Supplementary Methods

Primers for Gene Construction

ErbB2 was amplified by PCR using the following primers: 5'-cataagcttgctcaaacctgtgatttcctttacg-3' and 5'-cataagcttgctcaaacctgtgatttcctttacg-3' and the PCR product were introduced into the *Xho*I/ *Hind*III sites of pEYFP-N1 (Clontech). ErbB3 was amplified by PCR using the following primers: 5'-gcggtaccatgagggcgaacgacg-3' and 5'-gcgcggccgcgtttcgttctctgggcattag-3'. Citrine was mutated to a non-dimerizing version (A206K) by the Strategene QuikChange Site-Directed Mutagenesis Kit and amplified using 5'-ggtctagacatggtgagcaagg-3' and 5'-gcggggcccttacttgtaccagctcgtc-3' primers. The PCR products were then cloned into the *Xba*I and *Apa*I site (CitrineA206K) and *Kpn*I and *Not*I site (ErbB3) of pcDNA3 (Invitrogen).

Image Processing

Membrane segmentation. The background offset was removed by subtracting the mean intensity of a dark 20×20 pixel region outside the cell selected by visual inspection. For the automated analysis of EGF-QD and VFP-labeled receptor internalization a precise discrimination of the cell membrane from the cell interior was essential. In the bleaching corrected green intensity images, noise was removed by 2 successive applications of a 5×5 slice-by-slice median filter. Each image was normalized between its maximum and minimum and framed with a border of zeros. A labeling procedure (based on 4-connectivity) was used to identify connected components outside the cells with values below a threshold selected globally but specifically for each time series; thresholds varied between 7% and 17% depending on the dataset. Regions containing < 1/3 of the area of an image were removed from the region classified as outside the cell. The remaining region(s) defined the *outside* of the cells. These regions were inverted and used to initiate a slice-by-slice Euclidian 2D distance transform (DT). In the series of DT images, values corresponding to distances between 3-10 pixels to the *outside* were assigned to the *membrane* region and distances between 15-35 pixels to the *inside rim* region of the cell. This scheme yielded a reasonable segmentation as judged by visual inspection of many time series. The *inside rim* was preferred to the total interior (distance to outside ≥ 15) since the EGF-QD rarely internalized beyond this rim in studies at room temperature and thus a major fraction of unliganded green fluorescence (e.g. nascent protein in the Golgi) could be excluded from the analysis. Finally, the ratio of the fractions was determined, as described in Methods, to determine the relative fractions of internalized receptor and EGF-QD.

Estimation of the relative amount of internalization (Supplementary Figure). An EGF-QD-mask marking the pixels with red fluorescence was first generated by selecting the uppermost 4% of the pixel values in the red (EGF-QD) channel on a frame-by-frame basis. From the green channel (erbB-VFP receptor) a background value in the *inside rim* region of the cell, derived from the average green fluorescence in a lower (between 5 and 10% quantile of pixel intensities) red intensity region , was subtracted to correct for the non-negligible residual green fluorescence inside the cell.

To compute the relative amount of internalized red and green fluorescence, values inside the EGF-QD-mask were summed separately for the red and background-corrected green channel in the *membrane* and *inside rim* regions. From these sums the internalized EGF-QD (QD_{in}) and internalized erbB-VFP receptor (VFP_{in}) was determined as the fraction of their total sum in the membrane (QD_{mem} and VFP_{mem}). Normalization of the QD and VFP *inside rim* signals by the respective membrane signals eliminated the need to correct for photobleaching of the VFPs. This “internal calibration” approach was chosen to remove the influence of the amplifier gain as well as of background noise, to which cross-correlation would be sensitive.