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Supporting Information

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Engineering Functional Membrane–Membrane Interfaces by InterSpy

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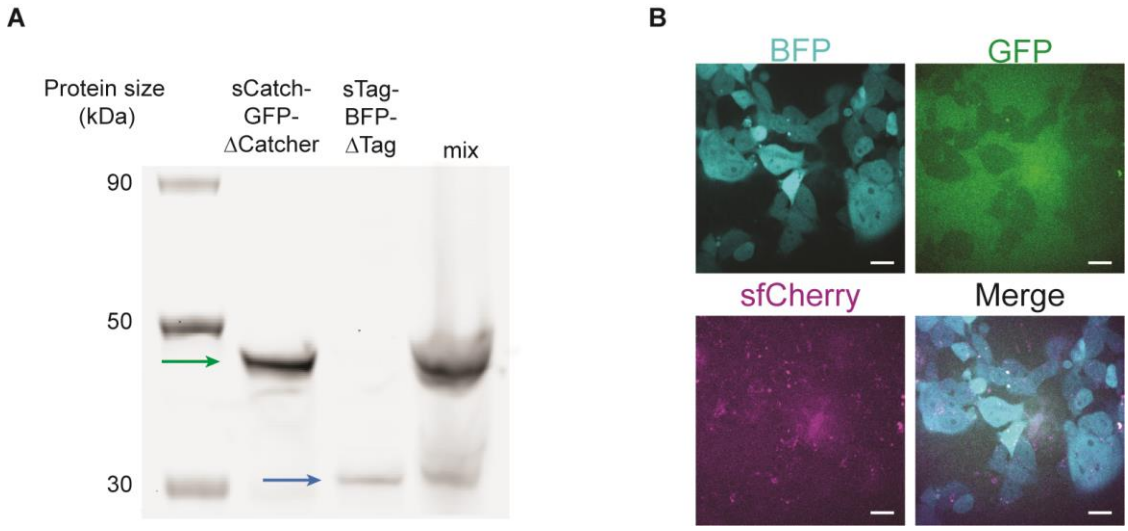

Figure S1: The crucial role of SpyTag/SpyCatcher domain in sfCherry reconstitution both in bulk CFE reactions and on cell membranes. **A)** In-gel fluorescence imaging of ladder (Lane 1), sCatch-GFP-ΔCatcher (Lane 2), sTag-BFP-ΔTag (Lane 3), and a mixture of sTag-BFP-ΔTag and sCatch-GFP-ΔCatcher (Lane 4). **B)** Representative confocal images of HEK293T cells expressing InterTag on their surface (identified with cytosolic expression of BFP (blue)) mixed with sCatch-GFP-ΔCatcher (green) leading to no sfCherry reconstitution (magenta). Scale bars: 20 µm.

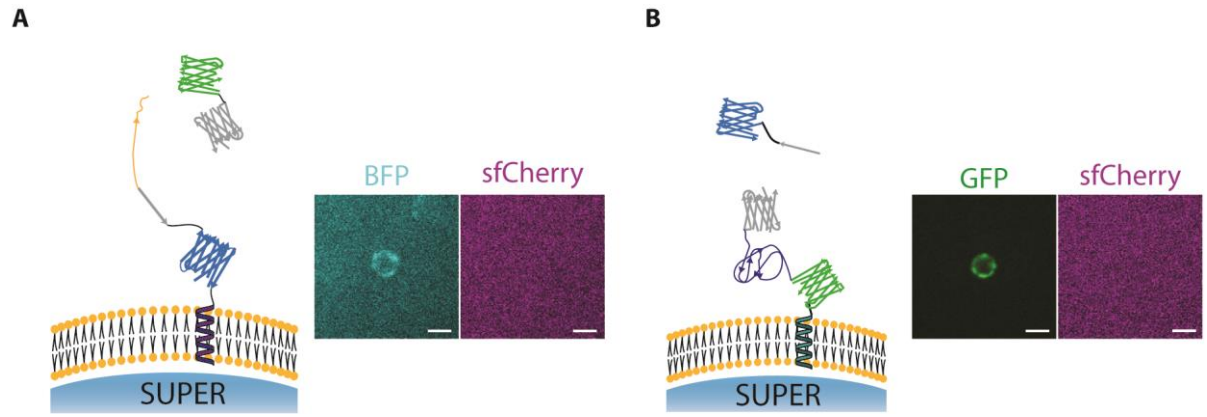

Figure S2: SpyTag/SpyCatcher interaction is indispensable for sfCherry reconstitution on SUPER templates. **A)** Representative confocal images of reconstitution of cell-free expressed InterTag-BFP (cyan) on the membrane of 5 µm SUPER templates mixed with sCatch-GFP-ΔCatcher showing lack of sfCherry (magenta) reconstitution. Scale bar: 5 µm **B)** Representative confocal images of reconstitution of cell-free expressed InterCatch-GFP (green) on the membrane of 5 µm SUPER templates mixed with sTag-BFP-ΔTag showing absence of sfCherry (magenta) signal. Scale bar: 5 µm.

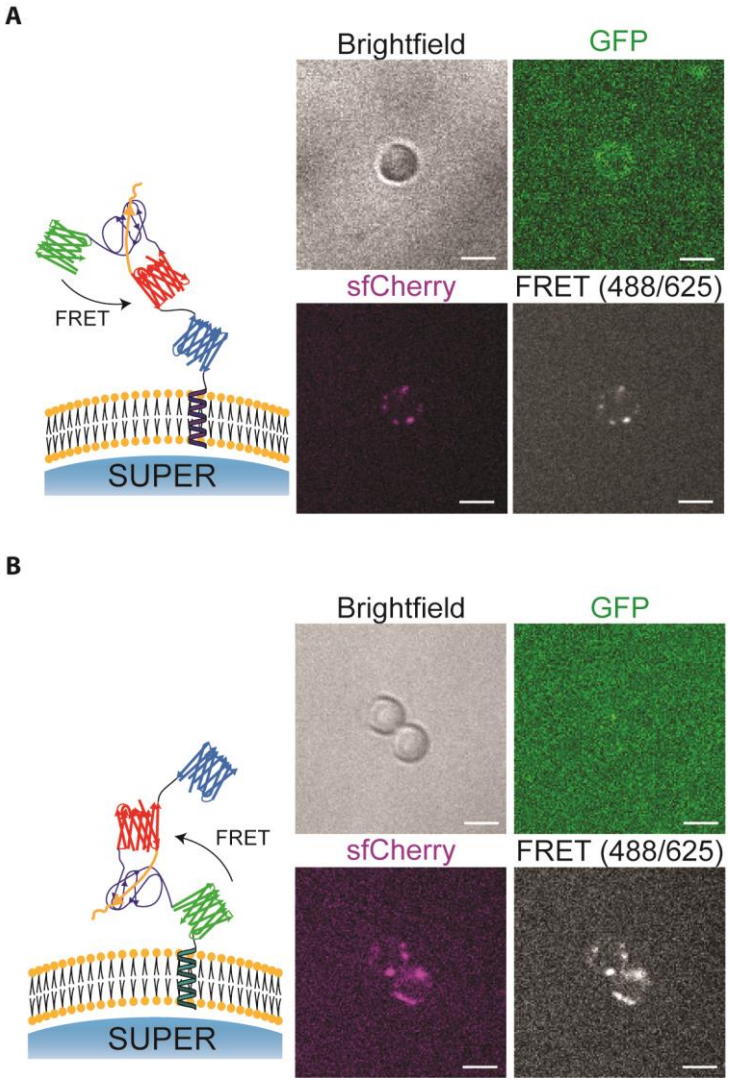

Figure S3: Fluorescence resonance energy transfer (FRET) between GFP and sfCherry on the membrane of SUPER templates. **A)** Representative confocal images of reconstitution of cell-free expressed InterTag-BFP on the membrane of 5 µm SUPER templates mixed with sCatch-GFP (green) and forming sfCherry (magenta), leading to FRET between GFP and sfCherry. Scale bar: 5 µm **B)** Representative confocal images of reconstitution of cell-free expressed InterCatch-GFP (green) on the membrane of 5 µm SUPER templates mixed with sTag-BFP and forming sfCherry (magenta), leading to FRET between GFP and sfCherry. Scale bar: 5 µm.

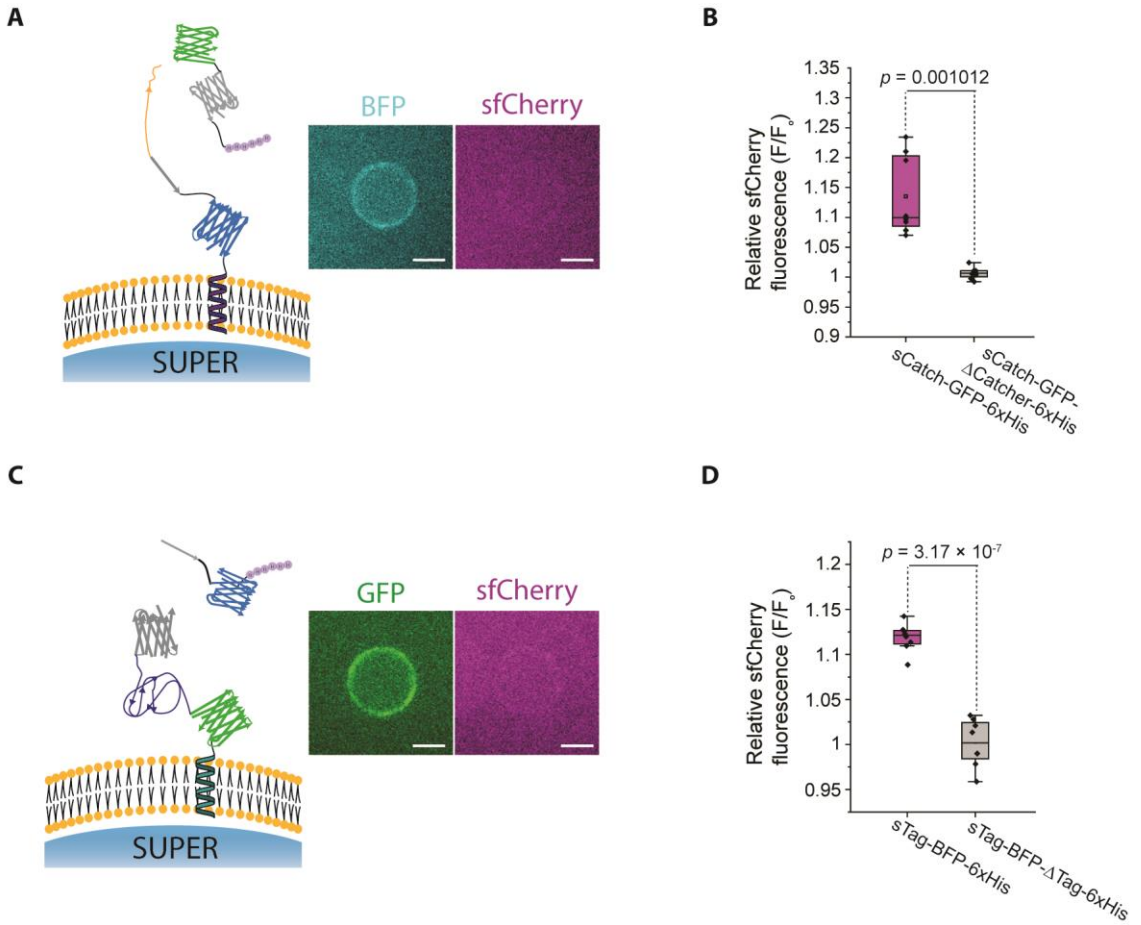

Figure S4: SpyTag/SpyCatcher interaction is indispensable for sfCherry reconstitution on 20 µm SUPER templates. **A)** Representative confocal images of bottom-up reconstitution of membrane proteins InterTag-BFP (cyan) on the membrane of 20 µm SUPER templates and no fluorescent sfCherry (magenta) formation when mixed with purified sCatch-GFP-ΔCatcher. Scale bars: 10 µm. **B)** Box plots comparing the relative sfCherry signal on the membrane of SUPER templates in the presence (magenta) or absence (gray) of SpyCatcher domain. The data shows the average ratio of sfCherry signal on the SUPER membrane to the background signal for 30 points along the bead periphery across 8 different beads (n=3). The box represents the 25–75th percentiles, and the median is indicated. The whiskers show the minimum and maximum data points. *p*-values are calculated using two-tailed t-test. **C)** Representative confocal images of bottom-up reconstitution of membrane proteins InterCatch-GFP (green) on the membrane of 20 µm SUPER templates and no functional sfCherry (magenta) formation when mixed with purified sTag-BFP-ΔTag. Scale bars: 10 µm. **D)** Box plots comparing the relative sfCherry signal on the membrane of SUPER templates in the presence (magenta) or absence (gray) of SpyTag domain. The data shows the average ratio of sfCherry signal on the SUPER membrane to the background signal for 30 points along the bead periphery across 8 different beads (n = 3).

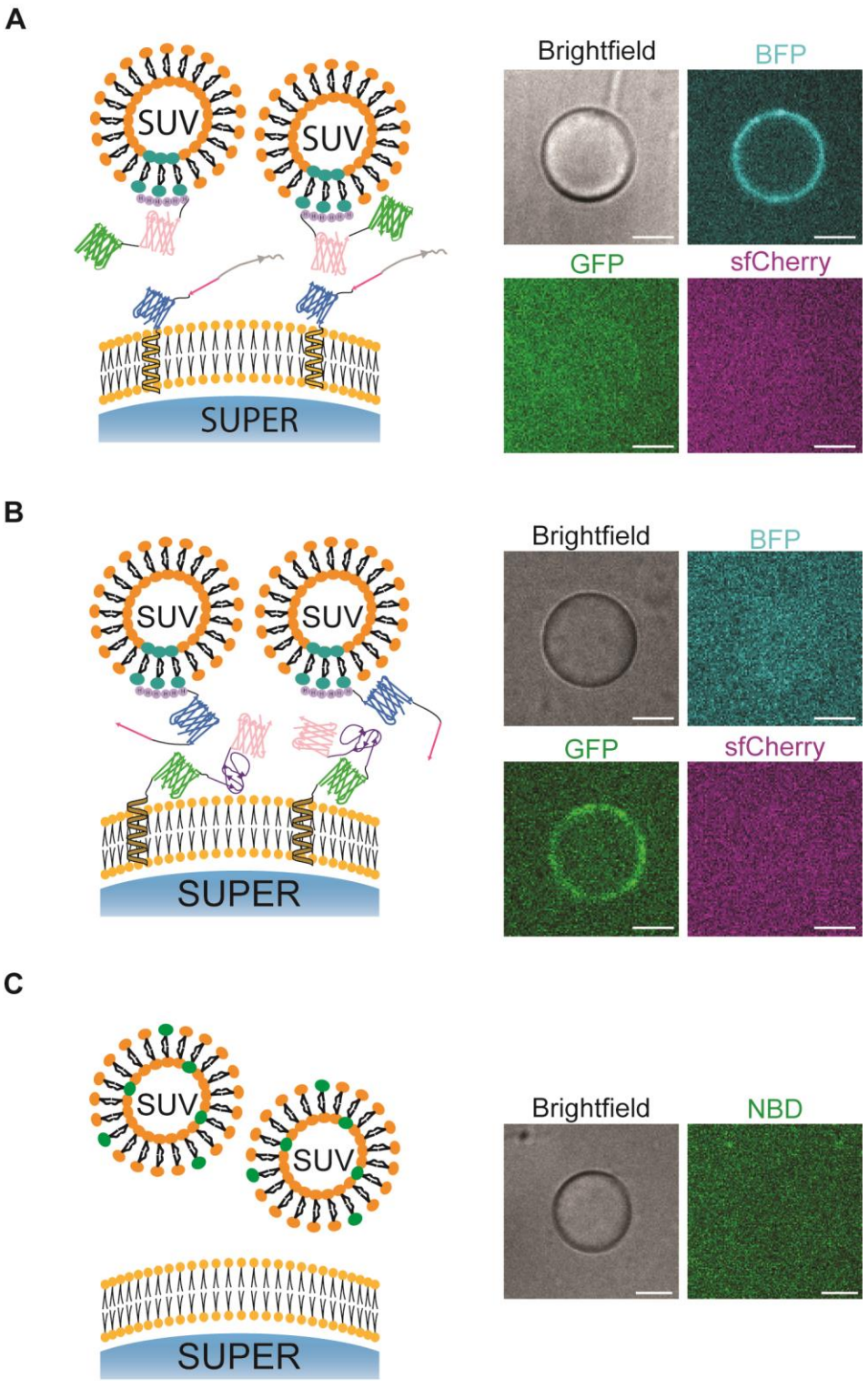

Figure S5: Membrane interface formation and fluorescent sfCherry reconstitution is solely dependent on SpyTag/SpyCatcher interaction. **A)** Representative confocal images of reconstituted InterTag-BFP on 20 µm SUPER templates (cyan) and no sfCherry formation (magenta) when mixed with SUVs carrying sCatch-GFP-ΔCatcher-6xHis (green). **B)** Representative confocal images of reconstituted

InterCatch-GFP on 20 µm SUPER templates (green) and no sfCherry formation (magenta) when mixed with SUVs carrying sTag-BFP-ΔTag-6xHis (cyan). **C)** Representative confocal images of unlabeled 20 µm SUPER templates (Brightfield) mixed with SUVs labelled with NBD. Scale bars: 10 µm.

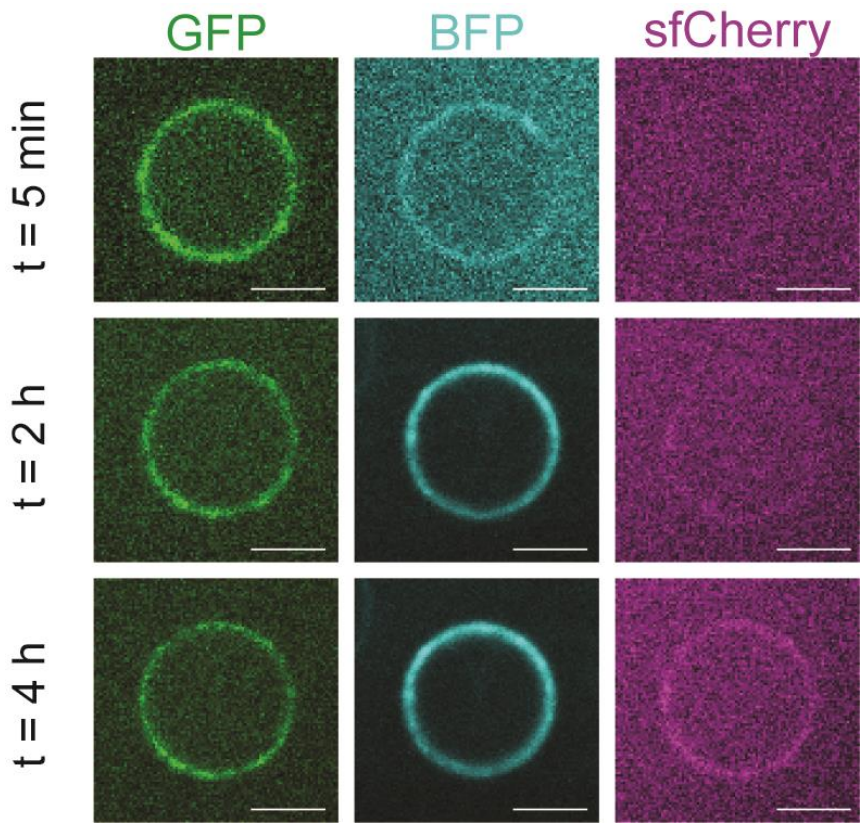

Figure S6: Reconstitution of fluorescent sfCherry mediated by SpyTag-SpyCatcher interaction over time. Shown are representative confocal images of cell-free reconstituted membrane protein InterCatch-GFP (green) on 20 µm SUPER templates and rapid localization of SUVs harboring sTag-BFP (cyan) before sfCherry formation (magenta). Scale bar: 10 µm.

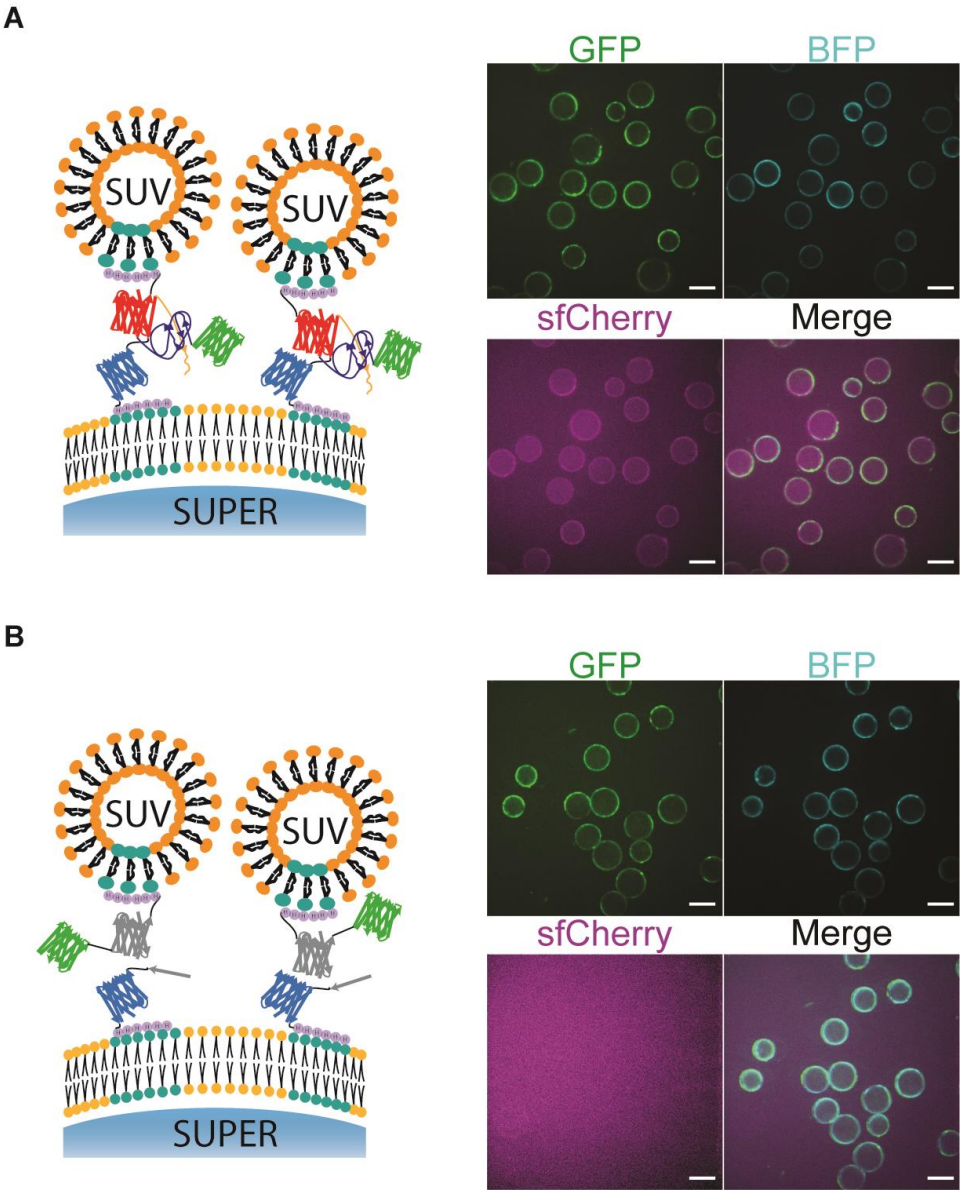

Figure S7: sfCherry reconstitution is relied on SpyTag/SpyCatcher bond formation when purified protein fragments create the membrane interface. **A)** Representative cohort images of reconstituted sfCherry (magenta) that fluoresces in the space between SUVs harboring sCatch-GFP (green) and 20 μm SUPER templates displaying sTag-BFP (cyan). Scale bars: 20 μm. **B)** Representative cohort images of no reconstituted sfCherry (magenta) in the space between SUVs harboring sCatch-GFP-ΔCatcher (green) and 20 μm SUPER templates displaying sTag-BFP (cyan). Scale bars: 20 μm.

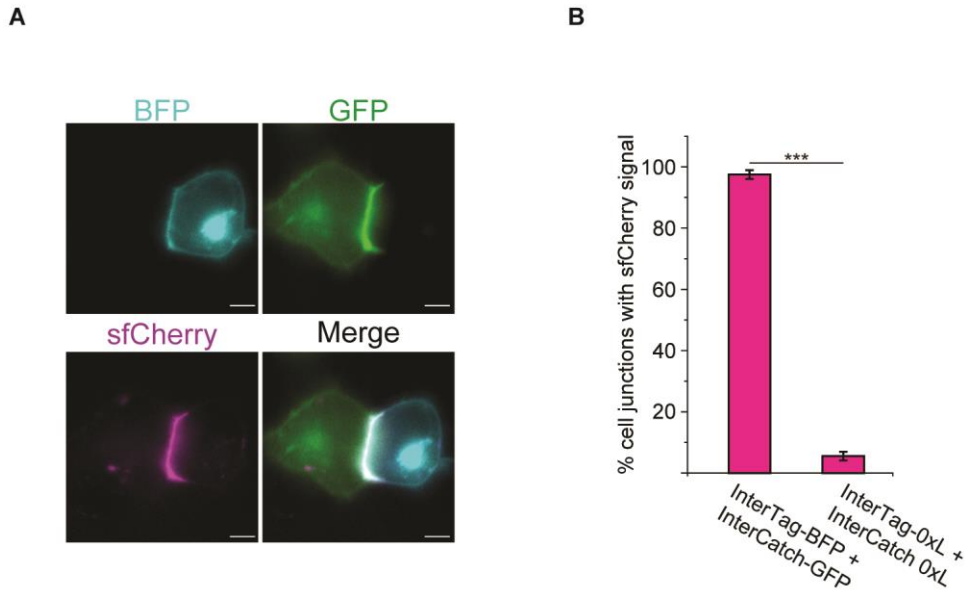

Figure S8: **A)** Representative single cell image of sfCherry (magenta) reconstituted through InterTag-BFP (cyan) and InterCatch-GFP (green). Scale bar: 5 µm. **B)** Plot showing percent of cell-cell junctions with reconstituted sfCherry signal for co-cultures involving InterTag-BFP and InterCatch-GFP and between InterTag-0xL and InterCatch-0xL. Data presented as mean ± SD, n=5, *p*-value is calculated using Fisher's exact test, and *** represent $p < 0.001$.

Table S1. Total number of cell junctions showing positive or negative fluorescence sfCherry signal in different linker combination groups. IC#:IT# represents co-culture of InterCatch #xL cells with InterTag #xL cells where # denotes the number of GGGGS linkers in each cell line.

Total number of GGGGS linkers	0	1		2		
Combinations	IC0:IT0	IC1:IT0	IC0:IT1	IC2:IT0	IC1:IT1	IC0:IT2
Positive	14	7	14	1	3	7
Negative	240	72	90	51	25	31

Total number of GGGGS linkers	3			4		
Combinations	IC3:IT0	IC2:IT1	IC1:IT2	IC0:IT3	IC3:IT1	IC2:IT2	IC1:IT3
Positive	2	23	61	24	17	100	73
Negative	59	30	56	20	23	57	77

Total number of GGGGS linkers	5		6
Combinations	IC3:IT2	IC2:IT3	IC3:IT3
Positive	164	132	272
Negative	72	65	100

Table S2. Constructs obtained from Addgene.

TfR-sfGFP-myc tag-SpyCatcher003	https://www.addgene.org/133451/
pcDNA3.1(+)_SpyCatcher-6aa-sfCherry(1-10)	https://www.addgene.org/117484/
pSFFV-SpyTag-sfCherry2(11)-TagBFP	https://www.addgene.org/117485/
SpyTag003-mKate2	https://www.addgene.org/133452/
pDisplay-LAP2-CFP-TM	https://www.addgene.org/34842/
pSBbi-GP	https://www.addgene.org/60511/
pSBbi-BP	https://www.addgene.org/60512/
pCMV(CAT)T7-SB100	https://www.addgene.org/34879/

Table S3. Primers used and constructs generated in this study

Oligos

- 1x GGGGS: 5’ GCTCTTCccgcgggaggcggtggatcggaacaGAAGAGC 3’
- 2x GGGGS: 5’ GCTCTTCccgcgggaggcggtggatcgggaggcggtggatcggaacaGAAGAGC 3’
- 3x GGGGS: 5’GCTCTTCccgcgggaggcggtggatcgggaggcggtggatcgggaggcggtggatcggaacaGAAGAGC 3’

Construct (Cloning method)	Component	Primer	Template plasmid
0xSBCatch (Gibson)	TfR	TfR_fwd: ccaagctggcctct*ggccaccatg*GATCAAGC TAGATCAGCATTCTC TfR_rev: gtttttgttcATAGCCCAAGTAGCCAAT CATAAATC	**TfR-sfGFP-myc tag-SpyCatcher003**
	SpyCatcher00 3 + sfC1-10	C3_sfC110_fwd: tgggctatGAACAAAAACTCATCTCAG AAGAG C3_sfC110_rev: caagcttggcctgacCTAGTCCTCGTTGTG GCTGG	**TfR_sfGFP_sC3_sf C110_Zeo**
	SB backbone (GFP)	SfiI digestion	**pSBbi-GP**
0xSBTag **(Gibson)**	IgK + SpyTag003 + sfC11	Igk_T3_sfC11_xTag_fwd: accccaagctggcctct*ggccaccatg*GAGACA GACACACTC CTGCTATGG Igk_T3_sfC11_xTag_rev: ttgttcggcGGTGCTGTGTCTGGCCTCG G	**pdgfr_BFP_sfc11_s T3_Zeo**
	PDGFR + Myc	pdgfr_xTag_fwd: cacagcaccGCCGAACAAAAACTCAT CTC	**pdgfr_BFP_sfc11_s T3_Zeo**

		pdgfr_xTag_rev: ccccaagcttggcctgacCTAACGTGGCTTCTTCTGCC	
	SB backbone (BFP)	Sfil digestion	**pSBbi-BP**
1x,2x,3x SBCatch (Golden Gate)	GGGGS Oligo	SapI digestion + **0xSBCatch**	
1x,2x,3xSBTag (Golden Gate)	GGGGS Oligo	SapI digestion + **0xSBTag**	
3xSB ΔTag (Golden Gate)		3xSB_NoTag_SapI_fwd: ttttttGCTCTTCaggctacaccatcgtgga 3xSB_NoTag_SapI_rev: ttttttGCTCTTCagccgtcaccagtggaacctg	**3xSBTag**
pT7-CFE1-InterCatch-GFP (Gibson)	Vector	BB-FWD: CACCACCACCACTAATAAAGATC BB-REV: CATATTATCATCGTGTTTTTCAAAGG	**pT7-CFE1-6xHis-HA**
	TfR-sfGFP-SpyCatcher003-sfCherry1-10	xCatch-FWD: TTTTCCTTTGAAAAACACGATGATAATATGATGATGGATCAAGCTAGATCAGC xCatch-REV: TCAGTCAGATCTTTATTAGTGGTGGTGGTGCTAGTCCTCGTTGTGGCTGG	**TfR-sfGFP-SpyCatcher003-sfCherry(1-10)**
pT7-CFE1-InterTag-BFP (Gibson)	Vector	BB-FWD: CACCACCACCACTAATAAAGATC	**pT7-CFE1-6xHis-HA**

		BB-REV: CATATTATCATCGTGTTTTTCAAAGG	
	SpyTag003-sfCherry11-tagBFP-PDGFR	xTag-FWD: TTTTCCTTTGAAAAACACGATGATAATATGCGTGGCGTTCCTCATATTG xTag-REV: TCAGTCAGATCTTTATTAGTGGTGGTGGTGCTAACGTGGCTTCTTCTGC	**IgK-SpyTag003-sfCherry11-tagBFP-PDGFR**
pET28b-sCatch-GFP (Gibson)	Vector	BB-FWD: CACCACCACCACCACCAC BB-REV: AAAAAACCTCCTTACTTTCTAGTCTCAAG	**pET28b_(R5Q5)(R5Q6)F20_His_Lys_RBS**
	sfGFP-SpyCatcher003-sfCherry1-10-6xHis	sCatch-FWD: TCTTGAGACTAGAAAGTAAGGAGGTTTTTTATGCGTAAAGGCGAAGAGC sCatch-REV: AGCCGGATCTCAGTGGTGGTGGTGGTGGTGGTCCTCGTTGTGGCTGGTGATG	**pT7-CFE1-InterCatch-GFP**
pET28b-sTag-BFP (Gibson)	Vector	BB-FWD: CACCACCACCACCACCAC BB-REV: AAAAAACCTCCTTACTTTCTAGTCTCAAG	**pET28b_(R5Q5)(R5Q6)F20_His_Lys_RBS**
	SpyTag-sfCherry11-tagBFP-6xHis	sTag-FWD: TCTTGAGACTAGAAAGTAAGGAGGTTTTTTatgcgtggcgttcctcatattg sTag-REV: AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCAGATCCTCTTCTGAGATGAG	**pT7-CFE1-InterTag-BFP**

pET28b-sCatch-GFP-ΔCatcher (Gibson)	Vector	BB-FWD: CACCACCACCACCACCAC BB-REV: AAAAAACCTCCTTACTTTCTAGTCTCAAG	**pET28b_(R5Q5)(R5Q6)F20_His_Lys_RBS**
	sfGFP-sfCherry1-10-6xHis	sCatch-dC-FWD: TCTTGAGACTAGAAAGTAAGGAGGTTTTTTatgcgtaaaggcgaagagc sCatchdC-REV: AGCCGGATCTCAGTGGTGGTGGTGGTGGTGGTCCTCGTTGTGGCTGGTGATG	**pT7-CFE1-InterCatch-GFP**
pET28b-sTag-BFP-ΔSpyTag (Gibson)	Vector	BB-FWD: CACCACCACCACCACCAC BB-REV: AAAAAACCTCCTTACTTTCTAGTCTCAAG	**pET28b_(R5Q5)(R5Q6)F20_His_Lys_RBS**
	sfCherry11-tagBFP-6xHis	sTagdT-FWD: TCTTGAGACTAGAAAGTAAGGAGGTTTTTTATGTACACCATCGTGGAGCAGTAC sTagdT-REV: AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCAGATCCTCTTCTGAGATGAG	**pT7-CFE1-InterTag-BFP**
pT7-CFE1-sCatch-GFP (Gibson)	Vector	BB-FWD: CACCACCACCACTAATAAAGATC BB-REV: CATATTATCATCGTGTTTTTCAAAGG	**pT7-CFE1-6xHis-HA**
	sfGFP-SpyCatcher003-sfCherry1-10	sCatch-FWD: TTTTCCTTTGAAAAACACGATGATAATATGATGCGTAAAGGCGAAGAGC sCatch-REV: TCAGTCAGATCTTTATTAGTGGTGGTGGTGCTAGTCCTCGTTGTGGCTG	**pT7-CFE1-InterCatch-GFP**

pT7-CFE1-sTag-BFP (Gibson)	Vector	BB-FWD: TAGCACCACCACCACTAATAAAG BB-REV: CATATTATCATCGTGTTTTTCAAAGG	**pT7-CFE1-6xHis-HA**
	SpyTag003-sfCherry11-tagBFP	sTag-FWD: TTTTCCTTTGAAAAACACGATGATAATATGCGTGGCGTTCCTCATATTG sTag-REV: GTCAGATCTTTATTAGTGGTGGTGGTGctaCAGATCCTCTTCTGAGATGAG	**pT7-CFE1-InterTag-BFP**
pT7-CFE1-sCatch-GFP-ΔCatcher (Gibson)	Vector	BB-FWD: CACCACCACCACTAATAAAGATC BB-REV: GGATCCACCCGAGCCA	**pT7-CFE1-6xHis-HA**
	sfGFP-sfCherry1-10	sCatch-dC-FWD: gatctgggttccggtggctcgggtggatccggatctagcggatctatggagg sCatch-dC-REV: TCAGTCAGATCTTTATTAGTGGTGGTGGTGCTAGTCCTCGTTGTGGCTG	**pT7-CFE1-InterCatch-GFP**
pT7-CFE1-sTag-BFP-ΔTag (Gibson)	Vector	BB-FWD: TAGCACCACCACCACTAATAAAG BB-REV: CATATTATCATCGTGTTTTTCAAAGG	**pT7-CFE1-6xHis-HA**
	sfCherry11-tagBFP	sTag-dT-FWD: TTTTCCTTTGAAAAACACGATGATAATATGTACACCATCGTGGAGCAG sTag-dT-REV: GTCAGATCTTTATTAGTGGTGGTGGTGctaCAGATCCTCTTCTGAGATGAG	**pT7-CFE1-InterTag-BFP**

Table S4. Calculated p-values for comparing statistical significance between groups in **Figure 5D**

	0x	1x	2x	3x	4x	5x	6x
0x		0.654	1	3.35E-21	1.11E-39	1.86-64	7.59E-70
1x			1	1.57E-10	1.55E-22	7.22E-40	1.48E-44
2x				4.83E-9	2.78E-18	3.08E-31	3.39E-35
3x					0.008	3.11E-12	4.61E-16
4x						0.0017	5.32E-6
5x							1
6x							