Fluoro Peroxynitrite

Cell Permeable Fluorescent Peroxynitrite Detection Kit

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Notes:

I. Introduction:

Peroxynitrite ($ONOO^-$) is produced via a diffusion reaction of nitric oxide and superoxide radicals. Endogenous Peroxynitrite is toxic to cells and effects mitochondrial function that can trigger cell death through nitration and oxidation reactions, however Peroxynitrite is also cytotoxic to invading pathogens. At the biochemistry level, peroxynitrite is involved in protein, lipids and DNA modification via oxidation and nitration reactions. Protein modification can lead to chemical and physical changes of protein properties. Furthermore, DNA modification can lead to DNA strand breaks and cell death (apoptosis). Peroxynitrite modification of lipids can modulate lipid-protein adducts formation.
Generation of peroxynitrite is attributed to pathogenic conditions in diseases such as neurodegenerative disorders, stroke, chronic inflammation, chronic heart failure, myocardial infarction and cancer. Redox related injuries are also related to peroxynitrite production like ischemia-reperfusion injury. Although peroxynitrite is a strong oxidant it reacts at a relative slow rate and can diffuse over a long range as far as on a cellular scale. These properties of peroxynitrite make it an interesting candidate for novel therapeutic interventions (1-6).

Cell Technology introduces NP3, a new and novel probe developed by Dr. Xin Li et.al at the Zhejian University, Hangzhou China. NP3 exhibits excellent sensitivity and specificity to peroxynitrite in live cells and animals and can readily pass the blood brain barrier (7).

Applications:

- Cell permeable In-situ monitoring of Peroxynitrite.
- Readily passes through blood brain barrior for neurological assessment.
- Study of Peroxynitrite levels in cellular stress, neurodegenerative diseases .
- Monitoring Peroxynitrite homeostasis in live cells and live tissue sections.

II. General Properties:

NP3 is quenched fluorogenic probes and when exposed to Peroxynitrite becomes fluorescent.
Probe NP3 is a Peroxynitrite ($ONOO^-$)specific probe that is cell permeable and detects the upregulated intracellular $ONOO^-$ with fluorogenic response. It is two-photon microscopy compatible. Its molecular weight is 362.1, with λex 405 nm.

General Protocol.
After culture on glass cover slips overnight, cells were treated with SIN-1, $ONOO^-$ or OGD for indicated time (or any other stimulations), then stained with **NP3** probe (5 μM) at 37°C for 30 min. Cells may be observed under confocal microscopy directly, or may be fixed in 4% PFA and imaged by confocal microscope. **NP3** fluorescence should be collected at 420-480 nm with λex 405 nm. This dye is compatible with propidium iodide for staining nuclei. This probe won't locate to a specific organelle but rather in the cytoplasm. Cells stained with **NP3** may also be observed under two-photo microscopy

III. Storage:

1. **One vial of Dried powder. Storage -20^{0}C. Reconstitute in anhydrous DMSO. Aliquot into single use vials and store at or below -20^{0}C.**

IV. Absorption, Fluorescent and Reactive properties of NP3

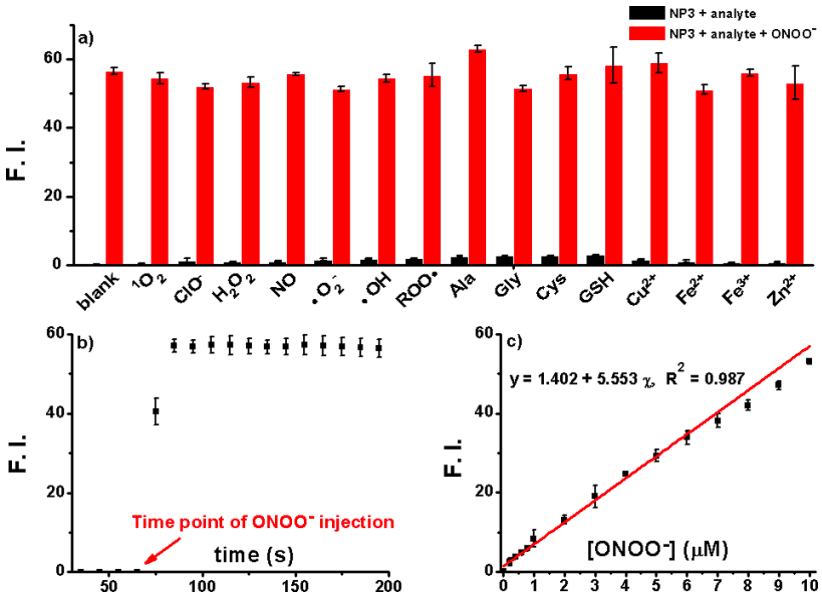

Figure 1. Characterization of fluorescent response of NP3 toward ONOO−. (a) Fluorescent responses of NP3 (5 μM) toward various analytes (10 μM). Data shown represent fluorescent intensity at 470nm, 30 min after addition of various analytes. (b) ONOO− (final concentration 10 μM) was quickly injected into a solution of NP3 (final concentration 5μM), and the fluorescent intensity at 470 nm was plotted against time. (c) Fluorescence enhancement of NP3 (5μM) at 470 nm as a function of ONOO− (0−10 μM) after 15 min of reaction. All data were acquired in PBS (10 mM, pH 7.4) with excitation at 375 nm.

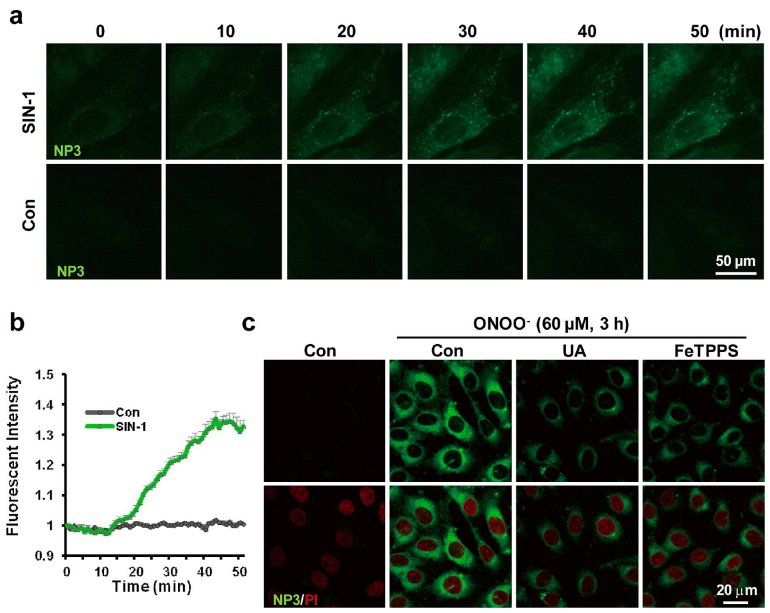

Figure 3. Characterization of ONOO− formation by NP3 in endothelial cells upon nitrosative stress. (a) Time-lapse series of single confocal plane images taken from living EA.hy926 endothelial cells. The cells were seeded on glass-bottom 6-well plates overnight and then preincubated with NP3 (5.0 μM) for 30 min, followed by stimulation with or without SIN-1 (0.5 mM). (b) Quantitative analysis of dynamic changes of NP3 fluorescence after SIN-1 (0.5 mM) treatment in panel a. Data are presented as a densitometric ratio change compared with control. (c) Effects of ONOO− scavengers uric acid (100 μM) and FeTTPS (1 μM) on changes in NP3 fluorescence in endothelial cells in the presence of ONOO− (60 μM). The ONOO− scavengers were preincubated for 1 h prior to ONOO− loading. Propidium iodide (PI) counterstaining indicates nuclear localization (red; λex 543 nm, λem 560–615 nm). NP3 fluorescence was collected at 420–480 nm with λex 405 nm.

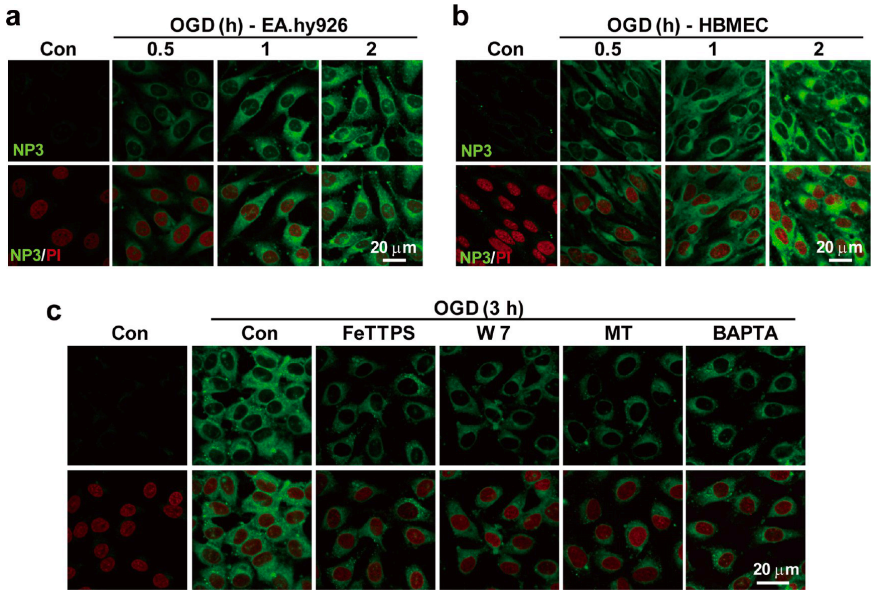

Figure 4. Oxygen-glucose Deprivation (OGD).Characterization of ONOO− formation by use of NP3 in endothelial cells upon OGD. (a, b) Representative confocal images show time dependent accumulation of NP3 fluorescence (green; λex 405 nm, λem 420−480 nm) in (a) EA.hy926 endothelial cells over 0.5−2 h following OGD exposure as well as in (b) human brain microvascular endothelial cells (HBMEC). PI counterstaining indicated nuclear localization (red, λex 543 nm, λem 560−615 nm). (c) The OGD-initiated NP3 fluorescence response to ONOO− was modulated by suppressing ONOO− formation. EA.hy926 endothelial cells were pretreated with FeTTPS (1 μM), W7 (1 μM), melatonin (10 μM), or BAPTA (1 μM) 1 h prior to OGD treatment to suppress the ONOO− signal.

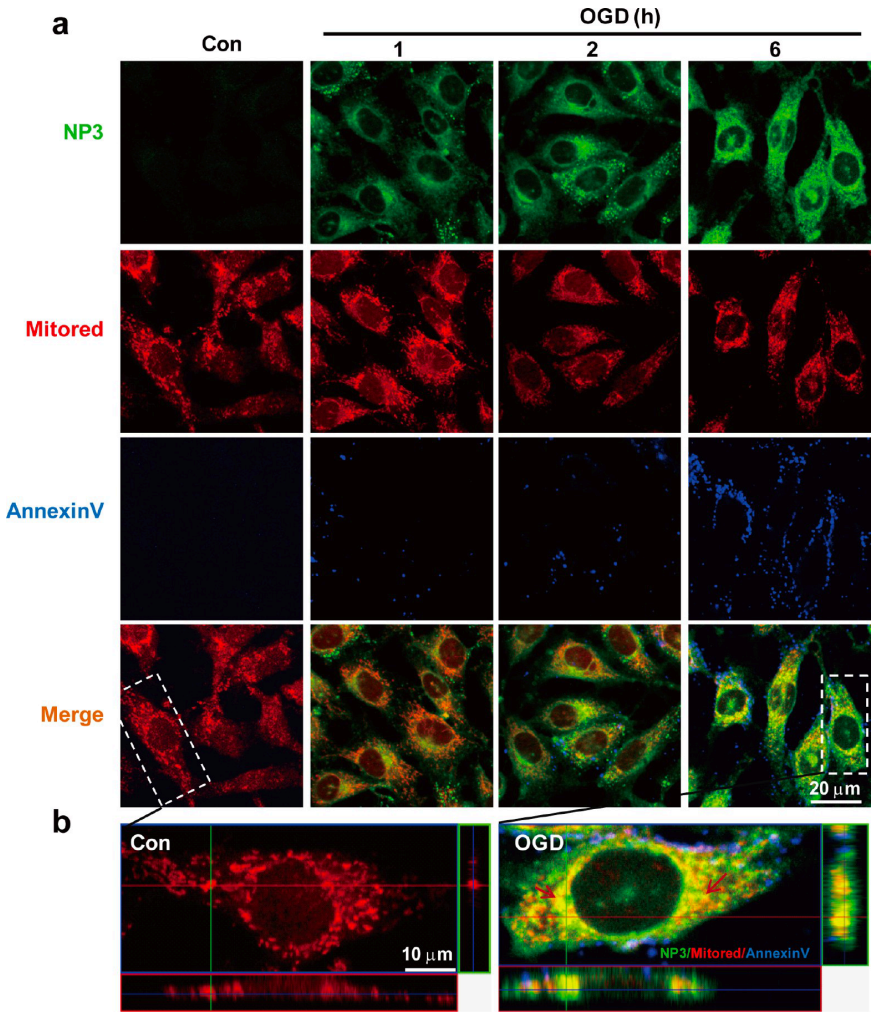

Figure 5. Distribution of NP3 fluorescence, examined by counterstaining with the mitochondria indicator MitoRed after OGD. (a) Representative confocal images show temporal changes of ONOO−-dependent NP3 fluorescence (green; λex 405 nm, λem 420−480 nm) and MitoRed (red; λex 543 nm, λem 560−615 nm), as well as the apoptosis maker annexin V (blue; λex 488 nm, λem 505−550 nm), following OGD treatment. (b) Orthogonal projections onto the x−z (upper) and y−z (right) planes are shown to confirm the colocalization of NP3 and MitoRed throughout endothelial cells as shown in panel a. after ischemia injury. All images were captured with a Zeiss LSM 510 confocal microscope.

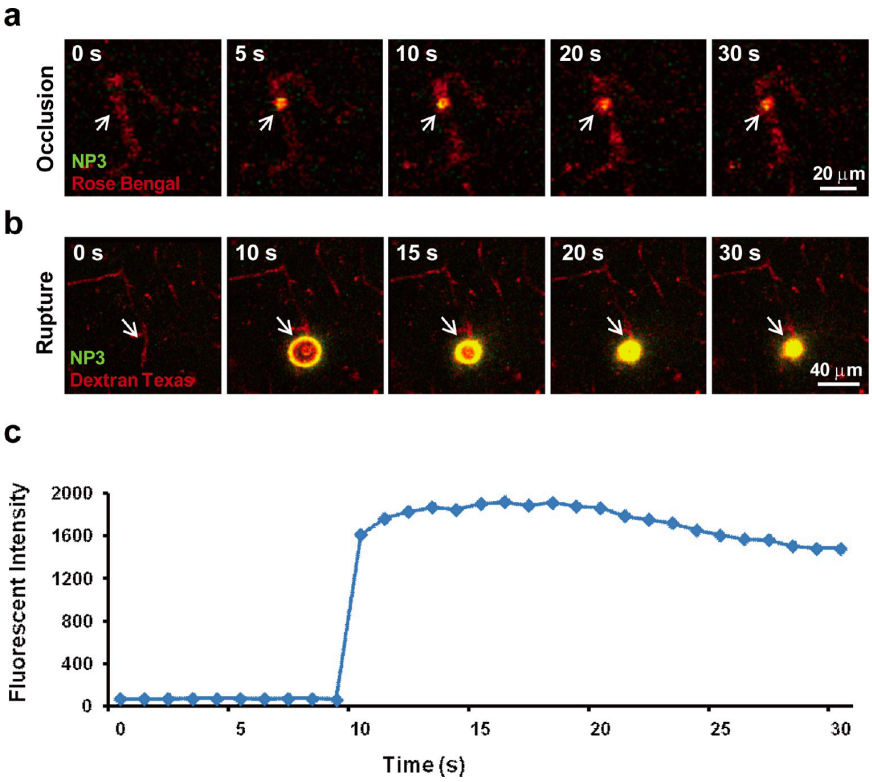

Figure 6. Real-time visualization of endogenous peroxynitrite fluxes after brain microvessel injury with a combination of NP3 and in vivo two-photon laser scanning microscopy. For two-photon imaging, cortical brain vessels 10–15 μm in diameter and 100 μm below the cortical surface were selected for imaging. (a, b) The time-series images are individual frames from a continuous time-lapse movie and show dynamic NP3 fluorescence elevation (arrows) following (a) rose bengal-induced vascular occlusion and (b) laser irradiation-induced vascular rapture in live mice. (c) Mean values of NP3 fluorescence intensity from panel b were measured to quantify the progressive ONOO− formation in cerebrovessels after ischemia. Emission was collected at 420–480 nm for NP3 fluorescence and 575–630 nm for dextran Texas or rose bengal upon excitation at 800 nm.

V. Warnings and Precautions:

1. **For Research use only. Not for use in diagnostic procedures**.
2. Practice safe laboratory procedures by wearing gloves, protective clothing and eyewear.

VI. Catalog Number

1. **Catalog # Fluoro-NP3: Part # XXXX:** 1 vial. Upon arrival store at -20^{0}C.

VII. Materials required but not supplied:

1. Black 96-well plates (clear bottom optional for bottom reading instruments).
2. Fluorescence plate reader.

3. Flow Cytometer or Fluorescence microscope.
4. Tubes, pipettes etc.
5. 1X PBS pH 7.0-7.4. or other suitable buffer.

VIII. General Information.

Probe NP3 is an $ONOO^-$ specific probe. It is easily cell permeable and detects the upregulated intracellular $ONOO^-$ with fluorogenic response. It is two-photon microscopy compatible. Its molecular weight is 362.1, with optimal λex 405 nm.

1. NP3 cell or tissue staining concentrations: 5-10 µM final.

2. In-vivo injection dose 10mg/kg.

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IX. Suspension Cell Preparation

1. Keep cell in log phase growth.
2. Day of the assay, wash cells 3 times with 1X PBS or other suitable media and resuspend cells in fresh media for your experimental protocol. Induce your experimental protocol.
3. Cell Staining. Harvest the cells and wash them 3 times in 1X PBS (pH 7.4). Adjust cell concentration to 1-2x10^6 cells/mL and add 5-10 µM (final concentration) to your samples. Incubate for 15-30 minutes at room temperature or 37^{0}C . Additional dyes or stains can be added at this time. After incubation wash cells with 1X PBS (pH 7.4) to remove residual dyes and resuspend cells in 1X PBS. Cells are now ready for fluorescence analysis.

Note: Each investigator should optimize cells concentration and dye concentration for their experiments.

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2. When we ready fluorescence do we wash the samples before reading or just read?
3. You mention add the NP3 dye, it this added directly to the media + cells or are the cells first washed into PBS (or other media) then loaded with dye before analysis?

X. Adherent Cell Preparation.

Note: Each investigator should optimize cells concentration and dye concentration for their experiments.

Culture cells on glass cover slips or other suitable container overnight and next treated according to your experimental protocol for indicated time. Do we wash cells at this time point? then stained with **NP3** probe (5 µM) at 37°C for 30 min. Cells may be observed under confocal microscopy directly or may be fixed in 4% PFA and imaged by confocal microscope. **NP3** fluorescence should be collected at 420-480 nm with λex 405 nm. This dye is compatible with propidium iodide for staining nuclei. This probe won't locate to a specific organelle but rather in the cytoplasm. Cells stained with **NP3** may also be observed under two-photo microscopy

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XI. Technical Notes

1. Pacher, Pál et al. "Nitric oxide and peroxynitrite in health and disease." *Physiological reviews* vol. 87,1 (2007): 315-424. doi:10.1152/physrev.00029.2006

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Natalia Ríos, ... Rafael Radi, in Nitric Oxide (Third Edition), 2017

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Rafael Radi From the Departamento de Bioquímica and Center for Free Radical and Biomedical Research, Facultad de Medicina, Universidad de la República, 11800 Montevideo, Uruguay

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*Patent Applications and Trade Marks pending.