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Dual-fluorescent bacterial two-hybrid system for quantitative Protein–Protein interaction measurement via flow cytometry

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ABSTRACT

Characterization of protein–protein interactions (PPIs) is essential for understanding cellular signal transduction pathways. However, quantitative measurement of the binding strength remains challenging. Building upon the classical bacterial adenylate cyclase two-hybrid (BACTH) system, we previously demonstrated that the relative reporter protein expression (RRPE), defined as the level of reporter expression normalized to that of the interacting protein, is an intrinsic characteristic associated with the binding strength between the two interacting proteins. In this study, we inserted fluorescent protein tdTomato in the chromosome as the reporter protein by CRISPR/Cas9 technology and employed a 12-amino acid tetracysteine (TC) to tag one of the interacting proteins, which can be further labeled by a membrane-permeable biarsenical dye. The combined use of tdTomato and TC-tag offers rapid and high-throughput analysis of the expression levels of both the reporter protein and one of the interacting proteins at the single-cell level by multicolor flow cytometry, which simplifies the quantitative measurement of PPI. The use of the as-developed RRPE-tdTomato-TC-BACTH approach was demonstrated in three demanding applications. First, binding affinities could be correctly ranked for discriminating interaction strengths with a tenfold difference or of the same order of magnitude. We demonstrate that the method is sensitive enough to discriminate affinities with a small difference of 1.4-fold. Moreover, residues involved in PPI can be easily mapped and ranked. Lastly, protein interaction inhibitors can be rapidly screened.

1. Introduction

Characterization of protein–protein interactions (PPIs) is essential in understanding and revealing cellular mechanisms [1–3]. Compared with *in vitro* approaches for PPI study, such as isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), and mass spectrometry, *in vivo* methods are based on genetic assays and do not require protein purification [4]. Two-hybrid systems of yeast or bacteria (Y2H or B2H) are among the most widely used *in vivo* approaches, which are based on the expression of a downstream reporter gene mediated by the binding between proteins of interest [5–8]. In particular, the signaling cascades of the two-hybrid methods enable the detection of weak and transient interactions [9]. However, the two-hybrid systems lack quantitation capability and are not suitable for interaction strength comparison of different pairs.

To achieve quantitative PPI analysis of the bacterial two-hybrid system, we previously developed an RRPE-BACTH method by using relative reporter protein expression (RRPE), defined as the level of reporter expression normalized to that of the interacting protein, in a bacterial adenylate cyclase two-hybrid (BACTH) system [10]. Through simultaneous flow cytometric measurement of one of the two interacting proteins and the β-galactosidase (β-gal) reporter protein of single bacteria upon dual immunofluorescence staining, the RRPE-BACTH method provides an efficient approach for the detection and affinity ranking of PPIs. However, immunofluorescent staining requires fixation and is time-consuming, and the sorted cells cannot be re-cultured.

To overcome these limitations, herein, fluorescent protein tdTomato was used as the protein interaction reporter, and one of the two

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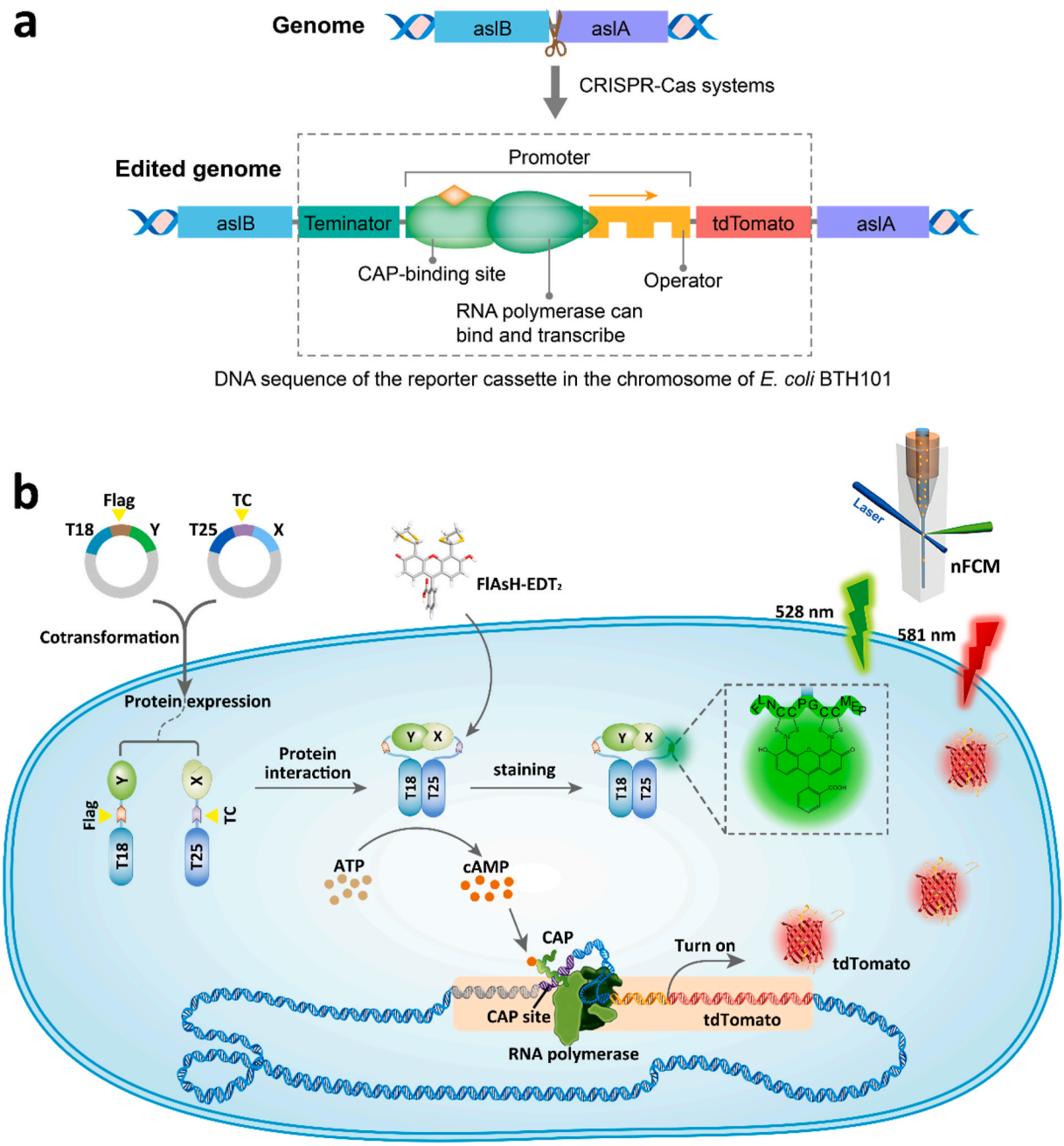

Fig. 1. Schematic depiction of the tdTomato-TC-BACTH system for PPI study at the single-cell level. (a) The tdTomato reporter cassette is inserted into the *asl* position in the chromosome of reporter strain through CRISPR/Cas9 technology to make an engineered tdTomato-BACTH reporter strain. (b) The mechanism of tdTomato-TC-BACTH system along with the simultaneous detection of both the interacting protein and reporter protein via flow cytometry.

interacting proteins was fluorescently labeled using the tetracysteine (TC) biarsenical system, so that the PPIs can be measured in real time. Moreover, the small-size TC-tag (12 amino acids containing FLNCCPGCCMEP) renders minimal interference with the natural interactions between the two interacting proteins and can be specifically labeled by a membrane-permeant fluorogenic biarsenical dye, such as fluorescein arsenical helix binder (FlAsH) [11,12]. The FlAsH/TC tag is a powerful tool for fluorescent labeling of proteins and has been widely used for *in vivo* protein labeling and cellular localization studies [13–17]. The RRPE for protein interaction quantification was confirmed in the present tdTomato-TC-BACTH system with great simplicity, and the measurement can be shortened from 5 h to less than 1 h. Based on the coiled-coil and the well-known TolB-Pal interactions as models, successful applications of the as-developed tdTomato-TC-BACTH method were demonstrated for the high-throughput (i) discrimination and ranking of the binding affinities for interaction strengths between tenfold and of the same order of magnitude, (ii) identification of important residues in PPI, and (iii) screening of PPI inhibitors.

2. Materials and methods

2.1. Materials and reagents

The TC-FlAsH In-Cell Tetracysteine Tag Detection Kit was purchased from Molecular Probes of Invitrogen, Inc. (Eugene, OR, USA). The *o*-nitrophenyl-*β*-galactoside (ONPG), isopropyl *β*-D-1-thiogalactopyranoside (IPTG), and X-gal were acquired from Sangon Biotech (Shanghai, China). The peptides of CPP-TBE and CPP-TBE-m used in this study are

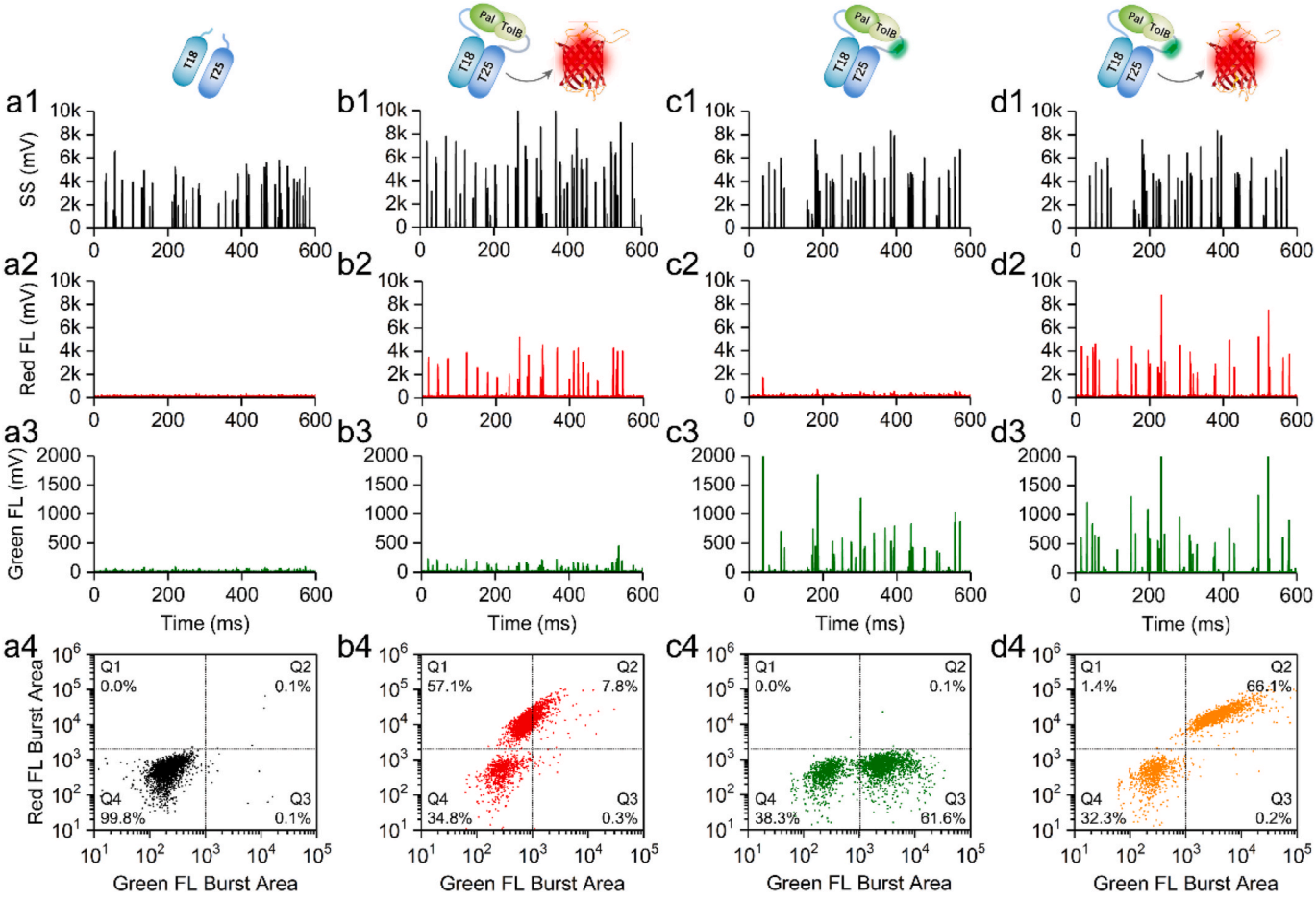

Fig. 2. Simultaneous measurement of the expression of the reporter protein and one hybrid protein of the tdTomato-TC-BACTH System by nFCM. (a, b, c, and d) Representative burst traces of side scatter (1), green fluorescence (2), and red fluorescence (3) and the bivariate dot-plots of red fluorescence burst area versus green fluorescence burst area (4) for *E. coli* BTH101-*tdTomato* co-transformed with plasmids of pUT18C/pKT25 (a), pUT18C-*pal*/pKT25-*tolB* (b), and pUT18C-*pal*/pKT25-TC-*tolB* (d). *E. coli* BTH101 co-transformed with plasmids of pUT18C-*pal*/pKT25-TC-*tolB* are shown in (c). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

presented in Table S1. The peptides were synthesized at China Peptides Co., Ltd (Shanghai, China) and dissolved in ultrapure water to achieve a stock solution of 5 mM. Other reagents were purchased from Sinopharm Chemical Reagent (Shanghai, China). Phosphate-buffered saline (PBS) was prepared using 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. All the buffers were filtered through a 0.22 μm filter and used within 3 weeks.

2.2. Bacterial strains and plasmids

The reporter strain *cya- E. coli* BTH101-*tdTomato* (F^-, cya^-99, araD139, galE15, galK16, rpsL1, hsdR2, mcrA1, and mcrB1) was constructed by inserting a 1741-bp DNA sequence encoding the *lac* promoter followed by the *tdTomato* gene into the *asl* position in the chromosome through clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology [18]. The primers used are listed in Table S3.

Escherichia coli ER2738 was used for the cloning experiments. The recombinant plasmids used in the present study are summarized in Table S2 and were verified by sequencing. Oligonucleotides were synthesized by Sangon Biotech and are listed in Table S3. To introduce the TC sequence FLNCCPGCCMEP into pKT25-*tolB*, oligonucleotide primers that are complementary to each other were synthesized with the sequences listed in Table S3 and annealed by heating at 95 °C for 5 min. The primers were then cooled to room temperature, and the products with cohesive ends were inserted into plasmid pKT25-*tolB* at the *Pst*I-*EcoR*I sites to obtain plasmids pKT25-TC-*tolB*. Plasmids with T25-TC-Kn were constructed likewise but with suitable primers. To introduce TolB-binding epitope (TBE) into the BACTH vector, the *pal* fragment was replaced by TBE fragment using Gibson Assembly [19] with primers containing TBE sequences. Other vectors expressing TBE mutants were constructed likewise but with suitable primers.

2.3. Bacterial cell culturing

The *E. coli* BTH101-*tdTomato* strains were transformed with recombinant plasmids expressing the T25 and T18 hybrids. Afterword, the transformation mixtures cultured in Luria-Bertani (LB) liquids containing 100 μg/mL ampicillin and 50 μg/mL kanamycin for 36 h with shaking at 250 rpm and 30 °C were directly analyzed via flow cytometry.

2.4. FlAsH staining

When the bacteria reached the exponential phase (with an OD600 of 0.5), 15 μL of bacterial solution was preloaded with 10 μM FlAsH-EDT_2. For the inhibition experiment, inhibiting peptide was added at the same time with a final concentration of 50 μM. The bacteria continued incubation for 8 h in the dark at 30 °C with vigorous shaking (250 rpm) to express the interaction protein together with FlAsH staining at the same time. After 8 h incubation in the dark at 30 °C with vigorous shaking (250 rpm), the mixture was centrifuged and washed twice with 0.5 mM BAL washing buffer. The bacterial cells were suspended in 50 μL of PBS

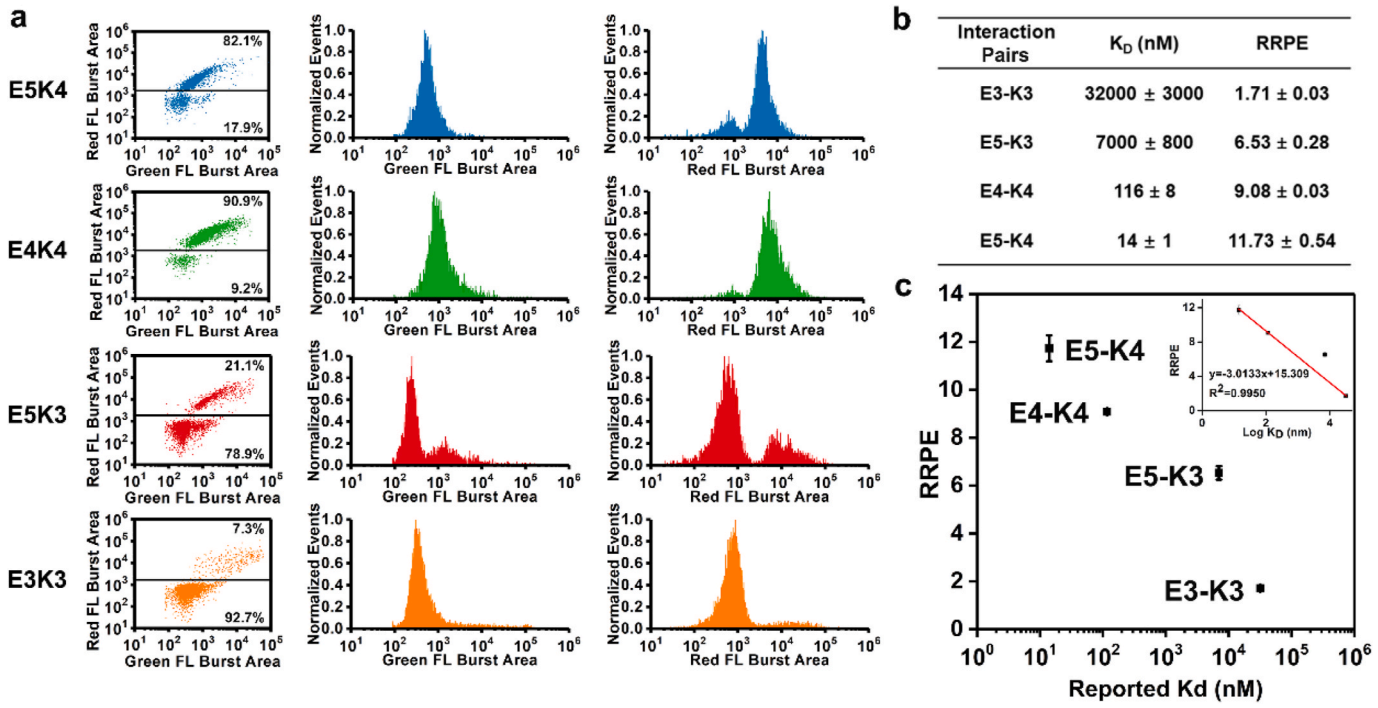

Fig. 3. RRPE measurement for four pairs of coiled-coil interactions using the tdTomato-TC-BACTH system via nFCM. (a) Bivariate dot-plots of tdTomato fluorescence versus TC-Kn green fluorescence and fluorescence distribution histograms for *E. coli* BTH101-*tdTomato* cells transformed with plasmid encoding En and TC-Kn helices. (b) Reported K_D and measured RRPE in this study of the coiled-coils interaction. (c) Correlation of the RRPE values measured via nFCM for coiled-coil interactions occurring in the tdTomato-TC-BACTH system with the literature-reported affinities. The error bars represent the standard deviation of three replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

for flow cytometry analysis.

2.5. Flow cytometry analysis

A laboratory-built nano-flow cytometer (nFCM) equipped with a solid-state 488 nm CW laser was used in present study [20,21]. The light emitted from individual bacterial cells was directed by the first dichroic beam splitter into two light paths. The reflected side-scattering light was directly detected by a photomultiplier tube. The transmitted light was then separated by the second dichroic beam splitter into two light paths for green (520/34 nm bandpass filter) and red (670/30 nm bandpass filter) fluorescence detection. The green and red channels were used to detect the fluorescence of FlAsH-EDT_2 and fluorescent protein tdTomato, respectively; 60 s of data acquisition were used for each sample. The fluorescence excitation and emission spectra of FlAsH-EDT_2 and tdTomato and the transmission spectra of the bandpass filters used for the green and red fluorescence detection are provided in Fig. S2.

3. Results and discussion

3.1. Design of the tdTomato-TC-BACTH system for quantitative measurement of PPI

The principle for the quantitative measurement of PPI is shown in Fig. 1. A tdTomato-BACTH reporter strain, *cya⁻ E. coli* BTH101-*tdTomato*, was constructed by inserting a DNA sequence encoding the *lac* promoter followed by the *tdTomato* gene into the *asl* position in the chromosome through CRISPR/Cas9 technology (Fig. 1a and Fig. S1). The *asl* loci in the *E. coli* chromosome has been confirmed as a high-expression location for reporter gene [22]. The negligible effect of *tdTomato* insertion was demonstrated with identical growth of wild type bacteria *E. coli* BTH101 and *E. coli* BTH101-*tdTomato* (Fig. S3). Plasmids carrying the hybrids with adenylate cyclase T25 and T18 domains were transformed into the reporter strain *cya⁻ E. coli* BTH101-tdTomato. A TC tag of 12 amino acids (FLNCCPGCCMEP) was inserted between the T25 domain and the hybrid protein to report the expression of the hybrid protein. The interaction of the two hybrid proteins results in reconstituted adenylate cyclase activity through functional complementation between T25 and T18 fragments, which leads to cyclic adenosine monophosphate (cAMP) synthesis. The produced cAMP interacts with the catabolite activator protein (CAP), and the cAMP/CAP complex binds to the promoter and turns on the transcription of the red fluorescence reporter protein tdTomato (Fig. 1b). The cell-membrane-permeant biarsenical dye FlAsH-EDT_2 (EDT = 1, 2-ethanedithiol) was then incubated with the sample to stain the hybrid proteins green via TC tag binding. The hybrid bacterial samples were then analyzed using the laboratory-built nFCM. Both the green fluorescence of the hybrid protein and the red fluorescence of the tdTomato reporter protein of single bacteria were measured simultaneously in a high-throughput manner. Thus, the RRPE can be easily calculated for single bacterial cells.

3.2. Simultaneous measurement of the expression of both reporter protein and interacting protein

To examine the feasibility of the proposed tdTomato-TC-BACTH system for PPI measurement, a laboratory-built nFCM was used for this study. The association of Pal and TolB is important for bacterial outer membrane integrity, and these two proteins were chosen as the PPI model [23]. Fig. 2(a–d) (1–3) shows representative burst traces obtained on the side scatter (SS), green fluorescence (FL), and red fluorescence channels for the engineered tdTomato-BACTH reporter strain upon FlAsH-EDT_2 staining. Fig. 2(a–d)4 shows the bivariate dot-plots of tdTomato reporter protein versus TolB expression (via TC-tag labeling). Quadrant gates were created for all the samples. For the blank control with *E. coli* BTH101-*tdTomato* co-transformed with pUT18C/pKT25 (no interacting protein expression), no discernible fluorescence bursts were detected on either the green or the red fluorescence channel (Fig. 2a2 and Fig. 2a3), and most cells (99.78%) fell in the Q4 region (Fig. 2a4).

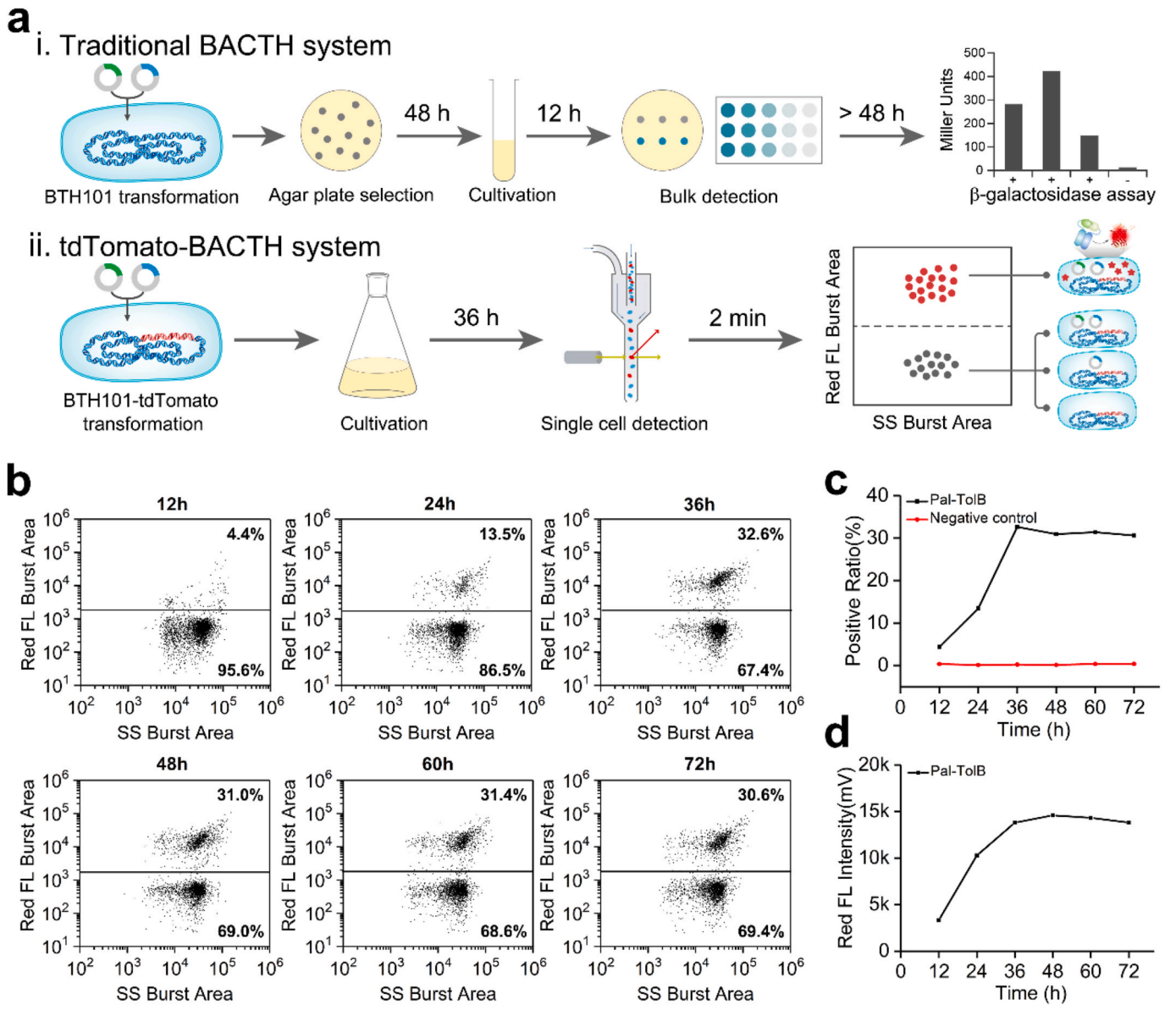

Fig. 4. FCM-based tdTomato-BACTH approach for rapid identification of target cells for PPI study. (a) Schematic diagrams of the FCM-based tdTomato-BACTH system (i) and the traditional BACTH system (ii). (b) Bivariate dot-plots of red fluorescence burst area versus side scatter burst area for *E. coli* BTH101-*tdTomato* transformed with plasmid encoding Pal and TolB and cultivated for different time periods. The population percentage (c) and MFI (d) of tdTomato-positive cells containing Pal-TolB as a function of cultivation time. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

These results indicate that both the nonspecific binding of FlAsH-EDT_2 and the false positive expression of the tdTomato reporter were negligible for the as-developed tdTomato-TC-BACTH system. A significant signal was observed on the red fluorescence channel for *E. coli* BTH101-*tdTomato* co-transformed with plasmid encoding interacting proteins without TC tags (pUT18C-*pal*/pKT25-*tolB*) owing to the expression of tdTomato reporter (Fig. 2b3). Moreover, it was found that 57.1% of cells fell in the Q1 region (Fig. 2b4). For the wild type *E. coli* BTH101 co-transformed with plasmid encoding interacting proteins with TC tag (pUT18C-*pal*/pKT25-TC-*tolB*), a significant signal on the green fluorescence channel was observed owing to the expression of the TC-tagged TolB protein (Fig. 2c2). Moreover, 61.6% of the cells fell into the Q3 region (Fig. 2c4). For the *E. coli* BTH101-*tdTomato* co-transformed with plasmid encoding interacting protein with TC tag (pUT18C-*pal*/pKT25-TC-*tolB*), distinct fluorescence bursts on both the green and red fluorescence channels were observed (Fig. 2d2 and Fig. 2d3). The bivariate dot-plot of Fig. 2d4 indicates that the percentages of positive cells (with concurrent expression of the interacting proteins and the tdTomato reporter) and negative cells (with either the expression of interacting protein or the expression of tdTomato but not both) were 66.1% (Q2 region) and 32.3% (Q4 region), respectively. This can be attributed to the all-or-none phenomenon observed during the bistability in the lactose utilization network [24,25] and the autoamplification positive feedback of cAMP on the expression of hybrid protein. That is, the positive feedback loop of the lactose utilization network leads to two stable states of tdTomato reporter expression in *E. coli*; one is fully activated with high expression, and the other is inactivated with only basal expression. The observed two populations of ON and OFF in the tdTomato-TC-BACTH system agreed well with our previous study, of which immunofluorescent staining was used to label both the β-galactosidase reporter protein and one of the interacting protein. Compared with immunofluorescent labeling, the fixation and permeabilization treatment can be eliminated to greatly speed up the sample analysis rate. Simultaneous quantitative analysis of the

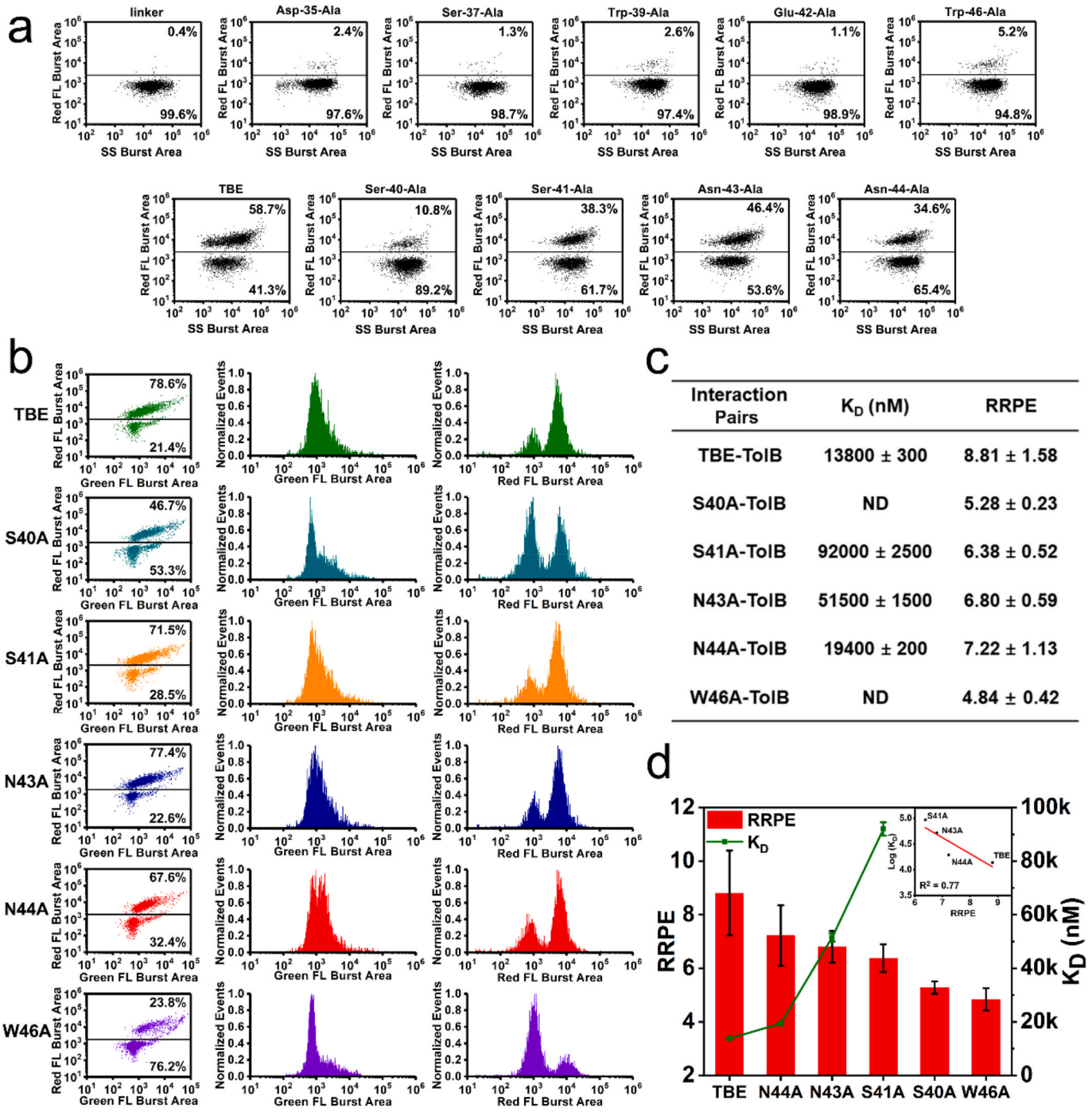

Fig. 5. FCM-based RRPE-tdTomato-TC-BACTH strategy for the quick mapping and ranking of key residues of PPIs. (a) Bivariate dot-plots of tdTomato fluorescence burst area versus side scatter burst area for *E. coli* BTH101-*tdTomato* transformed with plasmids carrying *tolB* and TBE mutants. (b) Bivariate dot-plots of tdTomato fluorescence burst area versus TC-tag green fluorescence burst area and the fluorescence distribution histograms for TolB interacting with wild type TBE and its mutants. (c) Literature reported K_D and RRPE measured in this study for the interactions between TolB and wild-type TBE or its mutants. (d) Comparison between the measured RRPE values and literature-reported K_D for different interactions. The error bars represent the standard deviation of three replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

expression of interacting protein and reporter protein can be accomplished in less than 1 h.

3.3. Evaluation of the RRPE-tdTomato-TC-BACTH approach for measurement of protein interaction strength

To investigate the quantification property of RRPE on estimating the interaction strength of protein pairs in the tdTomato-TC-BACTH system, we compared the interaction of four pairs of acid (En) and base (Kn) α-helices with various heptad repeats (n) that associate into coiled coils. The E coil and K coil interact through hydrophobic interactions and electrostatic attraction, and higher affinity is associated with a longer helix [26]. The dissociation constants (K_D) of the four coiled-coils measured via surface plasmon resonance (SPR) using a BIAcore instrument were 30 000 ± 3000, 7000 ± 800, 116 ± 8, and 14 ± 1 nM for the interactions of E3–K3, E5–K3, E4–K4, and E5–K4, respectively [26].

E. coli BTH101-tdTomato cells were transformed with plasmid encoding T18-*En* and T25-TC-*Kn* (n = 3, 4, 5) and plated. For each protein pair, three individual colonies were picked and inoculated into LB broth separately. Fig. 3a shows the bivariate dot-plots of tdTomato red fluorescence versus TC-Kn green fluorescence along with the fluorescence distribution histograms for these four interaction pairs. We calculated the RRPE with the median fluorescence intensity (MFI) of the positive population via ($MFI_{tdTomato,\ Positive}$-$MFI_{tdTomato,\ Negative}$)/($MFI_{TC\text{-}TolB,\ Positive}$-$MFI_{TC\text{-}TolB,\ Negative}$). The measured RRPE values were 1.71 ± 0.03, 6.53 ± 0.28, 9.08 ± 0.03, and 11.73 ± 0.54 for the interactions of E3–K3, E5–K3, E4–K4, and E5–K4, respectively (Fig. 3b). Fig. 3c shows that the measured RRPE exhibited a strong inverse linear correlation with the logarithm of the dissociation constant K_D of the four interaction pairs, demonstrating the ability of RRPE to quantify PPIs using the tdTomato-TC-BACTH systems. The RRPE-tdTomato-TC-BACTH approach can measure and rank a wide range of interactions with tight (K_d < 100 nM) to moderate (100 nM < K_d < 1000 nM) and to weak (K_d > 1000 nM) binding affinities.

3.4. Application of the FCM-Based tdTomato-BACTH approach for rapid identification of target cells for PPI study: elimination of agar plating

Traditional BACTH system normally consists of multiple steps, and the whole process from bacterial transformation, agar plate selection, and cultivation to beta-galactosidase assay spanned 4–5 days (Fig. 4a–i). Since FCM can quickly analyze rare target cells in a large number of surrounding cells, we aimed to directly identify target cells that have been successfully co-transformed with plasmid encoding interacting proteins, through tdTomato reporter fluorescence analysis by FCM. This way, the labor-intensive and time-consuming agar plating and colony selection steps can be bypassed to speed up the analysis rate of PPI study by the BACTH system (Fig. 4a–ii). To evaluate the feasibility of using the FCM-based tdTomato-BACTH approach for rapid PPI analysis, a time-course study on tdTomato reporter expression was conducted. The transformation mixtures cultured in liquid medium were harvested at different growth times and analyzed using FCM. The bivariate dot-plots of red fluorescence burst area versus side scatter burst area for *E. coli* BTH101-*tdTomato* transformed with plasmid encoding Pal and TC-TolB and cultivated for different time periods are displayed in Fig. 4b. Single-cell measurement via FCM indicates that after 12, 24, 36, 48, 60, and 72 h cultivation upon transformation, the fractions of cells expressing tdTomato were 4.4%, 13.5%, 32.6%, 30.1%, 31.4%, and 32.6%, respectively. We can see from Figs. 4c and 3d show that both the population of tdTomato-positive cells and the MFI of tdTomato reporter initially increased with the increase in cultivation time and reached a plateau around 36 h. For the negative control of *E. coli* BTH101-*tdTomato* transformed with empty vectors, the percentage of tdTomato-positive cells remained close to zero after prolonged cultivation (Fig. 4c and Fig. S4). These results indicate that the tdTomato reporter can hardly be triggered by self-activators; therefore, the false positive rate is negligible. These results demonstrate that the FCM-based tdTomato-BACTH approach can eliminate the conventional agar plating step and thus offer high-throughput identification of target cell; therefore, it has potential for high-throughput PPI study.

3.5. Quick mapping and ranking of key residues for PPI via FCM-Based RRPE-tdTomato-TC-BACTH strategy

The determination of key residues in PPIs is essential in understanding the molecular basis of recognition [27]. We sought to test the ability of the FCM-based tdTomato-BACTH approach for the rapid screening of the determinant amino acid residues of PPIs. A 16-residue TBE that is part of colicin E9 (ColE9) was subjected to alanine substitutions at various positions. These TBE mutations fall into two categories in the interaction with TolB: those that abolish binding, such as D35, S37, W39, E42, and W46A, and those that result in weak binding, such as S40A, S41A, N43A, and N44A [28,29]. Reporter strains transformed with plasmids carrying *tolB* and TBE mutants were grown in liquid medium for 36 h as described in the previous section and measured directly via FCM. Fig. 5a shows the bivariate dot-plots of tdTomato red fluorescence versus side scatter burst area for TolB interaction with various TBE mutations. Positive signals were observed for all the interaction pairs, even for the mutant pairs with interaction affinities too low to be detected through other methods (Fig. 5a). For example, for the mutants D35, S37, W39, E42, and W46A for which the interaction was reported to be abolished, the rates of positive population were 2.4%, 1.3%, 2.6%, 1.1%, and 5.2%, respectively. These values are significantly higher than the 0.4% for the negative control. Moreover, for the weak binding mutants S40A, S41A, N43A, and N44A, the rates of positive population were measured as 10.8%, 38.3%, 46.4%, and 34.7%, respectively. The results indicate that essential residues for PPIs can be easily discriminated via the FCM-based tdTomato-BACTH approach. In particular, the signaling cascade in the BACTH system ensures a higher sensitivity for the weak and transient interactions that are undetectable by traditional methods.

To rank the interaction strength of different TBE mutations with TolB, The RRPE values of four weak binding mutations (S40A, S41A, N43A, and N44A) and a strong binding mutant W46A were measured. Fig. 5b shows the bivariate dot-plots of tdTomato red fluorescence versus TC-TolB green fluorescence for these interaction pairs and the fluorescence distribution histograms. The measured dissociation constants K_D of these interactions were 13800 ± 300 μM, ND, 92000 ± 250 μM, 51500 ± 150 μM, 19400 ± 200 μM, and ND for wild-type TBE and mutants containing S40A, S41A, N43A, N44A, and W46A, respectively, determined by SPR using a BIAcore X biosensor (Uppsala, Sweden) [29]. The measured RRPE values in the present study were 8.81 ± 1.58, 5.28 ± 0.23, 6.38 ± 0.52, 6.80 ± 0.59, 7.22 ± 1.13, and 4.84 ± 0.42, respectively (Fig. 5c). The measured RRPE values and literature-reported K_D are plotted together in Fig. 5d, with the x-axis ranked in the sequence of RRPE values from high to low. For the five TBE mutants, the detected RRPE values were all smaller than that of the wild-type TBE. For the wild-type TBE and its three mutants with available K_D scores, similar to that of Fig. 3c, an inverse linear correlation was identified between the RRPE and the logarithm of the K_D (Fig. 5d). That is, a higher RRPE value was observed for a strong interaction or a lower K_D score. For mutants S40A and W46A of TBE, whose interactions with TolB were too weak to be measured via SPR, the newly developed FCM-based RRPE-tdTomato-TC-BACTH strategy can measure their RRPE values and rank their binding affinities. These results suggest that the FCM-based RRPE-tdTomato-TC-BACTH method is useful in providing a relative ranking for key residues of interacting proteins. Moreover, this method is sensitive enough to discriminate and rank not only the interaction strengths of the same order of magnitude but strengths with a difference as small as 1.4-fold (wild-type TBE-TolB versus mutant N44A-TolB).

3.6. Identification of PPI inhibitors using the FCM-Based RRPE-tdTomato-TC-BACTH approach

The inhibition of PPIs is an attractive strategy for developing drugs against various diseases [30–32]. In this study, we monitored the disruption of the Pal-TolB interaction with TBE to assess the ability of the FCM-based RRPE-tdTomato-TC-BACTH approach to identify PPI inhibitors. ColE9 uses TBE to competitively recruit TolB from the Pal-TolB complex, which causes local instability of the OM as a prelude to toxin import [29]. Structural comparison reveals that TBE and Pal bind at the same TolB site [33]. This TBE peptide is therefore a good candidate to inhibit Pal-TolB interaction [34]. However, TBE lacks cell permeability, which is essential for targeting intracellular proteins *in vivo*. To solve this issue, we conjugated the TBE peptide to a cell-penetrating peptide (CPP), $(RFR)_4XB$, which is an effective carrier for the intracellular delivery of molecules into *E. coli* [35]. The TBE

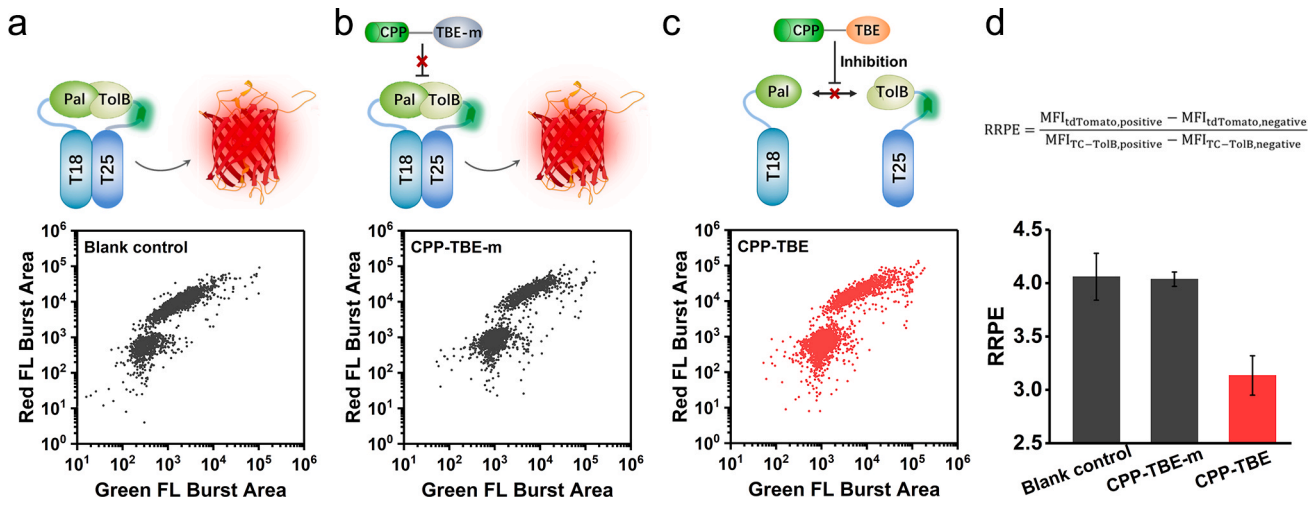

Fig. 6. FCM-based RRPE-tdTomato-TC-BACTH approach to identify PPI inhibitors. (a, b, and c) Bivariate dot-plots of tdTomato reporter protein versus TolB expression for *E. coli* BTH101-*tdTomato* transformed with plasmids of pUT18C-*pal*/pKT25-TC-*tolB* without the addition of any peptide (a, blank control), with the addition of CPP-TBE-m peptide (b, negative control), and with the addition of CPP-TBE (c). (d) Column chart of the RRPE measurements. The error bars represent the standard deviation of three replicates.

mutant (TBE-m) GASWGDGSSSGNNPWE was synthesized as a negative control, in which the key residues D35, S37, W39, and E42 of TBE were mutated to abolish the binding to TolB. The negligible effects of CPP-TBE on the bacterial growth, physiology and morphology were compared through growth curves and microscope imaging (Fig S5). Fig. 6(a–c) shows the bivariate dot-plots of tdTomato reporter protein versus TolB expression for *E. coli* BTH101-*tdTomato* transformed with plasmid encoding Pal and TolB without the addition of any peptide (a, blank control), with the addition of CPP-TBE-m peptide (b, negative control), and with the addition of CPP-TBE (c). Fig. 6d indicates that cells treated with CPP-TBE exhibited a much lower RRPE than both the blank control and the negative control; the RRPE values of the blank control, negative control, and the CPP-TBE-treated cells were 4.06 ± 0.22, 4.04 ± 0.07, and 3.14 ± 0.18, respectively. Thus, the RRPE value can reveal the inhibition effect of the inhibiting protein *in vivo*. Compared with researches studying the competitive inhibition of the TBE on Pal-TolB interaction [23,29,36,37], this is the first *in vivo* approach revealing the inhibition effect of TBE. Therefore, the FCM-based tdTomato-TC-BACTH approach holds a great potential for screening inhibitors of a given PPI.

4. Conclusions

In summary, we developed a FCM-based RRPE-tdTomato-TC-BACTH method for PPI measurement based on the simultaneous detection of the tdTomato reporter and the TC-tagged interacting protein. The major advantages of the present technique are its high sensitivity, great simplicity, and quantitative characterization of protein–protein interactions. Using multicolor flow cytometry and RRPE, we showed a good correlation between RRPE and reported binding affinities for several coiled-coil interactions and for TolB protein interacting with wild-type TBE and various TBE mutants. It is worth noting that the proposed RRPE method only provides a relative ranking for interaction pairs of the same interaction mechanism. The RRPE does not allow for the absolute quantification of protein interaction affinity. Because the En/Kn, TolB/TBE, and Pal/TolB interaction pairs are of different mechanisms, their RRPE values cannot be directly compared with each other for affinity ranking.

This method provides a quick way of screening and ranking key residues for PPIs. The ranking of coiled-coil interactions and protein interacting key residues demonstrates that the RRPE-tdTomato-TC-BACTH approach can readily distinguish interaction strengths with a ten-fold difference and the same order of magnitude. The method is sensitive enough to discriminate the interaction strengths with a 1.4-fold difference. We also showed that the RRPE-tdTomato-TC-BACTH approach can be used for the identification of protein interaction inhibitors. When combined with cell sorting, this FCM-based RRPE-tdTomato-TC-BACTH approach is expected to hold great potential for the high-throughput measurement of the binding strengths between designed library proteins and multiple target proteins, along with the rapid screening of inhibiting candidates. This platform can also be easily transferred to other hybrid systems for protein interaction quantification.

Credit author statement

L. Wu conceived the project. L. Wu, L. Su, and M. Deng performed the experiments and analyzed the data. X. Hong, M. Wu, M. Zhang, and E. Bouveret were helpful with the methodology. L. Wu and L. Su wrote the original draft. L. Wu and X. Yan acquired funding, supervised the project, and reviewed & edited the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xiaomei Yan declares competing financial interest as a cofounder of NanoFCM Inc., a company committed to commercializing the nanoflow cytometry (nFCM) technology.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122549.

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