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Fluorescent Hypoxia Sensor for Flow Cytometry

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ABSTRACT

Hypoxia is a condition where low levels of oxygen levels are present (1% to 2% O_2). Hypoxia play a wide role in physiological and pathological conditions, from developmental angiogenesis to tumor progression and evasion. Hypoxia also modulates a number of immune functions from promoting inflammation to suppressing adaptive immunity. The current method for detecting hypoxic cells relies on an immunochemical approach. Pimonidazole reacts with peptide thiols in hypoxic cells and the resulting adduct is detected with an anti-pimonidazole antibody. To increase the availability of hypoxia detection reagents, we have developed a fluorogenic hypoxia reagent (HR) for live cells. Using Jurkat leukemia cells incubated in hypoxic or normoxic conditions and stained with HR, we were able to clearly distinguish hypoxic cells from normoxic cells on a flow cytometer. We were also able to resolve cells incubated at differing levels of O_2 (10%, 5% and 2.5% O_2). To demonstrate that HR is compatible with other reagents, hypoxic cells were co-stained with a viability dye (SYTOX Red), a dye retained in intact mitochondria (TMRM) and with staining for a marker of apoptosis (annexin V). The results show that hypoxic cells excluded the dead cells dye while retaining TMRM, and were negative for annexin V staining at early time points. As expected, prolong incubation at hypoxia resulted in dead and apoptotic cells as evident in an increase in dead cells staining, a loss of mitochondrial integrity and an increase in annexin V$^+$ cells. In summary, we present a sensitive reagent for detecting hypoxic cells without the need for cellular fixation and permeabilization, while retaining it usage with other live cell flow cytometry detection reagents.

INTRODUCTION

Hypoxia has been shown to play a wide role in a variety of physiological and pathological conditions. These range from developmental angiogenesis to tumor progression and evasion. The current method of hypoxic cell identification has been a multi-step, antibody-based method. One of the fluorogenic hypoxia sensors for live cells that are currently available is reversible and does not lend itself well to flow cytometry applications. The Hypoxia Green Reagent is a novel, fluorogenic compound for measuring hypoxia in live cells. It is non-fluorescent when live cells are in an environment with normal oxygen concentrations and becomes fluorescent when oxygen levels are decreased. Green Hypoxia Reagent is a more sensitive reagent than pimonizadole, as pimonidazole adducts are formed at very low oxygen levels, whereas Green Hypoxia Reagent begins to fluoresce when oxygen levels are less than 10%.

MATERIALS AND METHODS

Hypoxia: Hypoxic growth conditions were created using a Heracell™ VIOS 160i CO_2 incubator. Using the on-board variable oxygen control, cells were grown in hypoxic (1% to 10% O_2). Normoxic cells, on the other hand, were incubated in 5% CO_2.

Staining with Hypoxic Sensor: Hypoxia Green Reagent was used at a final concentration of 1 µM, staining cells at a concentration of 1 x 10^6 cells/ml. Cell staining with the Hypoxia Green reagent was carried out in cells under their respective culture conditions for the duration between 30 minutes to 2 hours.

Flow Cytometry: Cells were analyzed using the Attune NxT Acoustic Cytometer. Cells were segregated from cellular debris using scatter parameters. Hypoxia Green signal was detected using the 488 nm laser channel with 530/30 nm bandpass (FITC, GFP channel). In some experiments, 561 nm laser excitation and 585/15 nm band pass detection was used (TMRM; tetramethylrhodamine, methyl ester for mitochondrial health). For SYTOX Red detection, 638 nm laser excitation was used with 670/14 nm band pass detection.

Microscopy: A549 cells were plated on MatTek dishes at a density of 100,000 cells/dish and incubated at 37° C overnight. The next day, culture media was changed, and cells were stained with 5 µM of Hypoxia Green Reagent. Subsequently, the cells were incubated at 20%, 5%, 2.5%, or 1% O_2 for 3 hrs. The cells were then washed twice with Live Cell Imaging Solution (LCIS) and imaged on a Zeiss 710 confocal microscope.

Pimonidazole mediated detection: Cells were treated with pimonidazole at a final concentration of 300 µM and incubated at 20% O_2 or 1% O_2 for 3 hrs. The cells were then fixed with formaldehyde (4%) and permeabilized with 0.2% Triton X-100. Staining with a primary antibody against pimonidazole and with an Alexa Fluor® 488 conjugated secondary antibody followed a standard Immunofluorescence protocol. Cells were imaged on a EVOS FL Auto Cell Imaging System using a GFP filter.

RESULTS

Figure 1. Spectrum of Hypoxia Green Reagent

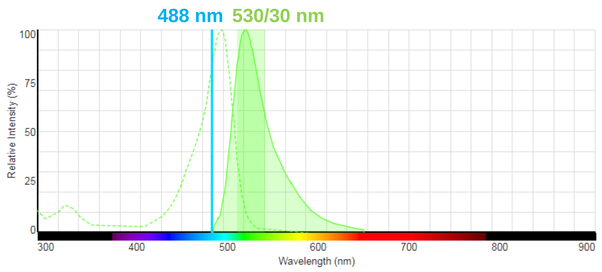

Figure 1. Spectrum of Hypoxia Green.
Excitation and emission spectra of Hypoxia Green Reagent fits into flow cytometer channel for FITC, Alexa Fluor 488 and GFP. Optimal excitation is with the 488 nm laser and detection is shown here with a 530/30 nm bandpass filter.

Figure 2. Response to hypoxia with Hypoxia Green Reagent

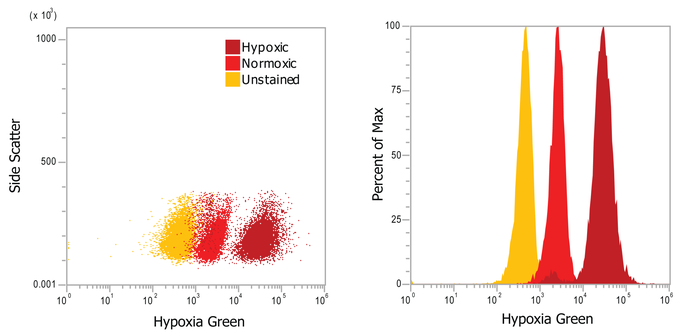

Figure 2. Hypoxic cells response with an increase in fluorescence in 530/30 nm channel (FITC, AF488, GFP) with Hypoxia Green Reagent.
Jurkat cells, a human leukemia cell line, were incubated at normoxic (20% O_2) or hypoxic levels of O_2, (1%) for 18 hours. After which, Hypoxia Green reagent was added to the cells and incubated for 3 hours at the respective O_2 levels. Cell were subsequently analysed on an Attune NxT Cytometer, with excitation at 488 nm and detection using a 530/30 nm filter.

Figure 3. Sensitive detection of hypoxia with Hypoxia Green Reagent

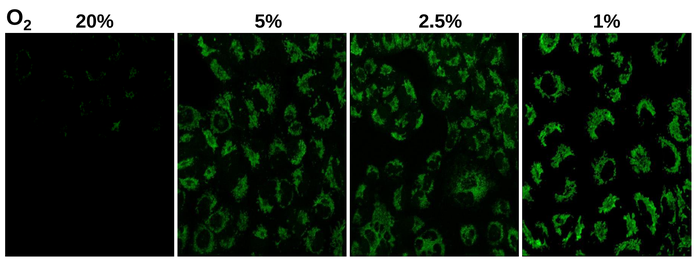

Figure 3. Hypoxia Green Reagent show response to decreasing amounts of O_2 starting at 5% O_2 in A549 cells
A549 cells, a human lung carcinoma line, were plated on MatTek dishes and incubated at 37°C overnight. Then, the cells were stained with 5 µM of Hypoxia Green Reagent and incubated at 20%, 5%, 2.5%, or 1% O_2 for 3 hrs. The resulting cells were imaged on a Zeiss 710 confocal microscope.

Figure 4. Differential response to decreasing O_2 with Hypoxia Green Reagent

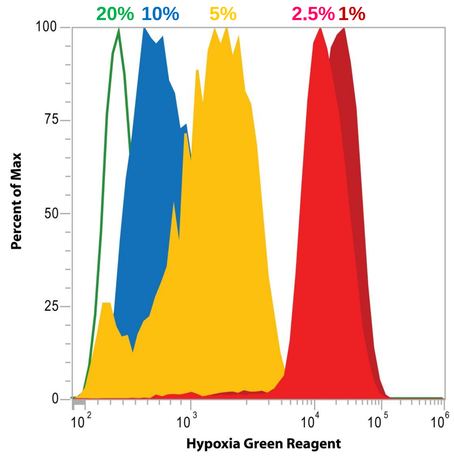

Figure 4. Cell incubated in decreasing amounts of O2 gave increasing signal with Hypoxia Green Reagent
Jurkat cells were incubated at normoxic (20% O_2) or decreasing levels of O_2, (10%, 5%, 2.5% and 1%) for 18 hours. After which, Hypoxia Green Reagent was added to the cells and incubated for 3 hours. At the end of the incubation period, cell were harvested and analysed on an Attune NxT Cytometer. Hypoxia Green Reagent was excited with a 488 nm laser and the signal was detected using a 530/30 nm filter. In the overlay, normoxic cells are denoted by the green outlined histogram, hypoxic cells are represented by the shaded histograms.

Figure 4. Performance benchmark to pimonidazole

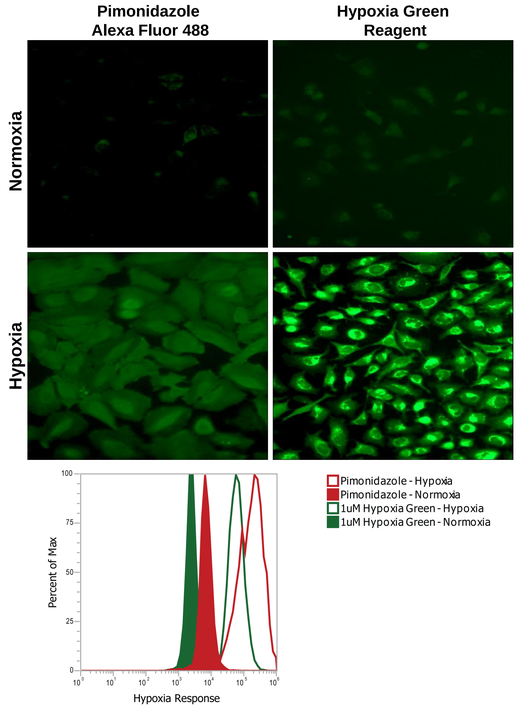

Figure 4: Performance of Hypoxia Green Reagent vs pimonidazole
HeLa or Jurkat cells, incubated at 20% O_2 or 1% O_2 for 3 hrs, were stained with either the Hypoxia Green Reagent or with anti-pimonidazole antibody. Cells were imaged on an EVOS™ FL Auto Cell Imaging System with a filter for GFP or were analysed with an Attune NxT Cytometer.

Figure 5. Multiplexing with Hypoxia Green Reagent

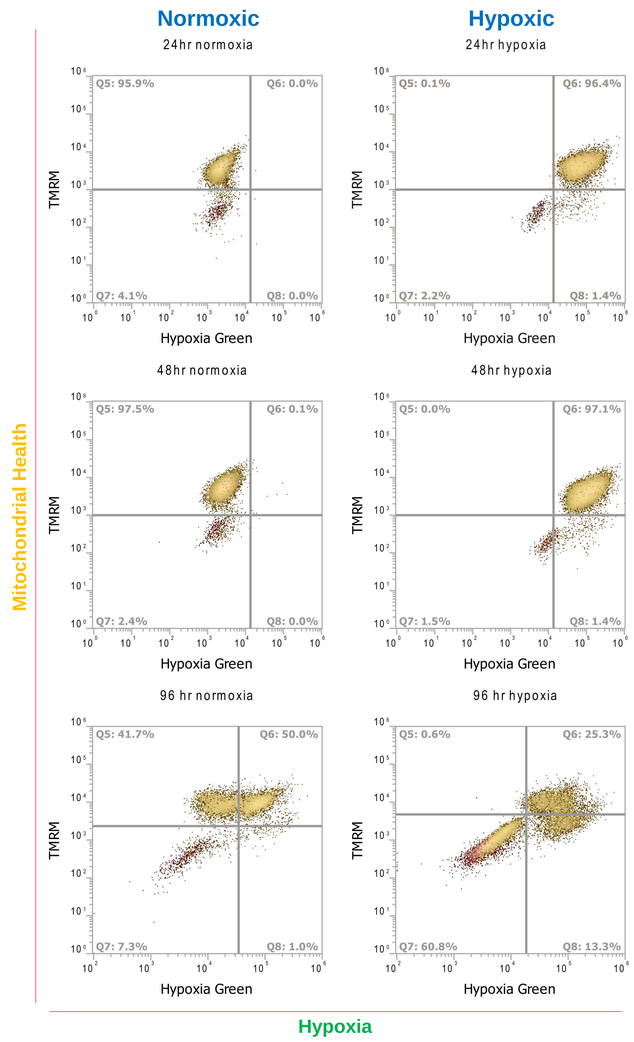

Figure 5. Detection of hypoxic cells was combined with mitochondrial health and viability analysis.
Prolonged incubation at 1% O2 gave rise to hypoxic phenotype (right column). Yellow shading in the dot plots are derived from SYTOX Red$^-$ (live cells) and red shading are SYTOX Red$^+$ (dead cells).
At 24 and 48 hours of treatment, cells retained mitochondrial functions as determined by the retention of TMRM signal.
At 96 hours of hypoxia, a reduction in TMRM signal suggesting loss of mitochondrial function in hypoxic cells, as well as an increase in cell death (lower left quadrant).
Cell were analysed on an Attune NxT Cytometer.

Figure 6. Multiplex with apoptosis and viability detection

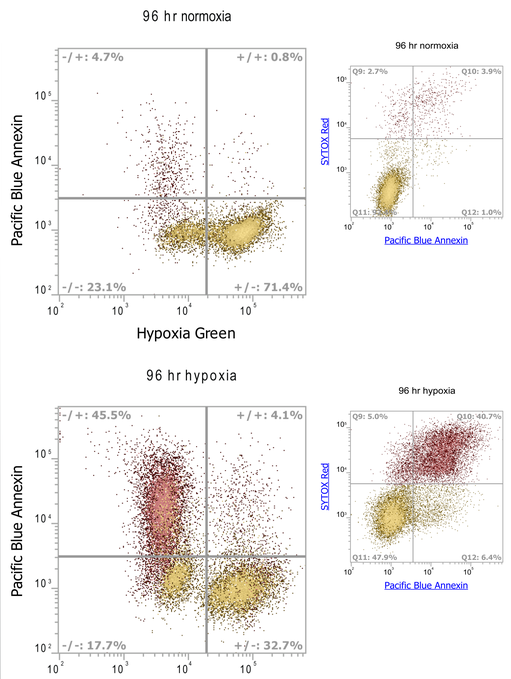

Figure 6:Multiplex staining of Hypoxia Green Reagent with Pacific Blue Annexin
96 hour incubation in 1% O_2 resulted in increase cellular apoptosis and death, where as 96 hour incubation in 20% O_2 resulted hypoxia response in a large population of cells.
Insert plots confirms late apoptotic nature of cells after 96 hours of hypoxia treatment.
Jurkat cells were incubated in 20% O_2 or 1% O_2 for 96 hours, and detection of apoptosis was with Annexin V staining, viability with SYTOX Red and hypoxia response with Hypoxia Green Reagent.

CONCLUSIONS

- Hypoxia Green Reagent has peak excitation at 490 nm and emission at 520 nm
- Hypoxia Green Reagent reveal distinct normoxic and hypoxic populations
- Works as an end point reagent in flow cytometry and imaging platforms.
- Hypoxia Green Reagent is sensitive to detect 10% O_2 to 1% O_2
- Equivalent performance to pimonidazole, and amenable to detection of hypoxic responses in live cells.
- Compatible in multiplex experiments with other functional reagents (Annexin V conjugates, live cell viability dyes, mitochondrial membrane potential sensor)

TRADEMARKS/LICENSING

Hypoxia Green reagent used:
Imaging: Image-iT™ Green Hypoxia Reagent, I14834
Flow cytometry: Hypoxia Green Reagent for Flow Cytometry, H20035

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