

Selective Cross-Linking of Interacting Proteins Using Self-Labeling Tags

Arnaud Gautier,[†] Eiji Nakata,[‡] Gražvydas Lukinavičius, Kui-Thong Tan, and Kai Johnsson*

Institute of Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

Received September 15, 2009; E-mail: kai.johnsson@epfl.ch

Abstract: We have designed molecules that permit the selective cross-linking (S-CROSS) of interacting proteins in cell lysates and the sensitive detection of the trapped complexes through in-gel fluorescence scanning. S-CROSS requires the expression of the putative interacting proteins as fusion to CLIP-tag or SNAP-tag, two protein tags that can be specifically labeled with synthetic probes. Bifunctional molecules that contain the substrates of the two tags connected via a fluorophore are used to selectively cross-link interacting proteins in cell lysate. The amount of trapped complex can be then quantified after SDS gel electrophoresis by in-gel fluorescence scanning. On the basis of a detailed kinetic analysis of the cross-linking reaction, we showed that the cross-linking efficiency can be used as an indicator of interaction between two proteins, allowing thereby the unambiguous identification of interacting protein pairs. We validated our approach by confirming a number of interactions through selective cross-linking and showed that it permits the quantitative and simultaneous analysis of multiple homotypic and heterotypic protein complexes and the differentiation between strong and weak protein–protein interactions.

Introduction

Protein–protein interactions play key roles in all biological processes from the regulation of signaling and metabolic pathways to the formation of multiprotein enzymatic complexes and cellular structures. Our understanding of these processes therefore requires the identification and characterization of the underlying protein–protein interactions. To map the so-called protein interactome, genome-scale studies based on the yeast-2-hybrid approach,¹ protein arrays,² or affinity purification combined with mass spectrometry³ have been undertaken. These studies have provided large sets of potentially novel protein–protein interactions that require validation by independent experiments and further characterization. To do so, a wide range of techniques is currently available.⁴ The most popular are: (i) cell-based assays employing autofluorescent proteins and Förster resonance energy transfer (FRET) measurements;⁵ (ii) protein complementation assays based on split protein sensors;^{6,7} and

(iii) immunoprecipitation of protein complexes from cell lysates (coimmunoprecipitation or co-IP).⁸

FRET-based assays permit the study of dynamics and localization of protein–protein interactions within cells. However, FRET measurements between two proteins tagged with autofluorescent proteins are technically demanding and generally have a low signal-to-noise ratio.^{9,10} In protein complementation assays, the two interacting proteins are expressed as fusion proteins to respectively two fragments of a reporter protein, a so-called split protein sensor, which is reassembled upon interaction of the two fusion proteins.⁶ Different reporter proteins have been used to design split protein sensors that reveal protein–protein interactions in living cells.¹¹ Split protein sensors are widely used now to study protein–protein interactions but have the limitations that the effective formation of active reporter depends on the geometry of the studied protein complex and that, with few exceptions,¹² the affinity of the fragments makes the formation of active reporter irreversible. The latter point prevents the dissociation of protein complexes and makes the approach less suited for studying dynamic interactions. Co-IP remains the most heavily used method to verify protein–protein interactions because of its ability to detect endogenous complexes and its technical simplicity. Its main limitation is that interactions, in particular weak and transient, can be broken up during the immunoprecipitation procedure. To address this problem, cross-linking strategies have been

[†] Present address: Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, United Kingdom.

[‡] Department of Life System, Institute of Technology and Science, Graduate School, The University of Tokushima, Minamijosanjimacho-2, Tokushima 770-8506, Japan.

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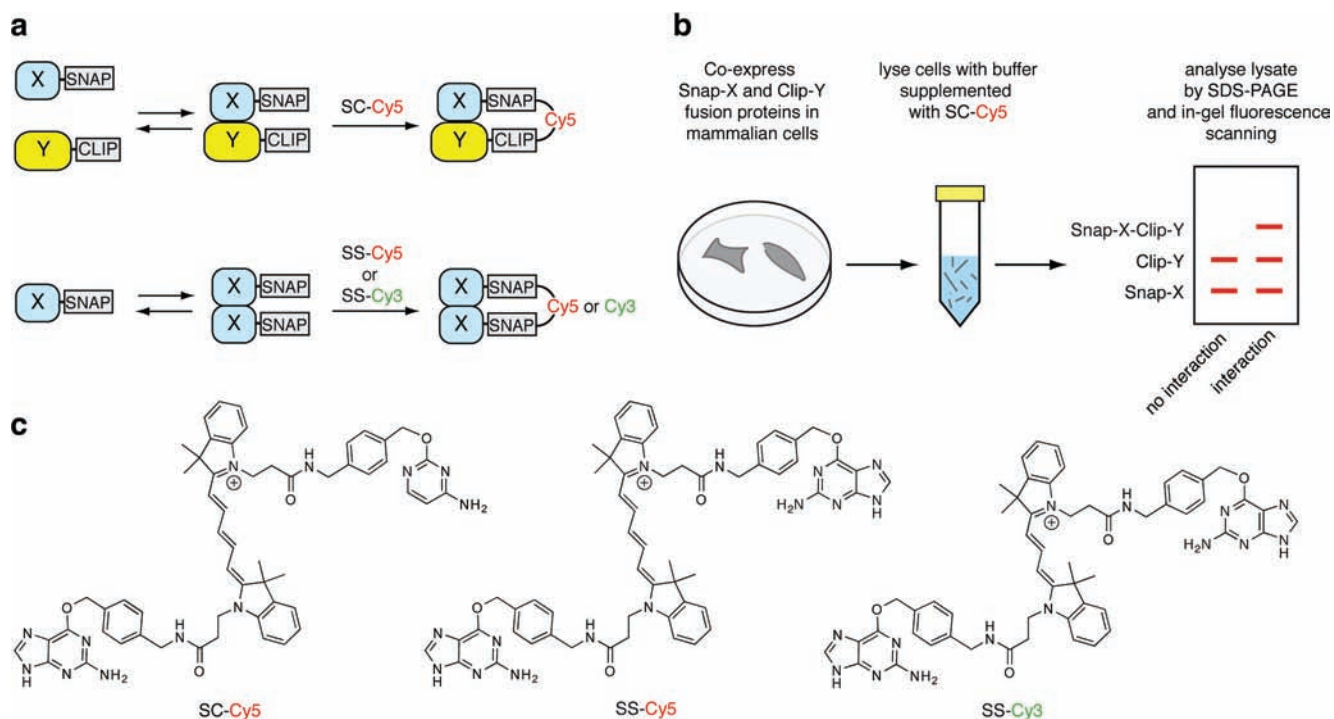


Figure 1. Specific cross-linking (S-CROSS) of interacting proteins. (a) Mechanism of S-CROSS: Cross-linking of CLIP-tag and/or SNAP-tag fusion proteins with bifunctional molecules in which the substrates of the two tags are connected via a fluorophore (either Cy5 or Cy3). This permits the irreversible trapping of homo- and heterotypic protein complexes. (b) Experimental protocol: Pairs of proteins are fused to SNAP-tag and CLIP-tag and coexpressed in mammalian cells. Cells are lysed in the presence of fluorescent bifunctional molecules, and the resulting lysate is then analyzed after SDS-PAGE by in-gel fluorescence imaging. It should be noted that the mobility of cross-linked, nonlinear proteins in SDS-PAGE can deviate from the mobility that would be expected for a linear protein of the same molecular weight. (c) Structures of the bifunctional molecules SC-Cy5, SS-Cy5, and SS-Cy3 synthesized for the cross-linking of protein complexes.

developed to capture complexes by covalent bonding.¹³ Bis-maleimide-based cross-linkers are now commercially available for the cross-linking of cysteine-containing proteins in living cells (BMOE and BHM, Pierce). However, this approach suffers from a very poor selectivity of the chemical reaction as any cysteine-containing protein can react with the cross-linker.¹³ Recently, alternative methods based on light-induced cross-linking of interacting partners have been introduced, using proteins that incorporate genetically encoded photo-cross-linker-containing amino acids^{14,15} or proteins that are fused to protein tags that can be labeled with photo-cross-linkers.¹⁶ Label transfer technologies that utilize a genetic tag fused to the protein of interest to transfer a chemical label (e.g., biotin) onto interacting partners have also been reported.^{17,18} However, so far few interactions have been studied using these techniques, and further studies are needed to evaluate their generality.

In summary, although there are numerous methods available for studying protein–protein interactions, there is a need for methods that permit the analysis of multiple interacting partners in a single experiment and the study of the stability of protein

complexes. Here, we introduce a technically simple method that fulfills these criteria and can be used in a variety of different organisms. This method enables the detection of protein–protein interactions in cell lysates through the selective cross-linking (S-CROSS) of protein complexes. S-CROSS is based on the coexpression of proteins fused to SNAP-tag¹⁹ or CLIP-tag,²⁰ two self-labeling tags that can be specifically and covalently labeled with synthetic probes. Protein–protein interactions are detected by lysing cells in the presence of bifunctional molecules in which the substrates of the two tags are connected via a fluorophore (Figure 1). The efficiency of the resulting cross-linking of the fusion proteins depends on their proximity and can be quantified after SDS gel electrophoresis by in-gel fluorescence scanning. The use of two self-labeling tags with nonoverlapping substrate specificity ensures selectivity and directionality of the cross-linking reaction between different proteins, facilitating the identification of multiple protein complexes. We have previously shown that the cross-linking of two SNAP-tag fusion proteins can be used as an indicator of protein proximity, but detection required Western blotting and experiments were restricted to SNAP-tag fusion proteins, which significantly limited the practicality of the approach.²¹

In this work, we demonstrate the following key features of S-CROSS: (i) the possibility to simultaneously analyze multiple interactions with high selectivity; (ii) the possibility to dif-

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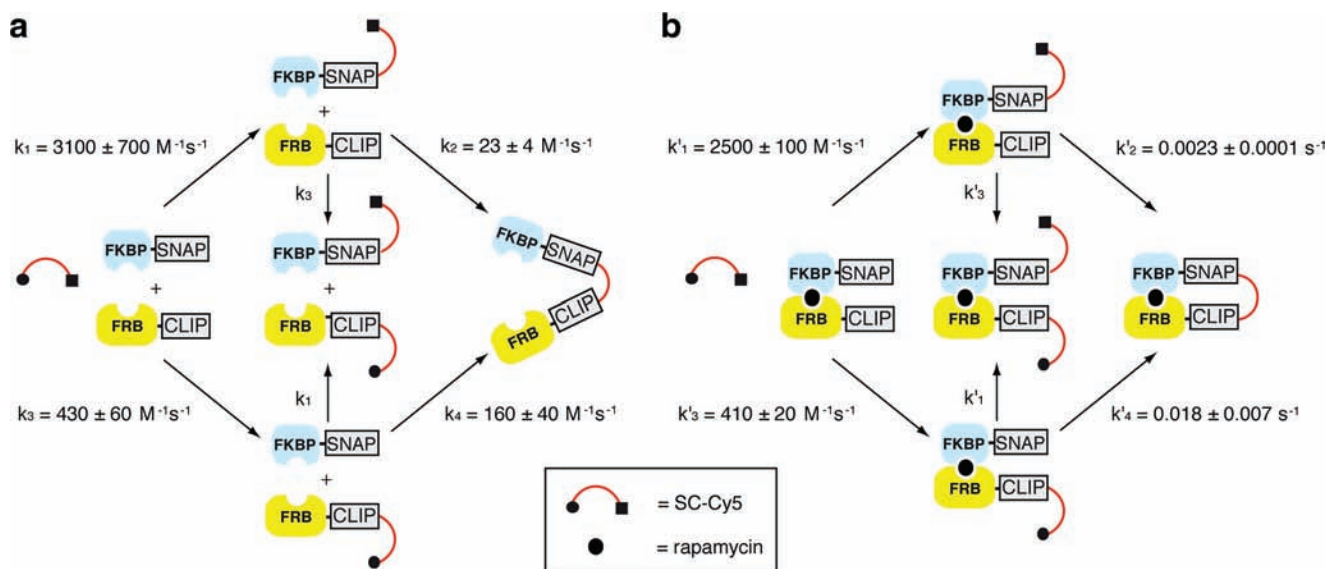


Figure 2. Inter- (a) and intramolecular (b) cross-linking reactions. The rate constants of the different steps of the intermolecular (a) and intramolecular (b) cross-linking reactions are reported. Rate constants are given with a 95% confidence interval. The effective molarity of the cross-linking reaction within the SNAP-FKBP-rapamycin-CLIP-FRB ternary complex was calculated by dividing the rate constants of the intramolecular cross-linking step by that of the intermolecular counterpart. We found an effective molarity of about 100 μM (ratio k'_2/k_2 or k'_4/k_4) (Supporting Information). Kinetic constants k_1 , k_2 , k_3 , and k_4 as well as k'_1 , k'_2 , k'_3 , and k'_4 were determined from experimental data shown in Figure S1 (Supporting Information). It should be noted that under these conditions SNAP-tag and CLIP-tag operate well below saturation with respect to substrate concentration, resulting in a linear relationship between the substrate effective concentration and the reaction rate.

ferentiate between strong and weak interactions; and (iii) the simplicity of the approach and the possibility to complement S-CROSS with other SNAP-tag- and CLIP-tag-based methods to characterize protein–protein interactions.

Results and Discussion

Design of Molecules for the Selective Cross-Linking of Interacting Proteins. The self-labeling protein tags SNAP-tag¹⁹ and CLIP-tag²⁰ are two engineered mutants of human *O*⁶-alkylguanine-DNA alkyltransferase (hAGT), which react specifically and rapidly with benzylguanine (BG) and benzylcytosine (BC) derivatives, respectively. We synthesized bifunctional substrates for the heterocross-linking of SNAP- and CLIP-tagged proteins (named SC-Cy5) and for the homo- and heterocross-linking of two SNAP-tag fusions (named SS-Cy3 and SS-Cy5) (Figure 1c). SC-Cy5 contains a BG and a BC subunit connected by a Cy5 dye, while SS-Cy3 and SS-Cy5 are made of two BG subunits tethered via a Cy3 and Cy5 dye, respectively. The syntheses of these molecules are described in the Supporting Information. The cyanine dyes allow for a sensitive detection by in-gel fluorescence imaging: the detection limit for labeled proteins in polyacrylamide gel is about 1–10 fmol (data not shown).

Kinetic Analysis of a Model Cross-Linking Reaction. To test if these cross-linkers can be used to detect protein–protein interactions, we studied the kinetics of the cross-linking reaction between two purified proteins in vitro. As a model system, we chose the rapamycin-inducible interaction between FKBP (the FK506 binding protein) and FRB (the FKBP-rapamycin binding domain of mTOR).²² FKBP and FRB form a stable dimer in the presence of rapamycin (K_D of 12 nM²³) but do not interact in the absence of rapamycin. FKBP was expressed as a SNAP-

tag fusion protein (SNAP-FKBP), FRB was expressed as a CLIP-tag fusion protein (CLIP-FRB), and the cross-linking of the two purified proteins by SC-Cy5 was studied in the presence and absence of rapamycin. The cross-linking reaction of SNAP-FKBP and CLIP-FRB with SC-Cy5 is a two-step process with two possible routes as outlined in Figure 2. The first route starts with the reaction of SNAP-FKBP with SC-Cy5 to yield SNAP-FKBP-Cy5-BC, which then reacts with CLIP-FRB. The second route starts with the reaction of CLIP-FRB with SC-Cy5 to give CLIP-FRB-Cy5-BG, which then reacts in a second step with SNAP-FKBP. We determined the rate constants of the different steps for the cross-linking in the presence and absence of rapamycin (Figure 2a,b). The individual rate constants in absence of rapamycin were determined for each step under pseudo first-order conditions (Figure S1a,b). The individual rate constants in the presence of rapamycin were determined from the analysis of reactions of preformed complex between SNAP-FKBP and CLIP-FRB complex at varying concentrations of SC-Cy5 using a multistep kinetic model (Figure S1c). Two important results emerged from these kinetic studies. First, the reaction of noninteracting CLIP-FRB or SNAP-FKBP with SNAP-FKBP-Cy5-BC or CLIP-FRB-Cy5-BG is roughly 20-fold slower than the reaction of the proteins with free SC-Cy5 (compare k_4 to k_1 and k_2 to k_3). The reason is most likely that the reactive groups BC and BG within SNAP-FKBP-Cy5-BC and CLIP-FRB-Cy5-BG are shielded by the protein to which they are attached. Consequently, when two noninteracting proteins are incubated with an excess of SC-Cy5, each protein reacts preferentially with SC-Cy5, and very little amount of cross-linked proteins is formed. For example, when incubating purified SNAP-FKBP and CLIP-FRB (each 0.2 μM) with 2 μM of SC-Cy5, no cross-linked product could be detected (Figure S2a,b). The second important result is that the cross-linking reaction is significantly faster when the two proteins interact because of high effective molarity of the tags within the protein complex. We calculated an effective molarity (M_{eff}) of about

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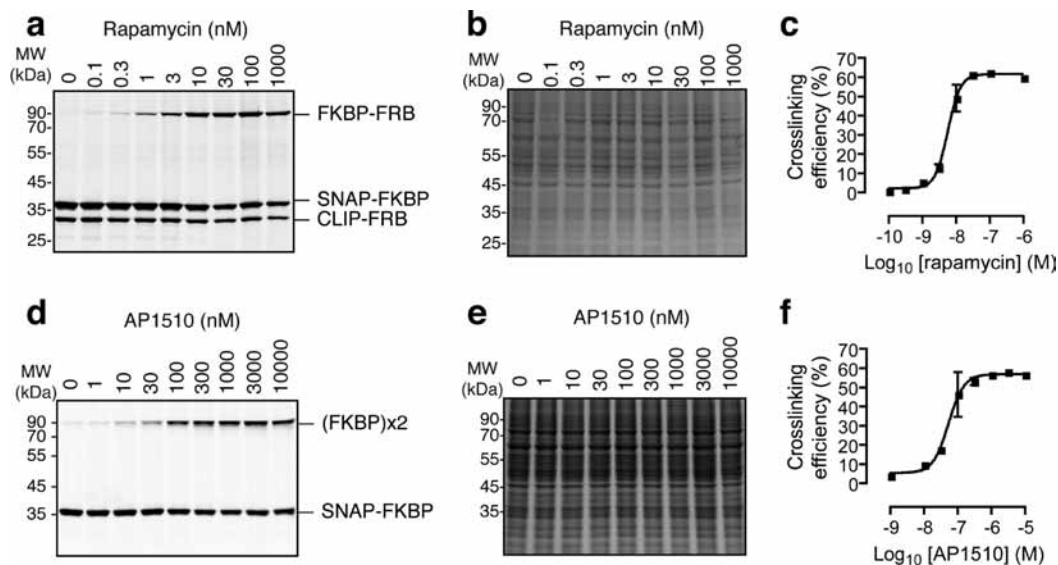


Figure 3. Detection of hetero- and homotypic interactions. (a–c) HEK 293 cells coexpressing SNAP-FKBP and CLIP-FRB were treated for 1 h with different concentrations of rapamycin prior to lysis in the presence of 4 μ M SC-Cy5. (a) Analysis of cross-linking by SDS-PAGE and in-gel fluorescence scanning. For clarity, in this and the following figure the names of the cross-linked proteins are given without the name of the tags. (b) Coomassie staining of gel (a). (c) Cross-linking efficiency (mean \pm SD of three independent experiments) determined in (a) versus rapamycin concentration. (d–f) HEK 293 cells expressing SNAP-FKBP were treated for 1 h with different concentrations of AP1510 prior to lysis in the presence of 1 μ M SS-Cy5. (d) Analysis of cross-linking by SDS-PAGE and in-gel fluorescence scanning. (e) Coomassie staining of gel (d). (f) Cross-linking efficiency (mean \pm SD of three independent experiments) determined in (c) versus AP1510 concentration. (c and f) Data were fitted with sigmoidal dose–response equations.

100 μ M for the cross-linking reaction of SNAP-FKBP and CLIP-FRB within the complex by dividing the rate constant of the cross-linking step within the protein complex by the rate constant of the cross-linking step between two noninteracting proteins (k'_2/k_2 or k'_4/k_4). The high effective molarity favors cross-linking over the competing reaction with another molecule of SC-Cy5. For example, when incubating purified SNAP-FKBP and CLIP-FRB (each 0.2 μ M) with 2 μ M of SC-Cy5 in the presence of rapamycin, 75% of cross-linked proteins and 25% of labeled monomers are formed, and the reaction is complete after 20 min (Figure S2c,d). This kinetic analysis explains why the cross-linking efficiency can be used as an indicator of interaction between two proteins. It should be noted that the effective molarity of the tags within a protein complex will depend on their proximity and relative orientation and will therefore differ between different protein complexes (vide infra). Despite the central importance of effective molarities in protein complexes, few values have been experimentally determined so far. However, the effective molarity measured here for the complex between SNAP-FKBP and CLIP-FRB is comparable to that measured for an inhibitor of human carbonic anhydrase (HCA) attached to SNAP-tag in a SNAP-mCherry-HCA fusion protein ($M_{\text{eff}} = 80 \mu\text{M}$).²⁴

Characterization of Heterotypic Interactions in Cell Extracts.

We next analyzed the rapamycin-dependent interaction between FKBP and FRB in mammalian cell extracts. SNAP-FKBP and CLIP-FRB were transiently coexpressed in HEK 293 cells, and the cells were incubated with varying concentrations of rapamycin. Cells were lysed in the presence of 4 μ M of SC-Cy5, incubated for 1 h at room temperature, and directly analyzed by SDS-PAGE and in-gel fluorescence scanning (Figure 3a,b). In this and the following experiments, we used cross-linker concentrations between 1 and 4 μ M so that they exceed the concentrations of the SNAP and CLIP fusion proteins in the

extract (typically between 5 and 500 nM, see Table S1), minimizing thereby nonspecific cross-linking (vide supra). Cross-linking efficiency increased 200-fold with increasing rapamycin concentration, from 0.3% in the absence of rapamycin to 60% at rapamycin concentrations above 30 nM. The EC₅₀ of rapamycin was determined to be 6 nM (Figure 3c), which is in agreement with previously determined values.²³

Characterization of Homotypic Interactions in Cell Extracts.

We next showed that S-CROSS can reveal homotypic protein complexes by cross-linking of SNAP-tag fusion proteins using SS-Cy5. HEK 293 cells expressing SNAP-FKBP were incubated with varying concentrations of AP1510, a synthetic molecule promoting the homodimerization of FKBP,²⁵ and lysed in the presence of SS-Cy5. The cross-linking efficiency increased from 2.5% in the absence of AP1510 to 60% at AP1510 concentrations above 300 nM (Figure 3d–f).

Simultaneous Detection of Multiple Interactions. The approach furthermore permits the simultaneous detection of multiple protein–protein interactions in a single experiment. To demonstrate this, we incubated HEK 293 cells coexpressing SNAP-FKBP and CLIP-FRB with a fixed concentration of AP1510 and varying concentrations of rapamycin. In the presence of AP1510 and absence of rapamycin, SNAP-FKBP should be mainly as a homodimer, whereas addition of rapamycin should shift the equilibrium toward the more stable SNAP-FKBP-CLIP-FRB heterodimer. Cells were lysed in the presence of both SC-Cy5 and SS-Cy3 to simultaneously trap the SNAP-FKBP-CLIP-FRB heterodimer and the homodimer of SNAP-FKBP; in-gel fluorescence scanning then permitted one to unambiguously distinguish the Cy3-cross-linked FKBP homodimer from the Cy5-cross-linked FKBP-FRB heterodimer (Figure 4a,b). The data demonstrate how addition of rapamycin leads to the formation of the FKBP-FRB heterodimer at the

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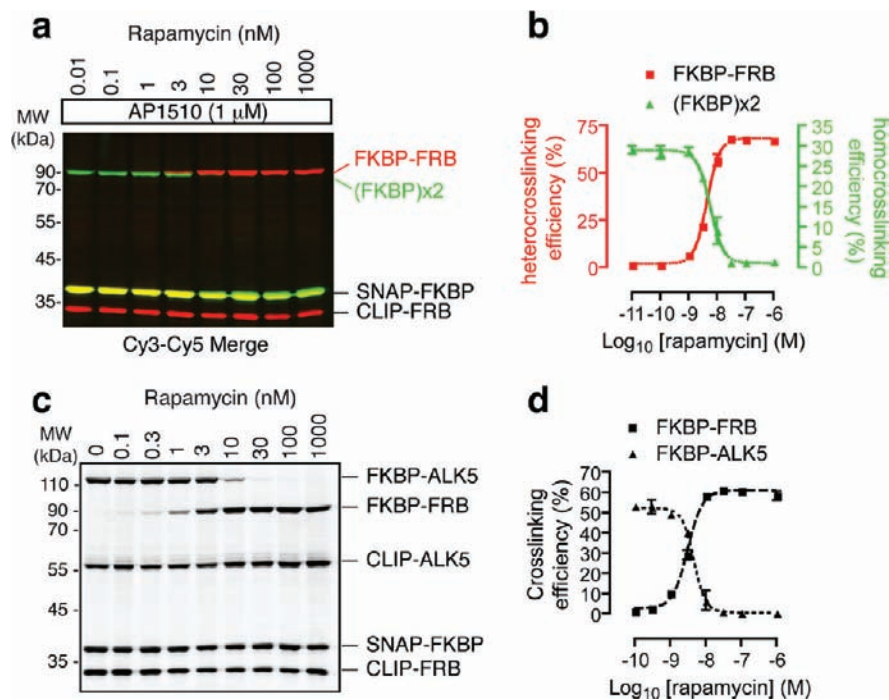


Figure 4. Simultaneous detection of several protein–protein interactions. (a,b) HEK 293 cells coexpressing SNAP-FKBP and CLIP-FRB were treated for 1 h with 1 μM AP1510 and different concentrations of rapamycin prior to lysis in the presence of 2 μM SC-Cy5 and 1 μM SS-Cy3. (a) Analysis of cross-linking by SDS-PAGE and in-gel fluorescence scanning; overlay of Cy3 (green) and Cy5 (red) channels. (b) Cross-linking efficiencies (mean ± SD of three independent experiments) determined in (a) versus rapamycin concentration. (c,d) HEK 293 cells coexpressing SNAP-FKBP, CLIP-FRB, and CLIP-ALK5 were treated for 1 h with different concentrations of rapamycin prior to lysis in the presence of 4 μM SC-Cy5. (c) Analysis of cross-linking by SDS-PAGE and in-gel fluorescence scanning. (d) Cross-linking efficiency (mean ± SD of two independent experiments) determined in (c) versus rapamycin concentration. (b and d) Data were fitted with sigmoidal dose–response equations.

expense of the homodimer of FKBP. This experiment illustrates moreover how the combined use of SC-Cy5 and SS-Cy3 enables the detection of different cross-linked protein complexes with the same mobility in SDS-PAGE.

The ability to simultaneously detect and quantify two interactions in one experiment is an important feature of S-CROSS. We therefore attempted to demonstrate more examples of such applications and in particular to extend S-CROSS to the simultaneous analysis of interactions between more than two proteins. A natural binding partner of FKBP is the transforming growth factor β type 1 receptor ALK5.²⁶ This interaction is disrupted by the rapamycin-dependent complex formation between FKBP and FRB.²² HEK 293 cells coexpressing SNAP-FKBP, CLIP-FRB, and the cytosolic domain of ALK5 fused to CLIP-tag were incubated with varying concentrations of rapamycin and lysed in the presence of SC-Cy5. The results illustrate how rapamycin disrupts the SNAP-FKBP-CLIP-ALK5 interaction and induces the formation of the SNAP-FKBP-CLIP-FRB complex (Figure 4c,d). It should be noted that it is possible to detect the exchange of SNAP-FKBP binding partners only because there is no interaction between the two tags prior to addition of the cross-linker. This is an important difference between S-CROSS and most split protein sensors.

We next applied S-CROSS to the tumor suppressor p53, a transcription factor involved in apoptosis, DNA repair, cell cycle arrest, and senescence. The activity of p53 in normal cells is regulated through its interaction with the E3 ubiquitin ligase Mdm2. Mdm2 controls p53 by binding its N-terminal transac-

tivation domain, promoting its ubiquitinylation and degradation by the proteasome. The p53–Mdm2 interaction can be disrupted by the Mdm2 antagonist nutlin-3a, a *cis*-imidazole analogue that binds to the p53-binding site of Mdm2.²⁷ We were able to detect the interaction of SNAP-Mdm2 and CLIP-p53 through S-CROSS in cell lysates and to demonstrate that prior incubation of the cells with nutlin-3a at concentrations above 1 μM disrupted the p53–Mdm2 interaction (Figure 5a,b). S-CROSS furthermore enabled us to analyze the oligomeric state of p53 and Mdm2 as well as the interaction between the two proteins simultaneously. p53 forms a tetramer (which is best described as a dimer of dimers²⁸), whereas Mdm2 forms dimers through a RING domain interaction.²⁹ By coexpressing p53 and Mdm2 as SNAP-tag fusions and cross-linking with SS-Cy5, we detected three different protein complexes in a single experiment: the two homotypic (SNAP-p53)₂ and (SNAP-Mdm2)₂ complexes and the heterotypic SNAP-Mdm2-SNAP-p53 complex; of these only the SNAP-Mdm2-SNAP-p53 complex was sensitive to nutlin-3a (Figure 5c). Deleting the tetramerization domain of SNAP-p53, yielding SNAP-p53Δ, prevented its efficient cross-linking with SS-Cy5 (Figure 5d) but did not affect its interaction with CLIP-Mdm2 (Figure 5e). These experiments illustrate the versatility of S-CROSS for the facile and simultaneous analysis of multiple protein–protein interactions, and the complementarity of SS-Cy5 and SC-Cy5 to discriminate hetero- and homocross-linked complexes.

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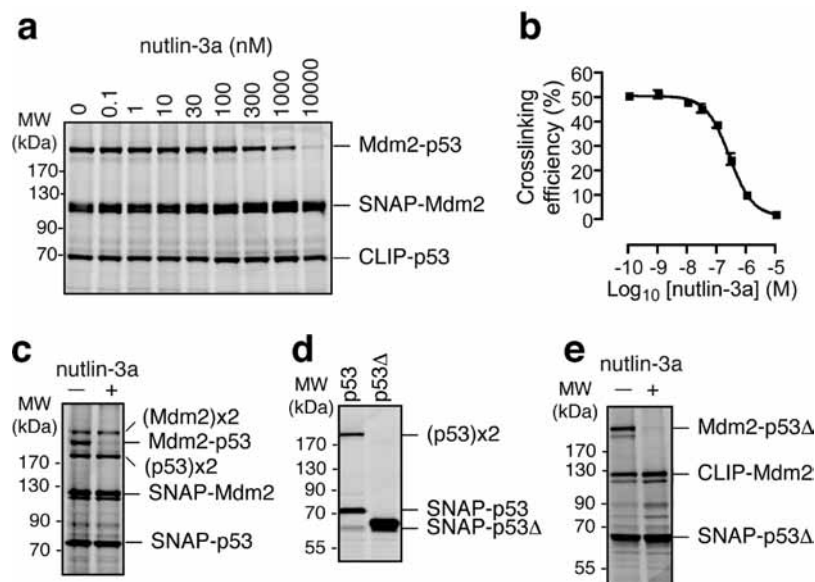


Figure 5. (a,b) HEK 293 cells coexpressing SNAP-Mdm2 and CLIP-p53 were treated for 1 h with different concentrations of nutlin-3a prior to lysis in the presence of 4 μ M SS-Cy5. For clarity, the names of the cross-linked proteins are given without the name of the tags. (a) Analysis of cross-linking by SDS-PAGE and in-gel fluorescence scanning. (b) Cross-linking efficiency (mean \pm SD of three independent experiments) versus nutlin-3a concentration. Data were fitted with a sigmoidal dose–response equation. (c) HEK 293 cells coexpressing SNAP-Mdm2 and SNAP-p53 were incubated with or without 10 μ M nutlin-3a for 1 h prior to lysis in the presence of 1 μ M SS-Cy5 followed by analysis of cross-linking by SDS-PAGE and in-gel fluorescence scanning. (d) HEK 293 cells expressing SNAP-p53 or SNAP-p53 Δ were lysed in the presence of 1 μ M of SS-Cy5 and subsequently analyzed by SDS-PAGE and in-gel fluorescence scanning. (e) HEK 293 cells coexpressing SNAP-p53 Δ and CLIP-Mdm2 were treated with 0 or 10 μ M of nutlin-3a for 1 h prior to lysis with 4 μ M of SS-Cy5 and subsequently analyzed by SDS-PAGE and in-gel fluorescence scanning.

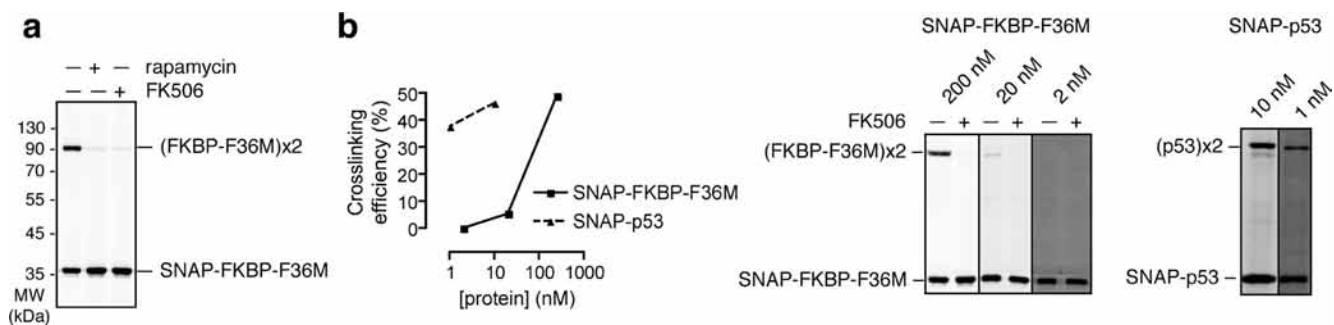


Figure 6. Detection of weak interactions. (a) HEK 293 cells expressing SNAP-FKBP-F36M were incubated with or without 1 μ M of different ligands (FK506, rapamycin) for 1 h and then lysed in the presence of 1 μ M SS-Cy5 and subsequently analyzed by SDS-PAGE and in-gel fluorescence scanning. The concentration of SNAP-FKBP-F36M in the extract was 250 nM. The ligands FK506 and rapamycin disrupt the homodimer and serve as control. (b) Efficiency of the cross-linking of SNAP-FKBP-F36M and SNAP-p53 tetramer with 1 μ M SS-Cy5 at different concentrations of fusion protein in lysates. Gels are shown on the right of the panel.

Detection of Weak Protein–Protein Interactions. Weak protein–protein interactions can be difficult to detect by conventional approaches such as affinity purification.³⁰ In contrast, S-CROSS permits the detection of weak interactions as the cross-linking reaction can trap a complex that is in rapid equilibrium with its monomers. As an example for a weak interaction, we characterized the homodimer formed by the FKBP mutant FKBP-F36M. The mutation F36M induces the dimerization of FKBP with an associated K_D of 30 μ M.³¹ Incubating lysates of HEK 293 cells expressing SNAP-FKBP-F36M with SS-Cy5 revealed that at SNAP-FKBP-F36M concentration of 250 nM the cross-linking efficiency reached 49% (Figure 6a), although, at that concentration, only 2% of the

complex should be present at the equilibrium if $K_D = 30 \mu$ M. This means that the protein complex is enriched during the cross-linking process. Modeling the influence of protein concentration, K_D , and effective molarity on the cross-linking efficiency revealed that the observed cross-linking efficiency for SNAP-FKBP-F36M can be rationalized by assuming an effective molarity for the cross-linking reaction within the homodimer of about 500 μ M (Figure S3). This value is comparable to the effective molarity experimentally determined for the cross-linking reaction within the SNAP-FKBP-CLIP-FRB complex (vide supra). The higher effective molarity value predicted in the case of SNAP-FKBP-F36M is consistent with the shorter distance between the N-termini in the homodimer of FKBP-F36M (38 Å) as compared to the FKBP-FRB heterodimer (49 Å) (Figure S3d). Our experiments and the modeling suggest that weak interactions with K_D of at least 30 μ M can be detected by S-CROSS if the effective molarity for the cross-linking reaction within the protein complex is greater than 100 μ M and

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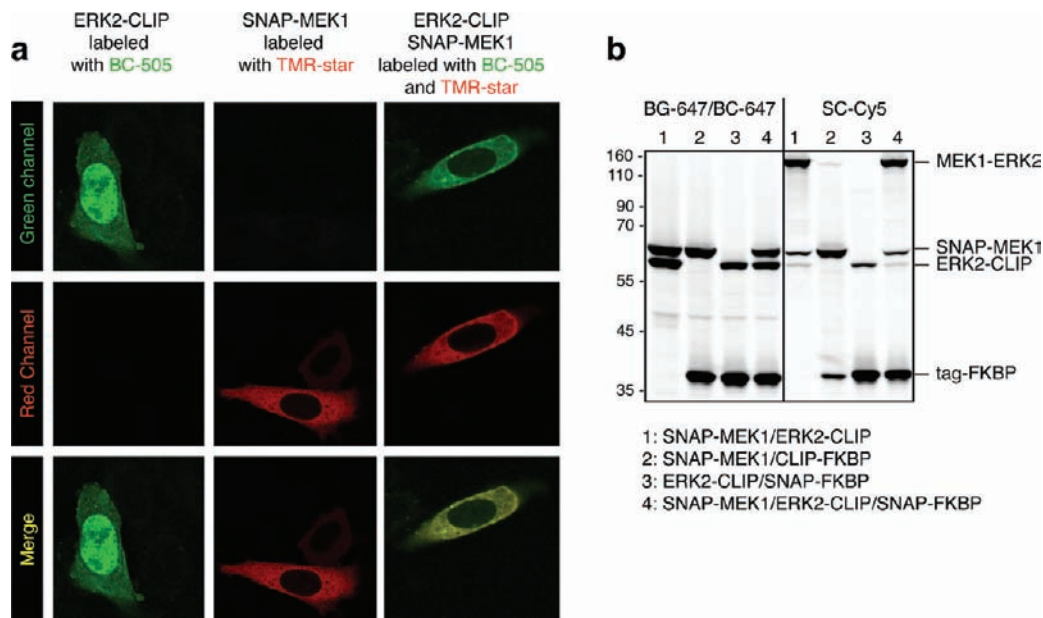


Figure 7. Characterization of the MEK1–ERK2 interaction by studying protein colocalization and selective cross-linking. (a) CHO-K1 cells expressing ERK2-CLIP were labeled for 30 min with BC-505 (10 μ M). Cells expressing SNAP-MEK1 were labeled for 30 min with TMR-star (2 μ M). Cells coexpressing ERK2-CLIP and SNAP-MEK1 were labeled for 30 min with BC-505 (10 μ M) and TMR-star (2 μ M). Cells were fixed with formaldehyde after labeling and imaged by confocal microscopy. Green channel: detection of ERK2-CLIP labeled with BC-505. Red channel: detection of SNAP-MEK1 labeled with TMR-star. Structures of TMR-star and BC-505 are shown in Figure S4. (b) HEK 293 cells coexpressing SNAP-MEK1/ERK2-CLIP were lysed in the presence of 4 μ M SC-Cy5 or in the presence of BG-647 and BC-647 (both 5 μ M) and subsequently analyzed by SDS-PAGE and in-gel fluorescence scanning; Cy5 channel shown. Control experiments were performed coexpressing the two proteins with FKBP fusions that should not interact with the two proteins: SNAP-MEK1 with CLIP-FKBP (lane 2), ERK2-CLIP with SNAP-FKBP (lane 3), and SNAP-MEK1, ERK2-CLIP, and SNAP-FKBP (lane 4).

the protein concentration in the sample is in the high nanomolar range (Figure S3c). Calculations showed that the cross-linking efficiency of a weak interaction should be strongly concentration-dependent at concentrations below the K_D . Indeed, dilution of SNAP-FKBP-F36M in the lysate led to a drastic drop of the cross-linking efficiency (Figure 6b). In contrast, the cross-linking efficiency in the case of the strong tetrameric SNAP-p53³² did not show any significant drop upon dilution to concentrations of about 1 nM (Figure 6b). The possibility to measure the cross-linking efficiencies of interacting proteins as a function of their concentrations is a convenient way to distinguish weak interactions from strong ones.

Combining S-CROSS with Other SNAP-tag and CLIP-tag-Based Methods. As SNAP- and CLIP-tag fusions can be labeled with various membrane permeable fluorescent probes in living cells, it is possible to complement S-CROSS experiments in lysates with colocalization studies²⁰ or FRET measurements³³ within cells. To illustrate this, we studied the interaction of the two kinases ERK2 and MEK1³⁴ by characterizing their colocalization through fluorescence microscopy and their physical interaction by S-CROSS (Figure 7). The formation of the stable complex between ERK2 and MEK1 results in a change of ERK2 localization, which is predominantly nuclear when not bound to MEK1 and predominantly cytosolic when bound to MEK1.³⁴ The fluorescence microscopy images shown in Figure 7a confirm how expression of SNAP-MEK1 influences the localization of ERK2-CLIP: ERK2-CLIP, which is predominantly localized in the nucleus when expressed alone, is localized in the cytosol

when coexpressed with SNAP-MEK1. To validate that the colocalization of the two proteins is due to a molecular interaction, we performed S-CROSS experiments on cells coexpressing SNAP-MEK1 and ERK2-CLIP. In these experiments, a cross-linking efficiency of 80% between the two proteins was observed (Figure 7b, lane 1). We furthermore carried out control experiments by coexpressing SNAP-MEK1 and ERK2-CLIP together with SNAP-FKBP as an internal control: we observed cross-linking between ERK2-CLIP and SNAP-MEK1 but not between ERK2-CLIP and SNAP-FKBP, demonstrating thereby the specific interaction between ERK2 and MEK1 (Figure 7b). The possibility to perform selective controls in the same cells and in parallel to the actual experiment is another important feature of S-CROSS that sets it apart from other methods to study protein–protein interactions.

Conclusion

We have introduced a new method named S-CROSS for the detection and characterization of protein–protein interactions in cell extracts by specific covalent cross-linking. This approach relies on the use of the self-labeling proteins SNAP-tag and CLIP-tag to specifically and directionally cross-link interacting proteins. The use of cross-linkers that incorporate fluorophores allows the sensitive detection of protein complexes by in-gel fluorescence imaging. This method should be applicable in all cells or organisms amenable to genetic manipulation. As compared to affinity purification methods, S-CROSS requires the expression of both partners as fusion proteins, but it is technically simpler and requires lower amount of material. Moreover, S-CROSS can be used to detect interactions of proteins expressed at level as low as 1 pmol/mg of total protein (see Table S1 for examples), which is in the range of expression

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levels found for endogenous proteins in mammalian cells.^{35,36} It should be possible to detect interactions of proteins expressed at even lower expression levels by combining S-CROSS with affinity purification to enrich the cross-linked proteins before SDS-PAGE analysis. A limitation shared with other tag-based approaches like FRET and protein complementation assays is that the interpretation of S-CROSS results can be biased if the geometry of the protein complex prevents the reaction of the tags, leading to false negatives. However, S-CROSS has features that make it attractive as compared to other techniques: (i) the ease with which multiple homotypic and heterotypic interactions can be simultaneously characterized, (ii) the ability to detect strong and weak interactions, and (iii) the possibility to exploit the versatility of chemical labeling to complement the cross-linking studies with live cell imaging or other fluorescence-based approaches.²⁰ In summary, S-CROSS is a versatile, sensitive, and simple method for the analysis of protein–protein interactions.

Materials and Methods

Synthesis. Detailed synthetic procedures and characterizations for all synthetic precursors are described in the Supporting Information.

General. SNAP-tag and CLIP-tag substrates were obtained from Covalys BioSciences and New England Biolabs. Rapamycin and FK506 were purchased from Sigma-Aldrich. AP1510 was obtained from ARIAD. Nutlin-3a was purchased from Cayman Chemical. SNAP-tag and CLIP-tag used in this work are 182-amino-acid mutants of the wild-type human *O*⁶-alkylguanine-DNA alkyltransferase in which the last 25 amino acids were deleted and the following mutations were introduced: for SNAP-tag,³⁷ K32I, L33F, C62A, Q115S, Q116H, K125A, A127T, R128A, G131K, G132T, M134L, R135S, C150Q, S151G, S152D, G153L, A154D, N157G, and S159E; and for CLIP-tag,²⁰ K32I, L33F, M60I, C62A, Y114E, Q115S, Q116H, A121 V, K125A, A127T, R128A, G131N, G132T, M134L, R135D, C150Q, S151G, S152D, G153S, A154D, N157P, and E159L. The fluorescence gel images were recorded with a Pharos FX molecular imager (Bio-Rad) and analyzed with Quantity One software (Bio-Rad). If not mentioned, experimental data were fitted with Prism software package (GraphPad Software). Simulations were performed with the DYNAFIT software.³⁸

Plasmid Constructions. For mammalian expression of SNAP-FKBP and CLIP-FRB, FKBP and FRB were fused to the C-terminus of SNAP-tag or CLIP-tag via a RSYPDVDPDYA linker. The genes encoding the fusion proteins were inserted between the *NheI* and *BamHI* sites of the pECFP-Nuc plasmid (Clontech). For the expression of SNAP-FKBP-F36M, FKBP-F36M was generated by PCR from FKBP and then fused to the C-terminus of SNAP-tag via a RSYPDVDPDYA linker. The gene encoding the fusion protein was inserted between the *NheI* and *BamHI* sites of the pECFP-Nuc plasmid (Clontech). For expression of SNAP-Mdm2 and CLIP-Mdm2, full length Mdm2 was fused to the C-terminus of SNAP-tag and CLIP-tag via a RS linker. The genes encoding the fusion proteins were inserted between the *NheI* and *BamHI* sites of the pECFP-Nuc plasmid (Clontech). For expression of SNAP-p53 and Clip-p53, full length p53 epitope-tagged at the C-terminus with a Flag-tag (DYKDDDD) was fused to the C-terminus of SNAP-tag or CLIP-tag via a PAG linker. The genes encoding the fusion proteins were inserted between the *EcoRI* and *BamHI* sites of the pSEMS1-SNAP26m plasmid (Covalys BioSciences). For

expression of SNAP-p53Δ, p53Δ (first 326 amino acids of p53) was fused to SNAP-tag via a PAG linker. The gene encoding the fusion protein was inserted between the *EcoRI* and *BamHI* sites of the pSEMS1-SNAP26m plasmid (Covalys BioSciences). For expression of CLIP-ALK5, the cytosolic domain of ALK5 (from amino acid 162 to 503) was fused to the C-terminus of CLIP-tag via a PAGYPDVPDYA linker. The gene encoding the fusion protein was inserted between the *EcoRI* and *XhoI* sites of pSEMS1-SNAP26m plasmid (Covalys BioSciences). For expression of SNAP-MEK1, MEK1 was fused to the C-terminus of SNAP-tag via a PAGIGAPGSSTSLYKKAGT linker. The gene encoding the fusion protein was inserted between the *EcoRI* and *XhoI* sites of pSEMS1-SNAP26m plasmid (Covalys BioSciences). For expression of ERK2-CLIP, ERK2 was fused to the N-terminus of CLIP-tag via a DIEFAS linker. The gene encoding the fusion protein was inserted between the *ClaI* and *SbfI* sites of the pCEMS1-CLIP10m plasmid (Covalys BioSciences). For expression in *E. coli* and Ni-NTA purification of (His)₆-SNAP-FKBP and (His)₆-CLIP-FRB, FKBP and FRB were fused to the C-terminus of SNAP-tag and CLIP-tag via a RSYPDVDPDYA linker. The genes encoding the fusions proteins were inserted between the *NdeI* and *BamHI* sites of the vector pET-15b (Novagen). Expression and purification of the fusion proteins was done as previously described.³⁹ All constructs were verified by DNA sequencing.

Cell Culture and Transfection. Human embryonic kidney (HEK)-293 cells were cultured in suspension in ExCell-293 medium (Sigma-Aldrich) supplemented with 4 mM L-glutamine (Lonza) at 37 °C in a 5% CO₂ atmosphere. Adherent Chinese hamster ovary (CHO)-K1 cells were cultured in Ham's F12 (Lonza) supplemented with 10% FBS (Lonza). HEK-293 cells were transiently transfected with polyethylenimine as previously described.²⁰ CHO-K1 were transiently transfected with Lipofectamine (Invitrogen) according to the manufacturer's protocol.

S-CROSS in Cell Extracts. At 24 h after transfection, samples of one million HEK-293 cells transfected with CLIP-tag and/or SNAP-tag constructs were lysed in 50 μL of buffer 1 (100 mM KH₂PO₄, 150 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, pH 7.0, protease inhibitor cocktail) or buffer 2 (50 mM HEPES, 25 mM NaCl, pH 7.2, protease inhibitor cocktail) supplemented with the cross-linker (see figure legends for concentrations) by performing three cycles of freezing and thawing. Total protein concentrations in cell extracts were determined by Bradford assay. Cell extracts were incubated for 1 h at room temperature before addition of SDS loading buffer and boiling at 95 °C. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel fluorescence scanning. The concentrations of the fusion proteins in cell extract were estimated by labeling an aliquot of lysate with 5 μM BG-647, a SNAP-tag substrate based on the Dyomics dye DY-647, and 5 μM BC-647, a CLIP-tag substrate based on the Dyomics dye DY-647, and comparison of the fluorescence intensity of the labeled monomers with that of a known quantity of recombinant SNAP-tagged protein labeled with BG-647. For each experiment, the lysis buffer, the quantity of extract loaded on gel, and the concentration of each fusion proteins in nM and pmol/(mg of total protein) are listed in Table S1 (Supporting Information). The concentration in nM gives the molar concentration of fusion protein in the lysate, while the concentration in pmol/(mg of total protein) is a measure of the expression level of the fusion protein. The cross-linking efficiency (CLE) was determined using the following equations and assuming that the different labeled species have the same fluorescence properties.

For the cross-linking between SNAP-tagged and CLIP-tagged monomers within a heterodimer:

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$$CLE^{\text{hetero}} = F^{\text{cross-linked heterodimer}} / (F^{\text{cross-linked heterodimer}} + qF^{\text{labeled SNAP-monomer}})$$

where $F^{\text{cross-linked heterodimer}}$ is the fluorescence intensity of the band corresponding to the cross-linked heterodimer, $F^{\text{labeled SNAP-monomer}}$ is the fluorescence intensity of the band corresponding to the labeled SNAP-tagged monomer, and q is the ratio [CLIP-tagged protein]/[SNAP-tagged protein] when [CLIP-tagged protein] < [SNAP-tagged protein], and is equal to 1 when [CLIP-tagged protein] ≥ [SNAP-tagged protein]. q was determined by measuring the ratio of the fluorescence intensities of the monomers labeled with BG-647 and BC-647 in a separate experiment (see above), assuming that the two labeled monomers have the same fluorescence properties.

For the cross-linking of SNAP-tagged monomers within a homodimer:

$$CLE^{\text{homo}} = 2F^{\text{cross-linked homodimer}} / (2F^{\text{cross-linked homodimer}} + F^{\text{labeled SNAP-monomer}})$$

where $F^{\text{cross-linked homodimer}}$ is the fluorescence intensity of the band corresponding to the cross-linked homodimer, and $F^{\text{labeled SNAP-monomer}}$ is the fluorescence intensity of the band corresponding to the labeled SNAP-tagged monomer.

Kinetic Analysis. Determination of the Rate Constants k_1 and k_3 of the First Step of the Intermolecular Cross-Linking Process. Recombinant (His)₆-SNAP-FKBP and (His)₆-CLIP-FRB (each 0.2 μM) were incubated with 2 μM SC-Cy5 in reaction buffer (50 mM HEPES, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, pH 7.2) at 24 °C. Aliquots were taken at different times, boiled at 95 °C for 5 min in SDS loading buffer containing 100 μM benzylguanine and 100 μM bromothenylcytosine (SNAP-tag and CLIP-tag blockers) to stop the reaction, and analyzed by SDS-polyacrylamide gel electrophoresis and in-gel fluorescence scanning. Experiments were done in duplicate. The reaction progress curve (fluorescence intensity of the labeled protein vs time) was fitted to a pseudo first-order reaction model. Second-order rate constants were then obtained by dividing the pseudo first-order constants by the concentration of cross-linker. Rate constants are given with a 95% confidence interval.

Determination of the Rate Constants k_2 and k_4 of the Second Step of the Intermolecular Cross-Linking Process. SNAP-FKBP-Cy5-BC and CLIP-FRB-Cy5-BG were prepared by incubating 1 μM of recombinant SNAP-FKBP and CLIP-FRB with 5 μM SC-Cy5 for 30 min at room temperature. The labeled proteins were then purified from excess SC-Cy5 using a centrifugal filter device (Microcon YM-50, Millipore). The washing step was repeated twice with reaction buffer (50 mM HEPES, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, pH 7.2) to remove all unreacted cross-linker. Solutions containing about 0.25 μM of SNAP-FKBP-Cy5-BC and CLIP-FRB-Cy5-BG were then incubated with either 5 μM CLIP-FRB or 5 μM SNAP-FKBP at room temperature. Aliquots were taken at different times, boiled at 95 °C for 5 min in SDS loading buffer containing 100 μM benzylguanine and 100 μM bromothenylcytosine (SNAP-tag and CLIP-tag blockers) to stop the reaction, and analyzed by SDS-PAGE and fluorescence gel imaging. Experiments were done in duplicates.

The reaction progress curve (fluorescence intensity of the cross-linked dimer vs time) was fitted to a pseudo first-order reaction model. Second-order rate constants were then obtained by dividing the pseudo first-order constants by the concentration of free protein. Rate constants are given with a 95% confidence interval.

Determination of the Rate Constants k'_1 , k'_2 , k'_3 , and k'_4 of the Intramolecular Cross-Linking Process. Recombinant (His)₆-SNAP-FKBP and (His)₆-CLIP-FRB (each 0.2 μM) were preincubated with 5 μM rapamycin to form the ternary complex, and then incubated with different concentrations of SC-Cy5 (1, 2, 4, 6, and 10 μM) in reaction buffer (50 mM HEPES, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, pH 7.2) at 24 °C. Aliquots were taken at different times (2.5, 5, 10, 15, 20, 40, and 80 min), boiled at 95 °C for 5 min in SDS loading buffer containing 100 μM benzylguanine and 100 μM bromothenylcytosine (SNAP-tag and CLIP-tag blockers) to stop the reaction, and analyzed by SDS-PAGE. Experiments were done in duplicates. The fluorescence intensities of all of the Cy5-labeled species (labeled SNAP-FKBP, labeled CLIP-FRB, and cross-linked SNAP-FKBP and CLIP-FRB) were determined for the different times and the five concentrations of cross-linker by in-gel fluorescence scanning. The reaction progress curves (fluorescence intensity vs time) of the cross-linked dimer formation and the direct labeling of SNAP- and CLIP-tagged monomers for the five cross-linker concentrations (set of 210 experimental points) were fitted with DYNAFIT software³⁸ to determine the rate constants using the model given in Figure 3b. This kinetic model assumes a tight ternary complex FKBP–rapamycin–FRB that does not dissociate during the reaction. The script used for fitting is given in the Supporting Information. Rate constants are given with a 95% confidence interval.

Microscopy. At 24 h after transfection, CHO-K1 cells seeded in μ-Dish (Ibidi) and transfected with CLIP-tag and/or SNAP-tag constructs were labeled with 10 μM BC-505, a CLIP-tag substrate based on the Dyomics dye DY-505, and/or 2 μM TMR-star, a SNAP-tag substrate based on tetramethylrhodamine, in culture medium for 30 min. After three washings with culture medium, cells were fixed with 4% formaldehyde solution for 20 min and washed twice with phosphate buffered saline. Cells were imaged with a confocal Leica SP5 white laser equipped with an oil immersion objective HCX PL APO 63×/1.40–0.60. Fluorescence emission was measured between 500–560 nm for DY-505 (excitation wavelength: 488 nm) and 560–660 nm for TMR (excitation wavelength: 540 nm).

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Supporting Information Available: Additional methods and materials, Figures S1, S2, S3, and S4, Table S1, and complete refs 1 and 3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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