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Flow Cytometric Single-Cell Analysis for Quantitative in Vivo Detection of Protein–Protein Interactions via Relative Reporter Protein Expression Measurement.

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Classification:

Major: Biological Sciences

Abstract:

Cell-based two-hybrid assays have been key players in identifying pairwise interactions, yet quantitative measurement of protein-protein interactions *in vivo* remains challenging. Here, we show that using relative reporter protein expression (RRPE), defined as the level of reporter expression normalized to that of the interacting protein measured in single cell, quantitative analysis of protein interactions in bacterial adenylate cyclase two-hybrid (BACTH) system can be achieved. A multicolor flow cytometer was used to measure simultanously the expression levels of one of the two putative interacting proteins and the β-galactosidase (β-gal) reporter protein upon dual immunofluorescence staining. Single cell analysis revealed that for every bacterial culture co-transformed with the two-hybrid plasmids, there exist two cell populations with or without the expression of interacting protein and reporter protein. Using Pal and TolB protein as the model of an interacting bait and pray pair, the RRPE was found to be constant regardless of the inoculation colonies and the cultivation time. Decreased RRPE was detected for TolB mutants with two N-terminal truncations (TolB $\Delta^{22\text{-}25}$ or TolB $\Delta^{22\text{-}33}$), suggesting that the RRPE was an intrinsic characteristic associated with the binding strength between the two interacting proteins. This hypothesis was verified with acid base coiled coils formed by two α-helices of various binding affinities, for which the measured RRPE progressively decreased as the affinity decreased. Several useful applications of our RRPE-BATCH method can be expected for the quantitative detection, strength comparison, and affinity ranking of pairs of interacting proteins.

Keywords: protein-protein interaction, yeast two-hybrid system, bacterial two-hybrid system, flow cytometry, binding affinity

Significance Statement

Assessing the intrinsic affinities for interacting proteins is of fundamental importance to explore and understand protein-protein interactions. Using a bacterial two-hybrid system (the BACTH system), we developed a quantitative method for the detection and affinity ranking of protein-protein interactions. By measuring the expression level of both the reporter protein and interacting protein at the single-cell level via immunofluorescent staining and flow cytometry, we found that the relative reporter protein expression (RRPE) is characteristic of the interacting protein pair and correlates with their binding affinity. This method can provide an efficient tool in prioritizing a large number of putative interacting proteins for following analyses.

56 **P**rotein-protein interactions are involved in virtually every cellular process, and their
57 study is crucial in revealing protein functions, deciphering protein interaction
58 networks, and identifying novel therapeutic targets (1, 2). Among numerous
59 methodologies developed for protein interaction study, the yeast two-hybrid (Y2H)
60 system is the most commonly used binary method for measuring direct physical
61 interactions between two proteins, and has been estimated to account for over 50% of
62 protein-protein interactions described in PubMed (3-5). This powerful *in vivo*
63 approach interrogates two proteins, called bait and prey, one fused to a DNA-binding
64 domain and the other fused to a transcriptional activation domain of a transcription
65 factor and expressed in yeast. If the two proteins interact in the system, they
66 reconstitute a functional transcription factor that induces the transcription of a reporter
67 gene, whose output can be measured as growth of yeast colonies on selective medium
68 or as blue coloration in a β-galactosidase (β-gal) assay. Although the Y2H system has
69 made significant contribution to the discovery of protein-protein interactions and the
70 interactome networks (6-8), both the false positive and false negative rates are
71 relatively high, and all the interactions are forced to occur in the yeast nucleus and are
72 thus not suitable for protein interaction involving membrane proteins and cytosolic
73 proteins (7, 9).

74 To overcome the limitations of Y2H system, a bacterial equivalent of the
75 two-hybrid system was developed based on functional complementation of the
76 catalytic domain of *Bordetella pertussis* adenylate cyclase (BACTH) (10, 11). This
77 leads to cAMP synthesis, which in turn, triggers the expression of several resident

genes such as the lactose or maltose operons. Particularly, this technique enabled the study of membrane proteins because cAMP is a diffusible molecule and the BACTH system does not require the hybrid proteins to be located in the nucleus as that of Y2H (12). However, the BACTH system as well as the Y2H system is not suitable for the quantitative measurement of pairwise protein interactions due to the lack of understanding of how the strength of the interactions correlate with the level of reconstituted reporters (13).

Flow cytometry is a well-established tool for the rapid, quantitative, and multiparameter analysis of single cells. Employing a codon-optimized yeast enhanced green fluorescent protein (yEGFP) as the reporter, Chen *et al.* developed a high throughput approach to study protein-protein interactions inside the cell via flow cytometric measurement (14). Through the development of a yeast surface two-hybrid (YS2H) system, Hu *et al*. reported quantitative flow cytometric measurement of protein-protein interactions via the secretory pathway (13). On the other hand, anchored periplasmic expression (APEx) bacterial two-hybrid system has been developed for the study of protein pairs on the basis of affinity or expression (15, 16). In both the YS2H and the APEx two-hybrid systems, the bait protein has to be produced at the surface of the cell, and the tag-fused prey protein has to be secreted in solution. Then, the strength of bait-pray interaction can be measured via antibody binding to the epitope tag appended to the prey protein. Compared to the surface bacterial two-hybrid system, the classic BACTH system is a well-established and much simpler approach for protein-protein interaction studies (10, 11). Particularly,

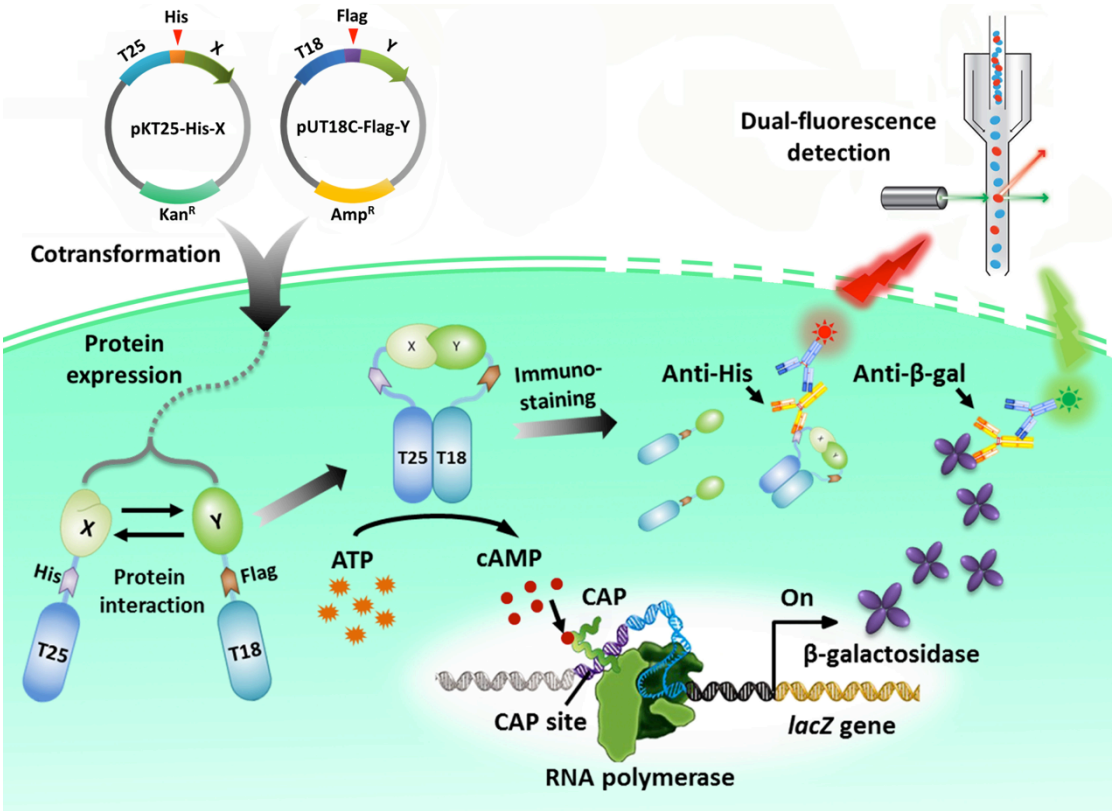
100 the signaling cascade in the BACTH system ensures higher sensitivity for the weak
101 and transient interactions. Therefore, a quantitative approach for the BACTH system
102 shall greatly advance the protein-protein interaction study due to the general
103 applicability of the BACTH system and its sensitivity for low affinity interaction.

104 Herein, we demonstrate that through flow cytometric detection and
105 immunofluorescent staining, the relative reporter protein expression (RRPE), defined
106 as the normalized report protein expression to that of the interacting protein in a
107 single cell, can be used to quantitatively estimate the binding strength between two
108 interacting proteins in the classical BACTH system. This feature allowed us to
109 confirm interacting pairs of proteins, investigate determinant residues in
110 protein-protein interaction, and compare interaction strength of different pairs. The
111 RRPE-BACTH method described here provides a practical and powerful method for
112 the rapid and quantitative *in vivo* measurement of protein-protein interactions.

113 Results and Discussion

114 **Design of the BACTH System for Flow Cytometric Analysis.** Scheme 1 illustrates
115 the experimental design of the flow cytometric BACTH system. Two compatible
116 plasmids carrying the hybrids with T25 and T18 domains respectively were
117 co-transformed into the reporter strain *cya*$^-$ *E. coli* BTH101. Between T25 or T18
118 domain and the hybrid proteins, His and Flag tags were inserted respectively, and
119 were used to follow the expression of the hybrid proteins. The interaction of the
120 hybrid proteins results in a functional complementation between T25 and T18
121 fragments, which reconstitutes the activity of adenylate cyclase and leads to cAMP

synthesis. The produced cAMP interacts with the catabolite activator protein (CAP) and the cAMP/CAP complex binds to the promoter and regulates the transcription of *lacZ* gene coding for the β-galactosidase (β-gal) reporter expression. β-gal was specifically labeled green with rabbit-anti-β-gal antibodies and FITC-conjugated goat anti rabbit IgG. Meanwhile, anti-His/Flag mouse monoclonal antibody and DyLight 649-conjugated goat anti mouse IgG were used to label the hybrid proteins red. Upon dual immunofluorescence labeling, the bacterial sample was analyzed by flow cytometer. FITC and DyLight 649 fluorophores can be excited by the 488 nm and 640 nm lasers respectively, and the emitted green and red fluorescence signals were detected concurrently on the FL1 and FL2 fluorescence channels. Therefore, for a bacterial two hybrid sample, the expression level of both the β-gal reporter and the hybrid proteins can be detected and quantified simultaneously.

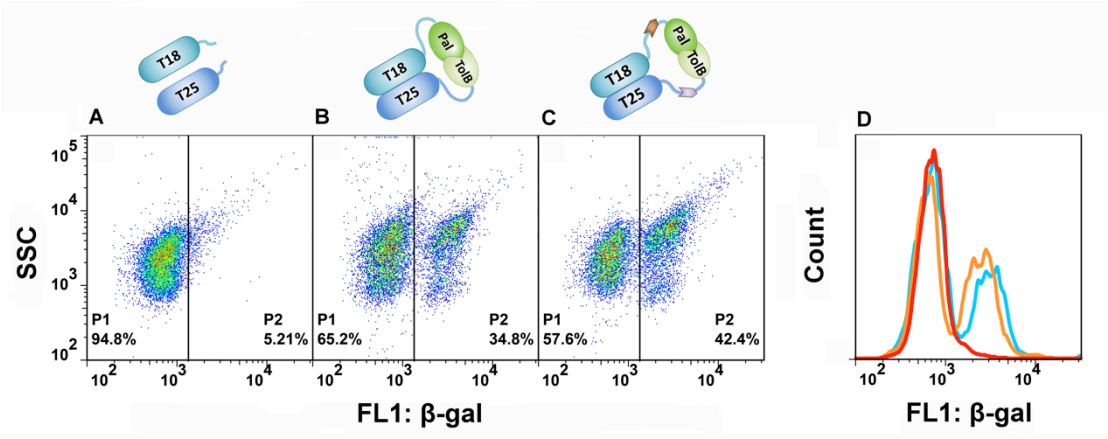


135 **Scheme 1.** Depiction of protein-protein interaction study at the single-cell level based
136 on the BACTH system, dual immunofluorescent staining, and flow cytometric
137 analysis.

138 **Flow Cytometric Detection of Protein-Protein Interaction by the BACTH System**
139 **via Immunofluorescent Staining of β-gal Reporter Protein.** Pal and TolB, two
140 proteins involved in maintaining the integrity of bacterial outer membrane were
141 chosen as the protein-protein interaction model (17). In the BACTH system, both the
142 interacting proteins and reporter proteins are produced in the cytoplasm of bacteria,
143 and the antibodies need to traverse the bacterial cell wall and membrane for target
144 staining. Therefore, much efforts have been devoted to optimize the
145 immunofluorescence labeling procedures including fixation, permeabilization, and
146 staining (see Materials and Methods). After immunofluorescent staining of β-gal
147 reporter protein with FITC, the samples were analyzed on the flow cytometer. Fig. 1
148 shows the bivariate dot-plots of side scatter intensity *versus* FITC fluorescence
149 intensity obtained for *E. coli* BTH101 co-transformed with plasmids
150 pUT18C-linker/pKT25-linker (negative control, no interaction),
151 pUT18C-*pal*/pKT25-*tolB* (interacting proteins without additional tag), and
152 pUT18C-Flag-*pal*/pKT25-His-*tolB* (tagged interacting proteins), respectively. Two
153 distinct populations with different green fluorescence intensity were observed for
154 bacterial samples co-transformed with plasmids containing the interacting Pal/TolB
155 pair proteins regardless the presence of Flag/His tag or not (Fig. 1, B and C). A
156 discriminant line between these two populations was drawn on the FL1 channel for

157 easy discrimination, defining two regions P1 and P2. For the negative control sample,
158 approximately 94.8% of the cells fall in the P1 region (Fig. 1A), whereas for cells
159 co-transformed with plasmids containing the interacting proteins with or without tags,
160 about 34.8% and 42.4% of the cells fall in the P2 region. The fluorescence
161 distribution histograms of these three samples are plotted in Fig. 1D. Comparable
162 median fluorescence intensities (MFI) for cells falling in the P1 region were observed
163 for all three samples, suggesting that for bacterial culture co-transformed with two
164 plasmids carrying Pal and TolB genes, there exists a large fraction of cells (about 40%)
165 in which the β-gal reporter protein cannot be detected. Meanwhile, events residing in
166 the P2 region can be ascribed to cells that co-express Pal and TolB interacting
167 proteins inside a single cell which leads to the expression of β-gal reporter. Because
168 the interaction between Pal and TolB is robust and well characterized, the fraction of
169 the cells that are similar to the negative control may be ascribed to the lack of
170 expression of either one or both of the interacting proteins. As plasmid loss is
171 excluded by the use of antibiotics in the culture media, this all-or-nothing
172 phenomenon can only be explained by the bistability in the lactose utilization network
173 of *E. coli*. In the BACTH system, the reporter gene and hybrid plasmids are both
174 regulated by the wild type P*lac* promoter (18). For cells falling in the P2 region, the
175 MFI are 3135 and 2539 for bacterial cultures transformed with interacting protein
176 genes without and with Flag/His tag, respectively, suggesting that tag insertion to the
177 C-terminal of the two proteins did not prevent the interaction. The observation of two
178 populations with completely different behaviors regarding reporter β-gal expression

179 highlights the importance and need of single-cell analysis for the BACTH system. In
180 contrast to the ensemble-averaged measurement by spectrophotometers, flow
181 cytometric analysis can reveal the inherent heterogeneity of bacterial populations in
182 β-gal expression that will provide more insights into the BACTH system.



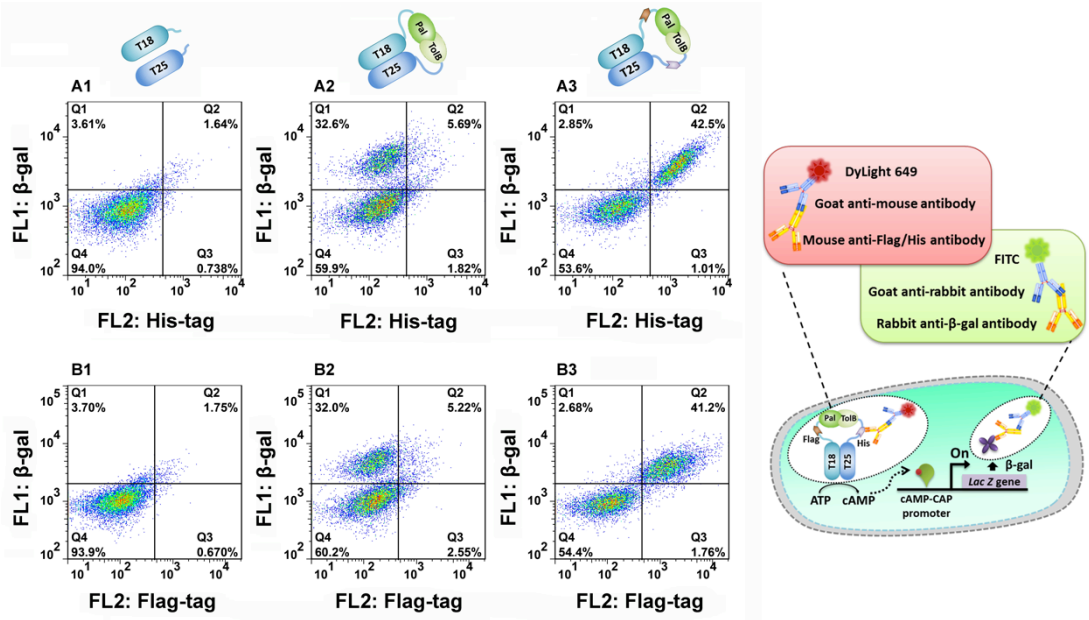
183

184 **Fig. 1.** Flow cytometric analysis of protein-protein interaction by the BACTH system
185 upon β-gal immunofluorescent staining. Bivariate dot-plots of side scatter intensity
186 (SSC) *versus* FITC fluorescence intensity (FL) for *E. coli* BTH101 co-transformed with
187 plasmids of A) pUT18C-linker/pKT25-linker, B) pUT18C-*pal*/pKT25-*tolB*, and C)
188 pUT18C-Flag-*pal*/ pKT25-His-*tolB*, respectively. D) FITC green fluorescence
189 distribution histograms of β-gal for *E. coli* BTH101 with no interaction (red, case A),
190 with expression of interacting proteins (blue, case B), and with expression of tagged
191 interacting (orange, case C).

192 **Simultaneous Measurement of the Expression of Interacting Protein and**
193 **Reporter Protein.** In order to study the relationship between the expression of β-gal
194 reporter protein and the expression of hybrid proteins, the Flag or His tag of one
195 interacting protein and the β-gal reporter protein were immunofluorescently labeled
196 with Dylight-649 and FITC, respectively. DyLight-649 was chosen to label the tag
197 fragment because it can be efficiently excited by the 640 nm laser, which avoids

198 spectral cross-talk with FITC. The green and red fluorescence signals were detected
199 on the FL1 and FL2 channel, respectively. Figs. 2A and 2B show the bivariate
200 dot-plots of β-gal reporter protein versus TolB expression (via His tag labeling) or Pal
201 expression (via Flag tag labeling), respectively. Quadrant gates were created for all
202 the samples. For the negative control with *E. coli* BTH101 co-transformed with
203 pUT18C-linker/pKT25-linker (no interacting protein expression), most cells (94.0%
204 and 93.9%) fall into the Q4 region (Figs. 2A1 and 2B1) with negligible fluorescence
205 on both the green and red fluorescence channels. For *E. coli* BTH101 co-transformed
206 with plasmids encoding interacting proteins but without tags
207 (pUT18C-*pal*/pKT25-*tolB*), 32.6% and 32.0% of the cells fall into the Q1 region due
208 to the expression of β-gal reporter protein. The two population phenomenon is similar
209 to the one observed in Fig. 1. For *E. coli* BTH101 co-transformed with plasmids
210 encoding interacting proteins with tags (pUT18C-Flag-*pal*/pKT25-His-*tolB*), 53.6%
211 and 54.4% of the cell population resides in the Q4 region (neither the expression of
212 β-gal nor the expression of interacting protein), while 42.5% and 41.2% of the cells
213 fall in the Q2 region indicating concurrent expression of the interacting proteins and
214 the β-gal reporter (Figs. 2A3 and 2B3). The similar ratios of Q2 reveal that only the
215 co-expression of the interacting proteins leads to the expression of the reporter gene.
216 Because expression of all of them are driven by cAMP in a positive feedback loop,
217 this establishes bistability. Clearly, using dual immunofluorescence staining,
218 simultaneous measurement of interacting protein expression and protein-protein
219 interaction (via reporter protein expression) can be successfully achieved at the

220 single-cell level using flow cytometry. It is worthy to note that although
221 pUT18C-Flag-*pal* and pKT25-His-*tolB* are two compatible plasmids, they have
222 distinct replication origins and different copy numbers (11). The plasmid of lower
223 copy number (pKT25-His-*tolB*) normally results in a lower level of protein expression,
224 which determines the rate of complex formation with its interacting protein partner.
225 Note that the same PMT voltage was used for the detection of His-tag and Flag-tag
226 signals, and the relatively higher signal for His tag is due to the higher affinity of
227 anti-his antibody.

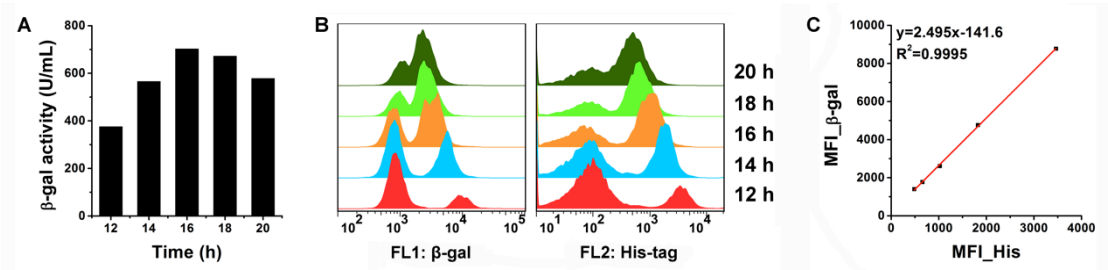


228

229 **Fig. 2.** Simultaneous measurement of the expression of interacting proteins and
230 reporter protein by flow cytometry. (A) Bivariate dot-plot of green fluorescence
231 intensity (β-gal) *versus* red fluorescence intensity (His-tag) for *E. coli* BTH101
232 co-transformed with plasmids of A1) pUT18C/pKT25, A2) pUT18C-*pal*/pKT25-*tolB*,
233 and A3) pUT18C-Flag-*pal*/pKT25-His-*tolB*, respectively. (B) Bivariate dot-plot of
234 green fluorescence intensity (β-gal) *versus* red fluorescence intensity (Flag-tag) for *E.*
235 *coli* BTH101 co-transformed with plasmids of B1) pUT18C/pKT25, B2)
236 pUT18C-*pal*/pKT25-*tolB*, and B3) pUT18C-Flag-*pal*/pKT25-His-*tolB*, respectively.

237 **Correlation between the Protein Interaction Strength and the Expression of**
238 **Interacting Proteins in a Single Cell.** As illustrated in Figure 1, in the BACTH
239 system, expression of the β-gal reporter protein is regulated by the production of
240 cAMP and thus by the interaction of two hybrid proteins (10). We examined the
241 relationship between the β-gal reporter protein expression and His-TolB (the plasmid
242 with lower copy number in the cell) expression by flow cytometry. When the cultures
243 of *E. coli* BTH101 reached a sufficient cell density (OD ~1.5) after 12 h cultivation,
244 these cultures were co-transformed with plasmids of
245 pUT18C-Flag-*pal*/pKT25-His-*tolB* every two hours. Before immunofluorescent
246 staining, β-gal activity of each sample was assayed by a classical Miller's protocol on
247 a spectrophotometer. Fig. 3A shows that β-gal activity increased from 375 at 12 h to
248 702 at 16 h and started to decrease slowly after then. Meanwhile, single cell
249 measurements by flow cytometry indicate that with the increase of cultivation time
250 from 12 h to 20 h, the fraction of cells expressing β-gal and His-TolB (Fig. 3B) kept
251 increasing from 7.5% to 75.5%. In contrast, the MFIs of positive cells decreased from
252 14500 to 2508 for β-gal signal (Fig. 3B1) and from 4155 to 495 for His-TolB signal
253 (Fig. 3B2), respectively. This phenomenon could be explained by the dilution of the
254 proteins inside a single cell upon cell division (19). When we plotted the MFI of β-gal
255 versus that of His-TolB after background signal subtraction for each protein, i.e.
256 ($MFI_{\beta\text{-gal, P2}}$-$MFI_{\beta\text{-gal, P1}}$) versus ($MFI_{\text{His-TolB, P2}}$-$MFI_{\text{His-TolB, P1}}$), a linear correlation with
257 R^2 of 0.9995 was obtained (Fig. 3C), which suggests that in the BACTH system,
258 expression of the β-gal reporter protein is linearly proportional to the expression of

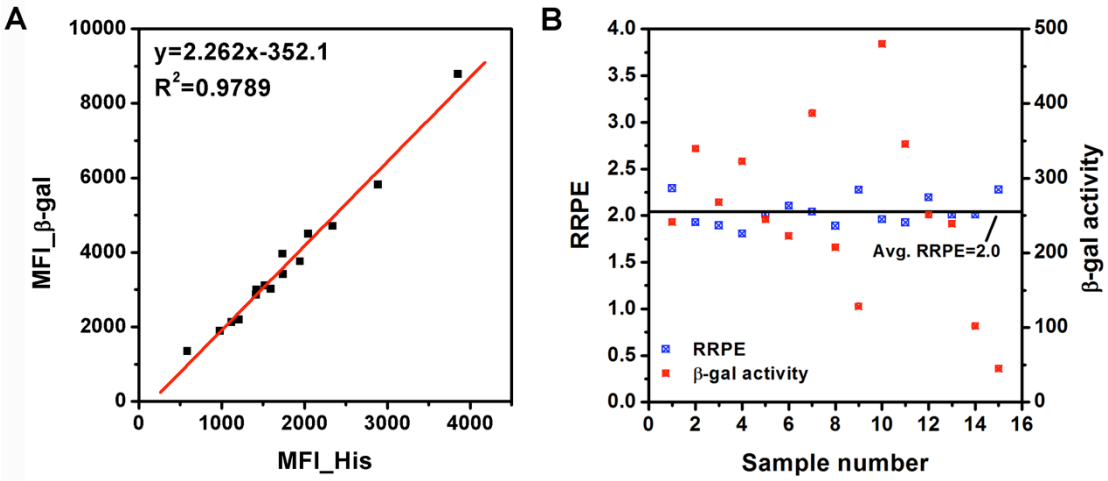
259 the interacting protein.

260 

261 **Fig. 3.** Enzymatic and flow cytometric analysis of the reporter protein β-gal for *E. coli*
262 BTH101 co-transformed with plasmids pUT18C-Flag-*pal*/pKT25-His-*tolB* at different
263 cultivation time. A) Column chart of β-gal activity measured with a classical Miller's
264 assay. B) Histograms of the fluorescence intensity distribution for β-gal and His-TolB
265 measured by flow cytometry. C) The correlation curve between the median
266 fluorescence intensities of β-gal and His-TolB after background subtraction.

267 The heterogeneity in the BACTH system has been well recognized because there
268 exist a big difference in plasmid copy number in different bacterial cells (11) and
269 stochasticity inherent in the biochemical process of gene expression (20). In order to
270 validate the generality of this observation, we first examined the correlation between
271 the expression of β-gal reporter protein and interacting protein for different colonies
272 at the same cultivation time. Fifteen different colonies were randomly picked from the
273 culture plate of *E. coli* BTH101 co-transformed with plasmids
274 pUT18C-Flag-*pal*/pKT25-His-*tolB* and inoculated in separate LB broth and cultivated
275 for 16 h. Fig. 4A shows the plot of β-gal expression versus that of interacting protein
276 His-TolB for different colonies, and a linear correlation was observed with R^2 of
277 0.9789. These data along with those reported in Fig. 3 suggest that for an interacting
278 protein pair, there is an important heterogeneity in the expression of both the hybrid

279 and reporter proteins, but that the expression of β-gal reporter protein exhibits a linear
280 proportion to that of the hybrid proteins regardless of different cultivation time for a
281 single colony or different colonies at the same cultivation time. Clearly, these results
282 demonstrate that the expression of the β-gal reporter protein is not only affected by
283 the affinity of the interacting protein pair but also by the expression level of the
284 hybrid proteins. Therefore, we propose to use relative reporter protein expression
285 (RRPE), defined as the normalized β-gal expression to that of the interacting protein,
286 to estimate the interaction strength of protein pairs. As shown in Fig. 4B, the
287 measured RRPE (blue dots) for the Pal-TolB interaction pair exhibits a constant value,
288 whereas the β-gal activity (red dots) is much more diverging among different
289 colonies.



290

291 **Fig. 4.** Flow cytometric analysis of the expression of β-gal reporter protein and
292 interacting protein His-TolB for bacterial samples inoculated with different single
293 colonies. A) The correlation curve between the median value of β-gal fluorescence
294 intensity and that of His-TolB. B) Plot of the relative reporter protein expression
295 (RRPE) versus β-gal activity for 15 bacterial cultures inoculated with different single
296 colonies randomly picked from the plate co-transformed with plasmids
297 pUT18C-Flag-*pal*/pKT25-His-*tolB*.

298 **Validation of the RRPE-BACTH Method for the Measurement of Protein**
299 **Interaction Strength.** To investigate the potential of using RRPE-BACTH method
300 for evaluating the strength of protein-protein interactions, we compared the Pal-TolB
301 interaction by using two mutated forms of TolB along with the wild type TolB. These
302 two mutated TolB proteins bear truncations, one with four residues deleted from the
303 N-terminus (TolB Δ^{22-25}) and the other with the entire N-terminal sequence deleted
304 (TolB Δ^{22-33}), which lower the binding affinity to Pal (21). The dissociation constants
305 K_D of these two truncated TolB proteins with Pal were reported to be 313 ± 15 nM
306 and 337 ± 18 nM, respectively, via ITC measurement at 30° C, which are about
307 tenfold higher than that of the wild type TolB (38 ± 3 nM). *E. coli* BTH101 cells were
308 co-transformed with pUT18C-Flag-*pal*/pKT25-His-*tolB*,
309 pUT18C-Flag-*pal*/pKT25-His-*tolB* Δ^{22-25}, or pUT18C-Flag-pal/pKT25-His-*tolB* Δ^{22-33}
310 plasmids and plated. Three individual colonies were picked and inoculated into LB
311 broth for each protein pair. These samples were immunofluorescence stained and
312 analyzed on the flow cytometer. Fig. 5A shows the representative bivariate dot-plots
313 of β-gal green fluorescence *versus* His-TolB red fluorescence and their fluorescence
314 distribution histograms for these three pairs. Fig. 5B indicates that the wild type TolB
315 exhibits the highest RRPE of 2.04 ± 0.21, and the mutated TolB Δ^{22-25} and TolB Δ^{22-33}
316 shared comparable RRPE values of 0.88 ± 0.07 and 0.91 ± 0.06, respectively. Hence,
317 the RRPE value follows the change in binding affinity of the TolB/Pal interaction,
318 with larger RRPE corresponds to higher binding affinity in the BACTH system.
319 Therefore, RRPE may be used to assess the binding affinity of protein-protein

interaction in the BACTH system.

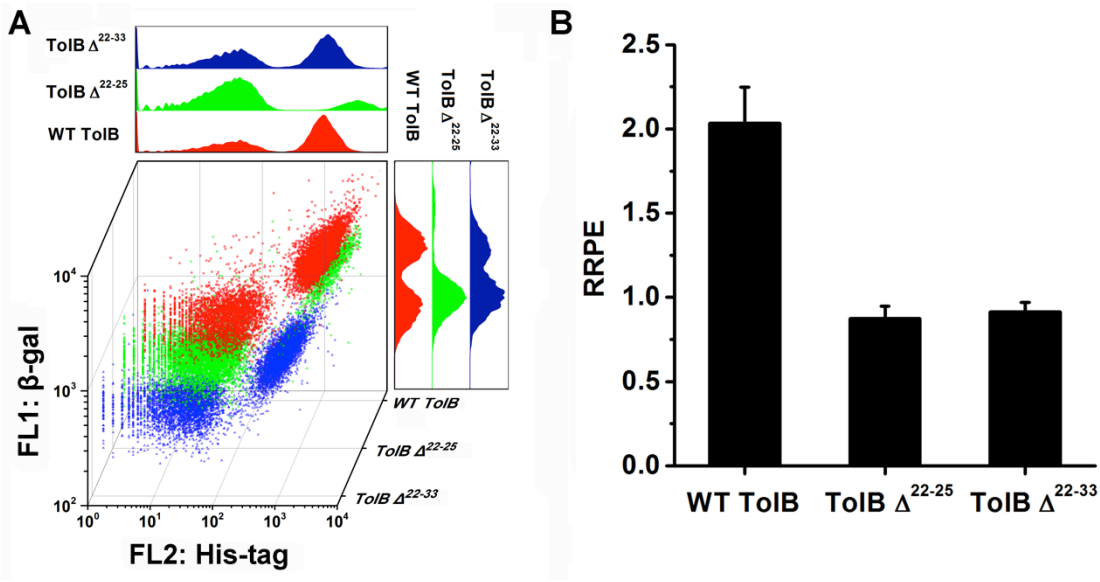
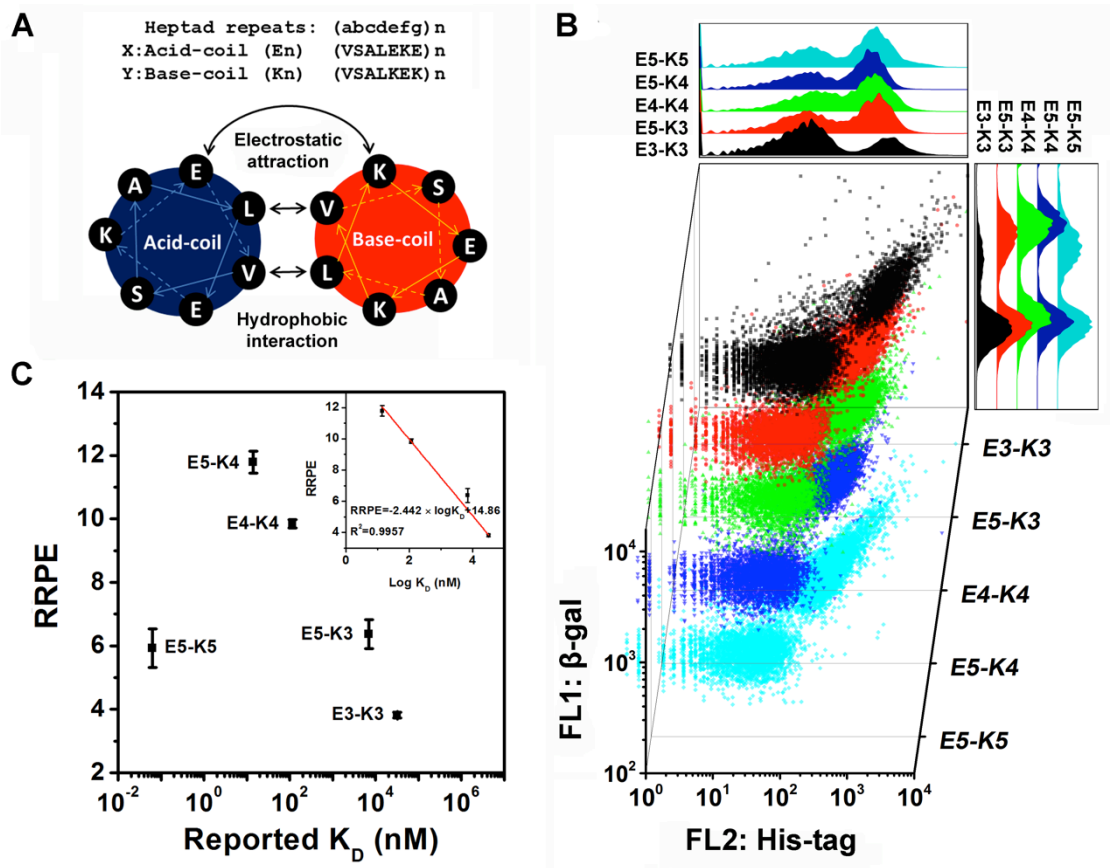


Fig. 5. RRPE measurement for Pal interacting with TolB and the two TolB mutants by the BACTH system and flow cytometry. (A) The bivariate dot-plots of β-gal green fluorescence *versus* His-TolB red fluorescence and the fluorescence distribution histograms for wild type TolB and its two truncated forms: TolB $\Delta^{22\text{-}25}$ and TolB $\Delta^{22\text{-}33}$. (B) Column chart of the RRPE for Pal interaction with TolB and the two truncated forms. The error bar represents the standard deviation of three replicates.

To further validate the applicability of the RRPE-BACTH method in affinity assessment, five pairs of acid (En) and base (Kn) α-helices with various heptad repeats (n) that associate into coiled coils were constructed into the BACTH system (Fig. 6A). E coil and K coil interacts through hydrophobic interaction at the interface and electrostatic attraction between the oppositely charged residues from the helix, and higher affinity is associated with longer helix (22). Among the five coiled-coils chosen in the present study, the dissociation constants (K_D) measured by surface plasmon resonance (SPR) using a BIAcore were 30000 ± 3000, 7000 ± 800, 116 ± 8, 14 ± 1, and 0.063 ± 0.005 nM for the interactions of E3-K3, E5-K3, E4-K4, E5-K4,

337 and E5-K5, respectively (22). *E. coli* BTH101 cells were co-transformed with
338 pUT18C-Flag-*En*/pKT25-His-*Kn* plasmids and plated. For each protein pair, three
339 individual colonies were picked and inoculated into LB broth separately. These
340 samples were immunofluorescence stained and analyzed on the flow cytometer. Fig.
341 6B shows the representative bivariate dot-plots of β-gal green fluorescence *versus*
342 His-Kn red fluorescence along with the fluorescence distribution histograms for these
343 five interaction pairs. The measured RRPE values were 3.8 ± 0.1, 6.4 ± 0.5, 9.9 ± 0.1,
344 11.8 ± 0.3, and 5.9 ± 0.6 for E3-K3, E5-K3, E4-K4, E5-K4, and E5-K5, respectively.
345 It should be noted that taking advantage of high sensitivity of the RRPE-BATCH
346 method, we can discriminate interactions with K_D lower than 10^4 nM. Fig. 6C shows
347 that the measured RRPE exhibited a strong correlation with the interaction affinity
348 from E3-K3 to E5-K4 except for E5-K5. This could be explained by the fact that
349 E5-K5 fits an interacting model with a relatively fast association and a very slow
350 dissociation, which is different from the other pairs (22). In contrast, in the BACTH
351 system, the synthesis of cAMP is irreversible and depends mainly on the rate at which
352 two coils associate (on-rate) to initiate complementation of T25 and T18. Because the
353 E5 coil was fused to T18 or T25 domain in the BATCH system, the on-rate of binding
354 with K5 could be reduced which leads to a lower binding affinity as demonstrated
355 with a decreased RRPE value (22). However, it needs to be pointed out that the
356 proposed RRPE method does not allow for the absolute quantification of protein
357 interaction affinity, because the BACTH system itself is an indirect method to detect
358 protein-protein interaction. Nonetheless, the good correlation between the measured

359 RRPE and the equilibrium dissociation constant reported in literature demonstrates
360 that the method is useful in providing a relative ranking of interaction strength for
361 protein variants in a given interacting pair of proteins.



362

363 **Fig. 6.** RRPE measurement for five pairs of coiled coil interactions using the BACTH
364 system and flow cytometry. (A) A schematic (adapted from the Fig. 1 by De
365 Crescenzo *et al*. (22)) of the acid (En)-base (Kn) coiled coils interaction with n
366 indicating the number of heptad repeats. (B) The bivariate dot-plots of β-gal green
367 fluorescence *versus* His tag red fluorescence intensity for *E. coli* BTH101 cells
368 co-transformed with plasmids of pUT18C-Flag-*En*/pKT25-His-*Kn*. (C) The correlation
369 of the RRPE values measured by flow cytometry for coiled coil interactions occurring
370 in the BACTH system with the affinity measured by SPR (22). The error bar
371 represents the standard deviation of three replicates.

372 **Conclusion**

373 We have developed a sensitive *in vivo* method for the quantitative measurement of
374 protein-protein interaction via the BACTH system and flow cytometry. Taking
375 advantage of the high-throughput and multiparameter measurement of single cell by
376 flow cytometry, the expression of reporter protein and interacting proteins can be
377 simultaneously measured and correlated at the single-cell level. It was found out that
378 for the BATCH system, for a bacterial culture inoculated even with a single colony
379 co-transformed with two plasmids encoding each of the two interacting proteins, there
380 exist two populations and a large heterogeneity for each population in protein
381 expression and reporter protein production, which would otherwise be masked by the
382 ensemble-averaged measurements. By measuring the expression level of interacting
383 protein and reporter protein for the population expressing β-gal, it was identified that
384 for an interacting protein pair, the value of RRPE is constant and is an intrinsic
385 feature. Moreover, a good correlation of the RRPE with the binding affinity of protein
386 pair was observed for Pal-TolB interaction with WT and mutant TolB proteins and for
387 several coiled-coil interactions. The RRPE method proposed here can not only be
388 used to validate existing protein interaction and finding new ones, but also to rank the
389 strength of interaction. It may further be used for highthroughput study of the binding
390 site of protein-protein complexes, selection of high-affinity antibodies, and screening
391 of peptide inhibitor libraries.

392

393 ## Materials and Methods

394 **Reagents and Chemicals.** Rabbit anti-β-galactosidase IgG was purchased from

395 Molecular Probes (Eugene, OR, USA). FITC-conjugated anti-His mouse monoclonal
396 antibody and goat anti-rabbit (GAR) IgG (H+L) were obtained from TransGen
397 Biotech (Beijing, China). DyLight-649-conjugated goat anti-mouse (GAM) IgG (H+L)
398 was purchased from EarthOx (San Francisco, CA, USA). Antibodies were diluted in 1%
399 fetal bovine serum (FBS) (obtained from Hyclone, Logan, Utah, USA) freshly
400 prepared in PBS before use. Enzymes used for molecular cloning were obtained from
401 TaKaRa Biotech (Dalian, China). Ortho-nitrophenyl-β-galactoside (ONPG),
402 lysozyme, GTE (50 mM Glucose, 25 mM Tris, 10 mM EDTA, pH 8.0), and X-gal
403 were purchased from Sangon Biotech (Shanghai, China). Paraformaldehyde (PFA)
404 stock solution (16%) was obtained from Alfa Aesar (Ward Hill, MA, USA). Other
405 reagents were purchased from Sinopharm Chemical Reagent (Shanghai, China). All
406 the buffers were filtered through a 0.22μm filter and used within three weeks.

407 **Bacterial Strains and Plasmids.** *E. coli* ER2738 was used for the cloning
408 experiments. The recombinant plasmids used in the present study are summarized in
409 Table S1 and were verified by sequencing. Oligonucleotides were synthesized by
410 Sangon Biotech and are listed in Table S2. Plasmid pKT25-His-*tolB* was constructed
411 by inserting the histidine tag (His-tag) into pEB362 at the *Pst*I/*EcoR*I sites. The *pal*
412 gene was digested with *EcoR*I and *Xho*I from pEB356 and inserted into the
413 *EcoR*I/*Xho*I sites of pEB1030 to produce pUT18C-Flag-*pal*. Genes of *tolB* $\Delta^{22\text{-}25}$ and
414 *tolB* $\Delta^{22\text{-}33}$ were amplified from plasmid pEB362 using suitable primers listed in Table
415 S2. The PCR products were cleaved by *EcoR*I and *Xho*I and cloned into the
416 *EcoR*I/*Xho*I sites of pKT25-His to yield plasmids pKT25-His-*tolB* $\Delta^{22\text{-}25}$ and

417 pKT25-His-*tolB* Δ^{22-33}. To introduce Kn and En sequences into each BACTH vectors,
418 oligonucleotide primers of Kn and En that are complementary to each other were
419 synthesized as listed in Table S2 and annealed by heating at 95 °C for 5 min.
420 Followed by cooling to room temperature, the products with cohesive ends were
421 inserted into plasmids pKT25-His and pUT18C-Flag at the *EcoR*I/*Xho*I sites to obtain
422 plasmids pKT25-His-Kn and pUT18C-Flag-En, respectively.

423 *E. coli BTH101* (*F-, cya-99, araD139, galE15, galK16, rpsL1, hsdR2, mcrA1,*
424 *mcrB1*) was used as the reporter strain of the BACTH system. Competent *E. coli*
425 *BTH101* strains co-transformed with two-hybrid plasmids bearing two different
426 antibiotic resistances were spread on Luria-Bertani (LB) plates containing 100 μg/mL
427 ampicillin, 50 μg/mL kanamycin, and 40 μg/mL X-gal at 30 °C. After incubating the
428 plates for 2 days, single colonies with successful co-transformation of two hybrid
429 plasmids were picked and inoculated in 2 mL of LB containing 100 μg/mL ampicillin
430 and 50 μg/mL kanamycin. Cultures were grown overnight with shaking (250 rpm) at
431 30 °C, unless specified otherwise. The harvested bacterial sample was adjusted to
432 OD_{600} ~1.0, immunofluorescently stained and analyzed on the flow cytometer.

433 **Immunofluorescent Staining.** To a 200 μL of the harvested bacterial cells, 8 μL of 1
434 M $NaPO_4$ (pH 7.4) and 40 μL of the primary fixative buffer (3 μL of 25%
435 gluteraldehyde per mL of 16% paraformaldehyde) were added and incubated at room
436 temperature for 15 min followed by 30 min on ice. The sample was washed twice
437 with 200 μL PBS and resuspended in 50 μL PBS. Then, 500 μL of ice cold 80%
438 methanol was added and the sample was treated for 1 h at room temperature. The

439 sample was washed twice with GTE buffer. The cells were permeabilized by
440 resuspending in 100 μL of 2 mg/mL lysozyme in GTE and incubated for 10 min at
441 room temperature. After washing twice with PBS, the cells were blocked in 100 μL 1%
442 FBS for 10 min. Then 20 μL of the suspension was centrifuged and resuspended in 40
443 μL of 5 μg/mL rabbit anti-β-gal antibody with/without 5 μg/mL mouse anti-His/Flag
444 antibody depending on the experimental requirement. After 1 h incubation at room
445 temperature, the sample was centrifuged and washed with PBS, then resuspended in
446 40 μL of 10 μg/mL FITC-conjugated GAR antibody with/without
447 DyLight-649-conjugated GAM antibody. The suspension was incubated for 30 min at
448 room temperature, centrifuged, and resuspended in 50 μL PBS. For flow cytometry
449 analysis, the sample should be diluted 500-fold with PBS before loading.

450 **Flow Cytometric Measurement.** A Becton Dickinson FACSVerse flow cytometer
451 equipped with 488 nm and 640 nm excitation lasers was used in this study. FL1
452 (527/32 nm band-pass filter) channel and FL2 (660/10 nm band-pass filter) channel
453 were used to detect the fluorescence of FITC and DyLight 649, respectively for the
454 immunofluorescently stained bacteria. A threshold value of 200 was set on FL1 to
455 eliminate non-bacterial particles. A total of 10000 events falling in the gated region
456 were collected for each sample. Data acquisition and analysis were carried out by
457 using BD FACSuite software. The data were analyzed by Flowjo 7.6.1 software (Tree
458 Star, Inc., Ashland, OR).

459 **Measurement of β-Gal Enzyme Activity by ONPG Colorimetric Assay.** A
460 protocol described in the literature was followed (12). Briefly, 200 μL of the

461 harvested bacterial cells (OD_{600}~1.0) were treated by 3 μL toluene and 3 μL 0.01%
462 SDS (shaking at 37 °C for 30 min). Then 1.8 mL PM2 buffer (70 mM $Na_2HPO_4 \cdot 12$
463 H_2O, 30 mM $NaH_2PO_4 \cdot H_2O$, 1 mM $MgSO_4$, 0.2 mM $MnSO_4$, pH7.0) with 100 mM
464 β-mercaptoethanol was added and mixed thoroughly. After that, 250 μL of the ONPG
465 substrate solution (4 mg/mL ONPG in PM2 buffer without β-mercaptoethanol) was
466 added to 1 mL of the mixture. The enzymatic reaction was carried out immediately on
467 a DU-800 spectrophotometer (Beckman Coulter) with measurement of $OD_{420\ nm}$. The
468 β-galactosidase activity corresponds to 200 × ($OD_{420\ nm,\ t2}$ - $OD_{420\ nm,\ t1}$) / (t2-t1) (min)
469 × 10. The factor 200 is the inverse of the absorption coefficient of o-nitrophenol,
470 while the factor 10 is the dilution factor.
471

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477 **Author contributions**

478 L.W., X.W., and X.Y. conceived and designed the research; L.W., X.W., T.L.,
479 and J.Z. performed research; L.W., X.W., and X.Y. analyzed data and wrote the
480 paper.

481 **Conflict of interest statement**

482 The authors declare no conflict of interest.

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