Table S3: Characterization of causes for the variability in EA-TIRFM anisotropy values; Related to STAR Methods; Steady-state fluorescence emission anisotropy measurements and analysis

Effect of Effective Numerical Aperture (NA) on emission anisotropy:

Microscope cofiguration	Effective NA	Anisotropy value on FN	Anisotropy value after mβCD treatment	Relative Aniso change	Calculated FRET efficiency (Fraction)
100X/TIRF	1.1	0.1739 (0.005)	0.1896 (0.005)	0.0157	0.25
100X/TIRF	0.85	0.1917 (0.005)	0.218 (0.004)	0.0263	0.37
100X/Confocal	0.73	0.1926 (0.006)	0.226 (0.01)	0.034	0.37

Effect of probe photophysical properties on emission anisotropy:

Probe used	Mean anisotropy value in Vin-/- cells (SD)	Mean anisotropy value in Vinculin add back cells (SD)	Relative anisotropy change
GFP-GPI (Figure 5B)	0.146 (0.006)	0.098 (0.003)	0.048
GFP-GPI (Figure S6B)	0.165 (0.006)	0.099 (0.005)	0.066
Alexa-568-FLAER (Figure 5F)	0.115 (0.003)	0.065 (0.003)	0.050
mRuby2-GPI (Figure 6D)	0.192 (0.0092)	0.157 (0.0032)	0.035
mRuby2-GPI (Figure S5E)	0.185 (0.0045)	0.138 (0.0063)	0.047

Probe used	Mean anisotropy value of cells on FN (SD)	Mean anisotropy value of cells on FN after mβCD treatment (SD)	Relative anisotropy change
GFP-GPI (Figure1D)	0.159 (0.002)	0.182 (0.004)	0.0233
YFP-GPI (Figure 1F)	0.174 (0.005)	0.189 (0.005)	0.0157