pH-Lemon, a fluorescent protein-based pH reporter for acidic compartments

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The supplementary information includes:

Material & Methods:
Cloning strategies for the generation of pH-Lemon constructs
Cell culture and transfection of HEK-293 cells for FLIM imaging
Calibration of pH-Lemon – GPI (Formula)

Tables, Figures & Movies:
Table S-1: Primers used to generate different pH-Lemon constructs.
Table S-2: Targeting sequences for generating differently targeted pH-Lemon constructs.
Figure S-1: Characterization of mTurquoise2, EYFP and their fusion constructs.
Figure S-2: Using fluorescence lifetime imaging microscopy (FLIM) for characterization of pH-Lemon in vitro and in situ.
Figure S-3: Visualization of pH-Lemon using array confocal laser scanning microscopy (ACSLM).
Figure S-4: Fluorescence lifetime imaging microscopy (FLIM) of HEK-293 cells expressing pH-Lemon – GPI.
Figure S-5: Fluorescence over-time imaging of Ca²⁺ and pH transients in HeLa cells.
Figure S-6: Treatment of HeLa cells with the vacuolar-type H⁺-ATPase (V-ATPase) inhibitor bafilomycin-A.
Movie S-1: Real-time imaging of pH-Lemon – GPI in HeLa cells over 10 minutes.
Movie S-2: Elevated cytosolic Ca²⁺ levels due to addition of an IP₃-generating agonist in pH-Lemon – GPI expressing HeLa cells.
Movie S-3: Effects of extracellular, non-permeabilizing buffer change on the vesicular pH of GPI vesicles.
Movie S-4: Addition of sodium azide and ammonium chloride to neutralize acidic vesicles in HeLa cells.

Cloning

Constructs for recombinant expression of pH-Lemon and SyphHer in E.coli were purchased from Gene Universal Inc. (Newark, USA) using pET-28a(+) vector and the flanking restriction enzyme sites Xhol and HindIII, with an internal EcoRI site separating the FPs. Primers for cloning were purchased ThermoFisher Scientific (Vienna, Austria). PCR reactions were performed using Herculase II fusion DNA polymerase (Agilent, Santa Clara, USA). Following the manufacturer’s guidelines for cloning, different pH-Lemon constructs for mammalian expression were generated using pcDNA3.1(−) vector (ThermoFisher Scientific). The correct sequence of respective constructs was confirmed by sequencing (Microsynth, Vienna, Austria). Mitochondrial targeting of pH-Lemon was achieved using an N-terminal tandem dimeric repeat of the COX8 targeting sequence. Cytosolic targeting of pH-Lemon was observed after addition of the lysine rich motif LPPLERLTL derived from GP41 human immunodeficiency virus, using HindIII-Stop-NES-EYFP_rev and EcoRI-EYFP_for Primers. Targeting pH-Lemon to the endoplasmic reticulum was achieved using the N-terminal targeting sequence of calreticulin (CaR) and the ER-retention sequence KDEL at the C-terminal end. Primers used for generation of ER targeted pH-Lemon comprised Nhel-CaR-mT2_for and HindIII-Stop-KDEL_rev. Localization of pH-Lemon at the outer mitochondrial membrane was achieved by attaching pH-Lemon to the N-terminal end of OMP25. pH-Lemon was localized to autophago(lyso)somes using LC3B sequence at the C-terminus of the sensor. Therefore, LC3B was cloned from HeLa cell cDNA using EcoRI-Lc3B_for and Xhol-Stop-Lc3B_rev primers and cloned into pcDNA3.1(+) vector (ThermoFisher). Subsequently, mTurquoise2 and EYFP were cloned into the vector using the primers Nhel-mT2_for, BamHI-Linker-mT2_rev, BamHI-EYFP_for and EcoRI-EYFP_rev. Finally, pH-Lemon GPl was generated using the membrane leading sequence and the glycosylation sequence of cadherin 13 at the N- and C-terminus, respectively, using sequentially the primers MLS1-XhoI-mT2_for and Nhel-MLS2_for, both in combination with EcoRI-Linker-mT2_rev. EYFP of pH-Lemon GPl was amplified using GPl-Kpn1-EYFP_rev and HindIII-Stop-GPl2_rev, both in combination with EcoRI-EYFP followed by ligation of GPl-mTurquoise2 PCR product into pcDNA3.1(−) vector. A detailed list of primer sequences used for cloning is presented in Supplementary Table 1.

Cell culture and transfection of HEK-293 cells for FLIM imaging

2 µg of sensor-encoding vector were introduced into exponentially growing human embryonic kidney cells (HEK 293, from ECACC, Sigma-Aldrich, Merck, Darmstadt, Germany) by a modified calcium-phosphate method. Cells were cultivated in MEM+GlutamaxTM, 10% (v/v) fetal calf serum, 1% (v/v) antibiotics/antimycotics, and 1% (v/v) non-essential amino acids (purchased from Gibco/Thermo Fisher Scientific, Darmstadt, Germany) in an humidified incubator at 37°C and 5% (v/v) CO2. Prior to measurements, cells were trypsinized and seeded on poly-L-lysine coated (0.1 mg/ml) coverslips.

Calibration of pH-Lemon - GPl

After perfusion of HeLa cells with extracellular buffers with pH 10.0 and pH 4.0, which were non-permeabilizing, the fluorescence intensities of mTurquoise2 and EYFP on the cell surface were measured. By using the formula below, the pH could be calculated.

\[
pH = K_D \times \frac{Ratio - Ratio_{min}}{Ratio_{max} - Ratio}
\]

Formula 1: Calculation of pH values using ratio values.
Table S-1: Primers used to generate pH-Lemon constructs

| Primer                          | Sequence                                                                 |
|--------------------------------|--------------------------------------------------------------------------|
| HindIII-Stop-NES-EYFP_rev      | TTTAAGCTTAGACGTCATCAGTTCTAATGGAGGCAACTTGTACAGCTCGTCCATGC                |
| EcoRI-EYFP_for                 | AAAGAATTCATGGTGAGCAAAGGGCA                                               |
| Nhel-CaR-mT2_for               | GCTAGCATGCTGTCGGCGTCCCCCTGCTGCTGGGCTGCTGCTGGGCAGGCGCGAGCACGAG            |
| HindIII-Stop-KDEL_rev          | TGGTAAAGCTTACAGCTCGTCCCTGAGCTACAGCTCGTCCATGC                             |
| Nhel-mT2_for                   | AAAGCTAGCTATGGTGAGCAAGGGCA                                               |
| BamHI-Linker-mT2_rev           | AGCGAGATCCCGCTACGGCCTTACGCTCGTCCATGC                                    |
| EcoRI-Lc3b_for                 | AAGGAATTCATGCCGTCGAGAGAAGACC                                              |
| XhoI-Stop-Lc3b_rev             | AGAATTCATGCCGTCGAGAGAAGACC                                                |
| BamHI-EYFP_for                 | AAAGAATTCATGCCGTCGAGAGAAGACC                                              |
| EcoRI-EYFP_rev                 | TTTGAAATTCCTTGATACGCTCGTCCATGC                                           |
| GPI1-XhoI-mT2_for              | CTGGTGCGTTCTCTGTGCCAGATGGCGTCTGCTGCTGAGATCCCTGCGATAGTTGAGCAAAAGGGCAAG   |
| Nhel-GPI2_for                  | AGATCTAGAATGCAGCGGAGAACTCCCGCTGTCGTTCTGCGTGATGCTGCATG                   |
| EcoRI-Linker-mT2_rev           | AGCGGAGATCCCGCTACGGCCTTACGCTCGTCCATGC                                    |
| GPI1-KpnI-EYFP_rev             | CTGGTGCGTTCTCTGTGCCAGATGGCGTCTGCTGCTGAGATCCCTGCGATAGTTGAGCAAAAGGGCAAG   |
| HindIII-Stop-GPI2_rev          | AGCAAGCTTACAGAAGCTGAAGGGGCTGAGGCCAGAAGCCAGCTGAGACGAGAAGGGCGAGCTGAGAGAAG |
| **Linker** | **GGTGGAGGCGGTAGC** |
| **Mitochondrial targeting sequence** | **ATGTCTGTTCTGACTCTCCTGTCCTCGGCGGCTCACAAGTTCCCGCAAGAGGACGCTCTCACCCGAGAGGGGACATGGGACCGGCTGCTCACTAGCTGCGGGGCTGACCAGCAGCCTAGGCGGGGCTGCAGGTCGCTGCAGGCTCCAGGGAAGCATCCAGTCTCTCGGGCATCCAAAGATCCACCAGCTGCCAG | **Mitochondrial targeting sequence** |
| **Dihydrofolate reductase leading sequence** | **ATGCAGCCGAGAACTCCGCTCGTTCTGCGTTCTCCTGTCCCAGGTGCTGCAGCCTAACATCTGCAGGATCC** | **Dihydrofolate reductase leading sequence** |
| **Polypeptide (C-terminal) for GPI-construct** | **GACTGCAACGCGGAGAGGAGGCTCAGGCTCCCTAGCTCGCTGCAGCAGCGCCTCTCCAGCTTAGCTTGTGCT** | **Polypeptide (C-terminal) for GPI-construct** |
| **ER targeting sequence** | **ATGCTGTCCCCGTCGCCCTGCGCTGCGCTGCTGGGCCCTGGGCACGCCGCC** | **ER targeting sequence** |
| **ER retention site** | **AAGGACGAGCTG** | **ER retention site** |
| **Outer mitochondrial membrane targeting sequence** | **CGAGGCGAGGAGAGCCGAGTGGAGTCTCTGTAGCAGCTCGGTGCTGCTGCCAG** | **Outer mitochondrial membrane targeting sequence** |
| **Nuclear exclusion sequence** | **TTGCCCTCCATTAGAAGGATGAAGTTGC** | **Nuclear exclusion sequence** |
| **LC3B** | **ATGCCGTCGGAGAAGACCTCAACGACAGGCGACCTCAGGAACAAGAAGTA GAAAGATGACGACTTATTGAGAGCAGCATCCAAACGATCCCCTGGTGATAA TAGAACCAGATAAGGTGAGAAGCAGCTCAGCTTGCTTGGATAAAAAAATGTT CATTGTACCTGAGACATCCATGAGCAGTGAGTGACATCGATCAGGAAATGAGG CGCTACAGCTATCAGCTAAATCGAGGCTTCCTCCTCTCGGTGTGGTAGACGACACA GCAGTGGTCAGCAGTCCTCAGGCCACAGCAATCTACAGAGGTTGAGTGGAGGAGAAGA GAATGGATTCCTGTACATGGTGCTATGCCTCCCAGGAGCGGGATGAAATTGTCAGTG** | **LC3B** |
Figure S-1. Characterization of mTurquoise2, EYFP and fusion constructs of both fluorescent protein (FPs). (a) Impact of pH on the fluorescence intensities of mTurquoise2 and EYFP. The two FPs were separately expressed in the cytosol of HeLa cells and the fluorescence intensities in different pH-environments were measured upon cell permeabilization using nigericin and monensin. Normalized fluorescence intensity of each cell at different pH is represented, n=3 independent experiments for EYFP, n=4 independent experiments for mTurquoise2. (b) Concentration response curve (CRC) of Cyto-pH-Lemon expressed in HeLa cells. The response of each cell expressing pH-Lemon to different pH-values is represented (n=3 independent experiments, 24 cells were analysed). Cells were permeabilized using nigericin and monensin. (c) CRC of purified pH-Lemon at different temperatures in vitro (n=3 ± SD). (d) Scheme of N-terminal EYFP and C-terminal mTurquoise2 construct. The flexible linker between the FPs is indicated in grey. (e) In vitro FRET-emission spectra of EYFP-mTurquoise2 in response to different pH values. (f) Corresponding concentration response curve (CRC) of the construct with an estimated EC50 of 6.2 (6.04-6.36). Data represents average ± SD, n=3. (g) Schematic drawing of a triple FP-construct between an EYFP flanked with one mTurquoise2 on each side. Flexible linkers between the two FPs are indicated in grey. (h) FRET-emission spectra of the triple FP-construct at various pH values in vitro. (i) In vitro emission spectra of mTurquoise2-EYFP-mTurquoise2 excited separately at 413 nm and 480 nm. Emissions were collected from 450-510 nm for mTurquoise2 and 510-580 nm for EYFP, respectively.
Using fluorescence lifetime imaging microscopy (FLIM) for characterization of pH-Lemon \textit{in vitro} and \textit{in situ}. (a) FLIM of recombinant pH-Lemon at pH 7.4 (blue trace) and pH 4.03 (red trace). Fluorescence decays are presented at $\lambda_{\text{exc}}=440$ and $\lambda_{\text{obs}}=475$ nm, respectively. (b) Gauss-curve fitted histograms of fluorescence lifetimes of pH-Lemon expressed in HEK293 cells at pH 7.01, pH 6.05 and pH 4.03. For permeabilization, cells were treated with nigericin and monensin. Fluorescence lifetimes are $2.477 \text{ ns} \pm 0.09948 \text{ ns}$ for pH 7.01, $2.818 \text{ ns} \pm 0.1501 \text{ ns}$ for pH 6.05, and $3.686 \text{ ns} \pm 0.117 \text{ ns}$ for pH 4.03. (c) Intensity image of HEK-293 cells expressing pH-Lemon – GPI at a pH of 4.0. (d) Intensity image of HEK-293 cells expressing pH-Lemon – GPI at a pH of 6.0. (e) Intensity image of HEK-293 cells expressing pH-Lemon – GPI at a pH of 7.0. (f) Respective FLIM image of HEK-293 cells at a pH of 4.0. (g) Respective FLIM image of HEK-293 cells at a pH of 6.0. (h) Respective FLIM image of HEK-293 cells at a pH of 7.0. Scale bars represent 5 $\mu$m.
Figure S-3. Visualization of pH-Lemon using array confocal laser scanning microscopy (ACLSM). (a) Pseudo-colored ratio image of a HeLa cell expressing outer mitochondrial membrane (OMM) pH-Lemon. pH-Lemon was fused with OMP25 for localization at the outer mitochondrial membrane (OMM). Scale bar and ratio scale are demonstrated in (b). (b) Pseudo-colored ratio image of a HeLa cell expressing cytosolic pH-Lemon (Cyto – pH-Lemon) generated by fusing the sensor to a nuclear exporting sequence (NES). Scale bar represents 10 µm. (c) Fluorescence images of mCherry-Golgi-7 (red, left panel) and mTurquoise2 of pH-Lemon – GPI (cyan, middle panel) are demonstrated. The overlay of the two images is shown in the right panel. Cells were fixed with Paraformaldehyde (PFA) for analysis. Scale bar represents 10 µm. (d) Pseudo-colored ratio image of pH-Lemon - GPI expressed in INS-1 cells. Scale bar represents 5 µm. (e) Pseudo-colored ratio image of pH-Lemon – GPI expressed in HEK-293 cells. Scale bar represents 5 µm. (f) Correlation analysis of summarized fluorescence intensities (mTurquoise2+EYFP fluorescence) and the respective ratio signal (EYFP/mTurquoise2) of the same fluorescence signals of single vesicles in HeLa cells. For analysis the same vesicles as demonstrated in Figure 3f, 3g were used. (g) Fluorescence images of HeLa cells expressing pH-Lemon – GPI. mTurquoise2 and EYFP fluorescence images and the pseudo-colored ratio images (mTurquoise2/EYFP) are demonstrated as moderate (upper panels) or high zoom (lower panels). The same cells are shown before (left images) and after treatment with NaN₃ and NH₄Cl for 5 minutes (right images) to neutralize the acidic vesicles (left upper panels, displayed in red). Scale bar of upper panels indicates 10 µm, the bar in the zoomed images represents 2 µm.
Figure S-4. Fluorescence lifetime imaging microscopy (FLIM) of HEK-293 cells expressing pH-Lemon – GPI. Representative fluorescence intensity (a - d) and FLIM images (e - h) of HEK-293 cells expressing pH-Lemon - GPI. Scale bars represent 5 µm.
Figure S-5. Fluorescence over time imaging of Ca\(^{2+}\) and pH transients in HeLa cells. (a) Representative single cell response of a HeLa cell loaded with Fura-2 for intracellular Ca\(^{2+}\) measurement (n=5 independent experiments, all cells responded as demonstrated). 100 µM ATP were administrated at the indicated time. (b) Left panel demonstrates a representative single cell response of a vesicle region as indicated in the right images (region of interest (ROI), white dashed line) of a HeLa cell expressing pH-Lemon – GPI upon application of different extracellular pH values (n= 5, 10 cells were analyzed, 10 cells responded as demonstrated). Right panel represents the corresponding pseudo-colored ratio images. Images are shown at an extracellular pH of 7.4 (left image), 10.0 (middle image) and 4.0 (right image). Scale bar represents 10 µm. (c) Time-lapse imaging of HeLa cells loaded with LysoTracker Red DND-99 to stain acidic vesicles. Left panel represents fluorescence over time curve of LysoTracker loaded cells upon transient treatment with NaN\(_3\) and NH\(_4\)Cl for the neutralization of acidic vesicles (n= 3 independent measurements, 45 cells were analyzed). Right panels show representative images of LysoTracker loaded cells at different time-points. Images are shown of cells under basal conditions (left image) during application of NaN\(_3\) and NH\(_4\)Cl (middle image) or after wash-out (right image). Scale bar in the right image represents 10 µm.
Figure S-6: Treatment of HeLa cells with bafilomycin-A increases the vesicular pH and prevents their re-acidification. (a) Schematic illustration of the effect of sodium azide and ammonium chloride, as well as bafilomycin-A on vesicular pH. Vacuolar-type H^+-ATPases (V-ATPases) located in the vesicular membrane generates an acidic vesicular pH by pumping protons into the vesicular lumen upon ATP consumption. Neutralizing agents like NaN₃ and NH₄Cl diffuse into the vesicular lumen, buffering the high H⁺ concentration by generating HN₃ and NH₄⁺, thus leading to neutralization of the vesicle. Treatment of cells with bafilomycin-A inhibits the V-ATPases, preventing H⁺ import. (b) HeLa cells treated with 0.5 µM bafilomycin-A for 70 minutes at 37°C (Baf-A) showed an increased average pH in the vesicular region compared to untreated DMSO cells (control). Data represents average ± SD, n=8 for both conditions, ***p<0.0001 using unpaired t-test. (c) pH over-time course of HeLa cells expressing pH-Lemon – GPI, either incubated with 0.5µM Baf-A (blue line) or with the same concentration of DMSO (control, grey line) for 70 minutes at 37°C. Buffers were exchanged using a gravity-based perfusion system. Vesicles were neutralized using a "2CA"-buffer containing NaN₃ and NH₄Cl, pH=9.0. Data shows representative single cell responses. (d) Representative pseudo-colored ratio-images of HeLa cells expressing pH-Lemon – GPI after incubation with DMSO or Baf-A using the same protocol as shown in panel c. Region of interest (ROI) is indicated as a dashed white line. Scale bar represents 10 µm. (e) Average pH values measured in the ROIs of DMSO treated control HeLa cells expressing pH-Lemon – GPI after incubation with DMSO or Baf-A using the same protocol as shown in panel c. Region of interest (ROI) is indicated as a dashed white line. Scale bar represents 10 µm. (f) Average pH measured in the ROIs of Baf-A treated pH-Lemon – GPI expressing HeLa cells at basal and neutralized conditions and after wash-out, according to the protocol demonstrated in (c) and (d). Data represent average ± SD, n=8, *p<0.05, ***p<0.0001 using one-way ANOVA with Tukey’s post-hoc test.
**Movie S-1:**

Pseudo-colored ratio – time-lapse movie of HeLa cells expressing pH-Lemon – GPI in the presence of a physiological extracellular buffer with pH 7.4. Images were acquired every 3 sec for a duration of 10 minutes.

**Movie S-2:**

Pseudo-colored ratio – time-lapse movie of HeLa cells expressing pH-Lemon – GPI upon intracellular Ca\(^{2+}\) mobilization using the IP\(_3\)-generating agonist ATP. At time point = 8:00 minutes extracellular Ca\(^{2+}\) was replaced by 100 µM EGTA (indicated as 0 mM Ca\(^{2+}\) in the video) using a gravity based perfusion system. Images of cells were acquired every 3 seconds.

**Movie S-3:**

Movie shows HeLa cells expressing pH-Lemon – GPI as pseudo-colored ratio images. After 2 minutes in the presence of a physiological buffer with pH 7.4, extracellular buffer was exchanged for a neutralizing buffer containing NaN\(_3\) + NH\(_4\)Cl (pH=9.0) via a gravity based perfusion system. After vesicle neutralization, the mixture was again exchanged for a physiological buffer, resulting in the re-acidification of the intracellular vesicles.

**Movie S-4:**

HeLa cells expressing pH-Lemon – GPI were perfused with physiological buffers of pH 7.4, followed by a buffer with pH 10.0 and pH 4.0 without permeabilizing the cell membrane. Cells were imaged every 3 seconds.

References
(i) Chen, C.; Okayama, H. High-efficiency transformation of mammalian cells by plasmid DNA. Molecular and cellular biology 1987, 7, 2745-2752.