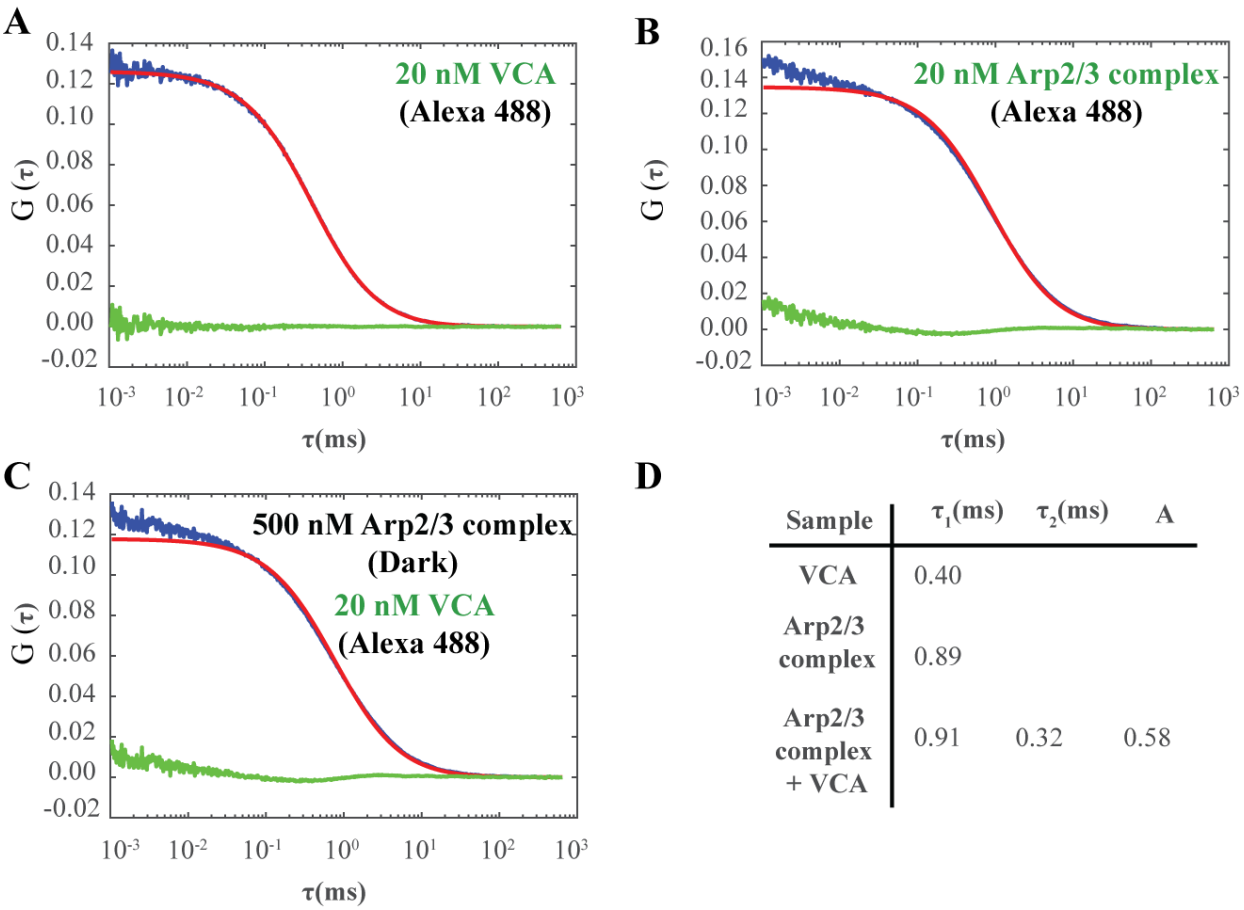
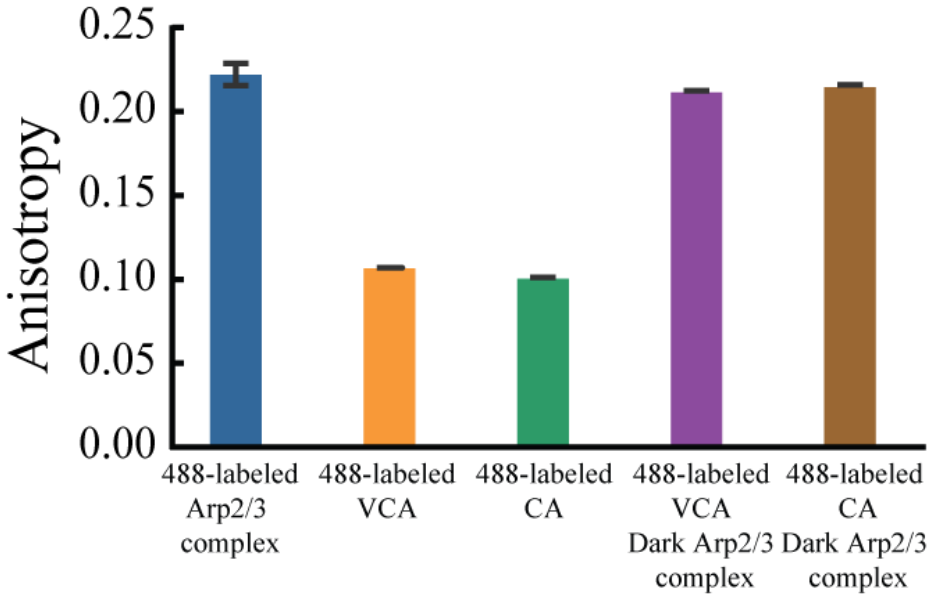
Supplemental Information

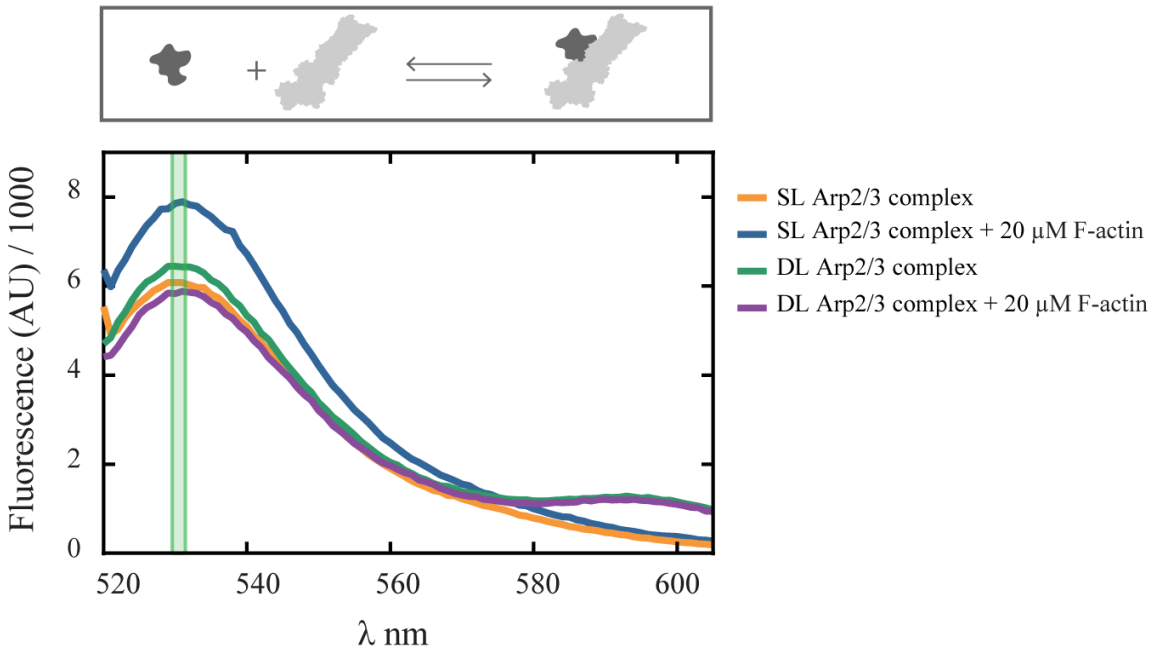


Sample	τ_1(ms)	τ_2(ms)	A
VCA	0.40		
Arp2/3 complex	0.89		
Arp2/3 complex + VCA	0.91	0.32	0.58

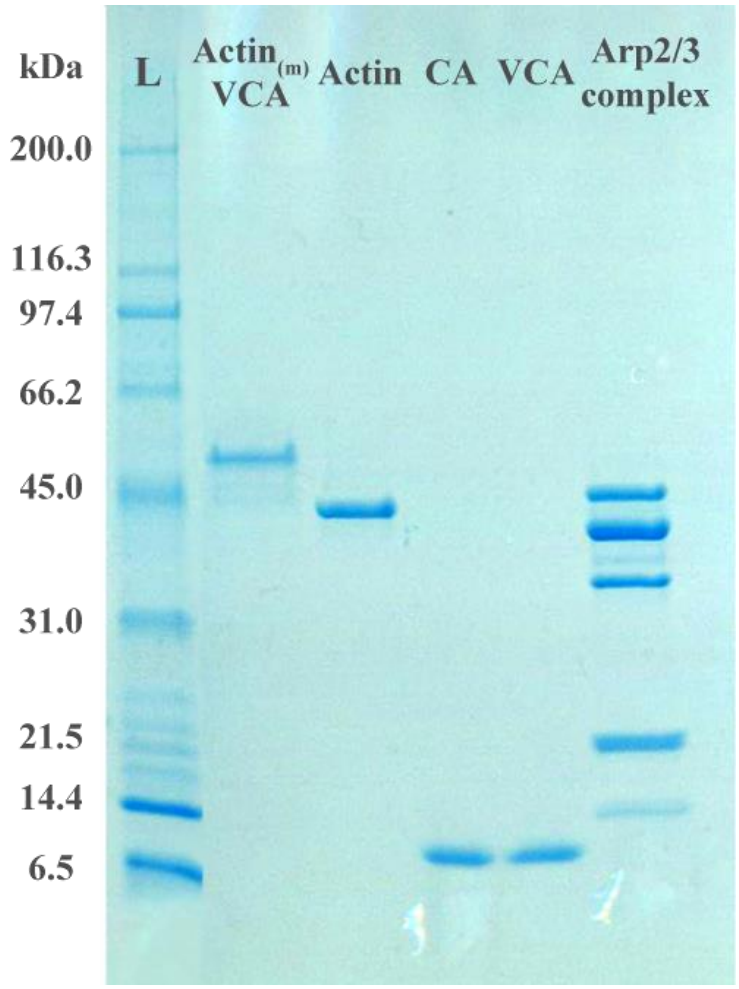
Supplemental Figure 1 – Fluorescence Correlation Spectroscopy shows that VCA binds to Arp2/3 complex in our experimental conditions. Conditions: Room temperature in KMET buffer (50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.2 mM ATP, 1 mM DTT, 10 mM Tris-HCl, pH 7.0) (**A**) Blue: 20 nM VCA labeled on an N-terminal cysteine with Alexa 488. Green: Residuals (**B**) Blue: 20 nM Arp2/3 complex labeled with Alexa 488 on ArpC3. Green: Residuals (**C**) Blue: 20 nM VCA labeled with Alexa 488 with 500 nM unlabeled Arp2/3 complex. Green: Residuals (**D**) Diffusion times (τ) in ms for 20 nM Alexa 488-VCA, 20 nM Alexa 488-Arp2/3 complex, and 20 nM Alexa 488-VCA with 500 nM unlabeled Arp2/3 complex. Abundance (A) indicates the fraction of the τ_1 species in the sample.



Supplemental Figure 2 – Fluorescence Anisotropy shows that VCA and CA bind Arp2/3 complex in our experimental conditions. Histogram with mean values and standard deviations from 4 measurements. Conditions: KMET buffer, 10 mM Tris-HCl, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, and 0.2 mM ATP. Samples: 100 nM Arp2/3 complex labeled on ArpC3 with Alexa 488; 50 nM Wsp1p-VCA and Wsp1-CA labeled on N-terminal cysteines with Alexa 488 alone or with 3 μM unlabeled Arp2/3 complex.



Supplemental Figure 3 – Measurement of FRET of bulk samples. All samples contained 20 nM Arp2/3 complex, either single-labeled (SL) on Arp2 with FlAsH-EDT_2 or double-labeled (DL) on Arp2 with FlAsH-EDT_2 and on Arp3 with Alexa 568, in KMEI buffer (10 mM imidazole, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 0.2 mM ATP). Arp2/3 complex was incubated overnight (~12 h) with 20 µM polymerized actin. FlAsH-EDT_2 was excited at λ 510 nm and emitted light was collected with a wavelength scan from 520 nm to 610 nm. Ligands increased the donor (FlAsH EDT2) intensity of SL Arp2-Arp3 and ARPC1-ARPC3, suggesting that binding affected the local environment of the fluorophores. We assume that the ligands have same effect on the emission of FlAsH-EDT_2 on both single- and double-labeled complexes when calculating ET_{eff}. Energy transfer efficiencies were calculated using Equation 1 (Materials and Methods). The fluorescence values used in this equation were the average fluorescence obtained from λ 530 nm to λ 532 nm (green bar) of single-labeled (for F_D) or double-labeled complexes (for F_{DA}). Figure 5 responds ΔET_{eff} values calculated by subtracting the ET_{eff} at 0 µM actin from the ET_{eff} at 20 µM actin. Reporting ΔET_{eff} allows us to compare the state of the complex with and without bound ligands.



Supplemental Figure 4 – SDS-polyacrylamide gel electrophoresis of purified proteins stained with Commassie Blue. Lane 1, Ladder of standard proteins; 2, actin-VCA; 3, actin; 4, Wsp1p-CA; 5, Wsp1p-VCA; 6, Arp2/3 complex.

Table S1 – Fluorescence anisotropy values for single-labeled Arp2/3 complexes.

Alexa 488	***Average (n=4)***	***Std Dev***
$ArpC1_{cys}$	*0.24*	*0.002*
$ArpC3_{cys}$	*0.22*	*0.002*
$Arp3_{cys}$	*0.22*	*0.001*
$Arp2_{cys}$	*0.21*	*0.001*

Alexa 594	***Average (n=4)***	***Std Dev***
$ArpC1_{cys}$	*0.21*	*0.003*
$ArpC3_{cys}$	*0.21*	*0.004*
$Arp3_{cys}$	*0.22*	*0.001*
$Arp2_{cys}$	*0.23*	*0.002*

Arp2/3 complex was labeled on single site on four subunits (Arp2cys, Arp3cys, ArpC1cys, and ArpC3cys) Alexa 488 or Alexa 594. Samples were each diluted to 100 nM concentration in KMET buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, and 0.2 mM ATP). Alexa 488 was excited at λ 490 nm and emitted light collected at λ 520 nm. Alexa 594 was excited at λ 590 nm and emitted light collected at λ 630 nm. Four measurements were performed per labeled construct.

Table S2 – Lifetimes Fluorescence of single-labeled Arp2/3 complexes

Subunit	***T1 Component***
$ArpC1_{cys}$	*4.09 ns*
$ArpC3_{cys}$	*4.10 ns*
$Arp3_{cys}$	*4.10 ns*
$Arp2_{cys}$	*4.20 ns*
Free dye	*4.10 ns*

Arp2/3 complex with a single Alexa 488 label on one subunit (Arp2cys, Arp3cys, ArpC1cys, or ArpC3cys) was each diluted to 40 nM in KMET buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, and 0.2 mM ATP). Samples were excited at 459 nm with a nano-LED and the emission at 517 nm was recorded. Fluorescence lifetime measurements confirm that quenching of the donor fluorophore does not affect the Förster radius under these conditions.

Table S3 - Energy transfer efficiency between probes measured by single molecule FRET and ALEX

		Energy Transfer Efficiency (ET_{eff})					***ALEX***				
Construct	***Ligand***	***Mean Peak***	***Mean Width***	***Minimum***	***Maximum***	***n***	***Mean Peak***	***Mean Width***	***Minimum***	***Maximum***	***n***
ArpC1-ArpC3	*None*	0.25	± 0.11	0.14	0.36	7	0.25	± 0.10	0.15	0.35	5
	VCA	0.28	± 0.13	0.16	0.41	4	0.30	± 0.10	0.19	0.39	1
	$Actin_{(m)}$-VCA	0.27	± 0.11	0.16	0.38	3	0.26	± 0.10	0.16	0.36	1
	CA	0.28	± 0.11	0.16	0.39	3	0.28	± 0.10	0.17	0.37	3
Arp3-Arp2	*None*	0.48	± 0.14	0.34	0.62	9	0.46	± 0.16	0.30	0.62	1
	VCA	0.61	± 0.09	0.52	0.70	4	0.62	± 0.10	0.52	0.72	1
	$Actin_{(m)}$-VCA	0.54	± 0.19	0.35	0.72	6	0.55	± 0.20	0.35	0.75	1
	CA	0.60	± 0.12	0.49	0.72	3	0.58	± 0.12	0.46	0.70	2

Mean values for the centers of the Gaussian distributions of FRET events and width calculations for a subset of measurements (n) on each sample obtained with smFRET and ALEX. Photon threshold was fixed at 30 for all calculations. Maximum and minimum ET_{eff} values were calculated by adding or subtracting the mean width value to the mean peak one. These numbers display the range of ET_{eff} values obtained. Measurements were made on 75 pM double-labeled Arp2/3 complexes ($Arp2_{cys}$-$Arp3_{cys}$ and $ArpC1_{cys}$-$ArpC3_{cys}$) in KMET buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, and 0.2 mM ATP) alone or with 20 µM Wsp1p-VCA, 2 µM actin-Wsp1p-VCA or 20 µM Wsp1p-CA.

Table S4 – ET_{eff} variation upon binding to different ligands by ensemble FRET

	$Arp2_{tetracys}$-$Arp3_{cys}$			$ArpC3_{tetracys}$-$ArpC1_{cys}$		
Ligand	***Mean***	***Standard Deviation***	***n***	***Mean***	***Standard Deviation***	***n***
2mM ATP	0.01	± 0.02	4	-0.05	± 0.04	4
20 μM *CA*	0.12	± 0.02	3	-0.06	± 0.05	4
20 μM *VCA*	0.16	± 0.02	4	0.01	± 0.04	3
20 μM *GST-VCA*	0.16	± 0.04	4	-0.04	± 0.04	4
2 μM $Actin_{(m)}$-*VCA*	0.15	± 0.03	4	-0.05	± 0.02	3
10 μM *F actin-VCA*	0.22	± 0.03	3	0.22	± 0.02	3
20 μM *F actin*	0.26	± 0.02	3	0.16	± 0.02	4

Labeled constructs were diluted to fixed concentrations of 20 nM in KMEI buffer (10 mM imidazole, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT). All samples had 0.2 mM ATP, except when testing the effect of ATP. For that experiment, ATP was either at 0 mM or 2 mM. Samples were incubated for 3 h at room temperature with a range of concentrations of VCA, CA, actin-VCA, GST-VCA, or a fixed concentration of polymerized actin and a range of concentrations of VCA. Complexes were incubated overnight (~12 h) with a range of concentrations of polymerized actin. Energy transfer efficiencies were obtained by comparing the donor intensities of double-labeled with single-labeled complexes (See supplemental methodology for details). Mean and standard deviations were calculated using different number of repetitions (*n*).

***Table S5 – Sequences of primers used for C-terminal tagging of Arp2/3 complex subunits*.**

Construct	***Primer Name***	***Sequence 5' – 3' (Bahler, Wu et al. 1998)***
ArpC1$_{cys}$	*ArpC1cysFw*	GACCTTACGCTGGGACTCCTGGCAATATTACTGCGTTTACCTCCAGCGGTACCGACGG ACGTGTTGTTTTGTGGACTCTTTGTTAGCGGATCCCCGGGTTAATTAA
ArpC1$_{cys}$	*ArpC1cysRv*	GATTGTTCAATTCTTTTATTCTATGAGATTAACATCTACGGAAATTCGATAATTGATT TGATAAGATTCATTTGCCGATATCTTTTGAATTCGAGCTCGTTTAAAC
ArpC3$_{cys}$	*ArpC3cysFw*	ATGTATATGCAACTGAAAAGGATCATCCCAGCAAGTGGTGGACTTGCTTCAGCAAGAG ACGTTTTATGAACAAAGCTTTGTGTTAGCGGATCCCCGGGTTAATTAA
ArpC3$_{cys}$	*ArpC3cysRv*	GATTGTAAGCCGCATTTGAAATAAAGGAAACGGAAAAACATAAAGGCTTTGAAATACA GTATGAGCCAAATTATTAATTAATAGACGAATTCGAGCTCGTTTAAAC
Arp3$_{cys}$	*Arp3cysFw*	CCTATTGCCATACCAAAGCAGATTACGAAGAATATGGTGCTTCCATTGCTCGTAGGTA CCAAATTTTTGGAAATTCTCTTTGTTAGCGGATCCCCGGGTTAATTAA
Arp3$_{cys}$	*Arp3cysRv*	GATTGTTCTAAGGCTAATGCCTGCTCCATTAAACTTTTCATTTTAATCAGTAAATCCT ATAGACGCAAAACTCATGATATATTTCAGAATTCGAGCTCGTTTAAA
Arp2$_{cys}$	*Arp2cysFw*	ACGATCACATGTGGGTGTCCAAGGCAGAATGGGAGGAGTATGGAGTACGTGCACTAGA CAAATTGGGTCCTAGAACTACTTGTTAGCGGATCCCCGGGTTAATTAA
Arp2$_{cys}$	*Arp2cysRv*	GATTGTAATTTATGAATCAAACTTCGTATAACTCAATTTACTAGCACATAAAAAGAAA GAAACAGAACATTAAACATAAATATACTGAATTCGAGCTCGTTTAAAC
ArpC3$_{tetracys}$	*ArpC3tetracysFw*	ATGTATATGCAACTGAAAAGGATCATCCCAGCAAGTGGTGGACTTGCTTCAGCAAGAG ACGTTTTATGAACAAAGCTTTGTGTTGTTGTTGTTAGCGGATCCCCGGGTTAATTAA
ArpC3$_{tetracys}$	*ArpC3tetracysRv*	GATTGTTGTTGTTGTAAGCCGCATTTGAAATAAAGGAAACGGAAAAACATAAAGGCTT TGAAATACAGTATGAGCCAAATTATTAATTAATAGACGAATTCGAGCTCGTTTAAAC
Arp2$_{tetracys}$	*Arp2tetracysFw*	ACGATCACATGTGGGTGTCCAAGGCAGAATGGGAGGAGTATGGAGTACGTGCACTAGA CAAATTGGGTCCTAGAACTACTTGTTGTTGTTGTTAGCGGATCCCCGGGTTAATTAA
Arp2$_{tetracys}$	*Arp2tetracysRv*	GATTGTTGTTGTTGTAATTTATGAATCAAACTTCGTATAACTCAATTTACTAGCACAT AAAAAGAAAGAAACAGAACATTAAACATAAATATACTGAATTCGAGCTCGTTTAAAC
ArpC1$_{cys}$	*ArpC1cysFwChk*	ATTATCTCAATTGCCTTTGCGT
ArpC1$_{cys}$	*ArpC1cysRvChk*	CACTTCTCAATTTTGGGGTCTC
ArpC3$_{cys}$	*ArpC3cysFwChk*	TATCAGTGAGTGCTTGGGAAGA
ArpC3$_{cys}$	*ArpC3cysRvChk*	GAGGTTTTAAAGCACAATTCGG
Arp3$_{cys}$	*Arp3cysFwChk*	AAACATTGTCCTTTCTGGTGGT
Arp3$_{cys}$	*Arp3cysRvChk*	CTCAATGAAAAAGCCTGGAAAC
Arp2$_{cys}$	*Arp2cysFwChk*	GTGAACAACCAGGTTTGAGTGA
Arp2$_{cys}$	*Arp2cysRvChk*	ACGTCACGGGAGTCTAGTCATT

Primers for PCR-based gene targeting in *S. pombe* and checking primers were generated using the Bahler Lab web-interface script: *"PPPP: Pombe PCR Primer Program C-terminal tagging"*. (url: http://bahlerweb.cs.ucl.ac.uk/cgi-bin/PPPP/pppp_c_term.pl)

Supplemental Materials and Methods

Fluorescence Anisotropy

We performed anisotropy measurements to check rotational freedom of the fluorophores attached to our different constructs and to corroborate VCA and CA were properly binding to the different Arp2/3 complexes. Measurements were made in a PTI Alpha-scan spectrofluorimeter (Photon Technology International, Santa Clara, California) (Marchand, Kaiser et al. 2001). For rotational freedom experiments, Alexa 488 and Alexa 594 single-labeled Arp2/3 complexes on all subunits (Arp2cys, Arp3cys, ArpC1cys, and ArpC3cys) were each diluted to 100 nM concentration in KMET buffer (10 mM Tris-HCl pH 7.0, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, and 0.2 mM ATP). Alexa 488 was excited at λ 490 nm and emitted light collected at λ 520 nm. Alexa 594 was excited at λ 590 nm and emitted light collected at λ 630 nm. Intensity scans at 1 s intervals were taken over 20 s, and the average anisotropy was calculated using the PTI Felix Software.

Fluorescence anisotropy, *r*, is defined as

$$r = \frac{(I_{VV} - G * I_{VH})}{(I_{VV} + 2G * I_{VH})} \qquad \text{(Equation S1)}$$

Where I_{VV} and I_{VH} are the parallel and perpendicular components of the polarized fluorescence light, respectively. I_{VV} and I_{VH} were measured at the same time using T-shaped light path. G is a correction factor defined as $G = I_{HV}/I_{HH}$. To calculate *G*, solutions were excited with horizontally polarized light (Vinson, De La Cruz et al. 1998).

To verify VCA and CA binding to Arp2/3 complexes, we used Alexa 488 labeled VCA or CA at a fixed concentration of 50 nM and 3 μM unlabeled Arp2/3 complex in KMEI buffer. Measurements and calculations were performed as described above.

Fluorescence Lifetime Measurements

To confirm that the Förster radius was not altered by quenching of the donor fluorophore, we measured fluorescence lifetimes with a TCSPC TD-Fluor Horiba Fluorolog 3 Time Domain Fluorimeter. The samples were 40 nM Arp2/3 complexes in KMET buffer with a single label on $Arp2_{cys}$, $Arp3_{cys}$, $ArpC1_{cys}$ or $ArpC3_{cys}$. We used a 459 nm nano-LED for excitation and 517 nm emission monochrometer. Slit widths were adjust to give an alpha measurement of 0.5 to 1.5% photon collection, and measurements continued until 10,000 photons were counted in the highest

bin. All traces were fit to single-exponential and the T1 component is reported.

Fluorescence Correlation Spectroscopy (FCS)

FCS measurements were made on an inverted Olympus IX-71 microscope (Olympus) (Trexler and Rhoades 2009) using an adjusted laser power in the 4-6 µW range. Fluorescence emission was collected through the objective and filtered using a long-pass dichroic (Z488RDC) and a band-pass filter (HQ600/200M- Chroma). Photons were passed through a 50-µm diameter optical fiber (OzOptics), which was directly coupled to an avalanche photodiode (Perkin-Elmer) (Metskas and Rhoades 2015). Experiments were done in passivated cover glasses with eight well chambers (Nunc). Each reaction contained 2 µM unlabeled Arp2/3 complex and 20 nM Alexa 488-labeled VCA. To obtain the experimental variation, 30 fluorescence autocorrelation traces lasting 15 s were recorded and averaged. The average curve was fitted with a standard single component diffusion model weighted by the inverse-square of the standard deviation (equation 4):

$$G(\tau) = \frac{1}{N}\frac{1}{1+\frac{\tau}{\tau_D}}\sqrt{\frac{1}{1+\frac{s^2\tau}{\tau_D}}} \qquad \text{(Equation S2)}$$

Where, $G(\tau)$, is the autocorrelation value as a function of τ, τ_D is the average diffusion time of the particles, s is the ratio of the radial to axial dimension of the focal volume, and N is the average number of fluorescent particles in the focal volume.

References

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