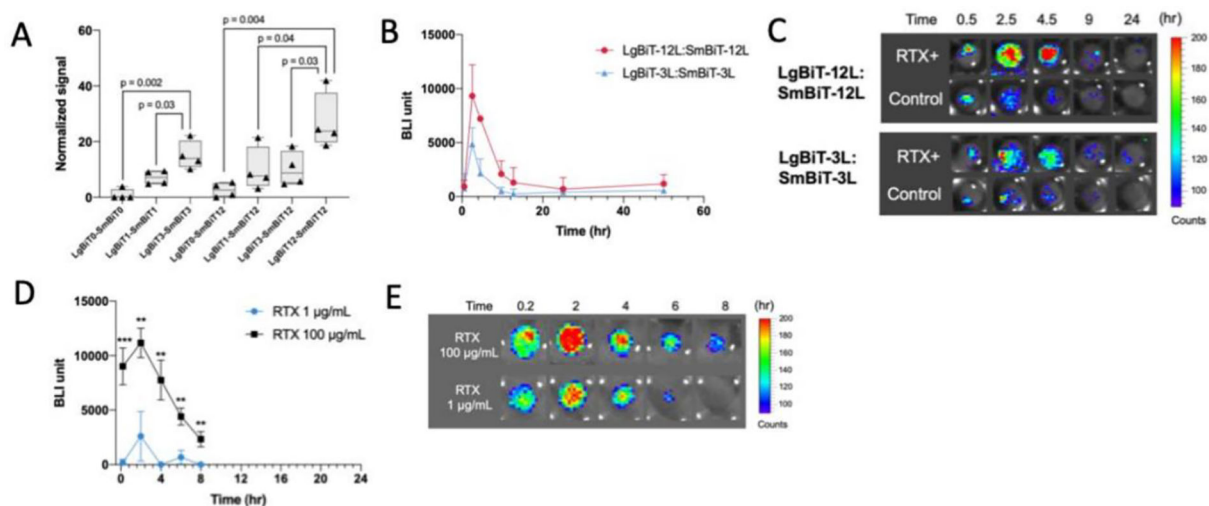


Figure 5. The biosensor system detected intercellular interaction in antibody dose-dependent manner in the 3D cell culture.

(A) LgBiT-12L: SmBiT-12L pair showed the higher sensitivity than other pairs. (B) Corrected bioluminescent signals indicated LgBiT-12L: SmBiT-12L pair showed higher sensitivity than LgBiT-3L: SmBiT-3L pair. At least three independent biologic replicates were performed per experiment. (C) Bioluminescent images of NK-CD16 and CD20⁺HeLa interactions at 0.5, 2.5, 4.5, 9, and 24 hour. (D) Rituximab-induced effector: target cell clustering was antibody concentration-dependent ($n=3$). (E) Bioluminescent images of NK-CD16 and CD20⁺HeLa interactions at 0.2, 2, 4, 6, and 8 hour. RTX = rituximab.

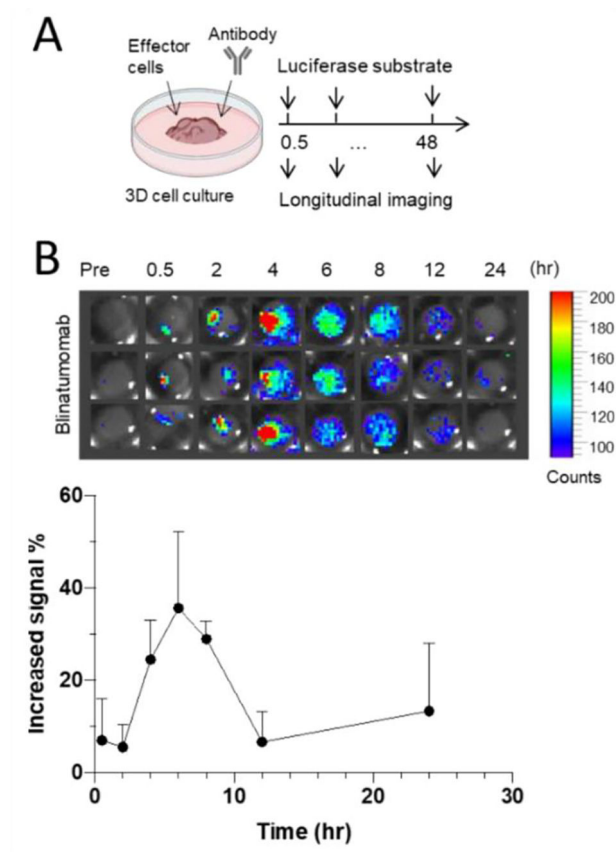


Figure 6. The system detected interaction between T and Raj B cells induced by blinatumomab. Raw data of bioluminescent signals from Jurkat T (CD3+) and Raj B (CD19) cell-cell interactions in the 3D cell culture system, which was visualized by LgBiT-3L: SmBiT-3L pair. Each data point represents one technical replicate. Error bars represent SD values. At least three independent biologic replicates were performed per experiment. (B) Bioluminescent images of at 0.2, 2, 4, 6, and 8 hour at 1 ng/ml blinatumomab.

Table 1.

Construction of the biosensor system with different lengths of spacers.

LgBiT		SmBiT	
Biosensor subunit structure	Length (nm)	Biosensor subunit structure	Length (nm)
N'–LgBiT–C'	0	N'–SmBiT–C'	0
N'–LgBiT–(GGGS) ₁ –C'	2	N'–SmBiT–(GGGS) ₁ –C'	2
N'–LgBiT–(GGGS) ₃ –C'	6	N'–SmBiT–(GGGS) ₃ –C'	6
N'–LgBiT–(GGGS) ₁₂ –C'	24	N'–SmBiT–(GGGS) ₁₂ –C'	24