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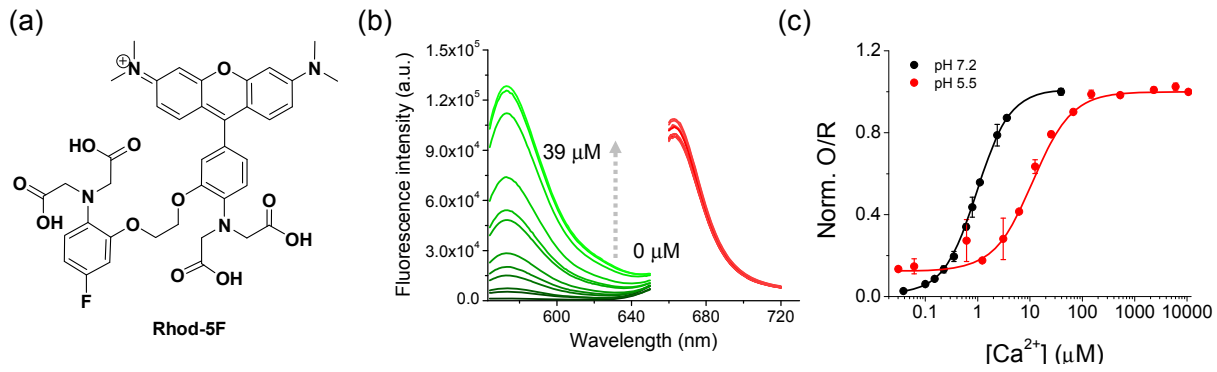
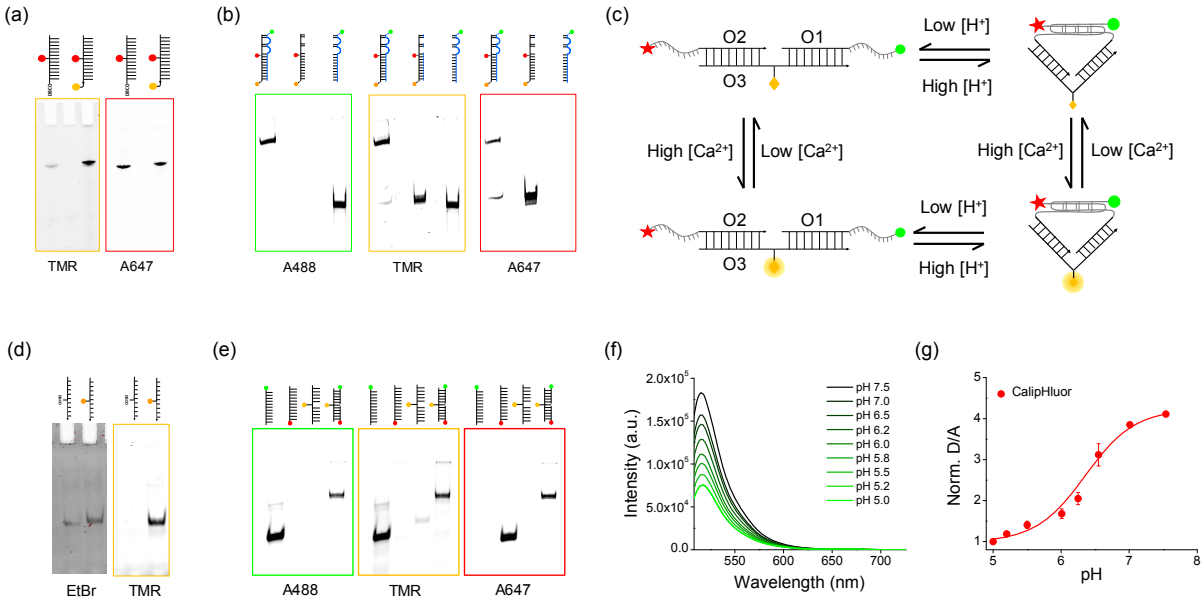


Supplementary Figure 1

In vitro characterization of Rhod-5F.

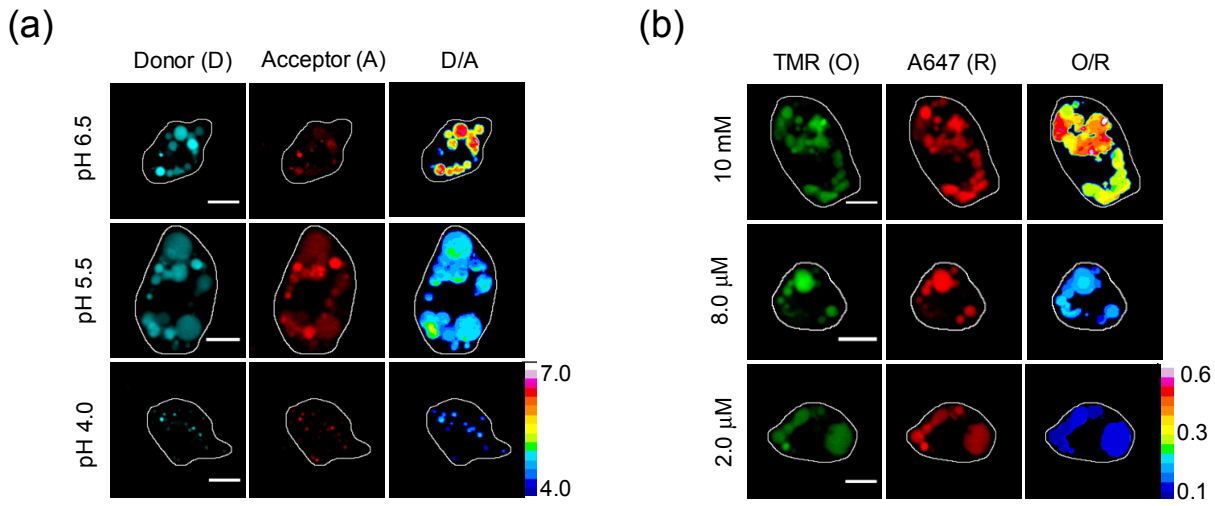
(a) Chemical structure of Rhod-5F. (b) Fluorescence emission spectra of Rhod-5F(green) and Alexa Fluor 647 (red) with increasing [Ca^{2+}] upon exciting Rhod-5F and Alexa Fluor 647 at 560 nm and 650 nm, respectively. (c) Normalized O/R ratio of Rhod-5F/Alexa Fluor 647 with increasing [Ca^{2+}] at pH 7.2 and 5.5. Error bar represents mean ± s.e.m. of three independent experiments. *bbb*



Supplementary Figure 2

Characterization of *CalipHluor*$_{Ly}$ and *CalipHluor*.

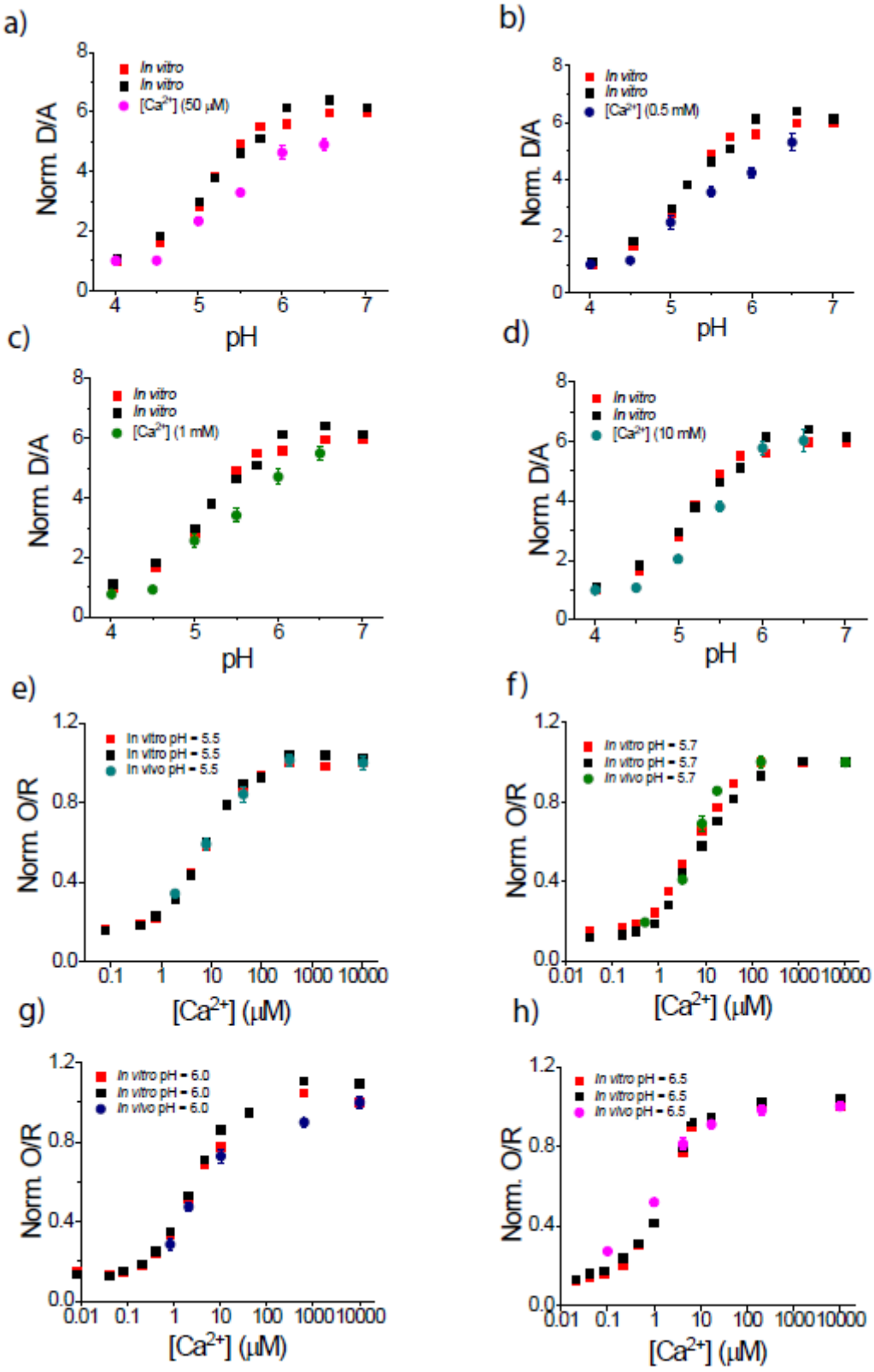
(a) Gel showing the conjugation of Rhod-5F to D2-DBCO strand. Gels were visualized in EtBr and TMR channels. (b) Native PAGE showing formation of *CalipHluor*$_{Ly}$. Gels were visualized in Alexa Fluor 488, TMR and Alexa Fluor 647 channels. (c) Schematic of working principle of *CalipHluor*. pH-induced FRET changes between Alexa Fluor 488 (donor, green sphere) and Alexa Fluor 647 (acceptor, red star) is used to report pH ratiometrically. A Ca^{2+} sensitive fluorophore (Rhod-5F, yellow diamond) and Alexa Fluor 647 report Ca^{2+} (at a given pH) ratiometrically by direct excitation of each dye. (d) Gel showing the conjugation of Rhod-5F to O3-DBCO strand. Gels were visualized in EtBr and TMR channels. (e) Native PAGE showing formation of *CalipHluor*. Gels were visualized in Alexa Fluor 488, TMR and Alexa Fluor 647 channels. (f) Emission spectra of *CalipHluor* at pH values ranging from 7.5 to 5.0 upon excitation at 488 nm. (g) Normalized ratio of fluorescence intensity of donor to that of acceptor (D/A) of *CalipHluor* as a function of pH. (D $_{ex}$ = 495 nm, $_{em}$ = 520 nm; A $_{ex}$ = 495 nm, $_{em}$ = 665 nm). Gels were performed twice independently. Error bar represents mean ± S.E.M of three independent experiments.



Supplementary Figure 3

In vivo performance of *CalipHluor$_{Ly}$*.

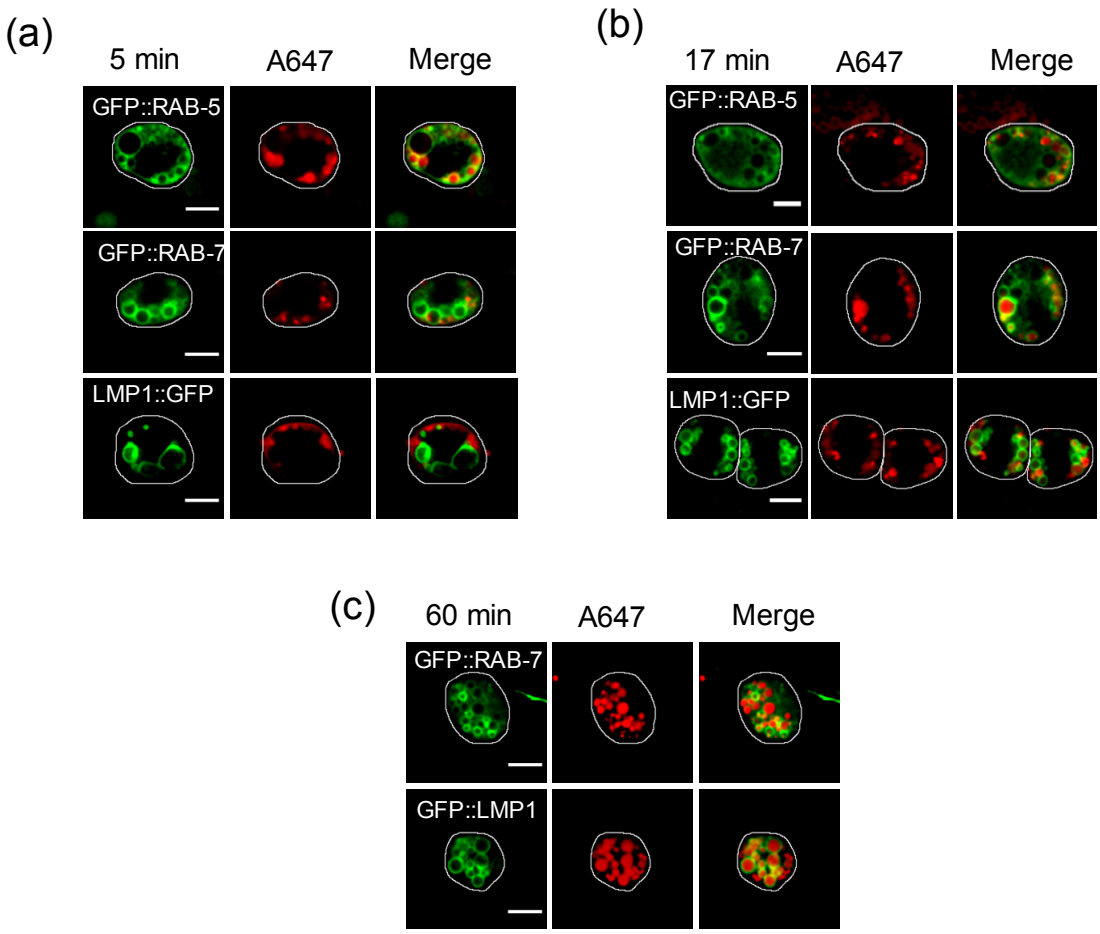
Representative pseudo color images of coelomocytes labeled with *CalipHluor$_{Ly}$* and clamped at the indicated (a) pH and (b) free [Ca^{2+}] at pH 5.5. Scale bar 5 m. Experiments were repeated three times independently with similar results



Supplementary Figure 4

Comparison of in vitro and in vivo pH and Ca^{2+} calibration profile of *CalipHluor*$_{Ly}$.

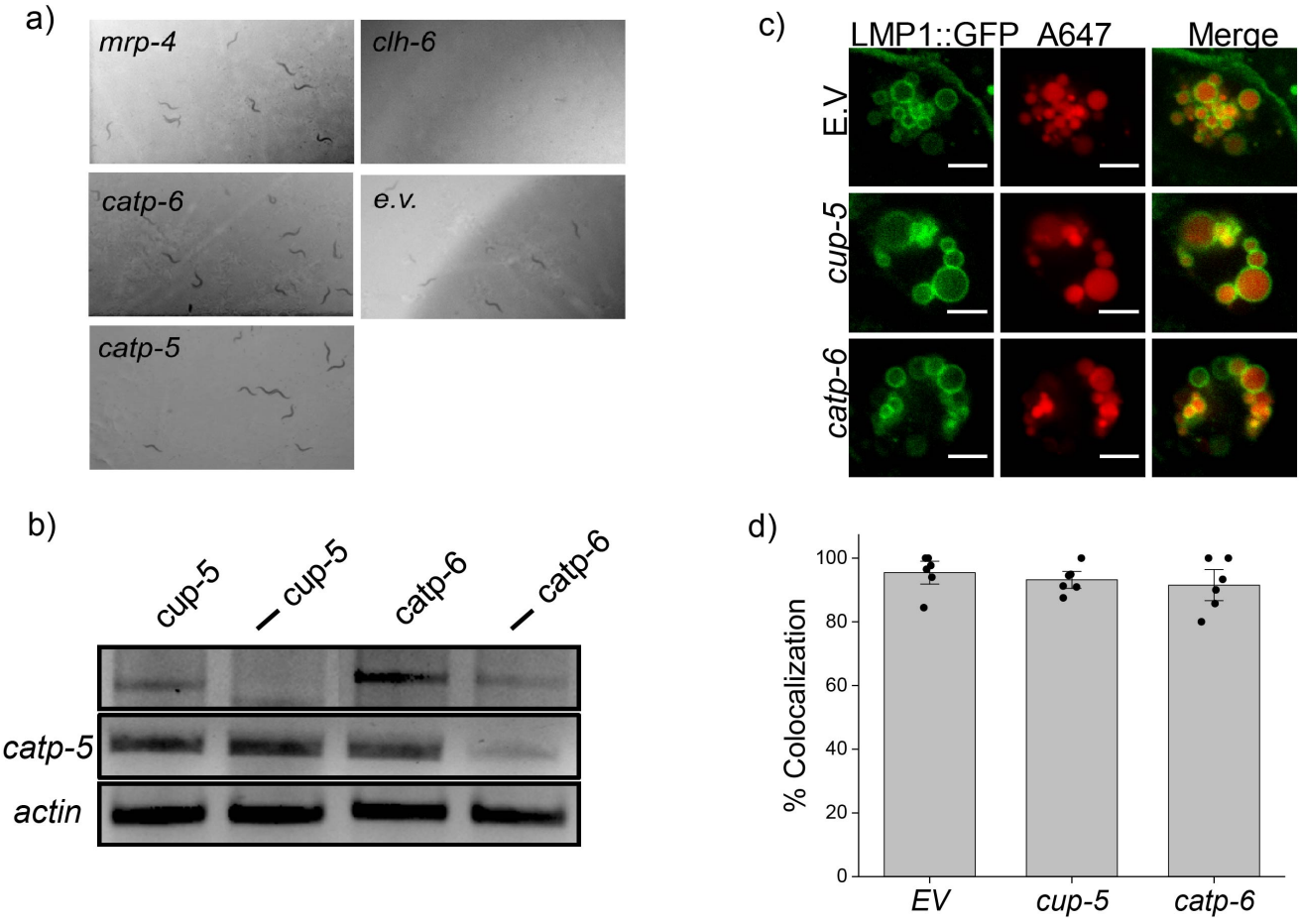
(a-d) D/A ratios of *CalipHluor*$_{Ly}$ as a function of pH clamped at different amounts of added [Ca^{2+}]. (e-h) Normalized O/R ratios of *CalipHluor*$_{Ly}$ as a function of free [Ca^{2+}] clamped at different pH points. For in vivo n = 10 worms; 15 cells and 50 endosomes were quantified; in vitro n = 2. Error bar represents mean $\pm$ s.e.m.



Supplementary Figure 5

Endocytic trafficking of *CalipHluor*$_{A647}$ in coelomocytes.

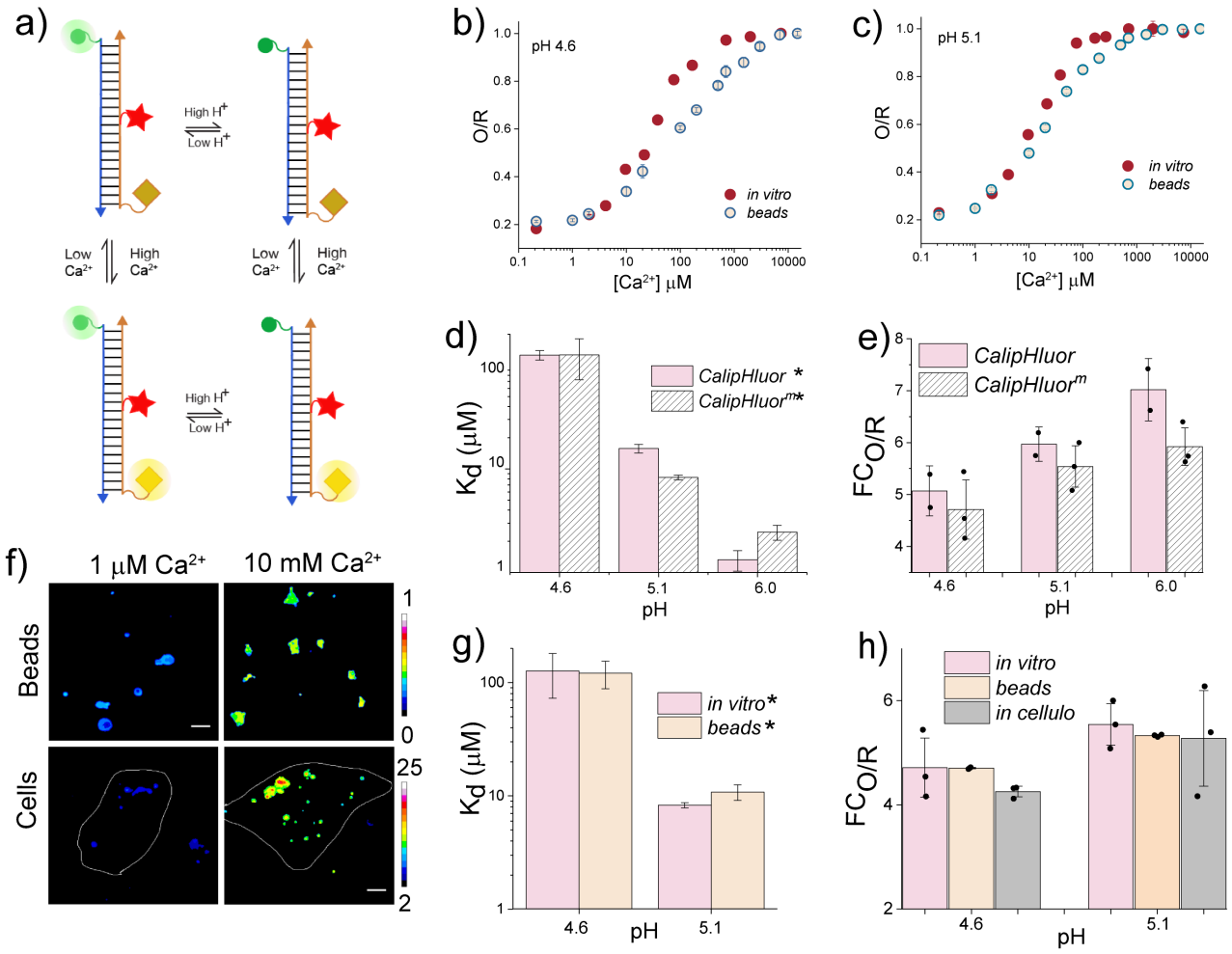
(a-c) Representative confocal images taken 5 min, 17 min, and 60 min following injection of *CalipHluor*$_{A647}$ in worms expressing GFP::RAB-5, GFP::RAB-7 and LMP-1::GFP. Scale bar 5 m. Experiment was performed once. *n* = 10 worms.



Supplementary Figure 6

Lethality rescue and RNAi controls.

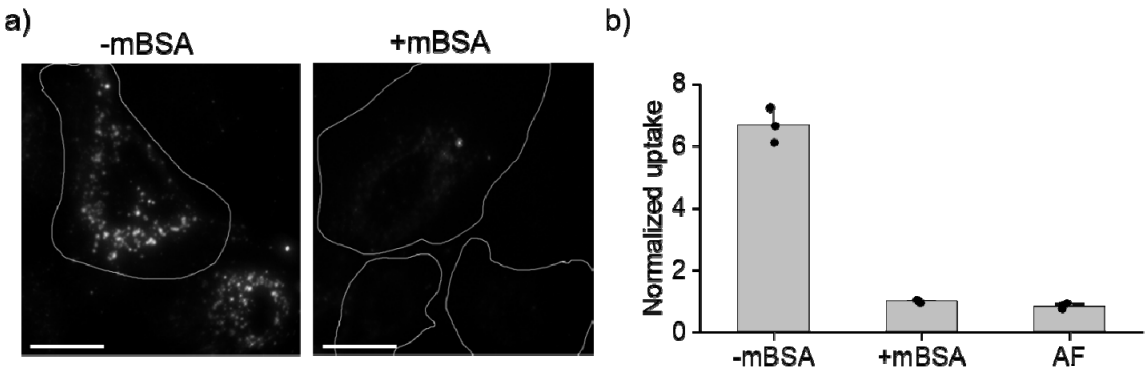
a) *catp-6* rescues lethality of *cup-5 +/-*. Representative images showing the number of progeny of *cup-5 +/-* worms in plates containing RNAi bacteria of *mrp-4* (positive control), *clh-6*, *catp-6*, *catp-5* and *e.v.* (control). Experiments were repeated twice independently with similar results. b) RT-PCR analysis of total RNA isolated from *C. elegans* pre- and post-RNAi. Lanes correspond to PCR-amplified cDNA of the indicated gene product isolated from wild type without RNAi treatment (denoted by gene name) and the corresponding dsRNA-fed worms (denoted as. '— gene name) c) Representative images of worms expressing LMP-1::GFP (green) in the background of various indicated RNAi, which were injected with $CalipHluor_{LyA647}$ (red) and imaged 60 mins post-injection. Scale bar: 5 µm. d) Quantification of colocalization between the $CalipHluor_{LyA647}$ and GFP in LMP-1::GFP worms. n = 10 cells; error bars represent mean ± s.e.m.



Supplementary Figure 7

Characterization of *CalipHluor^{mLy}*.

a) Schematic of the working principle of *CalipHluor^{mLy}*. An Oregon Green based pH sensor (green sphere), an ion insensitive Alexa Fluor 647 (red star) and a Ca^{2+} sensitive fluorophore (Rhod-5F, yellow diamond). Calibration curves comparing in vitro (red) and on beads (orange) calibration at; b) pH 4.6 and c) pH 5.1. Comparison of d) K_d and e) Fold change (FC) in O/R of *CalipHluor^{Ly}* (pink) and *CalipHluor^{mLy}* (black). f) Representative images Comparison of g) Fold change (FC) in O/R and h) K_d of *CalipHluor^{mLy}* in vitro (pink), on beads (orange) and in cellulo (gray). (*n* = 5 cells; 30 endosomes; *n* = 60 beads). Experiments were performed three times independently. *Error is obtained from Hill equation fit. Error bars represent mean ± s.e.m. Scale bar: 10 µm.



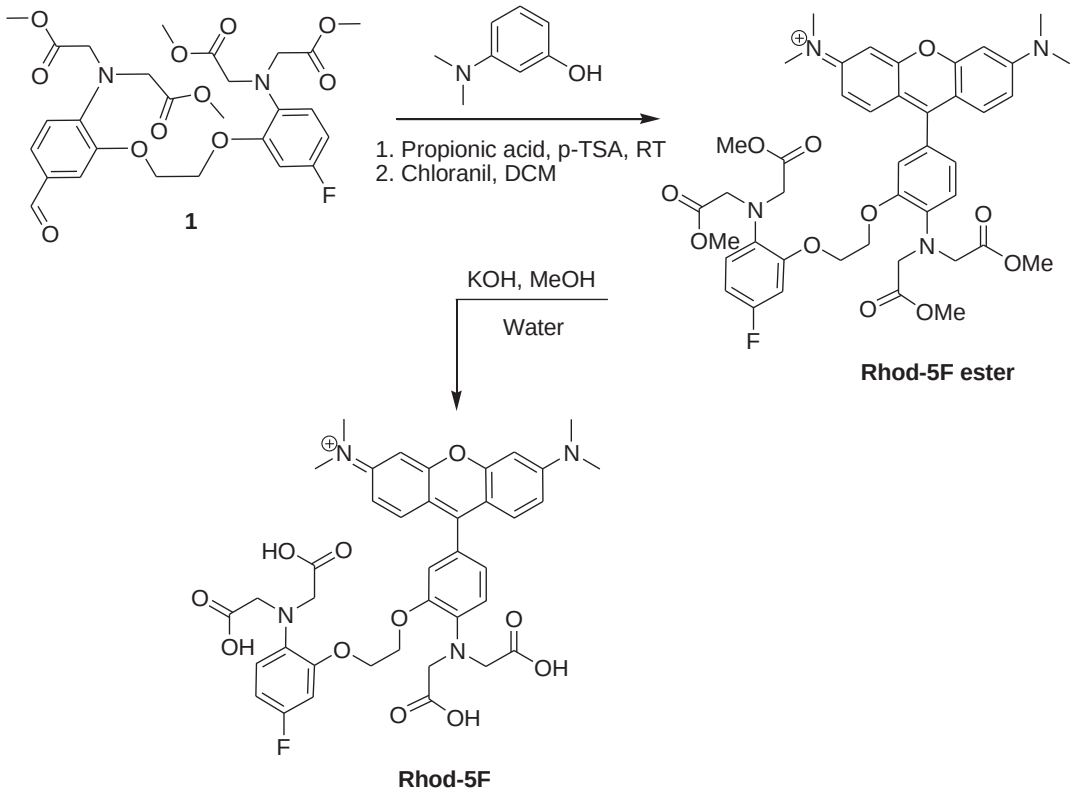
Supplementary Figure 8

Uptake of *CalipHluor*^{mLy} in fibroblast cells.

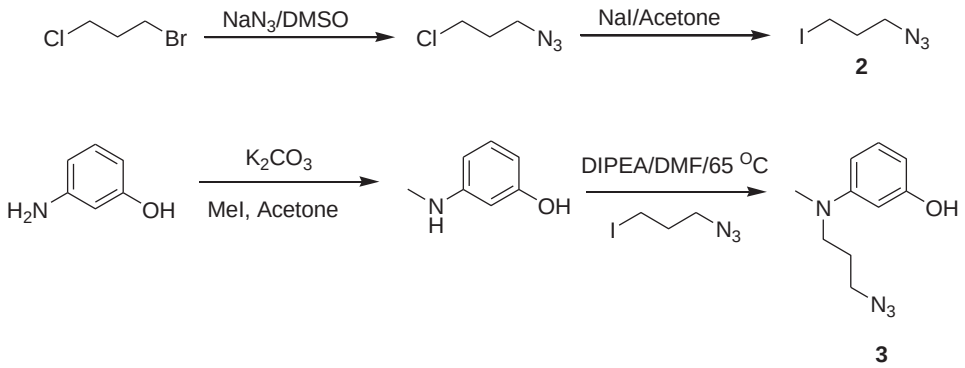
a)-b) *CalipHluor*^{mLy} internalization by primary human skin fibroblasts is competed out by excess maleylated BSA (mBSA, 10 μM), revealing that uptake is mediated by scavenger receptors. Cells are imaged in Alexa Fluor 647 channel. AF: autofluorescence. Scale bar: 10 μm. Experiments were performed in triplicate. Error bars indicate the mean of three independent experiments ± s.e.m. (n = 25 cells).

Contents

Scheme 1: Synthesis of Rhod-5F



Scheme 2: Synthesis of 3-((3-azidopropyl)(methyl)amino)phenol (**3**).



Scheme 3: Synthesis of Rhod-5F-N_3

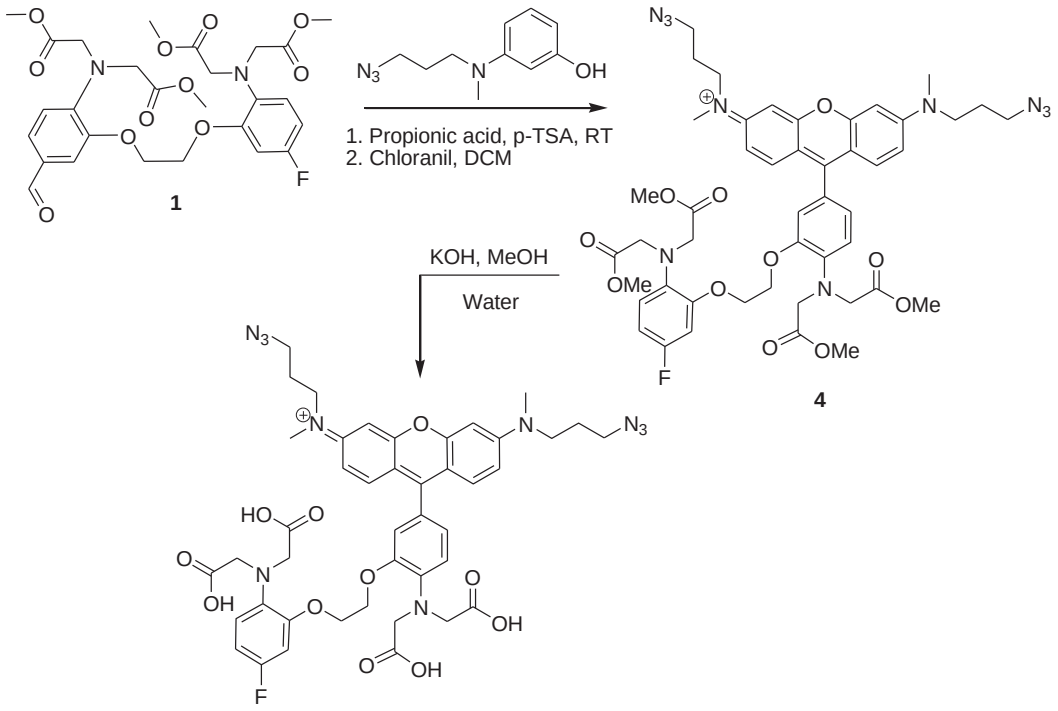


Table S1. Sequences used to form *CalipHluor*, *CalipHluor*$_{Ly}$ and *CalipHluor*^{mLy}. D1 and D2 were used to form *CalipHluor*$_{Ly}$; OG-D1 and D2 were used to form *CalipHluor*^{mLy}. Bromo cytosines in D1 are underlined and highlighted in red. O1-A488, O2-A647 and O3 strands were used to form *CalipHluor*. Complimentary sequences are highlighted in matching colors.

Strand	Sequence information
D1	5'-Alexa 488-CCC CTA ACC CCT AAC CCC TAA CCC CAT ATA TAT CCT AGA ACG ACA GAC AAA CAG TGA GTC-3'
D2	5'-DBCO-GAC TCA CTG TTT GTC TGT CGT TCT AGG ATA /iAlexa 647N/AT ATT TTG TTA TGT GTT ATG TGT TAT-3'
O1-A488	5'-Alexa-488-CCCCAACCCCAATACATTTTACGCCTGGTGCC-3'
O2-A647	5'-CCGACCGCAGGATCCTATAAAACCCCAACCCC-Alexa 647-3'
O3-DBCO	5'-TTA TAG GAT CCT GCG GTC GG/iDBCON/ GGC ACC AGG CGT AAA ATG TA-3'
OG-D1	5'-Oregon Green-AT AAC ACA TAA CAC ATA ACA AAA TAT ATA TCC TAG AAC GAC AGA CAA ACA GTG AGT C-3'

Table S2: Amount of free [Ca^{2+}] in clamping buffer at pH 5.5 was calculated using Maxchelator software.

Added calcium (μM)	Amount calcium added (μL)	Concentration of calcium added	Free [Ca^{2+}] (μM) in 50 μL
0	0	0	0
1	1	50 μM	3.89 E-2
2	2	50 μM	7.80 E-2
10	1	0.5 mM	3.89 E-1
20	2	0.5 mM	7.80 E-1
50	1	2.5 mM	1.9
100	2	2.5 mM	3.9
200	1	10 mM	7.9
500	1	25 mM	20.4
1E3	1	50 mM	43.1
2E3	2	50 mM	96.3
5E3	1	250 mM	360.3
10E3	2	250 mM	1.86 E3
20E3	2	500 mM	10.4 E03

Table S3: Mean pH and free [Ca^{2+}] in EE, LE and Ly of wild type (N2) worms, lysosomes of *catp-6*, *cup-5* +/- and *catp-6* RNAi in *cup-5* +/- worms using *CalipHluor*$_{Ly}$.

Worm	pH	Free [Ca^{2+}] (μM)
EE of N2	6.46 ± 0.07	0.3 ± 0.1
LE of N2	5.95 ± 0.02	0.3 ± 0.1
Ly of N2	5.30 ± 0.02	11 ± 0.8
Ly of *catp-6*	5.47 ± 0.03	1.6 ± 0.4
Ly of *cup-5* +/-	5.15 ± 0.01	40 ± 1.5
Ly of CATP-6 RNAi in *cup-5* +/-	5.50 ± 0.10	16 ± 4.9

Early endosome (EE), Late endosome (LE) and Lysosomes (Ly)

For all experiments n = 15 cells, 50 endosomes; data represent the mean ± s.e.m. Experiments were repeated thrice independently with similar results.