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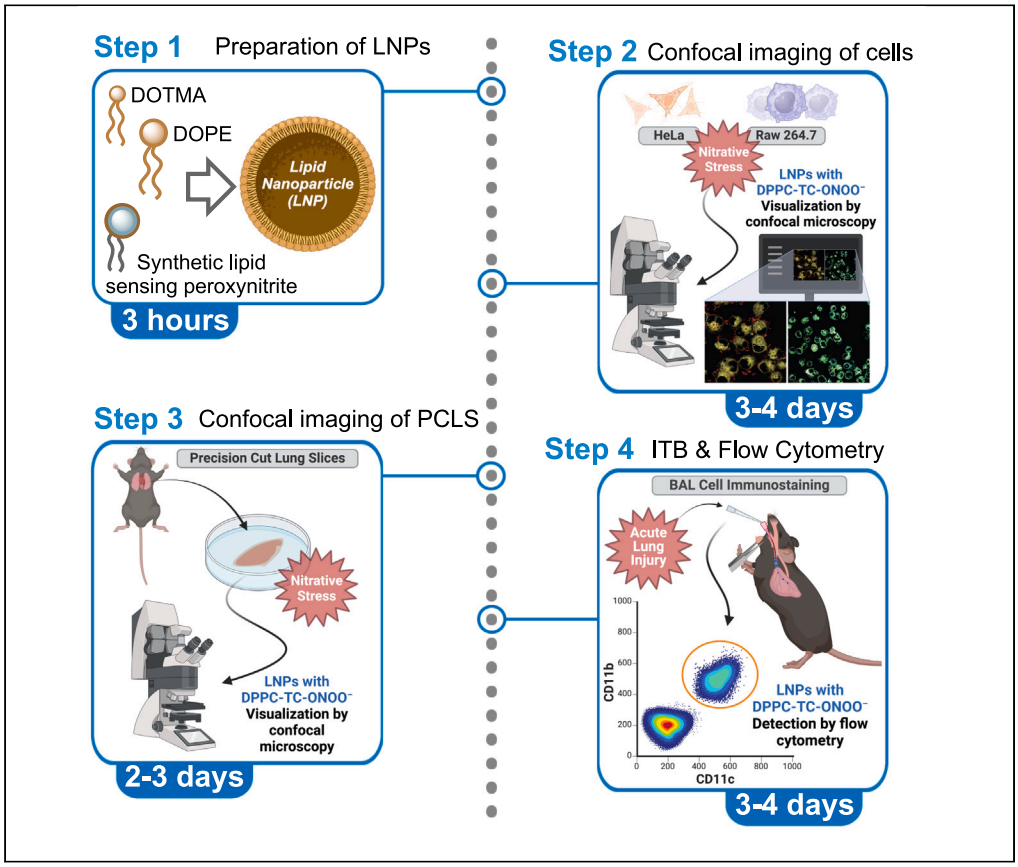
# Protocol for detecting nitrative stress in biological lipid membranes in murine cells and tissues




Detection of nitrative stress is crucial to understanding redox signaling and pathophysiology. Dysregulated nitrative stress, which generates high levels of peroxynitrite, can damage lipid membranes and cause activation of proinflammatory pathways associated with pulmonary complications. Here, we present a protocol for implementing a peroxynitrite-sensing phospholipid to investigate nitrative stress in murine cells and lung tissue. We detail procedures for sensing $ONOO^-$ in stimulated cells, both *ex vivo* and *in vivo*, using murine models of acute lung injury (ALI).

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

Tushar Aggarwal, Alyssa Bellomo, Emily R. Stevenson, Julia Herbert, Debra L. Laskin, Andrew J. Gow, Enver Cagri Izgu

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## Highlights

Probing peroxynitrite in biological lipid environments using DPPC-TC-$ONOO^-$, a synthetic lipid

Nitrative stress was detected by confocal fluorescence microscopy and flow cytometry

Steps for fluorescent staining of organelles in cultured murine cells

Steps for fluorescent detection of peroxynitrite in murine tissue

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# Protocol for detecting nitrative stress in biological lipid membranes in murine cells and tissues

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## SUMMARY

**Detection of nitrative stress is crucial to understanding redox signaling and pathophysiology. Dysregulated nitrative stress, which generates high levels of peroxynitrite, can damage lipid membranes and cause activation of proinflammatory pathways associated with pulmonary complications. Here, we present a protocol for implementing a peroxynitrite-sensing phospholipid to investigate nitrative stress in murine cells and lung tissue. We detail procedures for sensing $ONOO^-$ in stimulated cells, both *ex vivo* and *in vivo*, using murine models of acute lung injury (ALI).**

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

## BEFORE YOU BEGIN

Prior to performing the described biological procedures, four chemical compounds should be available at hand: (1) the peroxynitrite-sensing phospholipid (**DPPC-TC-$ONOO^-$**) (Figure 1A), (2) its fluorescently activated form that will serve as positive control (**DPPC-TC**) (Figure 1B), (3) 1,2-di-*O*-octadecenyl-3-trimethylammonium propane (**DOTMA**) (Figure 1C), and (4) 1,2-dioleoyl-*sn*-glycero-3 phosphoethanolamine (**DOPE**) (Figure 1D). **DOTMA** and **DOPE** serve as the major lipid components of lipid nanoparticles (LNPs). Step-by-step productions of **DPPC-TC-$ONOO^-$** and **DPPC-TC** are outlined by Wang and Dresel et al.[2]

We recommend researchers to use these compounds with special care to maintain their chemical integrity and function. Specifically, it is essential to keep the lipids dry (without solvent) and at ultra-low temperatures (e.g., −80°C) to minimize degradation (e.g., hydrolysis or side reactions from trace impurities) prior to use. Store the lipids containing a chromophore/fluorogenic motif, namely **DPPC-TC-$ONOO^-$** and **DPPC-TC**, in amber vials to protect them from light and minimize photobleaching. Aliquot the lipids into multiple batches to avoid multiple reusage and potential warming-cooling cycles. It is expected that the implementation of **DPPC-TC-$ONOO^-$** is not limited to murine cells and tissues. In accordance, procedural adaptions and optimizations to other biological models may be required.

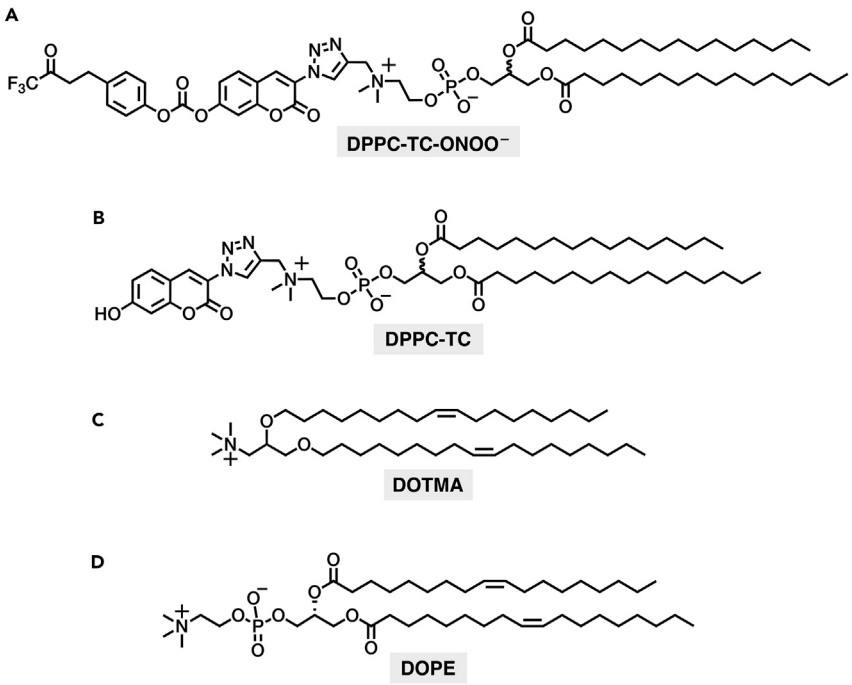


**Figure 1. Structures of the primary lipids used in the described methods**

(A) DPPC-TC-ONOO⁻: [1,2-Dipalmitoyl-*rac*-glycero-3-phosphocholine]–[triazole-coumarin]–[peroxynitrite sensor]; (B) DPPC-TC: [1,2-Dipalmitoyl-*rac*-glycero-3-phosphocholine]–[triazole-coumarin]; (C) DOTMA: 1,2-di-*O*-octadecenyl-3-trimethylammonium propane; and (D) DOPE: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

This protocol reports the prevalence of nitrative stress due to $ONOO^-$ and presumably other nitrative species. The scope of redox-active nitrogen and oxygen species is broad, and readers are therefore encouraged to refer to recent articles for a collective understanding of these species.[3,4]

### Institutional permission

This study involves the utilization of mice as experimental subjects. All animal experiments and procedures strictly adhere to the Rutgers University Institutional Animal Care and Use Committee (IACUC) and following the guidelines set by National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| FITC anti-mouse/human CD11b antibody (1:100 dilution) | BioLegend | Cat # 101206 |
| Alexa Fluor 647 anti-mouse CD11c antibody (1:100 dilution) | BioLegend | Cat # 117312 |
| Alexa Fluor 700 anti-mouse CD45 antibody (1:100 dilution) | BioLegend | Cat # 103128 |
| TruStainFcXanti-mouse CD16/32 (1:100 dilution) | BioLegend | Cat # 101320 |
| Chemicals, peptides, and recombinant proteins | | |
| Bleomycin | Santa Cruz Biotechnology | Cat # sc-200134B |
| Nitrogen mustard (mechlorethamine hydrochloride) | Sigma-Aldrich | CAS # 55-86-7<br>Cat # 122564 |
| 1-2-Dipalmitoyl-rac-glycerol | MilliporeSigma | CAS # 40290-32-2 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| 1,2-di-*O*-octadecenyl-3-trimethylammonium propane (DOTMA) | Avanti Polar Lipids | CAS # 104872-42-6 |
| 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) | Avanti Polar Lipids | CAS # 4004-05-1 |
| 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) | Avanti Polar Lipids | CAS # 384833-00-5 |
| eFluor780-conjugated fixable viability dye | Invitrogen | Cat # 65-0865-14 |
| ER-Tracker Green | Thermo Fisher Scientific | Cat #E34251 |
| MitoTracker Deep Red FM | Thermo Fisher Scientific | Cat #M22426 |
| CellLight Golgi-RFP | Thermo Fisher Scientific | Cat #C10593 |
| LysoTracker Deep Red | Thermo Fisher Scientific | Cat #L12492 |
| CellMask Deep Red Actin dye | Thermo Fisher Scientific | Cat # A57248 |
| Opti-MEM (minimum essential medium) | Thermo Fisher Scientific | Cat # 11058021 |
| Dulbecco's modified Eagle's medium (DMEM) | Corning | Product # 10-013-CV |
| Agarose, low gelling temperature | MilliporeSigma | Cat #9414 |
| Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F-12) | MilliporeSigma | Cat #D6434 |
| Penicillin/Streptomycin (10,000 U/mL) | Gibco | Cat #15140122 |
| Gentamicin solution (50 mg/mL) | MilliporeSigma | Cat #1397 |
| RIPA lysis and extraction buffer | Thermo Fisher Scientific | Cat # 089900 |
| Fetal bovine serum (FBS) | HyClone | Product # SH30396.03 |
| Tamoxifen (citrate salt) | MilliporeSigma | CAS # 54965-24-1 |
| Hank's balanced salt solutions (HBSS) 1X | HyClone | Product # SH30588.02 |
| Human IFN gamma recombinant protein (interferon-γ, or IFN-γ) | Gibco – Thermo Fisher Scientific | Product # PHC4031 |
| eBioscience lipopolysaccharide (LPS) | Invitrogen | Product # 00-4976-93 |
| Phorbol 12-myristate 13-acetate (PMA) | AdipoGen | Product # AG-CN2-0010-M001 |
| **Critical commercial assays** | | |
| (MTT) cell proliferation assay kit | ATCC | 30–1010K |
| Cytotoxicity Detection Kit$^{PLUS}$ (LDH) | Roche | Cat # 474493401 |
| Cell proliferation reagent (WST-1) | Roche | Cat # 11644807001 |
| **Experimental models: Cell lines** | | |
| HeLa | ATCC | CRM-CCL-2 |
| RAW264.7 | ATCC | TIB-71 |
| **Experimental models: Organisms/strains** | | |
| C57BL6/J WT mice (male and female, aged 8–12 weeks) | Jackson Laboratory | Strain # 000664 |
| **Software and algorithms** | | |
| ChemDraw 22.2.0 | PerkinElmer | v22.2.0 |
| GraphPad Prism | GraphPad | v8 |
| ImageJ | National Institutes of Health and the Laboratory for Optical and Computational Instrumentation | https://imagej.nih.gov/ij/ |
| Adobe Illustrator | Adobe | v2021 |
| Imarius x64 | Oxford Instruments | v9.0.1 |
| Leica LAS X | Leica Microsystems | https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/ |
| BioRender | BioRender | https://www.biorender.com/ |
| SoftMax Pro | Molecular Devices | https://www.moleculardevices.com/products/microplate-readers/acquisition-and-analysis-software/softmax-pro-software |
| Kaluza 2.1 | Beckman Coulter | https://www.beckman.com/flow-cytometry/software/ |
| **Other** | | |
| 35 mm microscopy dishes | | |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| 24-well non-treated cell plates | VWR | Cat # 10861-558 |
| BD Intramedic Luer-Stub adapters (20 G cannula) | Fisher Scientific | Cat # 22-04486 |
| BD disposable syringes with Luer-Lok tips – 1 mL | Fisher Scientific | Cat # 14-823-30 |
| BD SafetyGlide insulin syringe with permanently attached needle | Fisher Scientific | Cat #B328447 |
| Nunc Lab-Tek II chambered cover glass | Thermo Fisher Scientific | Cat # 155379 |
| Personna Double Edge Prep razor blades | VWR | Cat # 89237-604 |
| Stainless steel jump rings | Michaels | Item # 329298793324560390 |
| Round paint brush (size 2) | Michaels | Item #D257882S |
| LOOK 3-0 silk suture, non-absorbable | LOOK Surgical Specialties | SP117 |
| 15 mL conical centrifuge tubes | Falcon | 14-959-53A |
| Alabama R&D tissue slicer | Alabama Research and Development | Part # MD4000 |
| SpectraMax M3 microplate reader | Molecular Devices | |
| Leica SP8 confocal microscope | Leica Microsystems | |
| Gallios 10 color flow cytometer | Beckman Coulter | |

## STEP-BY-STEP METHOD DETAILS

### Seeding of HeLa cells

Timing: 2–3 weeks

1. Seed HeLa cells into a T25 flask (Figure 2, top).
   a. Culture HeLa cells.
   b. Once the cells reach 90% confluency, remove the medium and wash the cells with 5 mL sterile PBS.
   c. Add 3 mL of 0.05% Trypsin-EDTA to detach cells from the flask.
   d. Incubate for 3–5 min at 37°C with 5% $CO_2$ to allow for the detachment.
   e. Add 3 mL of DMEM supplemented with 10% v/v FBS and transfer cells to a 15-mL falcon tube.
   f. Pellet cells by centrifuging at 150 × *g* for 5 min at 25°C and aspirate the supernatant.
   g. Suspend cells with 1 mL of DMEM supplemented with 10% v/v FBS.
   h. Determine cell concentration using a hemocytometer.
   i. Seed 700,000 cells in 10 mL of DMEM supplemented with 10% v/v fetal bovine serum (FBS) into a T25 flask.
   j. Maintain cells at 37°C with 5% $CO_2$ until the cells reach 90% of confluency.

*Note:* Passage cells at least three times before experiments with the LNPs.

### Seeding of RAW264.7 macrophages

Timing: 2–3 weeks

2. Seed RAW264.7 cells into a 100 mm culture dish (Figure 2, bottom).
   a. Culture RAW264.7 cells.
   b. Once the cells reach 70% confluency, remove the medium and add 5 mL of prewarmed fresh media.
   c. Gently scape the cells using a cell scraper or a rubber policeman off the culture dish. Start from one edge and work your way across the surface.
   d. Transfer the detached cells to a 15-mL falcon tube.
   e. Pellet cells by centrifuging at 150 × *g* for 5 min at 25°C and aspirate the supernatant.
   f. Suspend cells with 5 mL of DMEM supplemented with 10% v/v FBS.

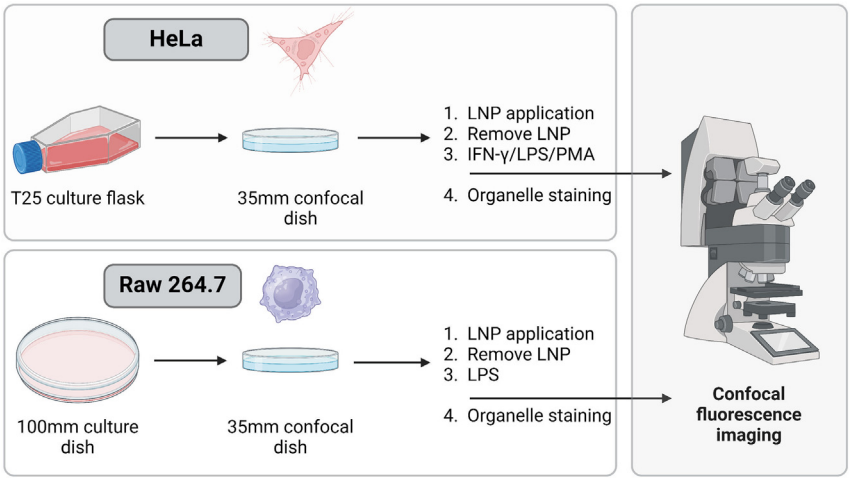


**Figure 2. Workflow of imaging mammalian cells stimulated for nitrative stress**

g. Seed $2 \times 10^6$ cells in 15 mL of DMEM supplemented with 10% v/v fetal bovine serum (FBS) and 1% penicillin/streptomycin into a 100 mm culture dish.
h. Maintain cells at 37°C with 5% $CO_2$ until the cells reach 70% of confluency.

*Note:* Passage cell culture at least three times before experiments with the LNPs.

## Preparation of lipid nanoparticles

Timing: 3 h

3. LNP Preparation Protocol.
   a. Prepare the stock solutions of **DPPC-TC-ONOO$^-$** (1 mM), DOTMA (5 mM), and DOPE (2.5 mM) in 1:1 (v/v) $CHCl_3$/MeOH solvent system.
   b. In a glass vial, add the stock solutions to make a **DPPC-TC-ONOO$^-$**/DOTMA/DOPE composition with 1:9.5:9.5 M ratio. This lipid ratio is used for each application.

*Note:* Desired LNP concentrations are 50 μM for cell studies, 75 μM for PCLS experiments, and 500 μM for intratracheal instillation. Here the LNP concentration is described as the added concentrations of each lipid. As an example, LNPs (75 μM) prepared for PCLS study require the following final lipid concentrations obtained after introducing sucrose solution (step d) and media (step h): [**DPPC-TC-ONOO$^-$**] = 3.75 μM; [DOTMA] = 35.6 μM; [DOPE] = 35.6 μM.

   c. Remove volatiles from the lipid mixture under reduced pressure. Insert the vial into a flask that can be connected to a vacuum line.
      i. Apply gentle vacuum to avoid the risk of uncontrolled suction of the lipid mixture.
      ii. Once the liquid level drops down near the bottom of the vial, then apply high vacuum.

*Note:* Make sure the liquid forms a residue that is spread over a large surface. To this end, it is important to keep the flask and/or vial straight up.

      iii. Continue high vacuum for 30 min.
      iv. Turn off the vacuum, disconnect the flask from the vacuum line, and recover the vial that now contains a thin film of the lipid mixture.
   d. Hydrate the thin film with 300 mM sucrose.

*Note:* Adjust the volume of sucrose to 40% of the total volume after mixing with the media for cell work and PCLS. Here the media will constitute for 60% of the final mixture.

*Note:* 300 mM sucrose solution has the same osmolarity as cell media, is cell compatible and has been shown in literature to form stable LNPs, which makes it an ideal solution for LNP preparation.

e. Resuspend the dry film in sucrose solution by shaking the vial.
f. Subject the resuspended mixture to sonication in water bath (room temperature, 40 kHz) for 60 min. Secure the vial using a foam of plastic holder inside the water bath.
g. Vortex the vial every 10 min to facilitate mixing and hydration.
h. Dilute the LNP solution with the desired biological medium (e.g., Opti-MEM) to a final LNP concentration of 50 μM for cell studies, 75 μM for PCLS experiments, or 500 μM for intratracheal instillation.

*Note:* In any case, the medium volume would constitute for the 60% of the final volume.

*Note:* It is advised to aliquot the stock solution of the lipids and store at −80°C. Avoid multiple freeze thaw cycles to avoid the degradation of the compounds.

This stepwise protocol outlines the preparation of LNPs using a mixture of DPPC-TC-$ONOO^-$, DOTMA, and DOPE, with specific lipid concentrations and processing conditions. The positive control sample must provide (theoretically) the maximum level of coumarin signal without $ONOO^-$. Therefore, a separate batch of LNPs should be prepared using the same protocol but with DPPC-TC in place of DPPC-TC-$ONOO^-$.

⚠ CRITICAL: Prepare fresh LNPs solution for each experiment.

Limitation: There could be batch to batch variation in the homogeneity of the LNP solution; no further characterization was done other than DLS analysis.

*Note:* We propose that a homogenous particle size distribution for the LNPs can be attained by utilizing an extruder kit and/or through size-exclusion chromatography. Further characterization of LNPs can be obtained via transmission electron microscopy (TEM).

### *In vitro* experimental protocol: Seeding of cells and incubation with LNPs

Timing: 1 day (for step 4)

Timing: 1 day (for step 5)

Timing: 15 h (for step 6)

Timing: 15 h (for steps 7–9)

4. Seed HeLa cells into a 35 mm glass microscope dish.
   a. Once the cells reach 90% confluency after 3 passage cycles, harvest the cells.
   b. Seed 50,000 HeLa cells in 400 μL media in a 35 mm microscopy dish.
   c. Maintain cells at 37°C with 5% $CO_2$ overnight.
5. Seed RAW264.7 cells into a 35 mm glass microscope dish.
   a. Once the cells reach 70% confluency after 3 passage cycles, harvest the cells.
   b. Seed 100,000 RAW264.7 cells in 400 μL media in a 35 mm microscopy dish.

c. Maintain cells at 37°C with 5% $CO_2$ overnight.

6. Incubation of the HeLa cells with the LNPs.
   a. Aspirate the media and transfer the LNPs solution directly to the cells and allow for 3-h incubation at 37°C with 5% $CO_2$ to ensure complete uptake of LNPs.
   b. After 3 h, replace the cell media with fresh Opti-MEM containing appropriate stimulants.
      i. For endogenous $ONOO^-$ generation in HeLa Cells, prepare a solution of IFN-g (100 ng/mL) and LPS (1 mg/mL) in Opti-MEM (250 μL).
      ii. Add the solution to HeLa cells.
   c. Incubate the samples for 12 h at 37°C with 5% $CO_2$.
   d. Decant the overnight media and add a solution of PMA (10 nM) in HBSS (250 μL) to the HeLa cells.
   e. Incubate the samples for 60 min at 37°C.
7. Incubation of the RAW264.7 cells with the LNPs.
   a. Aspirate the media and transfer the LNPs solution directly to the cells and allow for 3-h incubation at 37°C with 5% $CO_2$ to ensure complete uptake of LNPs.
   b. After 3 h, replace the cell media with fresh Opti-MEM containing appropriate stimulants.
   c. For Endogenous $ONOO^-$ generation in RAW264.7 Cells, prepare a solution of LPS (100 ng/mL) in Opti-MEM (250 μL).
   d. Add the solution to the cells.
   e. Incubate the samples for 12 h at 37°C with 5% $CO_2$.
8. For control experiments containing iNOS inhibitor, introduce 1400W (20 μM) into Opti-MEM.
9. Wash the cells with PBS and add the organelle tracker dye dissolved in HBSS.

*Note:* This detailed protocol outlines the sequential steps involved in the treatment of live cells with LNPs, media replacement, stimulation, and specific treatments for HeLa and RAW cells, including the inhibition of iNOS. The protocol concludes with the addition of organelle tracker for subsequent imaging.

### Fluorescent staining protocol for intracellular organelles in cultured cells

**Timing: 15–30 min (for step 10)**

**Timing: 30–60 min (for step 11)**

10. Concentration, incubation time and excitation/emission wavelength of each organelle tracker dye.
    a. ER-Tracker Green staining for Endoplasmic Reticulum: Apply ER-Tracker Green (1 μM) in 200 μL HBSS to cells and incubate at 37°C for 10 min. Excitation/Emission: 510/540 nm.
    b. MitoTracker Deep Red FM staining for Mitochondria: Apply MitoTracker Deep Red FM (250 nM) in 200 μL HBSS to cells and incubate at room temperature for 5 min. Excitation/Emission: 644/665 nm.
    c. CellLight Golgi-RFP BacMam 2.0 staining for Golgi Apparatus: Apply CellLight Golgi-RFP BacMam 2.0 (15 μL in Opti-MEM media) to cells and incubate at 37°C overnight. Excitation/Emission: 555/584 nm.
    d. LysoTracker Deep Red staining for Lysosomes: Apply LysoTracker Deep Red (100 nM) in 200 μL HBSS to cells and incubate at 37°C for 60 min. Excitation/Emission: 647/670 nm.
    e. CellMask Deep Red Actin Dye staining for Actin Filaments: Apply CellMask Deep Red Actin dye (1 μM) in 200 μL HBSS to cells and incubate at 37°C for 10 min. Excitation/Emission: 669/710 nm.
    f. Post-Incubation Steps.
       i. Remove the dye solutions carefully and wash cells three times with PBS.
       ii. Add 1 mL of HBSS to the cells prior to imaging.
11. Imaging of the sample on the confocal microscope (Figure 3).

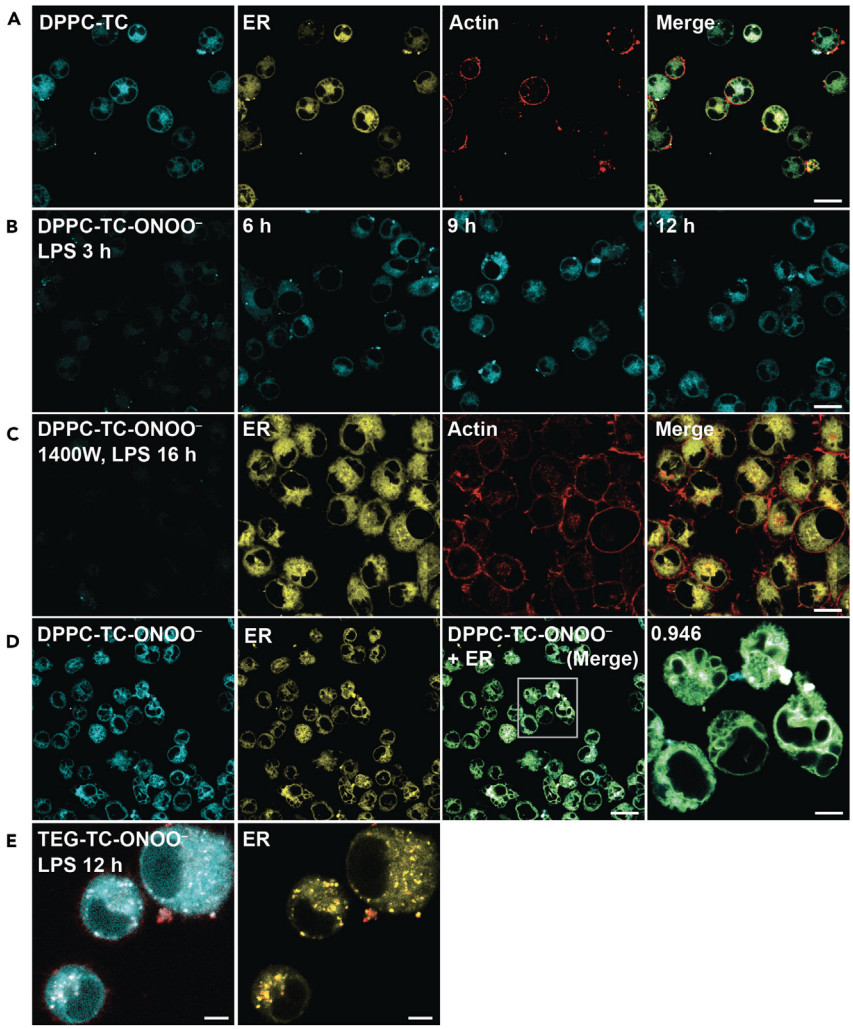


**Figure 3. Imaging of nitrative stress in live RAW246.7**

(A) Cells incubated with LNPs containing DPPC-TC (positive control) and left unstimulated.

(B) Cells treated with LNPs containing DPPC-TC-ONOO⁻, then stimulated with LPS. Time coarse images acquired after the addition of LPS (3–12 h).

(C) Cells treated with LNPs containing DPPC-TC-ONOO⁻, then incubated with 1400W and LPS.

(D) Quantitative colocalization study of stimulated cells.

(E) Cells treated with LPS and TEG-TC-ONOO⁻, the hydrophilic analog of DPPC-TC-ONOO⁻ whose *rac*-DPPC is replaced with triethylene glycol (TEG). No measurable correlation between TEG-TC-ONOO⁻ activation and ER tracker was found, underscoring the role of lipidation in probe design for targeting biological lipid compartments. Images for DPPC-TC, DPPC-TC-ONOO⁻ and TEG-TC-ONOO⁻ were acquired through the coumarin channel: 405/475 nm. Scale bars = (A–C) 20 μm, or (D–E) 5 μm.

a. Conduct imaging using an appropriate fluorescence microscope or confocal microscope.
b. Utilize the specific excitation/emission wavelengths for each stain as mentioned in step 11. **DPPC-TC-ONOO⁻** and **DPPC-TC** ex/em: 405/475 nm.

***Note:*** Adjust staining times and concentrations based on cell type and experimental requirements. Ensure proper removal of dye solutions and thorough washing steps to minimize background fluorescence. Handle dyes and stains according to manufacturer's protocols for optimal results: ER-Tracker Green, MitoTracker Deep Red FM, CellLight Golgi-RFP, LysoTracker Deep Red, or CellMask Deep Red Actin dye.

*Note:* We reported a cytotoxicity assessment of DPPC-TC-ONOO$^-$ and LNPs for HeLa and RAW264.7 cells using MTT assay.[1] Researchers wishing to use our probe and LNPs for studying other cell lines are advised to perform the MTT assay accordingly.

### *Ex vivo* experimental protocol: Preparation of murine precision cut lung slices (PCLS), NM exposure, and staining with LNPs

*Generation of PCLS samples*

Timing: 4 h (for steps 12–14)

12. Prepare all necessary reagents.
    a. Make 1.5 L 1X phosphate buffered saline (PBS) to collect PCLS while slicing.
    b. Prepare 20 mL of 1.5% low-gelling temperature agarose in culture medium (DMEM/F-12 ham supplemented with 1% penicillin/streptomycin and 50 μg/mL gentamicin). Warm the agarose in a 37°C water bath or incubator until ready to use.
    c. Chill reservoir assembly, embedding unit (on ice) and slicing buffer (1x PBS) (on ice) in 4°C.
    d. Fill 24-well plates with 0.5 mL culture medium and warm at 37°C.
13. Isolate lung tissue from mouse (Figure 4).
    a. Use 1 or 2 8–12-week C57BL6/J mice.
    b. Anesthetize mice with ketamine (135 mg/kg) and xylazine (30 mg/kg) via intraperitoneal injection.
    c. Cut the skin and peritoneum to open the abdominal cavity. Euthanize by exsanguination via the renal artery.
    d. Cut the diaphragm and expose the thoracic cavity.
    e. Perform a tracheotomy and insert a 20-gauge cannula the trachea. Secure cannula using surgical thread.
    f. Instill ~1 mL warmed (37°C) 1.5% low-gelling point agarose into the lung using 1 mL syringe until lung is fully inflated.

    *Note:* The accessory lobe fills last and can be used as an indicator that the lung is inflated.

    ⚠ CRITICAL: Ensure that agarose is free of air bubbles before loading. Maintain a fill pace of approximately 0.1 mL/s as filling the lung too fast can damage the tissue. Overfilling can also cause damage. To prevent uneven filling, do not insert cannula too far into the trachea. Uneven filling can also be due to cuts in the lung that occur during the necropsy.

    g. Prop the syringe upright using pins and allow agarose to congeal for approximately 10 min by placing the whole mouse at 4°C.
    h. After agarose is congealed, remove the syringe, and cut the lung out of the thoracic cavity.
    i. Separate the lung lobes by carefully cutting the connective tissue between lobes.
    j. Prepare the embedding unit and place lung lobes into plunger.

    *Note:* Fill with agarose until tissue is fully covered or top of the plunger.

    k. Allow agarose to congeal for approximately 10 min at RT while preparing for slicing.

    *Note:* Mice have lung lobes that can fully fit into the plungers. Lung tissue from other animals, such as rat, would have to be cored. We recommend slicing the left lung and the superior and inferior lobes of the right lung to generate slices that are uniform in size. Using this method, ~75 PCLS can be produced from 1 mouse.

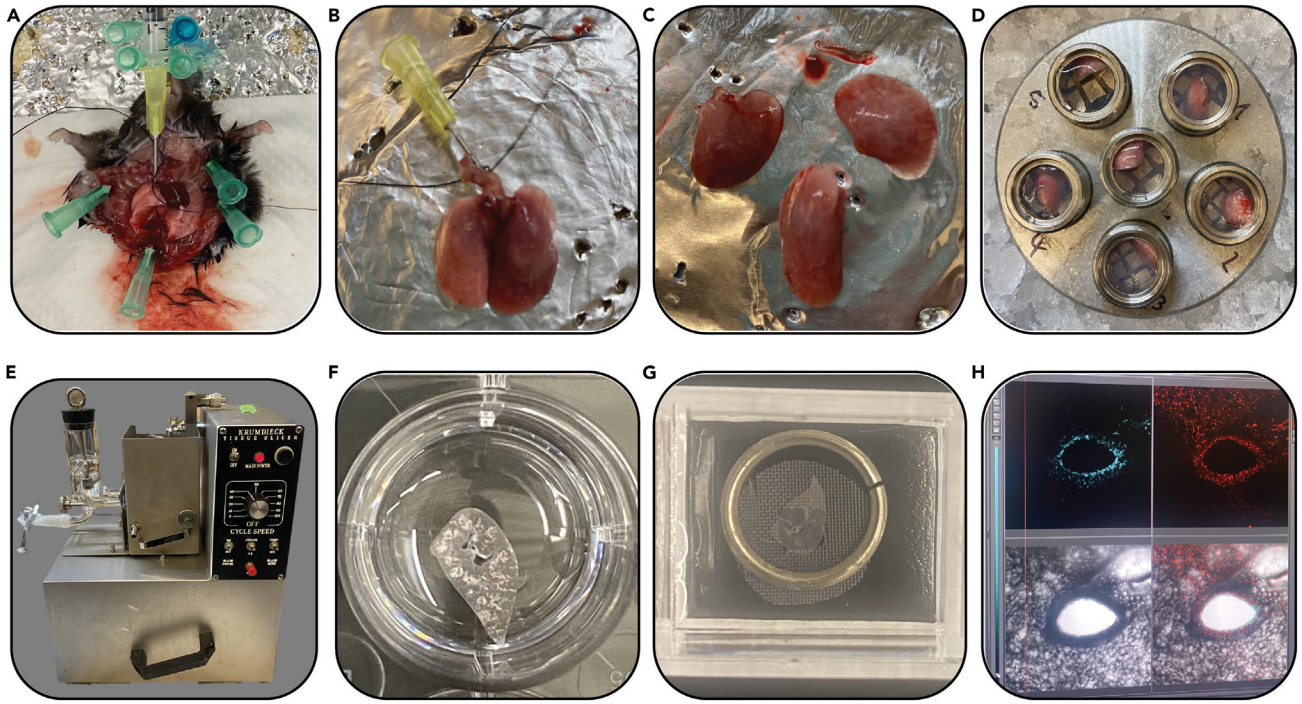


**Figure 4. Steps for generating PCLS, incubation with LNPs, and imaging**

(A) agarose instillation, (B) lung isolation, (C) lung lobe separation, (D) orientation in embedding unit, (E) Alabama R&D tissue slicer set up, (F) PCLS culturing, (G) mounting PCLS with mesh and metal rings, (H) imaging PCLS that have been incubated with LNPs on Leica SP8 confocal using LAS X software. This detailed protocol outlines the stepwise procedures for the *ex vivo* experiments involving mouse lung tissue (PCLS), including ethical considerations, tissue preparation, culture, exposure to NM and LNPs, imaging, and viability assessments.

⚠ CRITICAL: The orientation of the lung lobes in the plungers determines slice composition. To generate PCLS with cross-sectional airways, angle each lobe to follow the bronchioles.

14. Slice lung lobes using Alabama R&D Tissue Slicer.
    a. Prepare the reservoir assembly and glass trap according to manufacturer's instructions.
    b. Insert double-edged stainless steel razor blade.
    c. Fill reservoir with ~500 mL slicing buffer.
    d. Insert plunger with lung tissue.
    e. Slice at speed setting 40 to a thickness of ~300 μm.

    *Note:* Thickness setting can be set using a thickness gauge. This setting will change dependent on tissue types, additional weights added on top of the plunger, and between machines.

    f. When the tissue is fully sliced, stop the slicer, and collect buffer in a 500 mL beaker.
    g. Use a paint brush to lift PCLS from the buffer into each cell culture well.
    h. Repeat process until all lobes are sliced.

    *Note:* Between slicing experiments, the slicer needs to be taken apart and thoroughly cleaned according to manufacturer's instructions.

## Culturing and nitrogen mustard (NM) exposure of PCLS

Timing: 16 h (for step 15)

Timing: 27 h (for step 16)

15. Culture PCLS overnight.
    a. Place PCLS on a shaker in a 37°C incubator at 5% $CO_2$ and 90% humidity.
    b. After slicing, replace culture media (~500 μL for 24-well plates) immediately, and then 3 h after slicing.

*Note:* PCLS can be cultured for several weeks. The slicing protocol does not occur in a sterile environment; however, measures can be taken to prevent culture contamination, including performing experiments/media changes in a biosafety hood, antibiotics in culture medium, and autoclaving (tissue slicer) or disinfecting (70% ethanol) supplies (paint brush, metal rings, mesh) that come in contact with PCLS. A vacuum aspiration or a micropipette can be used to remove medium.

16. Expose PCLS to nitrogen mustard (NM).
    a. Prepare 50 μM NM solution in medium.
    b. Expose PCLS to 300 μL NM or Control (culture medium).
    c. Incubate on a shaker at 37°C for 1 h.
    d. Remove NM and wash with media twice.
    e. Incubate at 37°C for 24 h.

*Note:* NM is very toxic and can cause damage to the skin, eyes, and respiratory tract. Proper safety protocols must be followed.

### Incubation with LNPs and microscopy

Timing: 1.5 h (for step 17)

Timing: 1–4 h (for step 18)

17. Stain with LNPs and MitoTracker.
    a. Incubate PCLS with 75 μM LNPs for 1 h at 37°C.
    b. Remove LNPs and wash with medium.
    c. Incubate PCLS with MitoTracker Deep Red (1:1,000 dilution) for 2 min at room temperature.
    d. Remove MitoTracker and wash with medium.
    e. Move PCLS to 2-well chamber well containing 1 mL of medium.
    f. Use the paintbrush to orient the PCLS under the Sefar Nitex mesh (cut to fit into the well).
        i. To take images without the mesh in view, cut a small circle in the middle of the mesh.
        ii. Place the PCLS inside with the edges being held down by the mesh.
        iii. Place a metal jump ring on top of the mesh to hold it down.

⚠ CRITICAL: Be careful not to drop the ring directly on the PCLS.

18. Image PCLS airways using Leica TCS SP8 confocal microscope.
    a. Turn on 405 and 638 nm lasers and set detectors to image the airways and in the PCLS after focusing on a z-axis.

*Note:* You can take multiple z stack images of each sample and select one of the z stacks with best focus. It is sometimes difficult to find the best focal plane right away.

⚠ CRITICAL: PCLS are very fragile and small perturbations while handling can cause damage to the tissue. Gentle handling techniques (using a paint brush) are advised. This is especially important to note when securing PCLS down with mesh for imaging.

### *In vivo* experimental protocol: Intratracheal bleomycin (ITB) lung injury and LNP instillation

Timing: 3 days

19. Obtain 6- to 8-week-old C57BL6/J (Wild type, WT) (Jackson Laboratories, Bar Harbor, ME). Provide food and water *ad libitum* under standard housing conditions.

*Note:* For the purposes of protocol description, C57BL6/J mice have been described as the experimental model. Genotype may be altered with appropriate considerations for strain-specific differences.

20. Anesthetize mice using a whole-body isoflurane administration chamber.
21. After ensuring adequate anesthesia, administer a single 50 μL intratracheal instillation of either bleomycin (ITB, 3 U/kg) in DMSO/PBS (6:94 v/v) for the experimental group and administer DMSO/PBS (6:94 v/v) for the control group.
22. Observe mice for 10 min post-recovery to ensure complete dose retention.
23. Re-anesthetize mice 3 days later.
24. Administer a 50 μL intratracheal instillation of LNPs (0.5 mM) or sucrose (300 mM) as a control.
25. 3 h after the second set of instillations, perform euthanasia via a single intraperitoneal injection of xylazine (30 mg/kg) and ketamine (135 mg/kg), followed by exsanguination.
26. Expose the abdominal cavity and perform thoracotomy.
27. Conduct cardiac perfusion using 3 mL 1x PBS to minimize erythrocyte contamination during BAL collection.
28. Carefully exposing the trachea, a single small incision should be made through the superior portion of the trachea. Carefully, a 20-gauge cannula should be placed into the incision. Using surgical suture, secure cannula to trachea.
29. Slowly instill 1 mL ice-cold PBS through the cannula to collect BAL fluid. Repeat x5, adding the collected fluid from each sequential wash into a tube to collect a pooled sample from each animal.
30. Centrifuge the collected BAL fluid at 300 *g* for 8 min to pellet cells. Pelleted cells should be maintained on ice and stained for flow cytometry analysis.

*Note:* If samples are visibly contaminated with erythrocytes acquired during BAL fluid collection, lysis with an appropriate solution (i.e. ACK Lysis Buffer) compatible with downstream flow cytometric analysis may be used according to manufacturer's instructions.

This comprehensive protocol outlines the stepwise procedures for inducing acute lung injury in mice through intratracheal instillation of bleomycin, followed by secondary treatment with LNPs to quantify nitrative stress (Figure 5). The protocol includes dose optimization, sacrifice, and collection of bronchoalveolar lavage fluid for flow cytometry analysis.

### Immunostaining and flow cytometry analysis of BAL fluid cells

Timing: 4 h

The following protocol describes the immunostaining and data analysis workflow of BAL cells collected from mice treated with bleomycin/control and administered LNPs/control.

31. Resuspend cells in staining buffer to a volume of 100 μL.

*Note:* If using harvested cells for single stain controls, you may resuspend each individual sample in a higher volume (i.e. 120 μL). After resuspension, remove excess volume (i.e. 20 μL) from each tube and recombine in one "pooled" tube. These cells may then be used as single-color

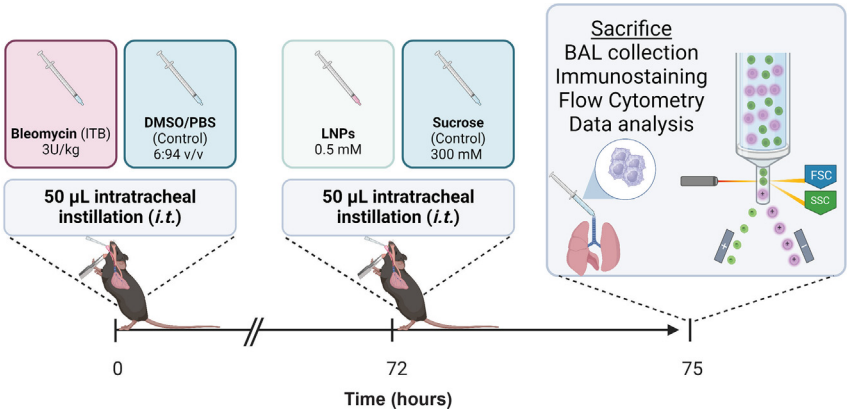


**Figure 5. Murine model of acute lung injury, LNP administration, and sample processing workflow**

Intratracheal instillation of bleomycin/control is to be followed by LNP/control 72 h after initial administration. Upon sacrifice, collection of brochoalveolar lavage (BAL) fluid should be performed, followed by cellular immunostaining for surface markers of interest. After initial determination of cells through forward scatter (FSC) and side scatter (SSC), live cells can be phenotyped based upon positive fluorescent signal detected for cell surface markers of interest. Downstream processing to determine fluorogenic signal of DPPC-TC signal *in vivo* within populations of interest should follow.

controls for each antibody used in the staining protocol for adequate compensation during flow cytometry analysis. In lieu of this process, commercially available compensation beads may be preferable for all single-color controls except for a viability control; cells harvested at the time of sacrifice are to be used for live/dead determination during the flow cytometric analysis.

32. Treat samples with TruStain FcX anti-mouse CD16/32 (Fc Block, 1:100) for 10 min at 4°C.

*Note:* This step inhibits non-specific binding during staining and analysis.

33. Stain cells with antibody cocktail for 30 min at 4°C: CD45, CD11b, and CD11c (1:100 each, or as determined by titration).

*Note:* Ensure samples are kept in the dark during this process.

34. Add 1 mL staining buffer to quench staining, and pellet cells by centrifugation for 6 min at 400 × *g*.
35. Resuspend cells in 1 mL of staining buffer and perform viability staining.

*Note:* Stain cells with eFluor 780-conjugated fixable viability dye (1:1000, or as determined by titration) in the dark for 30 min at 4°C. Only antibody cocktail-stained samples, along with a single-color control for viability should be stained with fixable viability dye. At this point in the protocol, single color controls for other antibodies (i.e., CD45) should be fixed and stored for later analysis. Add 1 mL staining buffer to wash cells, centrifuge, and aspirate.

36. Fix cells with 3% paraformaldehyde for 20 min at 4°C.
37. Wash fixed cells with cold PBS, centrifuge cells as in step 32 and resuspend cells in 100 µL PBS.
38. Utilize a multi-color flow cytometer for cell examination.

*Note:* This protocol and subsequent data analysis is modeled on the use of a Beckman Coulter Gallios 10-color flow cytometer.

39. Employ Kaluza software (Beckman Coulter) for data analysis (Figure 6).
40. Initially sort cells based on size and complexity using forward scatter (FS) and side scatter (SC) parameters (Figure 6A).

*Note:* Exclude doublets by discriminating on FS and FS Peak (Figure 6B).

41. Screen for viability by excluding cells that are positive for fixable viability dye from the analysis (Figure 6C).
42. Examine cells of myeloid origin by selecting for CD45-expressing cells (Figure 6D).
43. Analyze CD11b and CD11c expression in the cell population of interest to determine myeloid cell phenotype from BAL cells (Figure 6E).

*Note:* For the purposes of this analysis, BAL cells were stained with markers to determine myeloid lineage (CD45) and markers of tissue residency (CD11c) and migration (CD11b). Cells were phenotypically categorized based on the following expressional profiles: CD11c+/CD11b-: resident macrophage (Figure 6E, green box); CD11c+/CD11b+: migratory macrophages (Figure 6E, blue box); CD11c-/CD11b+: recruited macrophage (Figure 6E, red box).

*Note:* Additional targets for immunostaining can be added to this panel for further population discrimination pending the availability of conjugated antibodies and the laser array of the cytometer. Seeking technical guidance on flow cytometry panel building is advised.

44. Quantify DPPC-TC signal in population of interest (Figure 6F).

⚠ CRITICAL: To maintain maximum cell viability and to minimize photobleaching of the fluorescently activated lipid (DPPC-TC generated from DPPC-TC-ONOO$^-$), cells should be kept on ice and in the dark (i.e., ice bucket with lid, working in a dimly lit hood) throughout the immunostaining process. All centrifuges should be cooled to 4°C prior to use.

This flow cytometry sample preparation and analysis protocol provides a systematic approach for the examination and categorization of myeloid cells collected from BAL fluid. The steps include Fc Block treatment, antibody staining, viability assessment, fixation, and flow cytometry analysis for the quantification of DPPC-TC signal within macrophages.

## EXPECTED OUTCOMES

This protocol introduces an innovative application of LNPs for delivering a designer lipid-based ONOO$^-$ sensor into cells, tissues, and the lung lining *in vivo*. The method facilitates the direct detection of nitrative stress using confocal microscopy and flow cytometry, a phenomenon not previously documented in the literature. Our approach enables the identification of cytokine induced ONOO$^-$ stress within lipid-rich organelles, particularly the endoplasmic reticulum. PCLS exposed to NM *ex vivo* indicates the origin of nitrative stress as the vicinity of the airways. In an *in vivo* model of ITB-induced acute lung injury, quantifying the DPPC-TC signal within CD11b+ cells provide a statistically significant measure of nitrative stress compared to the control group.

## LIMITATIONS

First, current lipid delivery method does not validate the existence of probe affinity toward the ER. Our control studies with **DPPC-TC** and Liss-Rhod PE[1] suggest that ER localization is driven primarily by LNPs but not the nature of the lipid cargo. Therefore, LNP composition must be reconfigured for **DPPC-TC-ONOO$^-$** to localize at another lipid-rich subcellular target (organelle). Second, we currently have a poor mechanistic understanding of how cells uptake the LNPs carrying **DPPC-TC-ONOO$^-$** or **DPPC-TC**. We can only hypothesize that their cellular uptake might be via direct fusion due, in part, to the cationic nature of DOTMA, which constitutes nearly half of the LNP

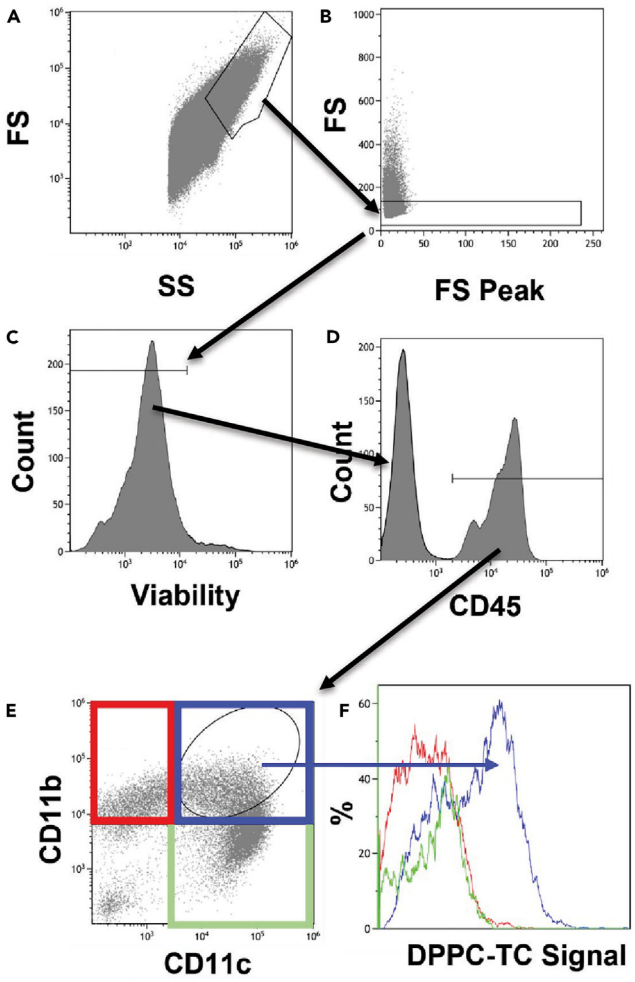


**Figure 6. Representative cell phenotyping workflow of BAL cells derived from wild-type mice instilled with DPPC-TC 3 h post bleomycin administration.**

(A) Preliminary identification of cells of interest was made based upon forward/side scatter.

(B) Double discrimination was performed to isolate single cells for analysis.

(C) Live/dead discrimination was performed to selected cells for subsequent analyses.

(D) Cells expressing the myeloid marker CD45 were identified.

(E) CD11b+/CD11c- (Resident macrophages; Red), CD11b+/CD11c+ (Migratory macrophages; Blue) and CD11b-/CD11c+ (Recruited macrophage; Green) cells were identified.

(F) Percentage of cells positive for DPPC-TC was determined within each macrophage populations characterized in 6E.

composition.[5,6] It would be critical to build an understanding of the mechanism(s) by which DPPC-TC-$ONOO^-$ is taken by mammalian cells, so that new probe designs or LNP compositions could be tested to accomplish either (i) a higher uptake efficiency, or (ii) targeting the plasma membrane.

The slicing technique used to prepare PCLS can also generate nitrosative stress. A proper untreated control is required to account for background $ONOO^-$ generation in this model.

The use of intratracheal bleomycin (ITB) is a well-established model of ALI, however, there are factors to consider when implementing this model for study. Technical expertise is required for this route of administration, and variability in injury resulting from this compound result from: technical experience of the administrator, batch-or lot-specific variation in bleomycin obtained commercially, anesthetic level of animal during administration, anatomical positioning post-administration.

Additionally, although C57BL/6J mice are highly sensitive to bleomycin-induced ALI,[7] individual variability in bleomycin hydrolase activity in the lungs may affect the injury observed. Researchers should ensure that compound preparation and instillation is consistent between animals in a study. Validation of ITB-induced injury should be confirmed histologically prior to performing experiments utilizing the probe-containing LNPs and performed on a batch or lot-specific basis with dose-response experiments.

## TROUBLESHOOTING

### Problem 1

DPPC-TC-ONOO$^-$ and/or DPPC-TC have poor solubility in organic solvents or aqueous buffer.

### Potential solution

First, use a mixture of chloroform and methanol (e.g., in 3:1 volumetric ratio) to dissolve the lipid at ~100 μM. The resulting mixture should go into a fully homogenous state with gentle shaking. The deuterated form of this solvent system ($CDCl_3$/d4-methanol) can be used for NMR spectroscopic authentication of DPPC-TC-ONOO$^-$ and DPPC-TC.

### Problem 2

The organelle tracker dye generates signal under more than one channel and/or images acquired have suboptimal signal intensity (Step-13).

### Potential solution

Avoid organelle tracker dyes with emission wavelengths that overlap with that of DPPC-TC, as this would lead to bleeding of fluorescence in each channel. If over-saturation of the signal is observed, dial down the dye concentration, or reduce the exposure time. You can also lower the incubation temperature from 37°C to room temperature.

### Problem 3

Images acquired from the PCLS have high levels of background signal (Step-20).

### Potential solution

Change the thickness of the slice to attain the optimum probe signal.

### Problem 4

Flow cytometry experiment results in either a non-detectable or high signal for cell surface markers and/or DPPC-TC (Step-46).

### Potential solution

Each fluorochrome used within a flow cytometry experiment must be properly titrated to the specific experiment and instrument used for analysis. The possibility of overlapping spectra from multiple fluorophores and from DPPC-TC should be considered when designing and testing antibodies to be used in proposed experiments. As stated, consultation with trained technical specialists is advised for the use of a flow cytometer. Additional references on proper experimental design and controls may be of use.[8,9]

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Enver Cagri Izgu (ec.izgu@rutgers.edu).

### Technical contact

Questions about the technical specifics of performing the protocol should be directed to the technical contact, Tushar Aggarwal (tushar.aggarwal@pnnl.gov).

### Materials availability

All unique/stable reagents generated in this study are available from the lead contact upon reasonable request with a completed Materials Transfer Agreement.

### Data and code availability

- Any primary data in this study can be requested from the lead contact.
- This study did not generate any new code.
- Any additional information to reanalyze the data can be requested from the lead contact.

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## AUTHOR CONTRIBUTIONS

T.A. conducted *in vitro* studies with cells and wrote the original version of the manuscript; A.B. conducted the PCLS studies and edited the manuscript; E.R.S. conducted the *in vivo* studies and edited the manuscript; J.H. designed the PCLS system for stimulation; D.L.L. supervised the PCLS work and edited the manuscript; A.J.G. designed the *in vivo* labeling studies and edited the manuscript; and E.C.I. conceptualized and oversaw the project, supervised *in vitro* studies, and wrote the original version of the manuscript.

## DECLARATION OF INTERESTS

E.C.I. is an inventor in a patent application filed by Rutgers University on the subject of this work.

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