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Hypoxia measurements in live and fixed cells using fluorescence microscopy and high-content imaging

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ABSTRACT

Hypoxia is an important phenomenon in many physiological processes and involved in many human diseases including cancers, cardiovascular diseases, and neurodegenerative diseases. The study of hypoxia has been complicated by the lack of sensitive dyes for measuring hypoxia at greater than 1% O_2. Here we describe a live cell–based method to conveniently measure hypoxia using Invitrogen™ Image-iT™ Green Hypoxia Reagent. Image-iT Green Hypoxia Reagent is a hypoxia-sensing fluorescent probe, and has excitation and emission peaks at 488 nm and 520 nm, respectively. The probe is sensitive to varying concentrations of oxygen and can detect O_2 concentrations as low as 5% in cells. Using this probe, we measured hypoxia in several cell lines, including A549, HeLa, and U2OS, using fluorescence microscopy, high-content imaging, and a fluorescence plate reader. Image-iT Green Hypoxia Reagent can be multiplexed with indicators for other important physiological parameters like mitochondrial membrane potential, apoptosis, and oxidative stress. Image-iT Green Hypoxia Reagent works well in detecting hypoxia in 3D tumor spheroids. Image-iT Green Hypoxia Reagent is a formaldehyde-fixable probe, and it facilitates sensitive, robust, and reproducible endpoint measurements of hypoxia in cells.

INTRODUCTION

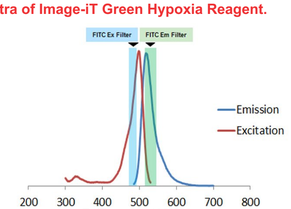
Image-iT Green Hypoxia Reagent is a novel fixable fluorogenic compound for measuring hypoxia in live cells. It is nonfluorescent when live cells are in an environment with normal oxygen concentrations, and becomes fluorescent when oxygen levels are decreased. Image-iT Green Hypoxia Reagent sustains its fluorescence when cells or tissue return to normal oxygen levels, allowing the cells or tissue to be fixed with minimal loss of fluorescent signal.

Features of Image-iT Green Hypoxia Reagent include:

- **Sensitivity**—indicates hypoxia in live cells by fluorescing in low-oxygen environments
- **Fixability**—sustains fluorescent signal when cells/tissue return to normal oxygen levels
- **Ease of use**—just add to cell culture medium and image

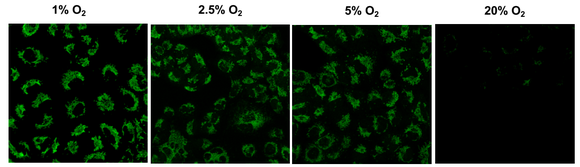
Image-iT Green Hypoxia Reagent is a fluorogenic compound that is live cell permeant and becomes fluorescent in environments with low oxygen concentrations. These properties make it a highly useful tool for detecting cells and tissues under hypoxic conditions. Image-iT Green Hypoxia Reagent is a very sensitive oxygen detector. Unlike pimonidazole adducts that respond only to very low oxygen levels, Image-iT Green Hypoxia Reagent begins to fluoresce when atmospheric oxygen levels are less than 5%. It responds quickly to such environments, and the fluorescence is sustained after the oxygen levels return to normal. These properties make Image-iT Green Hypoxia Reagent an ideal tool for detecting hypoxic conditions around tumors, 3D cultures, spheroids, and neurons.

Figure 1. Spectra of Image-iT Green Hypoxia Reagent.



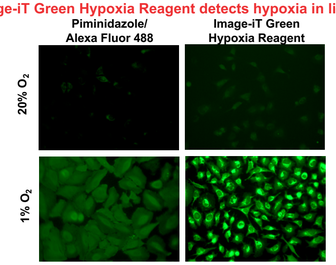
RESULTS

Figure 2. Sensitive detection of hypoxia with Image-iT Green Hypoxia Reagent.



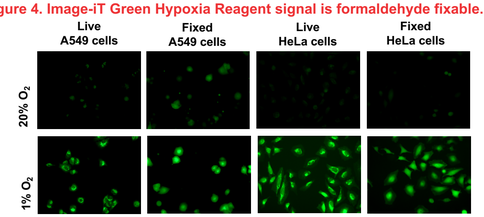
A549 cells were plated on MatTek™ dishes at a density of 1 x 10^5 cells/dish and left overnight at 37°C in a CO_2 incubator. The existing medium was removed from the cells and fresh growth medium containing Image-iT Green Hypoxia Reagent (5 µM final concentration) was added. Cells were incubated at 20%, 5%, 2.5%, or 1% O_2 for 3 hr. The cells were then washed twice with Invitrogen™ Live Cell Imaging Solution and imaged on a confocal microscope.

Figure 3. Image-iT Green Hypoxia Reagent detects hypoxia in live cells.



HeLa cells were plated on MatTek dishes at a density of 1 x 10^5 cells/dish and left overnight at 37°C in a CO_2 incubator. The existing medium was removed from the cells, and fresh growth medium containing Image-iT Green Hypoxia Reagent (5 µM final concentration) or piminidazole (300 µM) was added. Cells were incubated at 20% or 1% O2 for 3 hr. The cells were then washed twice with Live Cell Imaging Solution. The cells with Image-iT Green Hypoxia Reagent were imaged on the Invitrogen™ EVOS™ FL Auto Imaging System using a GFP filter. The cells with piminidazole were formaldehyde fixed (4%), detergent permeabilized (0.2% Triton™ X-100), and stained using a standard immunofluorescence protocol with a primary antibody against piminidazole and Invitrogen™ Alexa Fluor™ 488 secondary antibody, and then imaged on the EVOS FL Auto Imaging System using a GFP filter.

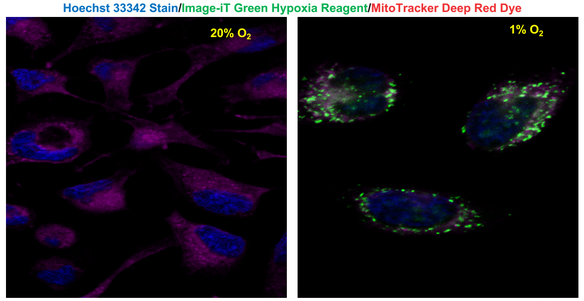
Figure 4. Image-iT Green Hypoxia Reagent signal is formaldehyde fixable.



A549 and HeLa cells were plated on MatTek dishes at a density of 1x10^5 cells/dish and left overnight at 37°C in a CO_2 incubator. The existing medium was removed from the cells and fresh growth medium containing Image-iT Green Hypoxia Reagent (5 µM final concentration) was added. Cells were incubated at 20% or 1% O_2 for 3 hr. The cells were then washed twice with Live Cell Imaging Solution and imaged on the EVOS FL Auto Imaging System using a GFP filter. The cells were then formaldehyde fixed (4%) for 15 min, washed twice with PBS, and again imaged on the EVOS FL Auto Imaging System using a GFP filter.

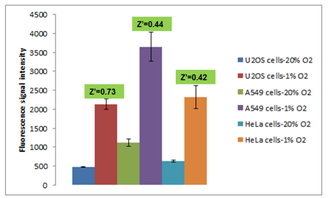
Figure 5. Detection of hypoxia and mitochondrial function in live cells.

Hoechst 33342 Stain/Image-iT Green Hypoxia Reagent/MitoTracker Deep Red Dye



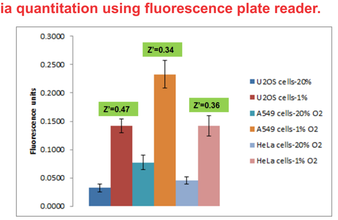
A549 cells were plated on MatTek dishes at a density of 1x10^5 cells/dish and left overnight at 37°C in a CO_2 incubator. The existing medium was removed from the cells, and fresh growth medium containing Image-iT Green Hypoxia Reagent (5 µM final concentration) was added. Cells were incubated at 20% or 1% O_2 for 3 hr. The cells were then washed twice with Live Cell Imaging Solution, stained with 50 nM Invitrogen™ MitoTracker™ Deep Red FM, and imaged on a confocal microscope.

Figure 6. Hypoxia quantitation using high-content imaging.



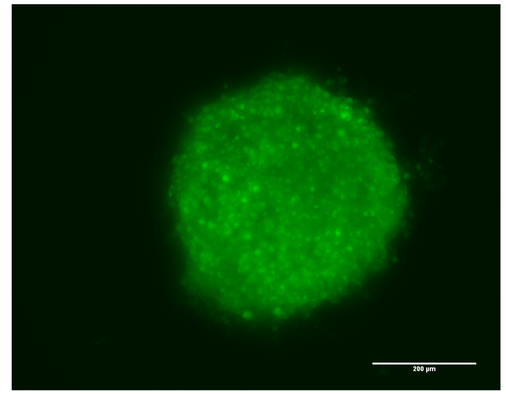
A549, HeLa, or U2OS cells were plated on a Greiner 96-well plate at a density of 7x10^3/well and left overnight at 37°C in a CO_2 incubator. The existing medium was removed from the cells and fresh growth medium containing Image-iT Green Hypoxia Reagent (5 µM final concentration) was added. Cells were incubated at 20% or 1% O_2 for 5 hr. The cells were then washed twice with Live Cell Imaging Solution, stained with Hoechst 33342 (2 µM), and imaged and analyzed on the Thermo Scientific™ CellInsight™ CX5 High Content Screening Platform.

Figure 7. Hypoxia quantitation using fluorescence plate reader.



A549, HeLa, or U2OS cells were plated on a Greiner 96-well plate at a density of 7x10^3/well and left overnight at 37°C in a CO_2 incubator. The existing medium was removed from the cells and fresh growth medium containing Image-iT Green Hypoxia Reagent (5 µM final concentration) was added. Cells were incubated at 20% or 1% O_2 for 5 hr. The cells were then washed twice with Live Cell Imaging Solution, stained with Hoechst 33342 (2 µM), and analyzed on the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader.

Figure 8. Detection of hypoxia in spheroids.



A549 cells were grown on a Thermo Scientific™ Nunclon™ Sphera™ 96-well U-bottom plate at a density of 1,000 cells/well. After 2 days of culture on these plates, the spheroids were stained with Image-iT Green Hypoxia Reagent and Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (blue) for 1 hr. The images were acquired on the EVOS FL Auto 2 Imaging System.

CONCLUSIONS

- Hypoxia is a very important phenomenon in many human diseases.
- Piminidazoles work well at ≤1% O_2; Image-iT Green Hypoxia Reagent works well at O_2 levels as high as 5%.
- Sensitive detection of hypoxia as an endpoint assay is enabled by Image-iT Green Hypoxia Reagent.
- Image-iT Green Hypoxia Reagent is formaldehyde fixable and has a much simpler workflow compared to hypoxia detection using piminidazoles.
- Image-iT Green Hypoxia Reagent has peak excitation and emission at 490 and 520 nm, respectively, and allows multiplexing with other functional reagents for a multiparametric study of hypoxia.

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