CELL TECHNOLOGY

hROS™

Selective Indicators for Highly Reactive Oxygen Species

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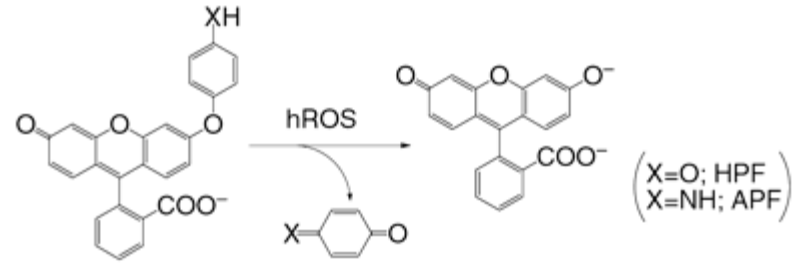
I. Introduction.

The two new novel probes, Aminophenyl fluorescein (APF) and Hydroxyphenyl fluorescein (HPF) developed by Tetsuo Nagano et. al. (1), are selective dyes for the detection of highly reactive oxygen species (hROS). The compounds themselves are not very fluorescent, however when reacted with hROS (hydroxyl radical:·OH, Peroxinitrite: $ONOO^-$ and hypochlorite: ^-OCl), APF and HPF exhibit strong dose dependent fluorescence. Furthermore, using these probes together, hypochlorite (^-OCl) can be selectively detected from hydroxyl radical:(·OH) and Peroxinitrite: ($ONOO^-$) . HPF/APF can be used to differentiate hROS from H202, nitric oxide (NO) and superoxide [1].

I. Assay Principle:



Reference 1.

Storage:

1. Aminophenyl fluorescein (APF) and Hydroxyphenyl fluorescein (HPF) should be stored at 4-8^{0}C. Protect from light until ready to use. The diluted material must be used immediately and discard any unused diluted material.

III. Warnings and Precautions:

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

IV. Kit Contents.

1. **Kit: Hydroxyl radical/Peroxinitrite Detection: Part# 5020**

    1. 1 vial: Part# 4012 HPF 5mM slution in DMF

2. **Kit: Hypochlorite Detection: Part# 5021**

    1. 1 vial : Part# 4011. APF: 5mM solution in DMF.
    2. 1 vial: Part # 4012. HPF: 5mM solution in DMF.

Materials and equipment required but not supplied:

1. Fluorescence plate reader / Fluorescent Microscope
2. Serum/BSA Free buffer

V. Preparation of reagent working solutions:

1. Cells should be loaded with HPF and APF in serum/BSA free media. HPF and APF can be diluted to a 10μM loading solution in: modified HBSS with 10mM Hepes, 1.0mM $MgCl_2$, 2.0 mM $CaCl_2$ and 2.7mM glucose or Krebs-Ringers phosphate buffer (with 114mM NaCl, 4.6mM KCl, 2.4mM $MgSO_4$, 1.0mM $CaCl_2$, 15mM NaH2PO4NaHPO4, pH 7.4).

Attention: As Phenol red may interfere with the assay, care should be taken to avoid using media containing Phenol red.

1. Table of Reactivity [1].

ROS	HPF Ex:499 Em:515	APF Ex:499 Em:515	DCFH-DA Ex:500 Em:520
Hydroxyl Radical: $^{\cdot}OH$	730	1200	7400
Peroxinitrite: $ONOO^-$	120	560	6600
Hypochlorite: ^-OCl	6	3600	86
Oxygen Radical: $^1O_2$	5	9	26
Superoxide: $O_2^{-\cdot}$	8	6	67
Hydrogen Peroxide : H_20_2	2	<1	190
Nitric Oxide: NO	6	<1	150
Alkylperoxyl Radical: $ROO^{\cdot}$	17	2	710
Autoxidation	<1	<1	2000

VI. Assay Protocol: Cells.

1. Rinse cells with modified HBSS or Krebs-Ringers phosphate buffer (as described above step V). *See Technical note 1 below.*
2. After the final wash, adjust cells to desired concentration (.1 to 1 x10^6 cells/mL) in HBSS or Krebs-Ringers phosphate buffer. Adherent cultures do not need detachment before loading the dye. Note: The optimal cell concentration may vary, and should be empirically determined by each investigator.

3. Loading the cells with APF / HPF. The final concentration of APF or HPF should be in the range of 1-10μM. For example dilute APF/HPF 1:10 in HBSS or Krebs-Ringers phosphate buffer to yield a 500μM stock solution. Add 2μL of the diluted APF / HPF to 100 μL of sample to yield a final concentration of 10μM.

Note: Each investigator should determine the optimal staining concentration for their particular application.
See Technical note 2 below.

5. Incubate cell for 30-60 minutes between 25-37^{0}C in the dark.
   Note: Each investigator should determine the optimal loading temperature and length of incubation for their particular application.

6. Without washing, activate cells according to your experimental protocol.

7. Measure fluorescence using a fluorescence plate reader or Fluorescent Microscope, utilizing excitation: 488nm and emission: 515nm.

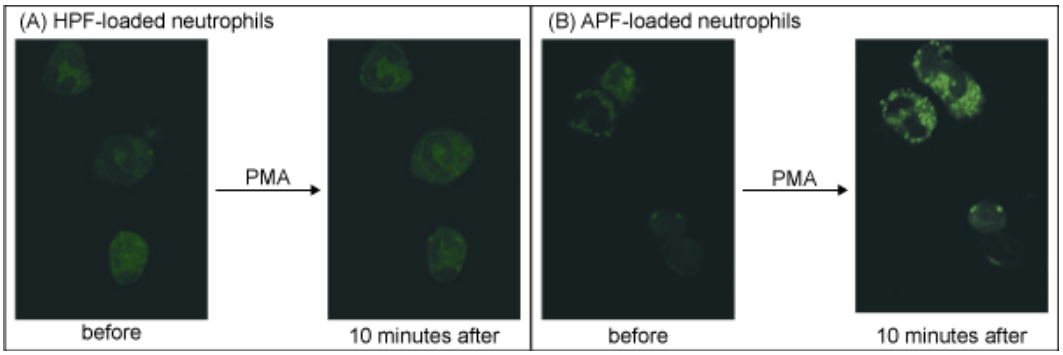


Figure 1: Detection of Hypochlorite ($^-$OCl) in neutrophils. Neutrophils were isolated from porcine blood, washed in Krebs-Ringers phosphate buffer (as described in step VI. above) and seeded in glass bottom dishes. The cells were then loaded with APF or HPF (10μM final) by incubation for 30 minutes at room temperature. The Dye-loaded neutrophils were stimulated with PMA (2ng/mL). Fluorescence images were acquired before and 10 minutes after stimulation. Excitation: 488nm emission: 505-550 nm barrier filters [1]

Technical Notes

1. DMSO is a scavenger of Hydroxyl Radical.
2. Hypochlorite can be detected by loading two samples, one with APF and the other with HPF. Hypochlorite production is visualized by increase in fluorescence of APF and no increase in fluorescence in HPF loaded cells.

References:

1 Ken-ichi Setsukinai, Yasuteru Urano, Katsuko Kakinuma, Hideyuki J. Majima , and Tetsuo Nagano. Development of Novel Fluorescence Probes That Can Reliably
Detect Reactive Oxygen Species and Distinguish Specific Species. THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 5, Issue of January 31, pp. 3170–3175, 2003