

## Supplementary Materials for

### Studying the Dynamics of SLP-76, Nck, and Vav1 Multimolecular Complex Formation in Live Human Cells with Triple-Color FRET

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Fig. S2. Flow cytometric analysis of single-labeled cell lines and of positive and negative control cells for 3FRET.

Fig. S3. FRET analysis of the interactions between Vav1, Nck, and SLP-76.

Legend for Movies S1 and S2

Table S1. Channels used for FRET analysis.

Table S2. Cell lines used as FRET controls.

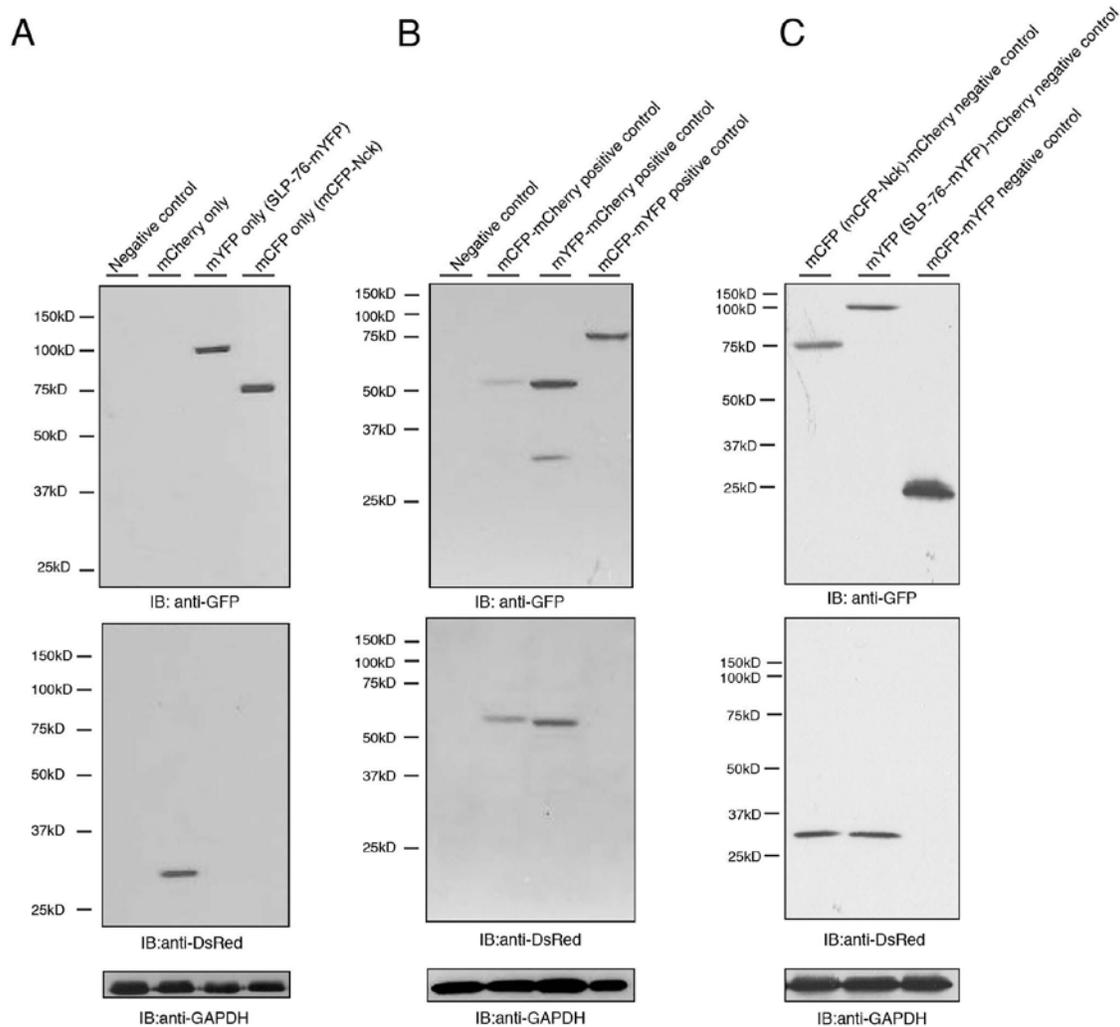
Table S3. 2FRET analysis of the interaction between Nck and Vav1 in the presence or absence of wild-type SLP-76.

#### Other Supplementary Material for this manuscript includes the following:

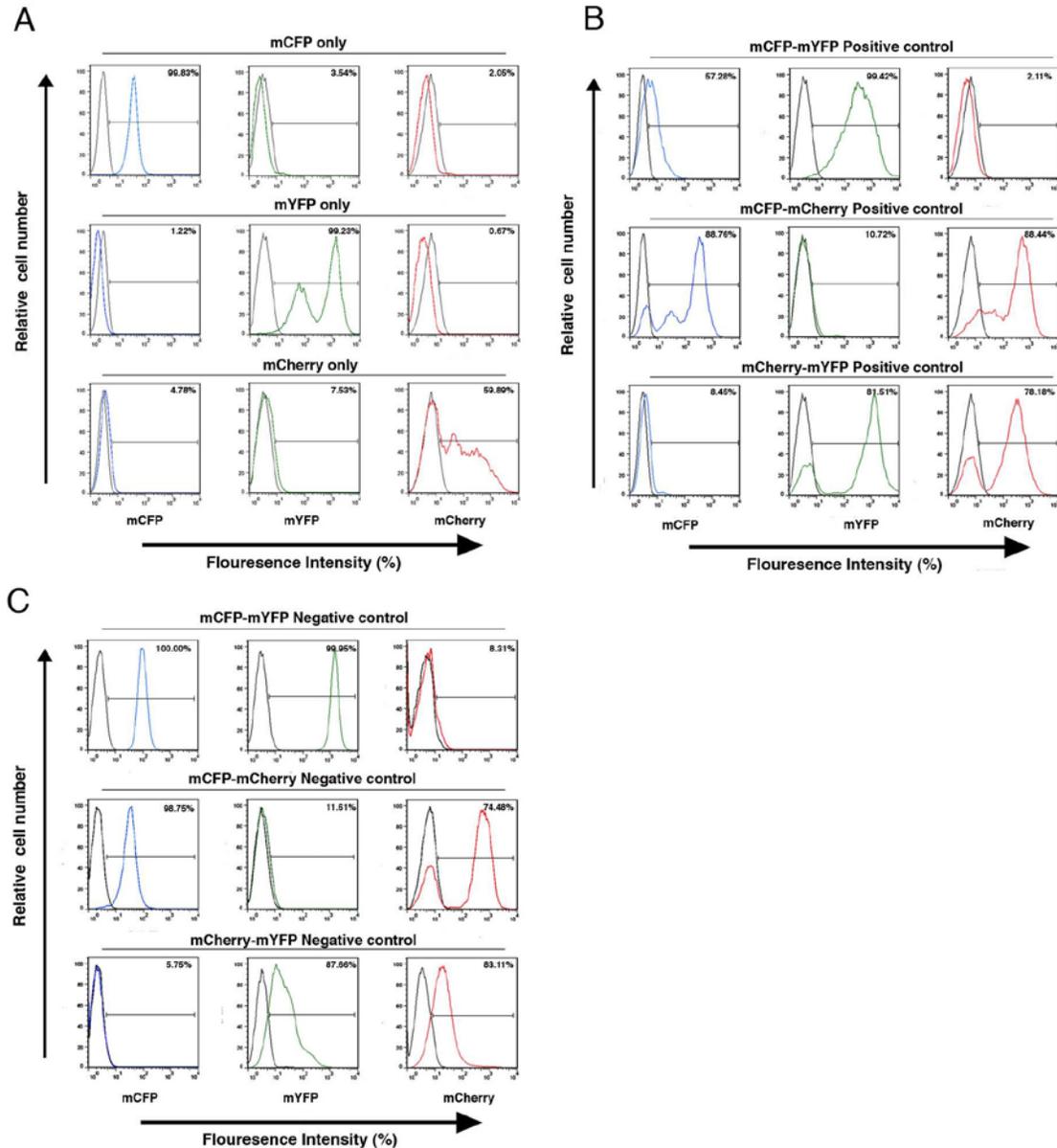
(available at [www.sciencesignaling.org/cgi/content/full/5/221/rs3/DC1](http://www.sciencesignaling.org/cgi/content/full/5/221/rs3/DC1))

Movie S1 (.mov format). Dynamics of Nck, Vav1, and wild-type SLP-76 in live T cells.

Movie S2 (.mov format). Dynamics of Nck, Vav1, and the SLP-76 Y3F mutant in live T cells.

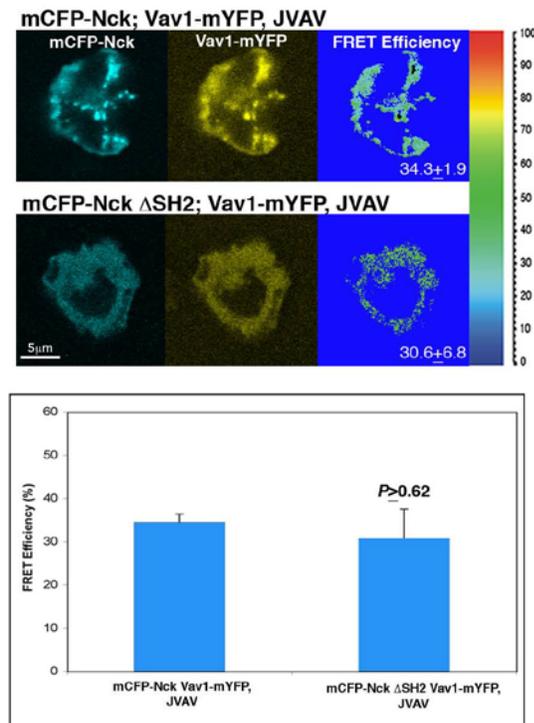


**Fig. S1.** Western blotting analysis of single-labeled cell lines and of positive and negative control cells for triple-color FRET. **(A)** Western blotting analysis of the single labeled cell lines and of the triple-color FRET positive and negative control cells. *Top panel*, Western blotting analysis with an antibody against GFP shows the presence of both mCFP-Nck (74 kD) and mYFP-SLP76 (103 kD). *Bottom panel*, mCherry protein (27 kD) was detected with an antibody against DsRed. **(B and C)** Western blotting analysis of the FRET control cell lines expressing coupled or uncoupled fluorescent proteins representing (B) positive and (C) negative controls, respectively, for energy transfer. *Top panels*, analysis with antibody against GFP detects mCFP and mYFP proteins; *middle panels*, antibody against mCherry; *bottom panels*, antibody against GAPDH, which served as a loading control. At least five independent experiments were performed.

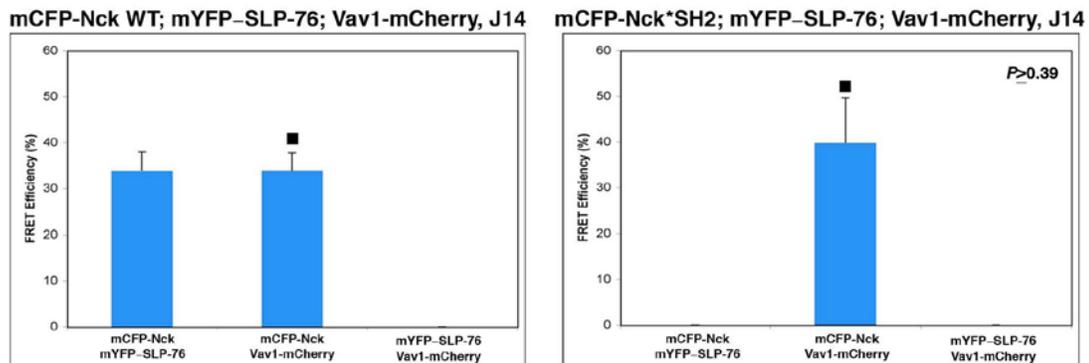


**Fig. S2.** Flow cytometric analysis of single-labeled cell lines and of positive and negative control cells for 3FRET. This analysis was performed to verify the fluorescence intensity of the FRET control cells. (A) Single-labeled cell lines. (B) Positive FRET control cell lines. (C) Negative FRET control cell lines. Blue, green, and red lines represent mCFP (excitation by mCFP laser, detection by mCFP emission filter), mYFP (excitation by mYFP laser, detection by mYFP emission filter), and mCherry (excitation by mCherry laser, detection by mCherry emission filter) fluorescence, respectively. Black lines represent untransfected cells, excited by the same lasers and detected by the same detectors and filters as were used for the transfected fluorescent cells. At least five independent experiments were performed.

A



B



**Fig. S3.** FRET analysis of the interactions between Vav1, Nck, and SLP-76. **(A)** Double-color FRET analysis was performed between Vav1-mYFP and either CFP-Nck WT or CFP-Nck  $\Delta$ SH2. **(B)** J14 cells were reconstituted with Vav1-mCherry, YFP-SLP-76, and either mCFP-Nck WT or mCFP-Nck R308K (SH2 mutant). 3FRET analysis was then performed. Graph shows the average FRET efficiencies of three independent experiments.

## **Movies**

Dynamics of Nck, Vav1, and wild-type SLP-76 (movie S1) were compared to the dynamics of Nck, Vav1, and the Y3F mutant SLP-76 (movie S2). Live SLP-76-deficient J14 T cells transfected to express mCFP-Nck, Vav1-mYFP, and mCherry-SLP-76 WT or mCherry-SLP-76 Y3F were dropped onto coverslips coated with stimulatory antibody against CD3 and visualized continuously by confocal microscopy. Five images, encompassing 0.5- $\mu\text{m}$  deep Z-stacks were collected every 110 s. Image slices corresponding to the coverslip were selected and exported as QuickTime movies. Playback rates are 100 $\times$  real time.

**Table S1.** Channels used for FRET analysis. Each cell was imaged with six different channels using the excitation lasers and the emission filters described in the table.

<b>Channel type</b>	<b>Excitation laser</b>	<b>Emission filter</b>
1. mCFP	458 nm	465-510 nm
2. mYFP	514 nm	530-600 nm
3. mCherry	594 nm	615 nm LP
4. mCFP-mYFP FRET	458 nm	530-600 nm
5. mCFP-mCherry FRET	458 nm	615 nm LP
6. mYFP-mCherry FRET	514 nm	615 nm LP

**Table S2.** Cell lines used as FRET controls.

Control Type		Protein expression	Molecular Weight (kD)	Cell Type
FRET calibration	mCFP	mCFP-Nck	72	*J14
	mYFP	mYFP-SLP-76	103	J14
	mCherry	mCherry	27	**E6.1
Positive Control	mCFP-mYFP	mYFP-mCFP-mYFP	81	E6.1
	mCFP-mCherry	mCFP-mCherry	54	E6.1
	mYFP-mCherry	mYFP-mCherry	54	E6.1
Negative Control	mCFP-mYFP	mCFP; mYFP	27; 27	E6.1
	mCFP-mCherry	mCFP-Nck; mCherry	72; 27	J14
	mYFP-mCherry	SLP-76-mYFP; mCherry	103; 27	J14

\*J14 cells are SLP-76-deficient Jurkat cells. \*\*E6.1 cells are wild-type Jurkat cells.

**Table S3.** 2FRET analysis of the interaction between Nck and Vav1 in the presence or absence of wild-type SLP-76. We performed FRET analysis of live cells dropped over a stimulatory coverslip. The average results of at least three independent experiments are presented.

<b>Cell Type</b>	<b>FRET Efficiency (%)</b>	<b>Standard error (<math>\pm</math>)</b>
Vav1-mCFP mYFP-Nck, E6.1	25.9	1.0
Vav1-mYFP mCFP-Nck SLP-76 WT, J14	25.6	7.5
Vav1-mYFP mCFP-Nck, J14	22.6	1.2