CyTRAK Orange™ TECHNICAL DATA SHEET

PRODUCT: CYTRAK Orange™
PRODUCT CODES: CO50050; CO50200; CO51000

PRESENTATION: aqueous solution.
STORAGE: store at 2-8 °C. DO NOT FREEZE

DESCRIPTION:
CYTRAK Orange™ is a novel orange fluorescing dye related to DRAQ5™ staining both nucleus and cytoplasm, with differential intensity (fig. 3). It is water-soluble and membrane permeant and can be used in LIVE or fixed cells in combination with other common fluorophores, especially GFP, FITC-labelled antibodies and far-red dyes. It is compatible with common cytometry and microscopy instruments.

Secondary cytoplasm staining allows definition of arrested / senescent cells based on higher integrated CyTRAK Orange™ fluorescence cf. healthy cells, by flow cytometry. The cytoplasmic signal is reliably weaker than the nuclear signal allowing automated compartment segmentation for high content imaging assays.

CyTRAK Orange™ "paints" organoids/mammospheres (Werner-Klein et al., 2020) to segment 3D microtissues for high content counting / sizing after drug perturbation.

NOTE: As a cell-permeant DNA intercalating probe, CyTRAK Orange™ may inhibit cell division in long-term assays and should be tested for any effect. It has been used to label endothelial cells in a 3-day trans-endothelial migration assay (Mierke, 2011).

APPLICATIONS:
- Flow Cytometry – live (or fixed)
  Nucleated cell gating (no lyse, no wash) (Dimmick et al., 2008)
  Arrested/Senescent cell identification (no lyse, no wash)
- Fluorescence Microscopy – live- (or fixed-) endpoint
  Counterstaining (Maiuri et al., 2008)
  Organoid / Spheroid "painting"
- HCS & Cell-Based Assay counterstaining
  Drug, RNAi, phenotypic screens, In-cell westerns, nucl:cyto segmentation

BEFORE STARTING:
Read the MSDS. Wear protective clothing, safety goggles and laboratory gloves. Check the concentration of CyTRAK Orange™ stated on the vial label.

MATERIALS OFTEN REQUIRED BUT NOT SUPPLIED:
PBS (azide-free), culture medium (CM), CM without phenol red* ("Imaging CM"), paraformaldehyde (FA), Triton-X 100, Tween-20, antibodies, blocking solution.

**Phenol red may introduce background in live cell imaging of CyTRAK Orange™ and any CM containing it should be exchanged for Imaging CM prior to the start of time-lapse or live-cell endpoint imaging.*

NOTE: make up the diluted (i.e. working conc$^{n.}$) CyTRAK Orange™ required for up to one day's lab work e.g. total volume required to image a batch of slides.

DETECTING CyTRAK Orange™ SIGNALS: (see figs. 1 & 2)
Flow cytometry: CyTRAK Orange™ is excited by blue or green laser sources but is not excited by red light. Detect using bandpass filters centred on 610 nm. CyTRAK Orange™ marks nucleated cells for gating (see fig. 4). CyTRAK Orange™ may be used to discriminate arrested / senescent cells based on increased brightness.
Microscopy / HCS Imaging Platform: CYTRAK Orange™ is optimally excited using blue/green wavelengths. Detect with filters centred on 610 nm. Segment nuclear and cytoplasmic compartments by first segmenting and masking nuclei, then apply a 'watershed' algorithm to detect cytoplasm. It can be used in multi-colour HCS, e.g. with AlexaFluor 350, GFP and AlexaFluor 647 (Sawada, et al., 2011).

*As no washing is required, CYTRAK Orange™ is added last. Use 200 µl per coverslip; 100 ul per 96-MTP well, 30 ul per 384-MTP well, 10 ul per 1536-MTP well.***

SPECTRAL CHARACTERISTICS:
Exλ_{max} 510 nm Emλ_{max} 610 nm

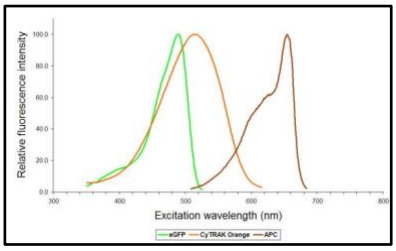

Fig. 1. Absorbance profile of CyTRAK orange™ compared to eGFP and APC

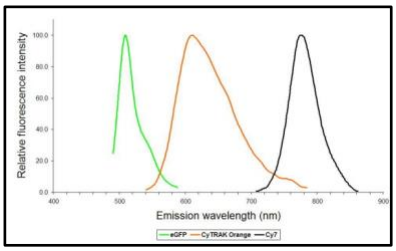

Fig. 2. Emission profile of CyTRAK Orange™ compared to eGFP and Cy7

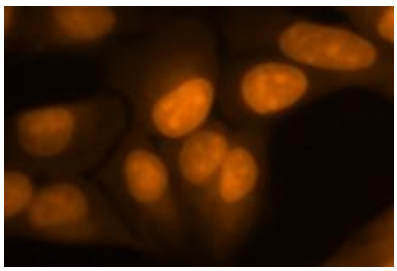
Fig. 3. CyTRAK Orange™ counterstaining of fixed U2OS cells, showing differential cytoplasmic staining

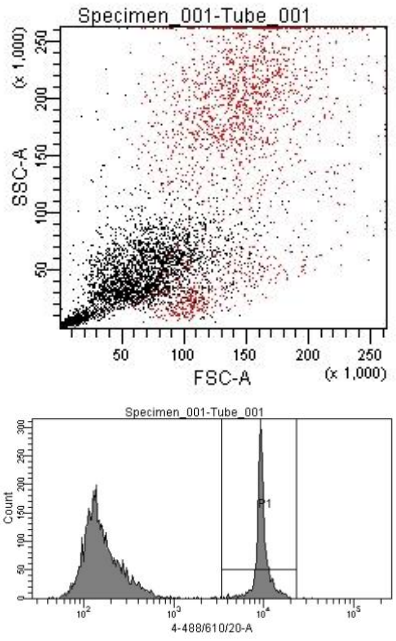

Fig. 4. Nucleated cell gating by CyTRAK Orange™ intensity of intact, unlysed bone marrow with retained forward and side scatter characteristics

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EXAMPLE PROTOCOLS

PROTOCOL 1:
NUCLEATED AND SENESCENT/ARRESTED CELL GATING BY FLOW OR IMAGING CYTOMETRY

1. Prepare cells for staining with CYTRAK Orange™: resuspend cells in appropriate buffer (PBS) at a concentration of ≤4 x 10^5 / ml in a test tube. For adherent cells estimate the number of cells based on confluence level or tissue section size.
2. Add CYTRAK Orange™ at 5-10 µM, final concentration. This will be an overlay for adherent cells / tissue sections, added to the well directly or in fresh medium following a wash step.
3. Gently mix, then incubate for 15-30 minutes at room temperature. n.b. protect from light. Staining is accelerated at 37ºC.
4. Analyze without washing. n.b. analyze live cells within 2 h. CyTRAK Orange™ stains live, fixed, permeabilized and dead cells.

PROTOCOL 2:
LIVE CELL COUNTERSTAINING FOR HCS IMAGING PLATFORM OR FLUORESCENCE MICROSCOPY

1. Wash and aspirate the slide or well.
2. Overlay cells with CyTRAK Orange™ - final concentration 5 µM in PBS, HBSS, **Imaging CM***. See guideline volumes above**
3. Incubate for 15-30 minutes at room temperature. For time-lapsed assays (e.g. studying translocation of an EGFP tagged protein) CyTRAK Orange™ may be added to the assay medium for the duration of the assay (typically 0.5 - 3 hr.) at 1 µM prior to any agonist / antagonist addition. n.b. protect from light. Staining is accelerated at 37ºC.
4. Analyze without further washing. CyTRAK Orange™ stains intact, live, permeabilized and dead cells. False colour CyTRAK Orange™ images orange for simplicity.

PROTOCOL 3:
MULTICELLULAR TUMOUR SPHEROID (MCTS) / ORGANOID / MAMMOSPHERE "PAINTING"

1. If necessary, replace CM with **Imaging CM***. Add CyTRAK Orange at 8-10 µM final concentration. Incubate for 1hr. at 37ºC.
2. If required, fix the tissue with FA at 4%, incubating for 1hr. at 37ºC. Image directly without further processing.

PROTOCOL 4:
FIXED CELL COUNTERSTAINING FOR HCS IMAGING PLATFORM OR FLUORESCENCE MICROSCOPY

A. SEPARATE FIXATIVE & COUNTERSTAIN (e.g. when external (immuno-)fluorescent stains are applied):

1. Prepare separate working solutions of 4% FA and 5 µM CyTRAK Orange™ in PBS.
2. Overlay slide or well with 4% FA. Incubate for 15-30 minutes at room temperature (RT) / 37ºC.
3. Gently aspirate FA, and wash with PBS.
4. Perform any necessary permeabilization, (immuno-)staining and blocking steps.
5. Wash with PBS to **remove any residual Triton X-100**, if used, and aspirate the sample. Overlay cells with CyTRAK Orange™. See guideline volumes above**. Incubate for 10-20 minutes at RT. n.b. protect from light.
6. Analyze without further treatment / washing. False colour CyTRAK Orange™ images orange for simplicity.

B. COMBINED FIXATIVE & COUNTERSTAIN (e.g. when expressed fluorescent protein is the only analyte):

1. Prepare separate working solutions of 8% FA and 10 µM CyTRAK Orange™ in PBS.
2. Overlay the slide or well with equal 0.5 volumes* of FA and CyTRAK Orange™ solutions (or pre-mix CyTRAK Orange™ and FA for a single overlay step). See guideline volumes above**
3. Incubate for 10-20 minutes at room temperature. n.b. protect from light.
4. Analyze without further treatment / washing. False colour CyTRAK Orange ™ images in orange for simplicity.

KEY REFERENCES:

Dimmick, I., et al. (2008) ISAC Conference; Poster
Maiuri, L., et al. (2008) J. Immunol. 180: 7697
Sawada, J. et al. (2011) HCA Conference; Poster
Mierke, C.T. (2011) J. Biol. Chem. 286: 40025
Mathis, et al. Leukemia (2013) 27(10): 1981
Edward, R. (2012) Meth. Enzymol. Vol. 505: 23
Werner-Klein, et al. (2020) Nat. Commun 11: 4977

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