Supporting Materials

Experimental Setup

Measurements were taken with a Zeiss 63x C-Apochromat water immersion objective (N.A.=1.2) at an excitation wavelength of 1000 nm and an average power, after the objective, of 0.3 mW. The data was acquired at a frequency of 20 kHz for a duration of ~60 seconds. Photon counts were detected by an avalanche photodiode (APD, SPCM-AQ-141, Perkin-Elmer, Dumberry, Quebec), recorded by a Flex02-01D card (correlator.com, Bridgewater, NJ), and analyzed with programs written in IDL 8.2 (Research Systems, Boulder, CO). For dual-channel measurements, a dichroic mirror with a center wavelength of 580 nm split the fluorescence emission into two detection channels. The green channel had an additional 84-nm-wide bandpass filter centered at 510 nm (Semrock, Rochester, NY) to eliminate the reflected fluorescence of mCherry.

Z-scans were performed by using an arbitrary waveform generator (Model No. 33250A, Agilent Technologies, Santa Clara, CA) to move a PZ2000 piezo stage (ASI, Eugene, OR) along the z-axis. The driving signal from the arbitrary waveform generator was a linear ramp function with a peak-to-peak amplitude of 2.4 V and a period of 10 seconds. The peak-to-peak voltage corresponded to 24.1 μm of axial travel with the cells occupying roughly 5 μm in the center of each pass. Data was acquired at a frequency of 20 kHz for either a few seconds for a single z-scan or over several minutes for sequential z-scans.

Sample Preparation and Plasmid Construction

The pEGFP-C1 and pEGFP-N1 plasmids were purchased from Clontech (Clontech, Mountainview,CA). The mCherry-C1 plasmid was cloned from the mCherry pRSET B plasmid which was a kind gift from Dr. R. Y. Tsien (University of California, San Diego). mCherry was amplified by PCR with a 5' primer that encodes an NheI restriction site and a 3'primer that encodes an XhoI site. The PCR fragment of mCherry was then ligated into the backbone of pEGFP-C1 (Clontech, Mountainview,CA). The EGFP-H-Ras plasmids were a kind gift from Dr. Mark Phillips (New York University School of Medicine). The MA domain of both HIV-1 Gag and HTLV-1 Gag were cloned from their full Gag sequence and amplified by PCR with a 5' primer that encodes an *XhoI* restriction site and a 3' primer that encodes an *EcoRI* site. The HIV-1 MA and HTLV-1 MA cDNAs were then ligated into the pEGFP-N1 plasmid. All sequences were verified by automatic sequencing.

All studies were performed using transiently transfected U2OS cells that were obtained from ATCC (Manassas, VA) and maintained in 10% fetal bovine serum (Hycolone Laboratories, Logan, UT) and DMEM medium. Cells were subcultered in eight-well coverglass chamber slides (Nalge Nunc International, Rochester, NY) 12 hours before transfection. Transient transfections were carried out using TransFectin (BioRad, Hercules, CA), according the manufacturer's instructions, 24 hours prior to measurement. Immediately before measurement, the growth medium was replaced with Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium (Biowhittaker, Walkerville, MD).

Brightness Analysis

The brightness from the FFS data was computed as previously detailed (1, 2). In addition, calibration measurements of the brightness of EGFP were performed in the thick section of 10-20 cells expressing EGFP. The average brightness λ_{EGFP} from this calibration experiment served as the normalization factor to convert an experimentally measured brightness λ into a normalized brightness $b = \lambda / \lambda_{EGFP}$.

SUPPORTING REFERENCES:

1. Chen, Y., J.D. Müller, S.Y. Tetin, J.D. Tyner, and E. Gratton. 2000. Probing ligand protein binding equilibria with fluorescence fluctuation spectroscopy. *Biophys. J.* 79: 1074–1084.

2. Sanchez-Andres, A., Y. Chen, and J.D. Müller. 2005. Molecular brightness determined from a generalized form of Mandel's Q-parameter. *Biophys. J.* 89: 3531–3547.