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 thiol in hypoxic cells and the resulting adduct is detected with an anti-pimonidazole antibody. To increase the availability of hypoxia detection reagents, the Hypoxia Green 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 an 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 fluorescent hypoxia sensors for live cells that are currently available is reversible and does not lend itself to flow cytometry applications. The Hypoxia Green Reagent is a novel, fluorescent compound for measuring hypoxia in live cells. It is non-fluorescent when oxygen levels are increased whereas Green Hypoxia Reagent begins to fluoresce when oxygen levels are decreased. Green Hypoxia Reagent is a method for detecting hypoxic cells relies on an antibody mediated detection:

MATERIALS AND METHODS
Hypoxia: Hypoxic growth conditions were created using a Heracell™ VKOS 160i CO2 incubator. Using the on-board variable oxygen control, cells were grown in hypoxia (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 \(\mu\)M 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 \(\mu\)M 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 \(\mu\)M 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 2. Response to hypoxia with Hypoxia Green Reagent

Figure 3. Sensitive detection of hypoxia with Hypoxia Green Reagent

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

Figure 5. Multiplexing with Hypoxia Green Reagent

Figure 6. Multiplex with apoptosis and viability detection

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/LICENSES
Hypoxia Green reagent used:
Imaging: Image-iT™ Green Hypoxia Reagent, 141834
Flow cytometry: Hypoxia Green Reagent for Flow Cytometry, H20035

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