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

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ABSTRACT

Hypoxia is an important phenomenon in many physiological processes and involved in many human diseases including cancer, cardiovascular and neurodegenerative diseases. The study of hypoxia has been complicated with the lack of sensitive dyes to measure hypoxia at greater than 1% O_2. Here, we describe a live cell-based method to conveniently measure hypoxia using an Image-iT™ Hypoxia Green Probe or Hypoxia Green Reagent for flow cytometry. The Image-iT™ Hypoxia Green Probe and Hypoxia Green Reagent for flow cytometry are hypoxia sensing fluorescent probe, and has excitation and emission peaks of 488 and 520 nm respectively. The probe is sensitive to varying concentrations of oxygen and can detect as low as 5% O_2 concentrations in cells. Using this probe, we measured hypoxia in several cell lines including A549, HeLa, U-2 OS and Jurkat using fluorescence microscopy, high content imaging, fluorescence plate reader and flow cytometry. The new Hypoxia Probe is multiplexible with other important physiological parameters like mitochondrial membrane potential, apoptosis and oxidative stress. Image-iT™ Hypoxia Green Probe and Hypoxia Green Reagent for flow cytometry work well in detecting hypoxia in 3D tumor spheroids. The Image-iT™ Hypoxia Green Probe and Hypoxia Green Reagent for flow cytometry are formaldehyde-fixable that allows end-point measurement of hypoxia in cells. The Image-iT™ Hypoxia Green Probe and Hypoxia Green Reagent for flow cytometry facilitate sensitive, robust and reproducible measurements of hypoxia in cells

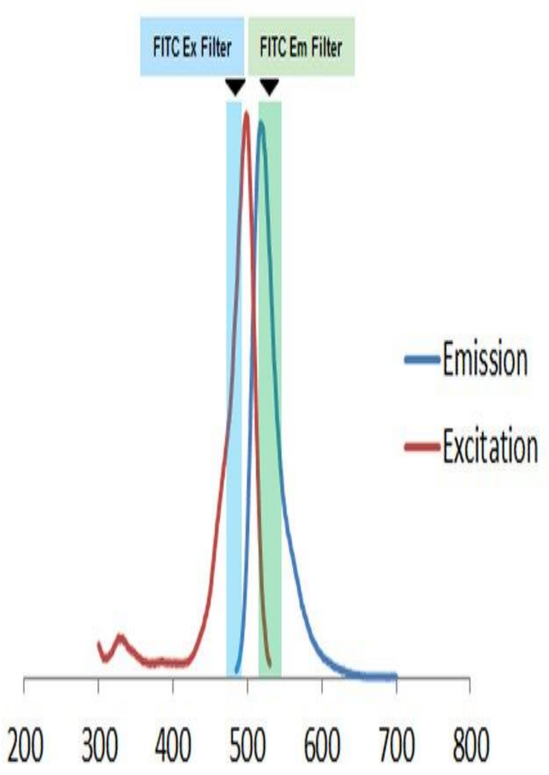
INTRODUCTION

Image-iT™ Hypoxia Green Reagent and Hypoxia Green Reagent for flow cytometry

Both Image-iT Green Hypoxia Reagent and Hypoxia Green Reagent for flow cytometry are novel, fixable fluorogenic compound for measuring hypoxia in live cells. They are non-fluorescent when live cells are in an environment with normal oxygen concentrations and becomes fluorescent when oxygen levels are decreased. Both probes sustain their fluorescence when cells/tissue return to normal oxygen levels, allowing the cells/tissue to be fixed with minimal loss of fluorescent signal.

Features of Image-iT Green Hypoxia Reagent include:
- Indicates hypoxia in live cells by fluorescing in low oxygen environments
- Fixable—sustains fluorescent signal when cells/tissue return to normal oxygen levels
- Easy to use—just add to cell culture media and image

Image-iT Green Hypoxia Reagent and Hypoxia Green Reagent for flow cytometry are fluorogenic compounds that are live cell permeable and becomes fluorescent in environments with low oxygen concentrations. These properties makes it a highly useful tool for detecting cells and tissue under hypoxic conditions. Image-iT Green Hypoxia Reagent or Hypoxia Green Reagent for flow cytometry are very sensitive oxygen detector. Unlike pimonidazole adducts that respond only to very low oxygen levels, both Image-iT Green Hypoxia Reagent and Hypoxia Green Reagent for flow cytometry begin 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, neurons, etc.



Ex/Em Max: 488/520 nm

RESULTS

Figure 2: Sensitive detection of hypoxia with Image-iT Hypoxia Green or Hypoxia green reagent for Flow Cytometry

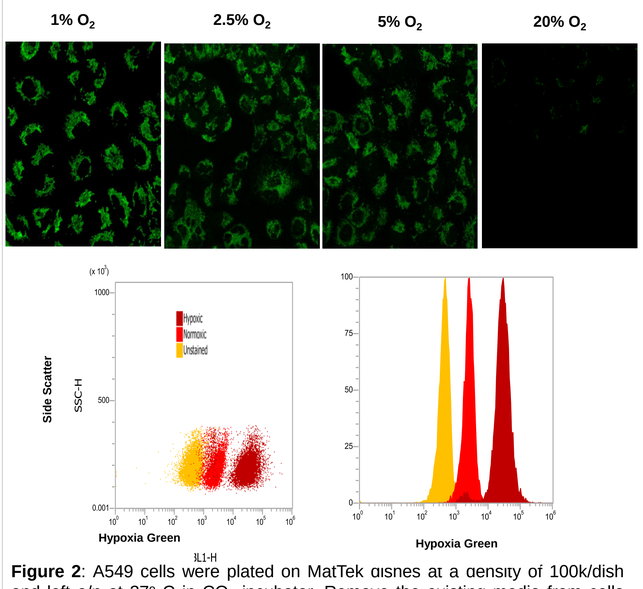


Figure 2: A549 cells were plated on MatTek dishes at a density of 100k/dish and left o/n at 37º C in CO_2 incubator. Remove the existing media from cells and add fresh growth media containing the Image-iT Green Hypoxia reagent at the final concentration of 5 µM. Incubate the cells at 20% O_2 , 5%, 2.5%, or 1% O_2 for 3 hrs. The cells were then washed 2x with Live Cell Imaging Solution (LCIS) and the cells were imaged on a Zeiss 710 confocal microscope. Hypoxic cells response with an increase in fluorescence in 530/30 nm channel (FITC, AF488, GFP) with Hypoxia Green Reagent. Jurket 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. Cell were analysed on an Invitrogen™ Attune™ NxT Cytometer, with excitation at 488 nm and detection using a 530/30 nm filter.

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

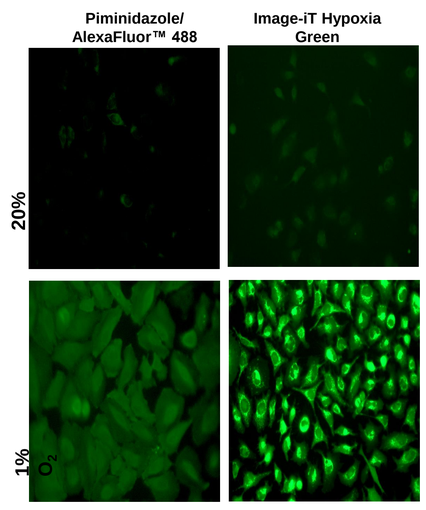


Figure 3: HeLa cells were plated on MatTek dishes at a density of 100k/dish and left o/n at 37º C in CO_2 incubator. Remove the existing media from cells and add fresh growth media containing the Image-iT™ Hypoxia Green reagent or Piminidazole at the final concentration of 5 µM and 300 µM respectively. Incubate the cells at 20% O_2 or 1% O_2 for 3 hrs. The cells were then washed 2x with Live Cell Imaging Solution (LCIS) and the cells with Image-iT™ Hypoxia Green reagent were imaged on a EVOS™ FL Auto Cell Imaging System using a GFP filter. The cells with Piminidazole were formaldehyde fixed (4%) and detergent permeabilized (0.2% Triton X-100) and stained using a standard Immunofluorescence protocol with primary antibody against Piminidazole and Alexa Fluor 488 secondary antibody and imaged on a EVOS FL Auto Cell Imaging System using a GFP filter.

Figure 4: Image-iT Hypoxia Green signal is formaldehyde-fixable

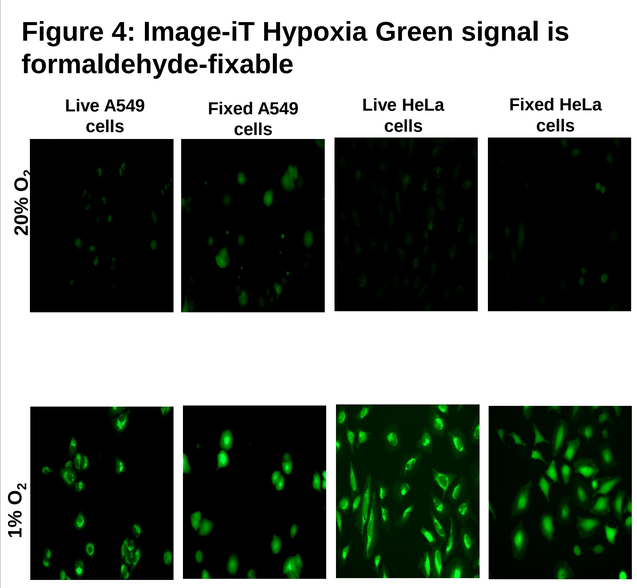


Figure 4: A549 cells were plated on MatTek dishes at a density of 100k/dish and left o/n at 37º C in CO_2 incubator. Remove the existing media from cells and add fresh growth media containing the Image-iT Hypoxia Green reagent at the final concentration of 5 µM. incubate the cells at 20% O_2 or 1% O_2 for 3 hrs. The cells were then washed 2x with Live Cell Imaging Solution (LCIS) and imaged on a EVOS FL Auto Cell Imaging System using a GFP filter. The cells were then formaldehyde (4%) fixed for 15 mins, washed 2x with PBS and then imaged on a EVOS FL Auto Cell Imaging System using a GFP filter.

Figure 5: Response to hypoxia with Hypoxia Green Reagent for flow cytometry

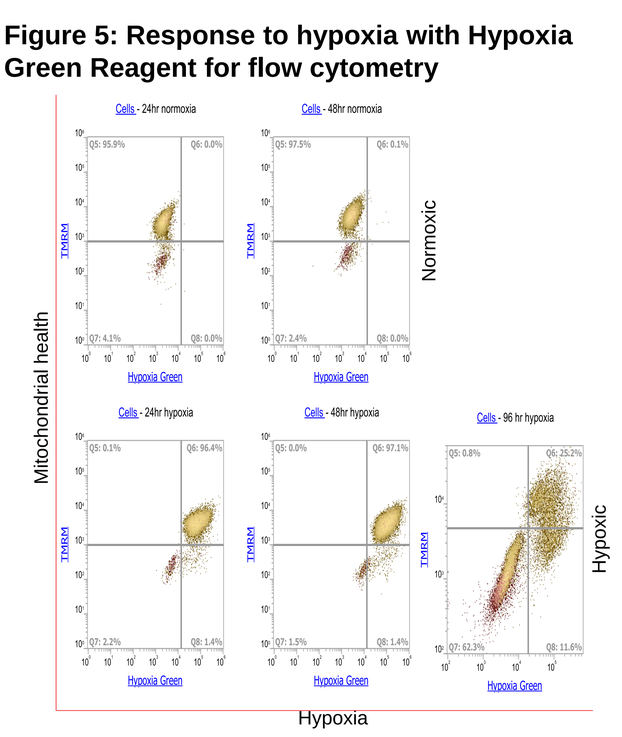


Figure 5: Detection of hypoxic cells was combined with mitochondrial health analysis. Prolonged incubation at 1% O2 gave rise to hypoxic phenotype (bottom row). At 24 and 48 hours of treatment, cells retained mitochondrial functions as determined by the retention of TMRM signal. At 96 hours of hypoxia, there was in greater loss in TMRM signal suggesting loss of mitochondrial function in hypoxic cells, as well as an increase in cells death (lower left quadrant). Yellow shading in the dot plots are derived from SYTOX Red$^-$ (live cells) and red shading are SYTOX Red$^+$ (dead cells). Hypoxia Green Reagent for flow cytometry allows for simultaneous detection of hypoxia and mitochondrial health. Jurket cells were incubated at normoxic (20% O_2) or hypoxic levels of O_2, (1%) for 24 and 48 hours hours. After which, Hypoxia Green reagent and TMRM were added to the cells and incubated for 3 hours. Cell were analysed on an Invitrogen Attune NxT Cytometer.

Figure 6: Hypoxia quantitation using high content imaging

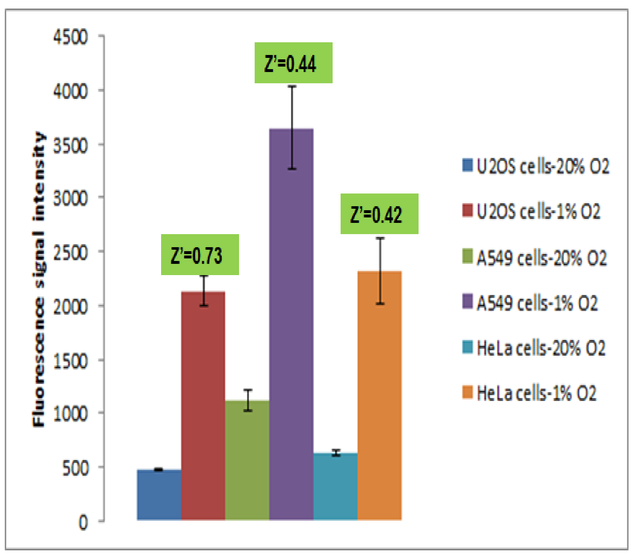


Figure 6; A549/HeLa/U2OS cells were plated on a Greiner 96-well plate at a density of 7k/well and left o/n at 37º C in CO_2 incubator. Remove the existing media from cells and add fresh growth media containing the Image-iT® Hypoxia Green dye at a final concentration of 5 µM. Incubate the cells at 20% O_2 or 1% O_2 for 5 hrs. The cells were then washed 2x with Live Cell Imaging Solution (LCIS), stained with Hoechst 33342 (2 µM), imaged and analyzed on the Thermo Scientific™ CellInsight™ CX5 HCS instrument.

Figure 7: Hypoxia quantitation using fluorescence plate reader

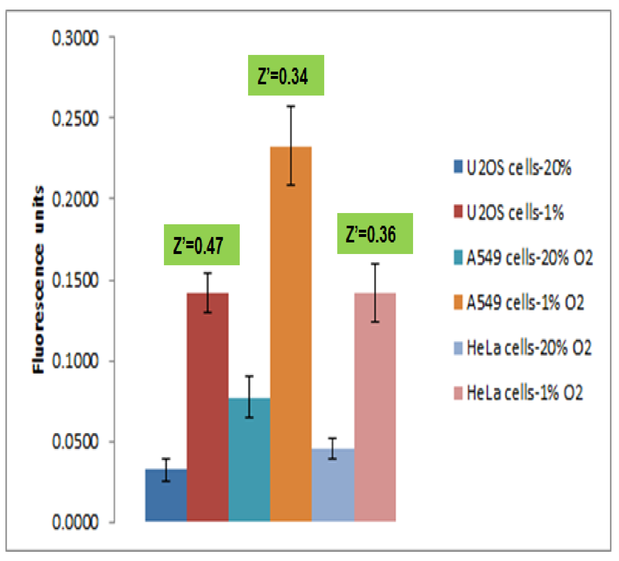


Figure 7: A549/HeLa/U2OS cells were plated on a Greiner 96-well plate at a density of 7k/well and left o/n at 37º C in CO_2 incubator. Remove the existing media from cells and add fresh growth media containing the Image-iT Hypoxia Green reagent at a final concentration of 5 µM. Incubate the cells at 20% O_2 or 1% O_2 for 5 hrs. The cells were then washed 2x with Live Cell Imaging Solution (LCIS), stained with Hoechst 33342 (2 µM), analyzed on the Thermo Scientific™ Varioskan™ LUX multimode microplate reader.

Figure 8: Detection of hypoxia in spheroids

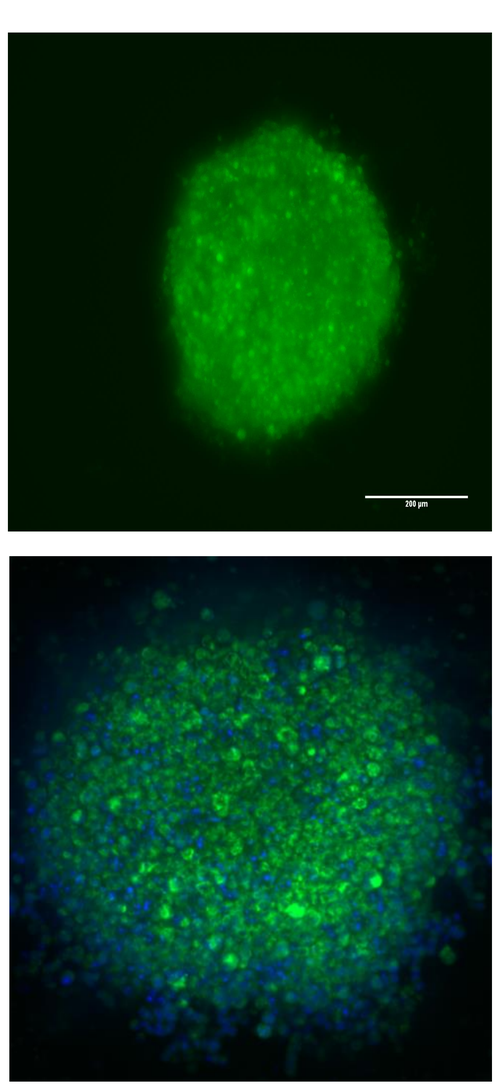


Figure 8: A549 cells were grown on a Nunclon Sphera 96-well U-bottom plates at a a density of 1000 cells/well. After two days of culture on Nunclon Sphera 96-well U-bottom plates, the A549 spheroids were stained with Image-iT Hypoxia Green Probe and NucBlue Live ReadyProbes reagent (blue) for 1 hr. The images were taken on a EVOS FL Auto II Cell Imaging System. (top) or CellInsight CX7 High content platform (Bottom)

CONCLUSIONS

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

TRADEMARKS/LICENSING

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