BIOSTATUS

HypoxiTRAK™: PRESERVING AND REPORTING THE CELLULAR EXPERIENCE OF HYPOXIC ENVIRONMENTS

Background

The role of hypoxia on cancer and stem cell behaviour is an expanding area of research presenting a renewed focus for drug and biomarker development. Low oxygen stress conditions can change the biological behaviour and pharmaco-dynamic responses of tumour cells due to the hypoxia-induced activation of cell protective and proliferation mechanisms.
HypoxiTRAK™ is a novel biomarker ideal for use in flow cytometry and imaging to report (i) the degree of hypoxic experience of cells (ii) identify the functionally hypoxic fraction of cells within in a population.

Product summary

- HypoxiTRAK™ is a far-red fluorescent molecule that readily enters live cells. Its spectral characteristics are shared with DRAQ5™
- HypoxiTRAK™ is converted to a metabolite, under hypoxic conditions, which persists in cells for several days allowing a simple determination of the extent of hypoxia, without antibody staining
- HypoxiTRAK™ is non-toxic and suited to continuous exposure that allows the generation of hypoxia dependent changes in cell biology
- The HypoxiTRAK™-metabolite is designed to halt cell cycle progression, preserving the absolute number of hypoxia-experiencing cells
- Cells sensitive to cycle arrest induced by HypoxiTRAK™-metabolite may undergo cell death - the *non-viable cell fraction* is a simple surrogate readout of the hypoxia experience of cells

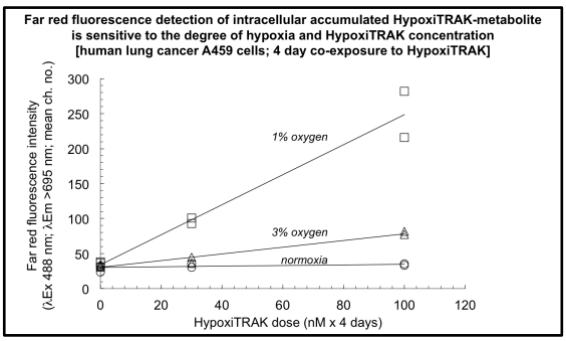

Figure 1. ANALYSING CELLULAR EXPERIENCE OF HYPOXIA USING HypoxiTRAK™ AND CONVENTIONAL FLOW CYTOMETRY
Analysis of degree of hypoxic experience over 4 days in cells co-exposed to HypoxiTRAK™. HypoxiTRAK™ added directly to monolayer cell cultures prior to incubation at different levels of oxygenation. Cells harvested using conventional method and analysed by directly by flow cytometry using 488 nm laser excitation and emission detection preferably >695 nm wavelengths.

Concept

The far red fluorescent HypoxiTRAK™ molecular probe is designed to convert exclusively in hypoxic cells to a bioactive metabolite that retains the parent molecule fluorophore but which now accumulates in cells to provide a positive and persistent far red fluorescent signature (figs. 2, 3, 7). The bioactive metabolite of HypoxiTRAK™ induces cell arrest, marking and 'freezing' the cells that are hypoxic within a cell population (figs. 5, 6). This enables direct read-out of a hypoxic cell fraction without the need for further manipulation; for example, no antibody-conjugate detection system is required.

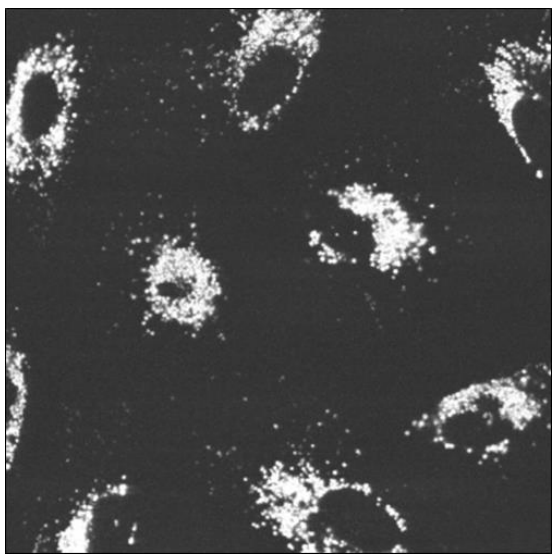

Figure 2. CELLULAR ACCUMULATION OF HypoxiTRAK™-METABOLITE
Confocal imaging of hypoxia-generated HypoxiTRAK™-metabolite accumulation in the cytoplasmic compartment (exc. 633 nm / Em. 680/20 nm; image shows a field of nine A549 cells previously exposed to 100 nM HypoxiTRAK™ for 4 days under 1% oxygen).

Retrospective sensor for biologically relevant levels of hypoxia

The HypoxiTRAK™ probe is designed to activate by bio-reduction at biologically relevant levels of hypoxia, given that *in vivo* tumour cells can occupy hypoxic niches with lower median oxygen levels (~1 % oxygen; pO_2 7.5 mmHg) compared to normal tissues (~5.5% oxygen; pO_2 42 mmHg) while tumour cores may maintain a less than 0.1% oxygen (pO_2 1 mm Hg) environment. The hypoxia sensing range for HypoxiTRAK™ is relevant to biomarker and hypoxia-targeting drug development. Since oxygenation levels can vary in both *in vivo* and *in vitro* models with regard to extent and duration, HypoxiTRAK™ provides a retrospective/historical reporting of the integrated hypoxic experience of a cell – it is a sensor of the degree of hypoxia experienced by each cell within a cell population both in vitro and in vivo (fig. 1).

Exploiting HypoxiTRAK™ spectral properties

HypoxiTRAK™ also provides a means of assessing the degree of hypoxia by simple assays for growth arrest or apoptosis. HypoxiTRAK™ bio-activation resulting in prolonged cell cycle arrest can be further detected by an increase in side scatter by flow cytometry. Since no fixation is required, HypoxiTRAK™ bio-activation can also provide a convenient method of negative selection for cells not experiencing significant levels of hypoxia within heterogeneous populations including 3D culture systems.

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HypoxiTRAK™ shares spectral characteristics with the related live-cell permeant DNA dye DRAQ5™, which labels all nucleated cells. Thus, DRAQ5™ can be used as a convenient standard to positively mark all DNA containing cells within a sample in parallel with an analysis for the hypoxic fraction.

Stable and convenient sensor

HypoxiTRAK™ is typically present during the period of hypoxia under investigation. The physicochemical properties of HypoxiTRAK™ facilitate permeation throughout populations of cells in 3-D culture and tissues thereby enhancing its entry into hypoxic niches and reducing retention upon cell isolation, providing an excellent signal-to-noise ratio (fig. 3). HypoxiTRAK™ is therefore ideal for studies over several days. HypoxiTRAK™ read-out is direct with no requirement for cell processing. HypoxiTRAK™ shows low perturbation properties in the absence of bio-activation while the unique far-red fluorescence signature offers compatibility with other end-point assays for hypoxia that use fluorescence signatures.

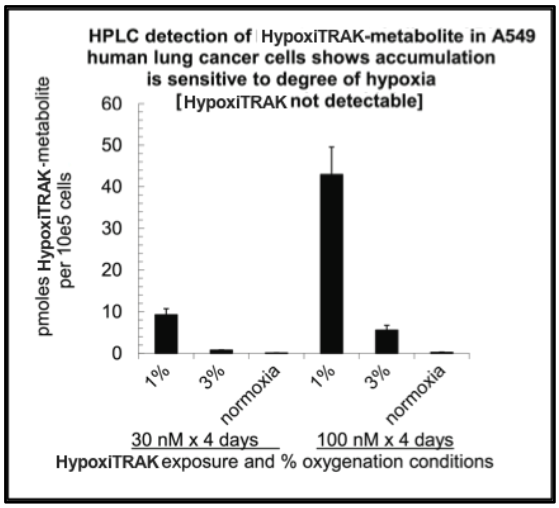
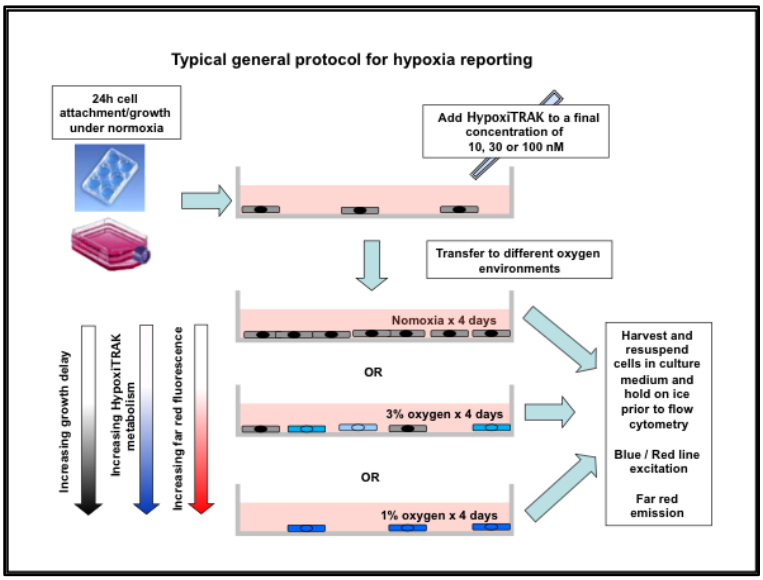


Figure 3. SELECTIVE ACCUMULATION OF HypoxiTRAK™-METABOLITE UNDER HYPOXIA
HypoxiTRAK™-metabolite accumulates as a function of the degree of hypoxia while intracellular HypoxiTRAK™ is below the detection limit

Example protocol



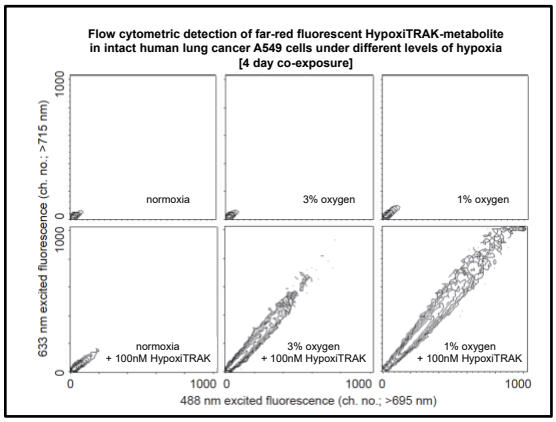


Figure 4. HypoxiTRAK™ LASER OPTIONS
Hypoxia generated HypoxiTRAK™-metabolite can be excited by blue- and red-line lasers

Before starting:

Read the MSDS. Wear protective clothing, safety goggles and laboratory gloves. Check the concentration of HypoxiTRAK™ stated on the vial label.

Materials often required but not supplied:

Phosphate-Buffered Saline (PBS), culture medium, Paraformaldehyde, Antibodies, Blocking Solution, Plastic-ware.

****NOTE:** retained HypoxiTRAK™-metabolite inhibits cell division – marking and preserving cells that have experienced hypoxic conditions.

Detecting HypoxiTRAK™ signals: (see Figs. 2, 3 & 4)

Flow cytometry: HypoxiTRAK™-metabolite should be blue-excited (488 nm) and detected using a 660/16 nm channel. Exposure of cells to the unconverted HypoxiTRAK™ is confirmed by red-excitation (635 nm) and detection with a 670 nm longpass channel.

Microscopy / HCS Imaging Platform: HypoxiTRAK™ is optimally excited using yellow / red wavelengths. It is detected with far-red filters above 660 nm. It is spectrally compatible with a wide range of visible range chromophores e.g. FITC, R-PE.

To report hypoxia, HypoxiTRAK™ is added to the culture medium at the appropriate time in the protocol. It does not need to be replaced for analysis of the cells.

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1. Prepare cell cultures with treatments, if any, to be subjected to hypoxia (t = 0): monolayer in early growth phase or suspension culture at approximately 2 x 10^5 cells/ml. If required harvest a parallel culture at the start of the experiment to established culture density.

2. Add HypoxiTRAK™ directly to cultures: initially use a final concentration range of 10, 30 or 100 nM HypoxiTRAK™ to test for dynamic range with respect to the prevailing culture density and extent of hypoxia.

3. Incubate cultures under the selected hypoxic conditions for chosen periods (t = 2-5 days) during which biologically relevant changes to cell behaviour are under study.

4. Analysis:
   For adherent cultures – wells can be imaged as required in real-time, or cells harvested by an appropriate method, washed to remove debris and re-suspended in cold medium prior to conventional flow cytometry.
   For suspension cultures - samples can be used directly without any processing and analysed by flow cytometry.

   Samples (e.g. suspension cells, adherent cells, cryo-preserved sections) requiring further staining for immunofluorescence should be fixed with 4% formaldehyde for 5 minutes. Care should be taken to limit PBS washes to a minimum to avoid wash-out of HypoxiTRAK™-metabolite, prior to immune-staining.

   Cell enumeration at this point will reveal reciprocity between cell proliferation capacity, viability and HypoxiTRAK™ bio-activation.

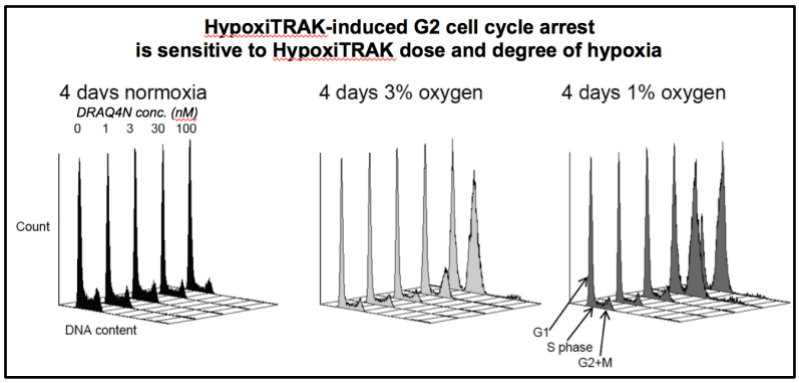

Figure 5. HYPOXIA-GENERATED HypoxiTRAK™-METABOLITE INDUCES CELL CYCLE ARREST
Hypoxia-induced HypoxiTRAK™-metabolite accumulation is detectable by simple DNA content analysis of cell cycle arrest

WHAT YOU SHOULD EXPECT TO SEE

In cells that have experienced functional hypoxia, HypoxiTRAK™ accumulates in the cytoplasm (see fig. 2) and can be detected by fluorescence microscopy or by flow cytometry (figs. 1, 3, and 4). Flow cytometry data may be best displayed using CDF. As a far-red fluorescing dye HypoxiTRAK™ signals are spectrally separated from the majority of visible-range chromophores.

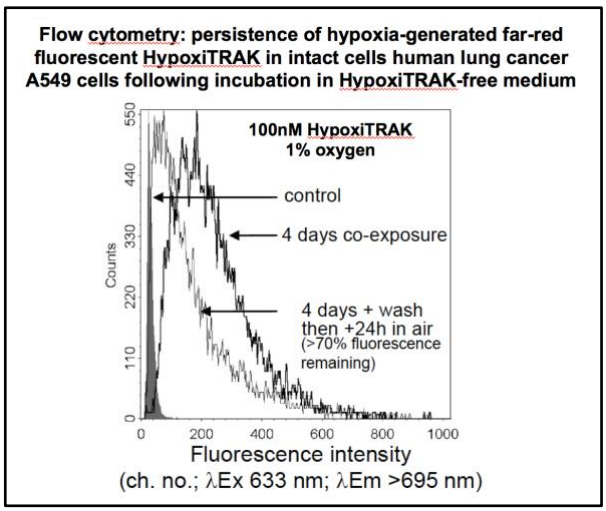

Figure 7. HypoxiTRAK™-METABOLITE PERSISTENCE
Hypoxia-generated HypoxiTRAK™-metabolite shows intra-cellular persistence following return of cells to normoxia & HypoxiTRAK™-free culture medium

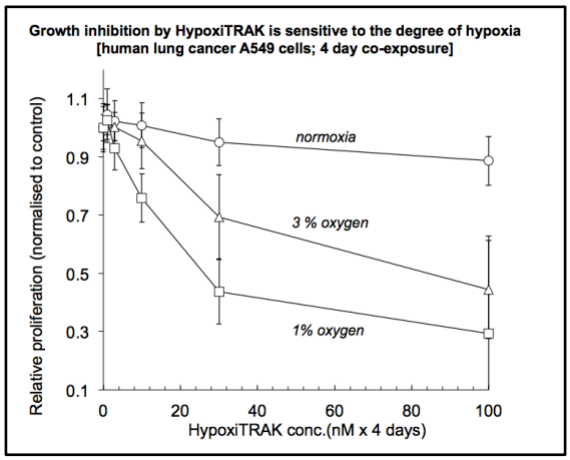

Figure 6. HYPOXIA-GENERATED HypoxiTRAK™-METABOLITE DELAYS GROWTH
Hypoxia-induced HypoxiTRAK™-metabolite accumulation by simple analysis of growth delay: relative number of population doublings over 4-day hypoxia exposure period compared to the normoxia control.

FOR FURTHER INFORMATION PLEASE CONTACT:
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