Figure S1. **Additional characterization of pH-sensitive red FPs.** (A) pH titration curves of additional pH-sensitive red FPs mentioned in this study. Error bars represent SEM. (B) Modeled location of the M163K mutation in pHOran4 (based on the crystal structure of mOrange; PDB ID 2H5O). The FP is viewed from the axis of the β-barrel. Secondary structures are shown in cartoon form. The chromophore and the mutated residue are in stick representation with carbon colored orange in the chromophore and gray in mutated residue. Nitrogen is shown in blue and oxygen in red. (C) Modeled location of the K163Y mutation in pHuji (based on the crystal structure of mCherry; PDB ID 2H5Q). The chromophore and the mutated residue are in stick representation with carbon colored magenta in the chromophore and gray in the mutated residue. Nitrogen and oxygen are colored as in B.
Figure S2. Sensitivity of SEP, pHoran4, and pHuji in HeLa cells with collapsed pH gradients. (A) TIRF images of a HeLa cell transfected with pDisplay-pHuji at pH 7.4, 5.0, and then 7.4 plus NH$_4$Cl (50 mM), which reveals intracellular vesicles that are normally acidic (yellow arrowheads), whereas diffuse fluorescence of proteins on the plasma membrane remains unchanged. (B) Fluorescence ratio of cells transfected with the indicated pDisplay-FP, in NH$_4^+$-containing versus control solution ($n = 3$–5 cells). The ratios do not differ from unity, showing that no acidic intracellular organelle contributes to pDisplay-FP fluorescence. (C) Images at pH 7.4 and then pH 5.0 of the same cell as in A after 2-min incubation with digitonin (50 µg/ml). Note the presence of bright puncta, which suggest that intracellular organelles were also permeabilized by digitonin and hence at the same pH as the bath solution. At pH 5.0, similar to untreated cells, pHuji fluorescence is largely, but not completely, decreased. (D) Fluorescence ratios of cells at pH 5.0 versus 7.4 in control conditions or in the presence of digitonin ($n = 3$–4 cells). The fluorescence ratios were not significantly different in control and in digitonin ($P > 0.85$). Error bars represent SEM.

Figure S3. Scission events recorded in cells cotransfected with TfR-SEP and TfR-pHoran4. (A) Mean TfR-pHoran4 fluorescence of detected terminal (orange) and nonterminal (brown) scission events (137 and 237 events, respectively, in nine cells) at pH 7.4 (top) and 5.0 (bottom) aligned to their time of scission. The black lines indicate 95% confidence intervals for significant enrichment. (B) Example of an event co-detected with TfR-SEP and TfR-pHoran4.
Figure S4. The new detection algorithm shows increased performance as compared with the original one. (A and B) Examples of detection of scission events correctly detected with TfR-SEP only (A) or TfR-pHuji only (B). (C) Example of a scission event wrongly detected with SEP only using the original algorithm, despite a large fluorescence increase in the red channel (TfR-pHuji raw pH 5.0). This false negative resulted from a high level of background fluorescence before the time of detection that precluded the detection of the individual vesicle at time 0. The principle of detection of the new algorithm is to subtract the five preceding images for each frame to detect local increases of fluorescence (minus background) regardless of the surrounding noise. The obtained images are then thresholded to segment and track the thus detected objects (segmented). (D) Frequency of TfR-SEP (green) and TfR-pHuji (red) events detected with the original method (left) versus the new one (right). The new method detects more events with both labels (**, P < 0.01; ***, P < 0.001). (E) Proportion of scission events, in n = 5 cells, detected in both channels (yellow) or with either TfR-pHuji (red) or TfR-SEP (green) using the original method (left) versus the new one (right). The proportion of matched events is statistically increased using the new method (P = 0.012). Error bars represent SEM.
Figure S5. Detection of CCVs and differential sorting of TfR-SEP and pHuji-β2AR in the same cell. (A) Portion of a HeLa cell cotransfected with TfR-SEP and pHuji-β2AR, before (top) and during (bottom) application of isoproterenol (20 µM). (B) Fluorescence in the green and red channels of segmented clusters at pH 7.4 (a) and detected CCVs at pH 5.0 (b) during application of isoproterenol in a representative cell. Yellow dots, objects detected in both channels (201 clusters [a] and 9 CCVs [b]); green dots, objects detected in the SEP channel only (47 clusters [a] and 321 CCVs [b]); red dots, objects detected in the pHuji channel only (71 clusters [a] and 13 CCVs [b]). Black dashed lines show linear regression of all TfR-SEP objects (a, R = 0.83) and CCVs (b, R = 0.44). Green and red lines show the lower limits of SEP and pHuji object detection, respectively. (C) Frequency of events represented as cumulative number of events detected with TfR-SEP (left) and pHuji-β2AR (right) during the course of the experiment, normalized at the end (17 min). Dotted lines are regression lines of the data before agonist application, extrapolated to the entire recording. (D) Example of scission events detected in both channels (a) or in the green channel only (b) during agonist application.

Table S1. Emission peak at different pH values

| Protein      | pH 5.0 | pH 7.2 | pH 10.0 | Peak shift (from pH 5.0 to 10.0) |
|--------------|--------|--------|---------|---------------------------------|
| SE-phluorin  | nm     | nm     | 512     | 3                                |
| pHoran1      | 509    | 512    | 512     | 3                                |
| pHoran2      | 558    | 561    | 562     | 4                                |
| pHoran3      | 600    | 598    | 598     | 2                                |
| pHuji        | 584    | 576    | 568     | 16                               |
| mNectarine   | 580    | 568    | 562     | 18                               |
| pHTomato     | 578    | 578    | 562     | 16                               |
| mCherry-TYG  |        |        |         |                                  |

Table S2. Mutations of the pH-sensitive red FPs developed in this study

| Protein | Template | Mutations                      |
|---------|----------|--------------------------------|
| pHoran1 | mOrange  | E160K/G196D/K184E/E218V        |
| pHoran2 | mOrange  | N98S/T127S/E160K/K184V/G196D   |
| pHoran3 | mOrange  | E160K/K161W/M163G/K184V/G196D/E218V |
| pHoran4 | mOrange  | M163K                          |
| A-9     | mApple   | I161G/K163H                    |
| A-17    | mApple   | I161G/K163F                    |
| A-47    | mApple   | I161G/K163V                    |
| pHuji   | mApple   | K163Y                          |
| mCherry-TYG | mCherry | M66T                           |
Video 1. **NIH-3T3 cell transfected with TfR-SEP corresponding to Fig. 4 A.** Cells were recorded under TIRF illumination on a microscope (IX71; Olympus) while the extracellular medium was switched from pH 5.0 to 7.4 every 2 s (ppH) for 6 min. Only the images recorded at pH 5.0 are shown (0.25-Hz time lapse, accelerated 40 times). Note the high number of putative endocytic events (suddenly appearing fluorescent dots).

Video 2. **Same as Video 1 for the NIH-3T3 cell transfected with TfR-pHTomato corresponding to Fig. 4 A.** Note the moving fluorescent intracellular organelles and the low number of putative endocytic events (suddenly appearing fluorescent dots).

Video 3. **Same as Video 1 for the NIH-3T3 cell transfected with TfR-pHoran4 corresponding to Fig. 4 A.** Note the high number of putative endocytic events (suddenly appearing fluorescent dots).

Video 4. **Same as Video 1 for the NIH-3T3 cell transfected with TfR-pHuji corresponding to Fig. 4 A.** Note the high number of putative endocytic events (suddenly appearing fluorescent dots).