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Supplemental Information

Single-Color Fluorescence Lifetime Cross-Correlation Spectroscopy In Vivo

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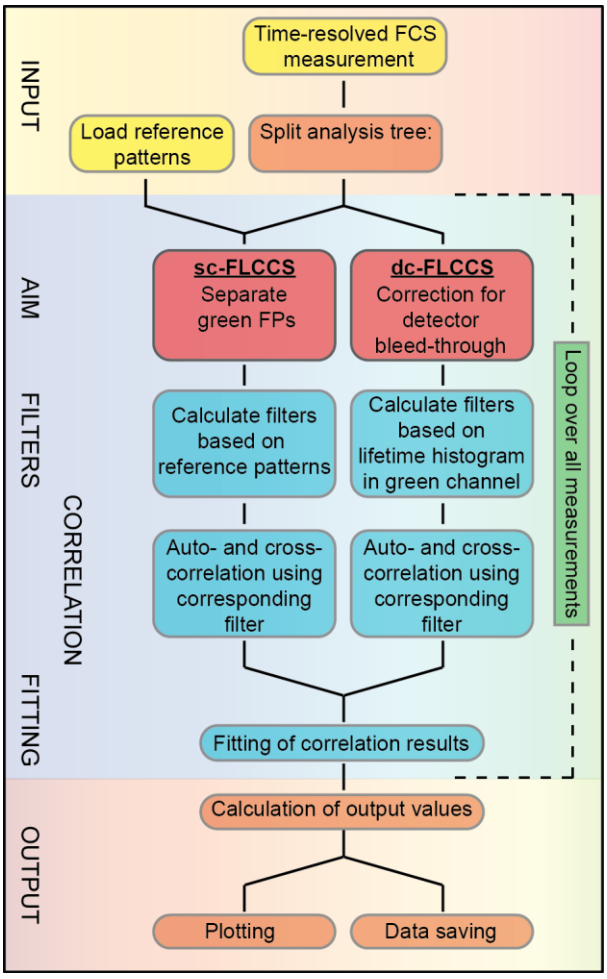

Fig. S1 Schematic illustration of the analysis pipeline for both sc-FLCCS and dc-FLCCS. Main difference is in the calculation of the weighting filters.

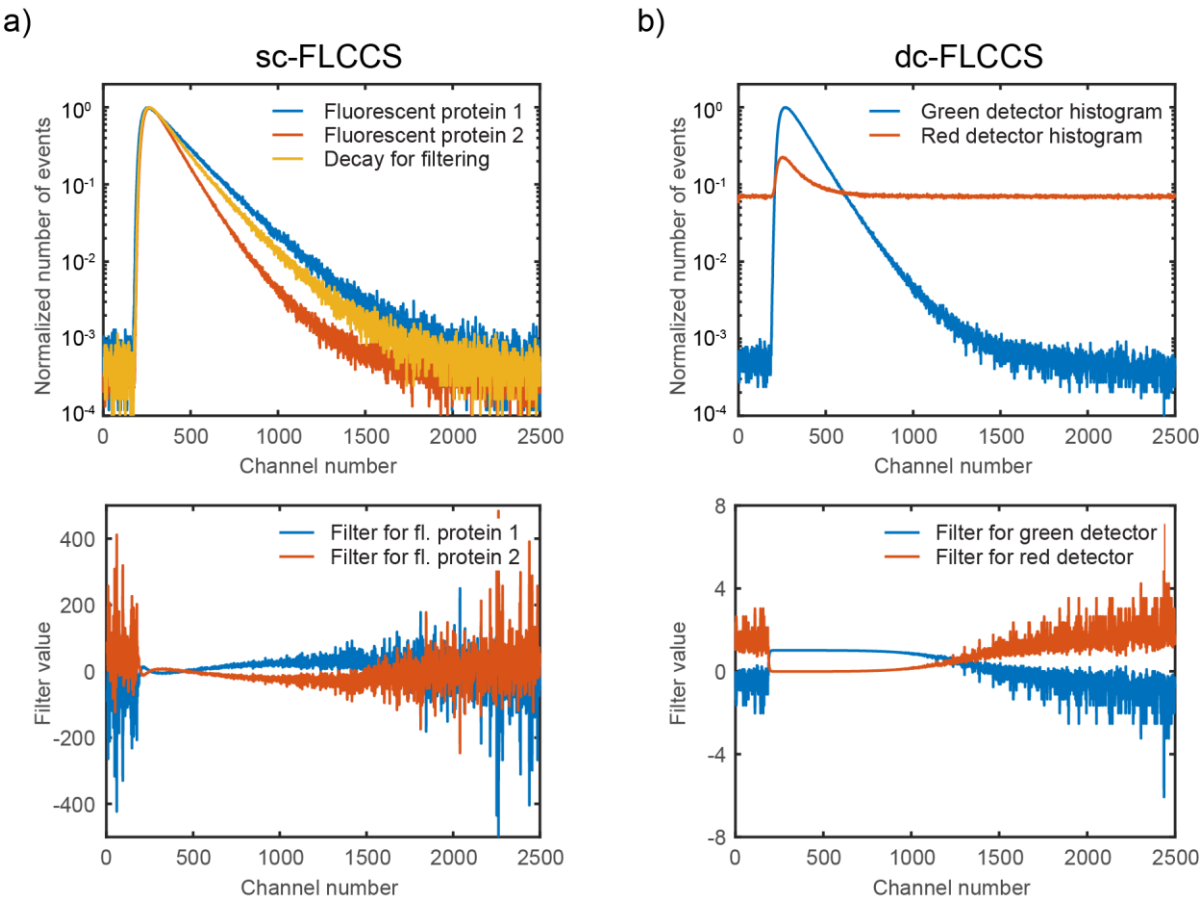

Fig. S2 Illustration of the calculation of weighting filters for sc-FLCCS and dc-FLCCS. **a)** Fluorescence lifetime histograms of both fluorescence proteins and the lifetime histogram of the measurement which is supposed to be filtered (top) are used to derive the weighting filters (bottom). b) Lifetime histograms of the green and red channels (top). Only the green laser was pulsed. The histogram-like pattern which is above the overall red signal in the red channel corresponds to the fluorescence bleed-through. Only the lifetime histogram from the green channel was used to calculate the weighting filters (bottom).

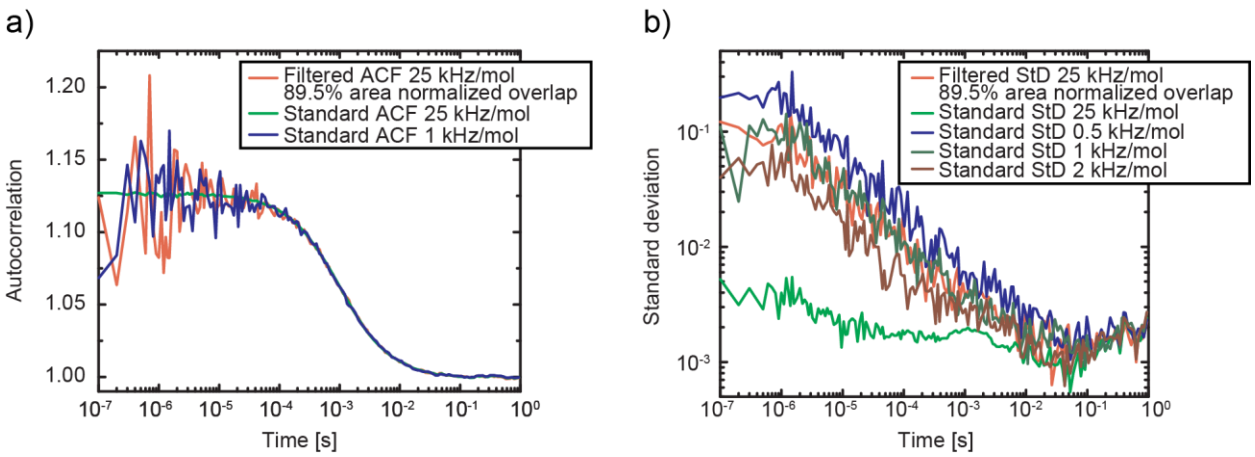

Fig. S3 **(a)** A comparison of simulated autocorrelation functions (ACF) for standard calculation with molecular brightness 25 kHz/mol (green), filtered ACF for lifetime difference 4 ns versus 3 ns (89.5% normalized area overlap) (orange) and standard ACF with molecular brightness 1 kHz (blue) **(b)** A comparison of standard deviations for each time point of simulated autocorrelation functions estimated by splitting the 180 s long simulation into 10 equal 18 s long pieces. Green curve is standard deviation for standard calculation with molecular brightness 25 kHz/mol, blue for 0.5 kHz/mol, olive for 1 kHz/mol, brown for 2 kHz/mol and orange is standard deviation for filtered ACF for lifetime difference 4 ns versus 3 ns (89.5% normalized area overlap). Simulation indicates that pattern overlap 90% for FLCS corresponds to decrease in SNR corresponding to 25x lower molecular brightness.

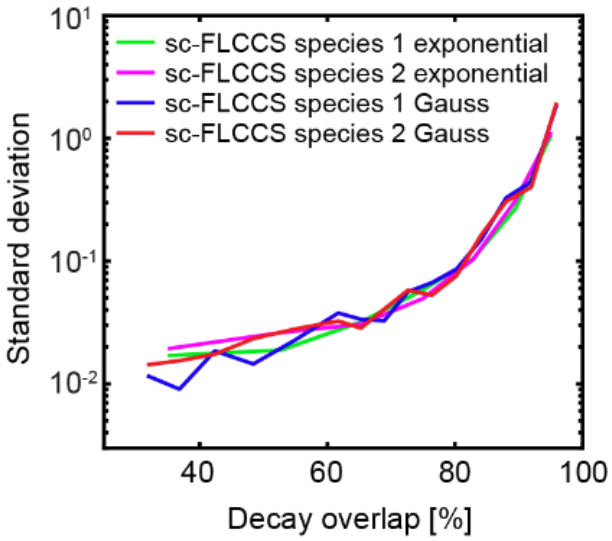

Fig. S4 The shape of fluorescence histograms used for calculation of filters does not affect the sc-FLCCS filtering. MC simulations provided the data assuming either gaussian or exponential histogram profile. Use of different shapes of histogram profiles did not show any difference in the quality of resulted auto-correlation functions.

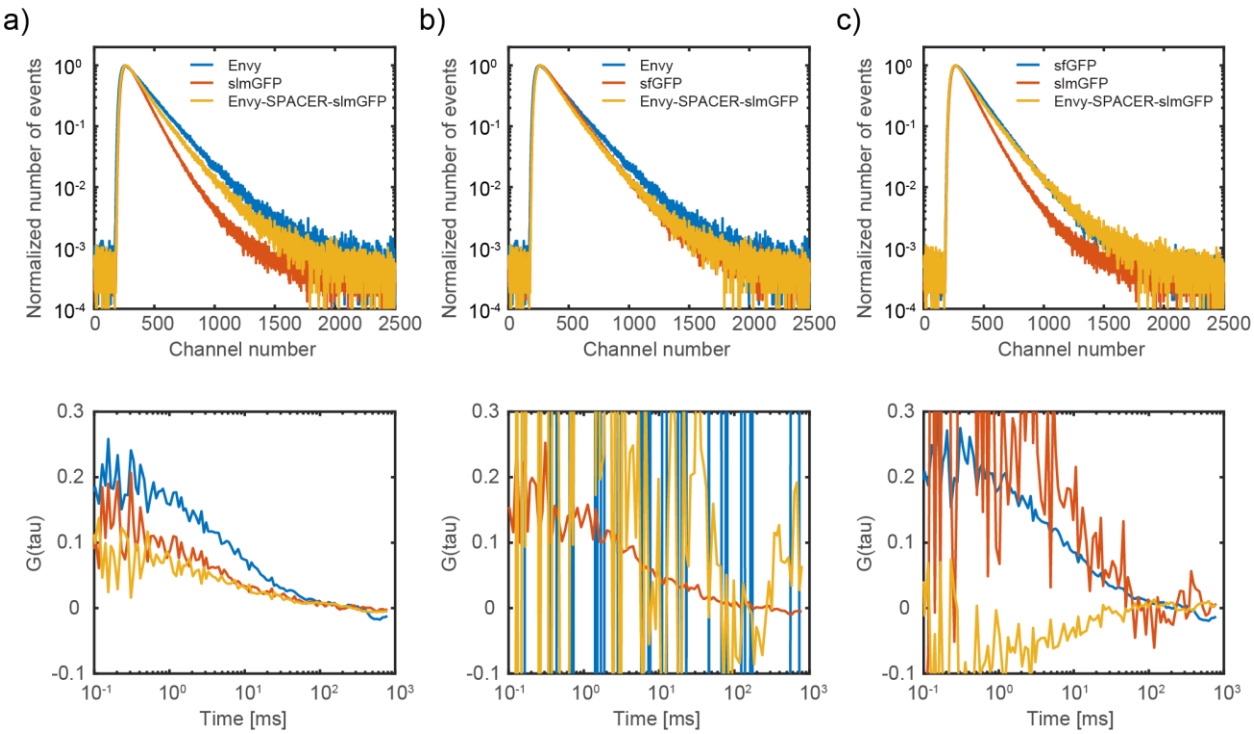

Fig. S5 *In silico* testing of sc-FLCCS approach by varying the fluorescence lifetime histograms used for determination of the correlation function. This was tested on the positive strain, where Envy and slmGFP was one translational unit spaced by the protein Don1. a) Correct Envy and slmGFP histograms were used for the calculation of weighting filters. Proper filtering resulted in correct auto- and cross-correlation functions. b) Fluorescence lifetime histogram of slmGFP was replaced by the one from sfGFP. Since sfGFP histogram overlaps with the experimental histogram (top), the only positive correlation function resulted from sfGFP filtered correlation. c) Envy histogram was replaced by the sfGFP histogram. Again, the only meaningful correlation function resulted from sfGFP filtered correlation.

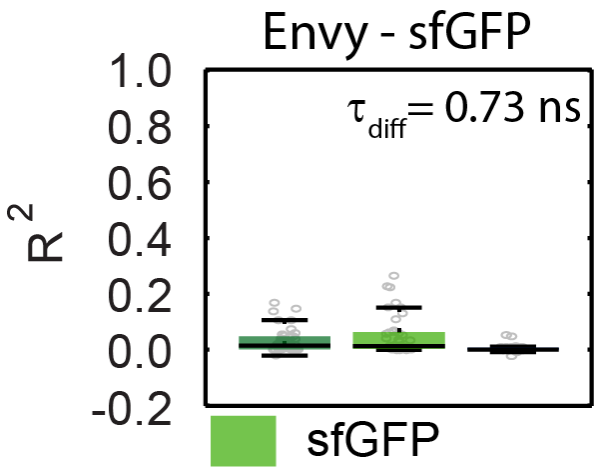

Fig. S6 The quality of the lifetime filtering is represented by the R^2 values of the fit of auto- and cross-correlation curves from the strains where the Don1 (SPACER) was tagged with Envy and sfGFP (additional to Figure 3b). The difference in average fluorescence lifetimes between FPs (τ_{diff}) is listed in the upper right corner.

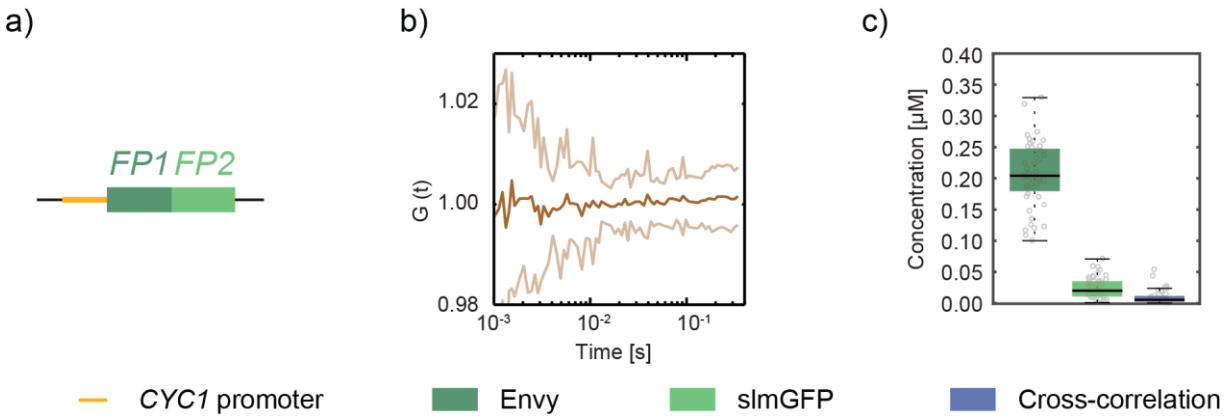

Fig. S7 **(a)** Schematic illustration of the strain with maximal FRET. **(b)** Summary of cross-correlation curves for the full FRET strain. Solid dark line corresponds to the mean curve, light borders denote standard deviations. **(c)** Concentrations of individual proteins in FRET control as determined by sc-FLCCS.

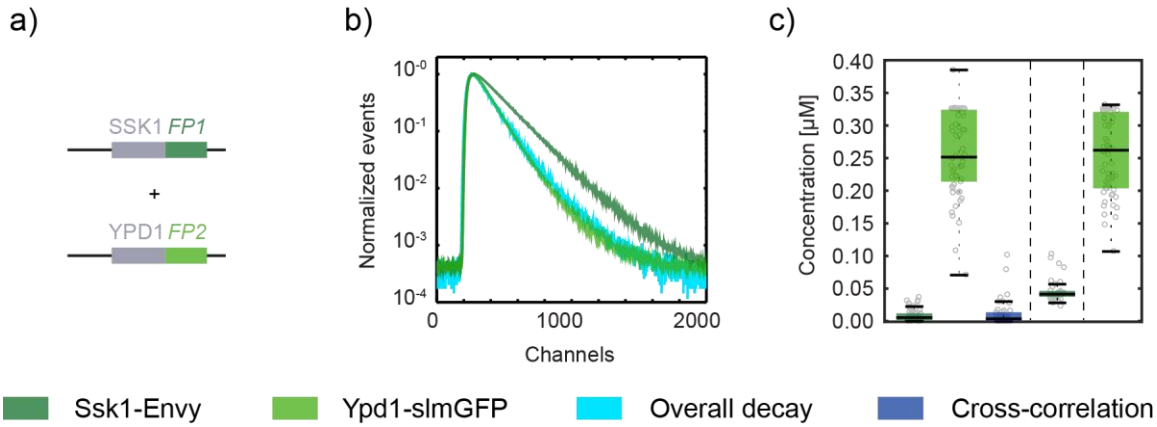

Fig. S8 **(a)** Schematic illustration of the strains in the abundance control. **(b)** Fluorescence decay histograms of single tagged strains (dark and light green curves) and the strain with combination of both tagged proteins with endogenous promoters as depicted in **(a)** (cyan curve). **(c)** Overall concentrations resulted from auto-correlation and cross-correlation curves for each diffusing species and their complexes as determined by the sc-FLCCS analysis. Dashed lines separate individual strains.

Strains used

Strain	Background	Description	Appearance
YDK62	ESM356	PDC1-slmGFP-hphNT1	Table 1
YDK80	ESM356	PDC1-myeGFP-KanMX	Table 1
YDK77	ESM356	PDC1-sfGFP-KanMX	Table 1
YMaM968	ESM356	pRS415-GPDpr-UBI-M-mCherry-sfGFP_cp3-KanMX	Table 1
YMaM969	ESM356	pRS415-GPDpr-UBI-M-mCherry-sfGFP_cp7-KanMX	Table 1
YMaM970	ESM356	pRS415-GPDpr-UBI-M-mCherry-sfGFP_cp8-KanMX	Table 1
YMaM715	ESM356	pRS415-GPDpr-Ubi-M-mCherry-sfGFP(no sf)-KanMX	Table 1
YDK73	ESM356	PDC1-mNeonGreen-KanMX	Table 1
YMaM717	ESM356	pRS415-GPDpr-Ubi-M-mCherry-Clover(F64L)-hphNT1	Table 1
YDK60	ESM356	PDC1-Clover-hphNT1	Table 1
YMaM712	ESM356	pRS415-GPDpr-Ubi-M-mCherry-sfGFP(L64F)-KanMX	Table 1
yMaS181	ESM356	pRS415-GPDpr-mCherry-DON1-Envy-HIS3MX6	Table 1
yMaS182	ESM356	pRS415-GPDpr-mCherry-DON1-GFPgamma-HIS3MX6	Table 1
yMaS180	ESM356	pRS415-GPDpr-mCherry-DON1-Ivy-HIS3MX6	Table 1
yMaS171	ESM356	PDC1-NowGFP-KanMX	Table 1
yST332	BY4741	YPD1-myeGFP-natNT	Figure 1
yMaS177	ESM356	YPD1-NowGFP-KanMx	Figure 1
yMaS184	ESM356	YPD1-GFPgamma-HIS3MX6	Figure 1
yMaS185	ESM356	YPD1-Ivy-HIS3MX6	Figure 1
yMaS233	ESM356	YPD1-sfGFP-hphNT1	Figure 1
yMaS234	ESM356	YPD1-slmGFP-hphNT1	Figure 1, Figure 4
yMaS238	ESM356	YPD1-Envy-HIS3MX6	Figure 1
yMaS208	ESM356	NatNT2-CYC1pr-Envy-DON1-3myeGFP-KanMx	Figure 3
yMaS231	ESM356	NatNT2-CYC1pr-Envy-DON1-slmGFP-hphNT1	Figure 3
yMaS203	ESM356	NatNT2-CYC1pr-GFPgamma-DON1-3myeGFP-KanMx	Figure 3
yMaS230	ESM356	NatNT2-CYC1pr-GFPgamma-DON1-slmGFP-hphNT1	Figure 3
yMaS209	ESM356	NatNT2-CYC1pr-Ivy-DON1-3myeGFP-KanMx	Figure 3
yMaS270	ESM356	NatNT2-CYC1pr-Ivy-DON1-slmGFP-hphNT1	Figure 3
yMaS263	ESM356	NatNT2-CYC1pr-Envy-Don1 + KanMx-CYC1pr-slmGFP-Ste11	Figure 3
yMaS245	ESM356	Ssk1-Envy-HIS3MX6 + Ypd1-slmGFP-hphNT1	Figure 4
yMaS239	ESM356	Ssk1-Envy-HIS3MX6	Figure 4
yMaS262	ESM356	NatNT2-CYC1pr-slmGFP-Envy	Figure 4
yKH193	ESM356	Rpn7-3mCherryDCU-KanMx + Pre6-slmGFP-hphNT1	Figure 4
yMaS242	ESM356	Rpn7-Envy-HIS3MX6 + Pre6-slmGFP-hphNT1	Figure 4
yMaS255	ESM356	Rad23-3mCherryDCU-KanMX + Pre6-slmGFP-hphNT1	Figure 5
yMaS264	ESM356	Rad23-3mCherryDCU-KanMX + Rpn7-Envy-HIS3MX6 + Pre6-slmGFP-hphNT1	Figure 5