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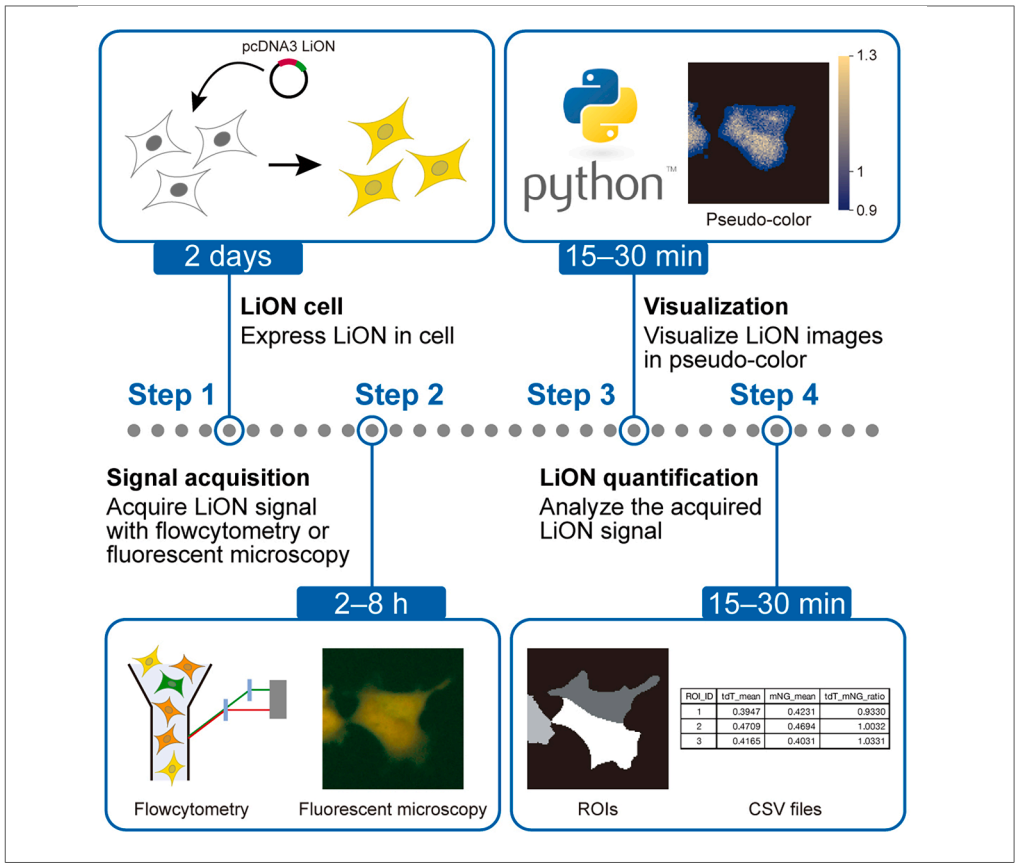
Protocol to assess intracellular labile iron and oxygen at single-cell resolution using a genetically encoded ratiometric reporter



Here, we present a protocol to acquire, visualize, and quantify intracellular labile iron and oxygen signals at single-cell resolution using LiON (labile iron and oxygen notifier), a genetically encoded ratiometric reporter. We describe steps for fluorescence microscopy and flow cytometry acquisition, ratio-map visualization, and region of interest (ROI)-based quantification. We then detail procedures for single-cell time-lapse tracing.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Ayato Maeda,
Toshiro Moroishi

contact@med-sci.jp (A.M.)
moroishi.toshiro@tmd.ac.jp (T.M.)

Highlights

Guidance on the usage of labile iron and oxygen notifier (LiON)

Guidance on ratio image generation and ROI-based fluorescence quantification

Instructions for cell segmentation and single-cell tracking

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Protocol to assess intracellular labile iron and oxygen at single-cell resolution using a genetically encoded ratiometric reporter

Ayato Maeda[1,2,*] and Toshiro Moroishi[1,3,*]

[1]Division of Cellular Dynamics, Medical Research Laboratory, Institute of Integrated Research, Institute of Science Tokyo, 1-5-45 Yushima, Tokyo 113-8510, Japan

[2]Technical contact

[3]Lead contact

*Correspondence: contact@med-sci.jp (A.M.), moroishi.toshiro@tmd.ac.jp (T.M.)
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SUMMARY

Here, we present a protocol to acquire, visualize, and quantify intracellular labile iron and oxygen signals at single-cell resolution using LiON (labile iron and oxygen notifier), a genetically encoded ratiometric reporter. We describe steps for fluorescence microscopy and flow cytometry acquisition, ratio-map visualization, and region of interest (ROI)-based quantification. We then detail procedures for single-cell time-lapse tracing.

For complete details on the use and execution of this protocol, please refer to Maeda et al.[1]

BEFORE YOU BEGIN

This protocol describes how to acquire, visualize, and quantify the LiON signal in cultured cells using fluorescence microscopy and flow cytometry. LiON is a genetically encoded ratiometric reporter designed for time-course monitoring of intracellular bioavailable iron and oxygen at single-cell resolution. The LiON cassette consists of the hemerythrin-like (Hr) domain of human F-box and leucine-rich repeat protein 5 (FBXL5) fused to the red fluorescent protein tdTomato (tdT), followed by a self-cleaving P2A peptide and the reference fluorophore mNeonGreen (mNG) (Hr-tdT–P2A–mNG). The P2A sequence enables stoichiometric co-expression of the Hr-tdT sensor and mNG from a single transcript, allowing normalization for expression level and cell-to-cell variability. The LiON construct is cloned into a mammalian expression vector (pcDNA3.1 (+)) under the control of the CMV promoter for transient expression in cultured cells and is available from Addgene (Plasmid #252093), where full sequence information and vector maps can be accessed.

Innovation

LiON repurposes the iron- and oxygen-sensitive Hr to regulate the stability of a fused tdTomato protein and uses a P2A-linked mNeonGreen as an internal normalization control. This design yields a ratiometric readout (tdTomato/mNeonGreen) that is compatible with live imaging, time-course analysis, and flow cytometry, and that captures cell-to-cell heterogeneity in iron/oxygen status.

Institutional permissions (if applicable)

All procedures in the publication presenting data supporting this protocol (Maeda et al.[1]) were approved by the Safety Committee for Experiments Using Genetically Modified Organisms of the Institute of Science Tokyo. All experiments were conducted in accordance with institutional biosafety guidelines under appropriate containment conditions. Individuals intending to replicate

or adapt any part of this protocol are advised to seek approval from their relevant institutional review boards or ethics committees, in accordance with local and national regulations.

Prepare perturbation reagents and instrument settings

Timing: 1–3 h

1. Prepare fresh working solutions of ferric ammonium citrate (FAC; 0–100 μg/mL in culture medium) and deferoxamine (DFO; 0–100 μM in culture medium). Other modulators used in the LiON study include hemin (iron loading), 2,2′-bipyridyl (Bpy; iron chelation), and deferasirox (DFX; iron chelation).

   Note: Culture medium should be prepared according to the specific requirements of the cells being used (e.g., Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum for HEK293A cells).

2. If oxygen perturbation is planned, equilibrate incubators or chambers to 21%, 10%, or 1% O_2 at 37°C with 5% CO_2 before starting.
3. For microscopy, select filter sets/lasers that separately excite and detect tdTomato and mNeonGreen.

   Note: Define exposure/laser power and detector gain to avoid saturation in either channel.

4. For flow cytometry, configure channels that robustly capture tdTomato and mNeonGreen fluorescence; establish compensation if spectral overlap is detected, and define a consistent gating strategy (FSC/SSC, singlets, live cells).

Establish LiON-expressing cells

Timing: 2 days

5. Generate a transient LiON-expressing cell line (e.g., HEK293A) by lipofection.
   a. Seed an appropriate number of cells on 24-well plate (e.g., HEK293A: 2.0×10^4 cells per well)
   b. After 24 h, change the medium and transfect 500 ng of pcDNA3 LiON with Polyjet (SignaGen Laboratories, SL100688) according to the manufacturer's protocol (https://signagen.com/DataSheet/SL100688.pdf).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ferric ammonium citrate (FAC)	Sigma-Aldrich	Cat# F5879
Deferoxamine mesylate (DFO)	abcam	Cat# ab120727
Deferasirox (DFX)	Combi-Blocks	Cat# QA-8243
2,2′-Bipyridyl (Bpy)	Wako	Cat# 042-04241
Chlorohemin (Hemin)	TCI	Cat# H0008
PolyJet	SignaGen Laboratories	Cat# SL100688
Experimental models: Cell lines		
HEK293A	Invitrogen	Cat# R70507

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pcDNA3 LiON	Addgene	Cat#252093
Software and algorithms		
Fiji (ImageJ2, v2.14.0/1.54f)[2]	Schindelin, et al., 2012	https://imagej.net/software/fiji
Python	Python.org	PRID: SCR_008394
FlowJo v10.8.1	BD Biosciences	N/A
Cellpose v3.1.1.2[3]	Stringer et al., 2021	https://www.cellpose.org/
Trackpy v0.7[4]	Allan et al., 2025	https://doi.org/10.5281/zenodo.18203938
Other		
Fluorescent microscope	KEYENCE	BZ-X800
Flow cytometer	Beckman Coulter	CytoFLEX
Hypoxia incubator	ASTEC	Cat# APM-30DR

STEP-BY-STEP METHOD DETAILS

Acquisition of LiON signal

Timing: 2–8 h (depending on treatment duration)

Quantify LiON as the ratio of tdTomato to mNeonGreen fluorescence intensity. (tdT/mNG). Acquire both channels with matched settings across conditions to enable quantitative comparisons.

1. Fluorescence microscopy.
   a. 24 h after transfection, replace medium with pre-warmed culture medium containing vehicle, FAC, DFO, or other iron perturbation reagents.

   Note: Typical dose ranges used for characterization are FAC 0–100 μg/mL and DFO 0–100 μM.

   b. If performing oxygen perturbation, transfer cells to a pre-equilibrated oxygen-controlled environment (21%, 10%, or 1% O_2).
   c. Set microscope acquisition parameters for tdTomato and mNeonGreen (excitation, emission, exposure/laser power, gain).

   Note: Confirm that neither channel is saturated in representative fields.

   d. Acquire paired tdTomato and mNeonGreen images.

   Note: For time-lapse experiments, acquire at fixed intervals (e.g., every 1 h) while maintaining 37°C and 5% CO_2.

   e. Save raw data in a lossless format (e.g., 16-bit TIFF) and record acquisition settings in metadata or a lab notebook.

   ⚠ CRITICAL: Use identical acquisition settings for all experimental groups within an experiment. Changing exposure/gain between groups invalidates direct ratio comparisons.

2. Flow cytometry.
   a. Apply perturbation reagents as above (FAC, DFO, and/or oxygen conditions) in culture plates. Collect cells at the planned time point.
   b. Harvest cells using gentle dissociation with Trypsin-EDTA and neutralize with culture medium.

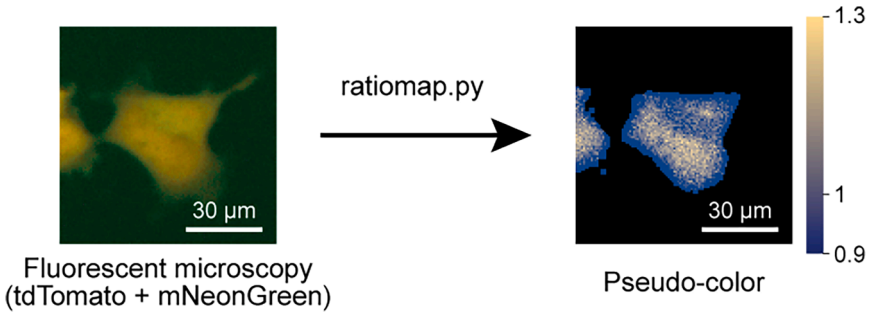

Figure 1. Schematic workflow for converting fluorescence images to pseudo-color ratio maps

Schematic illustration of the procedure used to convert fluorescence images into pseudo-color representations. Raw fluorescence images acquired from tdTomato and mNeonGreen channels (left) are processed using the ratiomap.py script to generate pixel-wise ratio maps, which are subsequently visualized as pseudo-color images (right).

Scale bars, 30 μm.

c. Acquire data: gate on cells by FSC/SSC, then on singlets (FSC-A vs FSC-H or equivalent). Optionally gate out dead cells using a dead cell staining dye such as 4′,6-diamidino-2-phenylindole (DAPI).

d. Record tdTomato and mNeonGreen fluorescence for each event. Using FlowJo, export per-event intensities in csv format for downstream ratio calculation.

Visualization of LiON signal (pseudo-color imaging)

Timing: 15–30 min per dataset

3. Load paired tdTomato and mNeonGreen fluorescence images as TIFF files using a Python-based image processing pipeline (ratiomap.py) (Figure 1).

```
>python ratiomap.py *.tif \
> --mode autorounded --ticks 3 --outdir out \
> --colors "#1D2671, #FFE08A" \
> --mask-mode max --mask-scale 0.9 --mask-dilate 1
```

Note: The script supports multi-page and multi-series TIFF files and automatically resolves full-resolution image planes.

4. Inspect image dimensions, bit depth, photometric interpretation, and samples per pixel upon loading.

Note: Only image pairs with identical spatial dimensions are processed further to ensure valid pixel-by-pixel operations.

5. Suppress background noise and unreliable ratio values by generating an optional mask from one channel.
   a. Generate the mask by performing intensity scaling and thresholding, optionally applying Gaussian blurring, and conducting morphological operations (dilation and erosion).
   b. Hold mask parameters constant across all images within a dataset.

6. Convert both tdTomato and mNeonGreen images to floating-point arrays to allow accurate arithmetic operations and avoid integer truncation.
7. Generate ratio images on a pixel-by-pixel basis by dividing tdTomato intensity values by mNeonGreen intensity values.

   Note: Exclude division by zero and masked pixels from downstream visualization and range calculation.

8. Determine a shared minimum and maximum ratio range across all images within the same batch. Use this global range to standardize visualization and ensure direct comparability between experimental conditions.
9. Convert ratio images into pseudo-colored maps using predefined color palettes or user-defined color.
10. Generate colorbars with fixed tick positions and numeric labels corresponding to the shared ratio range.

Quantification of LiON signal

Timing: 15–30 min per dataset

11. Analyze fluorescence images using a custom Python pipeline (roi_LiON.py).

```
>python roi_LiON *.tif
```

Load paired single-channel tdTomato and mNeonGreen fluorescence images as matched image sets.

Note: Ensure each pair represents the same field of view.

12. Automatically segment cell ROIs using Cellpose with the cyto3 pretrained model.

Note: Perform segmentation on a single reference channel to generate pixel-wise cell masks for each image.

Note: Use the cyto3 model to identify whole-cell cytoplasmic boundaries rather than nuclei, enabling robust extraction of cellular fluorescence signals.

13. Use Cellpose-derived masks as shared ROIs and apply them identically to both tdTomato and mNeonGreen images from the same field.
14. Perform fluorescence quantification for both channels over the same cellular area to eliminate bias due to channel-specific segmentation.
15. Extract pixel intensities separately from the tdTomato and mNeonGreen images within each ROI.
16. Apply background correction uniformly according to the script settings, either by excluding non-ROI pixels or by subtracting a defined background level.

Note: Keep all processing parameters constant across images and experimental conditions.

17. Calculate summary statistics for both channels for each segmented cell, including mean fluorescence intensity and, where applicable, additional metrics such as area or integrated intensity.

Note: Use these values as cell-averaged signals derived from all pixels within each ROI.

18. Calculate the tdTomato/mNeonGreen fluorescence ratio on a per-cell basis using mean intensities obtained from the shared ROI rather than pixel-by-pixel division.

Note: Use this approach to reduce noise amplification and ensure that ratio values reflect whole-cell fluorescence balance.

19. Compile quantitative results from all cells and images into summary tables (CSV format) containing per-cell fluorescence values and ratios.

Note: Use these outputs for downstream statistical analysis and figure generation.

Single-cell trace of the LiON signal (time-lapse)

Timing: 60 min per dataset

20. Perform time-lapse fluorescence imaging using BZ-X800 microscopy equipped with an environmental control chamber that maintains physiological temperature, CO_2 concentration, and humidity.
    a. Acquire time-lapse sequences by sequentially imaging TRITC and GFP channels for tdTomato and mNeonGreen, respectively, at each time point within the same field of view.
    b. Keep imaging parameters, including objective lens, magnification, numerical aperture, exposure time, excitation intensity, binning, and camera settings, constant throughout the entire experiment and across all conditions.
    c. Fix the temporal resolution (frame interval) to enable direct comparison of fluorescence dynamics over time.
    d. Save single-channel tdTomato and mNeonGreen images at every time point for each field of view to generate paired fluorescence image stacks representing the same cells tracked over time.
21. Analyze fluorescence images using a custom Python pipeline (timelapse_tracking.py).

```
>python timelapse_tracking.py time-lapse.tif \

> --axes THWC \

> --save-labels --save-overlay --save-roi-csv
```

22. Automatically define ROIs to enable quantitative single-cell fluorescence analysis using Cellpose.
    a. Perform cell segmentation with the pretrained cyto3 cytoplasmic model optimized for identifying whole-cell boundaries rather than nuclei.
    b. Apply Cellpose to a reference fluorescence channel at each time point to generate pixel-wise segmentation masks corresponding to individual cells and define the cytoplasmic area of each cell at that specific time point.
23. Track individual cells across time-lapse frames after segmentation using a centroid-based tracking algorithm implemented in Python following principles established in the trackpy framework.
    a. Calculate centroid coordinates for each segmented cell at every time point.
    b. Link cells between consecutive frames based on nearest-neighbor association using a defined maximum displacement threshold to account for cell movement.
    c. Assign a consistent cell identifier to the same cell across frames to enable longitudinal tracking throughout the time-lapse sequence.

d. Perform tracking independently of fluorescence intensity and rely solely on spatial information derived from segmentation masks to ensure robust tracking despite temporal changes in fluorescence intensity or cell morphology.

24. Extract pixel fluorescence intensities within each shared ROI.

Note: Handle background fluorescence uniformly by excluding non-ROI pixels or applying a consistent background subtraction strategy, and fix all segmentation, tracking, and background-handling parameters across datasets.

25. Calculate mean fluorescence intensity values separately for tdTomato and mNeonGreen for each cell and time point.
26. Optionally calculate tdTomato/mNeonGreen ratios on a per-cell, per-time-point basis using per-cell mean fluorescence intensities obtained from shared ROIs.

Note: Derive ratio values from ROI-averaged intensities rather than pixel-wise division to enable quantitative tracking of fluorescence intensity changes and reporter ratios within individual cells over time.

27. Aggregate quantitative outputs into tabular formats (CSV files) containing cell identifiers, time points, fluorescence intensities, and calculated ratios.

Note: Use these data for downstream statistical analysis and visualization.

28. Keep all image acquisition parameters and analysis settings constant across experiments to ensure reproducibility.

Note: Use Cellpose-based shared ROIs to allow unbiased comparison of LiON signals within the same cells and across time, which is critical for longitudinal fluorescence analyses.

EXPECTED OUTCOMES

Using this protocol, LiON-expressing cells should show reproducible changes in tdTomato/mNeonGreen ratios in response to iron and oxygen perturbations. In iron-loading conditions (e.g., FAC), tdTomato/mNeonGreen increases; in iron-chelation conditions (e.g., DFO, Bpy, or DFX) or hypoxia (e.g., 1% O_2), the ratio decreases. Flow cytometry should capture broad per-cell distributions of LiON ratios, reflecting heterogeneous iron/oxygen states across the population. Time-lapse microscopy should show gradual ratio changes over hours (for example, an increase within 6 h of FAC treatment and a decrease during DFO treatment), with variable response kinetics among single cells (Figure 2).

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Export quantitative outputs generated by the image analysis pipeline as comma-separated value (CSV) files containing per-cell measurements for each time point, including cell identifiers, time indices, mean tdTomato fluorescence intensity, mean mNeonGreen fluorescence intensity, and calculated tdTomato/mNeonGreen ratios.
2. Perform all statistical analyses using the exported CSV files without further modification of image analysis parameters.

Note: Subject data to quality control procedures prior to statistical testing by excluding cells with incomplete trajectories, segmentation failures, or tracking discontinuities.

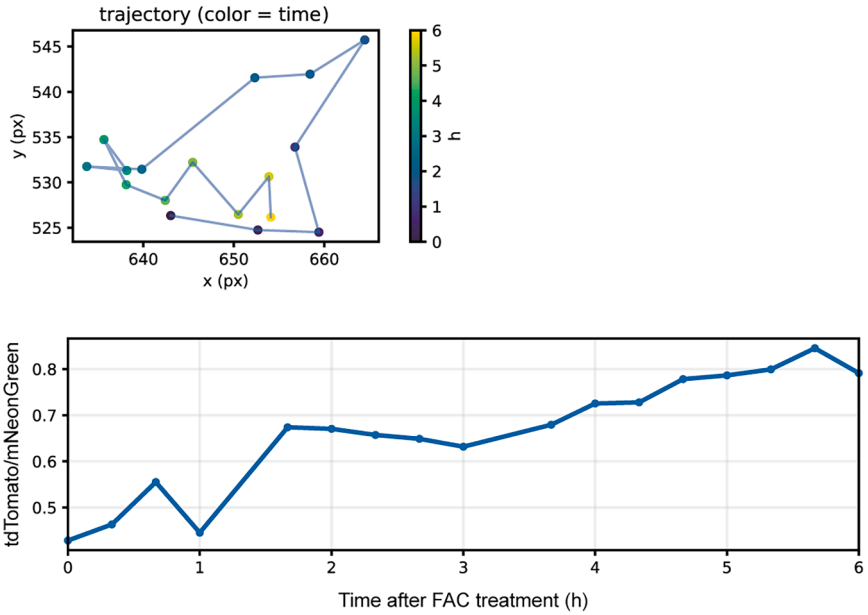

Figure 2. Single-cell trajectory analysis and temporal changes in LiON fluorescence ratio after FAC treatment

Representative two-dimensional migration trajectory of a single cell after FAC treatment.

Each dot represents the centroid position of the cell at an individual time point, and colors indicate elapsed time after FAC addition (0–6 h).

Consecutive positions are connected to visualize the migration path.

Time-course changes in the tdTomato/mNeonGreen fluorescence intensity ratio for the same single cell.

Fluorescence intensities were quantified within the tracked cell region at each time point, and the ratio was plotted as an indicator of intracellular iron/oxygen-related status. FAC treatment induced a gradual increase in the tdTomato/mNeonGreen ratio over time at the single-cell level.

Note: Identify cells exhibiting extreme fluorescence values indicative of segmentation artifacts or cell death using predefined criteria, such as intensity thresholds or interquartile range–based outlier detection, and remove them where appropriate.

3. Conduct analyses at both the single-cell level and the population level according to the experimental question.
   a. Analyze fluorescence intensities and tdTomato/mNeonGreen ratios longitudinally for each tracked cell at the single-cell level to assess temporal dynamics, response kinetics, and intracellular variability over time.
   b. Aggregate per-cell values across cells within the same experimental condition and time point at the population level and calculate summary statistics, including mean, median, standard deviation, and standard error of the mean (SEM), for each group.

Note: The choice of statistical tests, the definition of n (e.g., number of cells or independent experiments), and the distinction between biological and technical replicates should be determined by individual researchers based on their experimental design and data characteristics.

LIMITATIONS

LiON is a dual-input reporter: both iron and oxygen regulate the stability of the Hr-fused tdTomato. Therefore, LiON cannot by itself distinguish whether a reduced ratio reflects decreased iron, decreased oxygen, or both. When testing iron perturbations, keep oxygen constant and include matched vehicle controls.

LiON provides a relative ratiometric readout and is not intended for absolute quantification of intracellular iron.

TROUBLESHOOTING

Problem 1

Low or no LiON signal in live cells (related to step 5 in "before you begin").

Potential solution

Increase the plasmid volume for transduction or change transfection method.

Confirm microscope filter sets and laser lines match the excitation/emission spectra of tdTomato and mNeonGreen.

Reduce laser power and exposure time and minimize repeated imaging of the same field.

Problem 2

Abnormally high or saturated ratio values (related to step 1).

Potential solution

Adjust detector gain and exposure to avoid pixel saturation in both channels (tdTomato and mNeonGreen).

Apply intensity thresholding to exclude pixels or ROIs with extremely low fluorescence before ratio computation.

Problem 3

Large cell-to-cell variability unrelated to experimental conditions (related to step 1).

Potential solution

Exclude dead cells using morphological criteria or viability dyes where appropriate.

Manually inspect and validate automated ROI segmentation results, especially at image borders or in crowded regions.

Problem 4

Poor reproducibility between imaging sessions (related to step 1).

Potential solution

Record and reuse identical microscope settings (laser power, gain, exposure, binning) across experiments.

Include reference samples (e.g., vehicle, FAC, or DFO) in each imaging session to monitor system stability.

Problem 5

tdTomato/mNeonGreen ratio changes not correlated with iron or oxygen manipulation (related to step 1 and 2).

Potential solution

LiON potentially reflects the proteasome activity because Hr-tdTomato degradation is dependent on proteasome.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Toshiro Moroishi (moroishi.toshiro@tmd.ac.jp).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to the technical contact, Ayato Maeda (contact@med-sci.jp).

Materials availability

Plasmids generated in this study are available through Addgene (plasmid #252093, Addgene: pcDNA3 LiON).

Data and code availability

All custom Python scripts are available on GitHub (Github: https://github.com/AYTMED/STAR_PROTOCOLS_LiON) and are archived on Zenodo (Zenodo: https://doi.org/10.5281/zenodo.18203938).

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization, A.M. and T.M.; methodology, A.M. and T.M.; investigation, A.M.; formal analysis, A.M. and T.M.; writing – original draft, A.M. and T.M.; writing – review and editing, A.M. and T.M.; funding acquisition, T.M.; resources, T.M.; supervision, T.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this study, the authors used ChatGPT 5.2 to improve English grammar and text presentation. After using this tool, the authors reviewed and edited the manuscript as needed and take full responsibility for the content of the publication.

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