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Manual staining of WTC cells with CellMask Deep Red and NucBlue Live for live-cell visualization of the plasma membrane and DNA

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Required reagents

- Complete, phenol red-free mTeSR1 culture media, referred to in this protocol simply as "phenol-red free mTeSR": 400 ml basal media with provided 100 ml 5x supplement (Stem Cell Technologies custom order) with added 1% penicillin streptomycin (Gibco #15070-063)
  Refer to page 16 of the Stem Cell Technologies technical manual about preparation, storage and shelf life of this media. Warm mTeSR1 media at room temperature only! Never warm mTeSR1 media in a 37ºC water bath!
- CellMask Deep Red Plasma membrane stain (ThermoFisher #C10046)
  Avoid multiple cycles of freeze-thawing CellMask Deep Red Plasma membrane stain. We recommend aliquoting CellMask Deep Red Plasma membrane stain into single use volumes. CellMask Deep Red Plasma membrane stain should be stored at -20ºC and protected from light. We experienced some variability in the fluorescent signal intensities between different CellMask Deep Red Plasma membrane stain lots (see Figure 1). Therefore, the final concentration of CellMask Deep Red used should be optimized depending on the lot number (see section "CellMask Deep Red lot concentration optimization procedure" for details). This protocol is optimized for lots 1730970, 1813792, 1853335 and 1900978, which we recommend to use at 1x, 5x 3x and 3x, respectively (see Table 1).
- NucBlue Live Ready Probe Reagent (ThermoFisher #R37605)
  NucBlue Live is a ready to use solution of Hoechst 33342, which is stable at room temperature and should be protected from light.

- Healthy, mature WTC colonies on 24-well or 96-well glass bottom plates with high performance #1.5 cover glass as specified in the SOP "Plating of WTC Cells on Glass Bottom Imaging Plates for Imaging Purposes"

Protocol information

- For our imaging purposes, we usually only stain two to three wells in a 24-well plate and five to eight wells in a 96-well plate at a time. This provides us with a sufficient number of healthy and mature colonies for the 2.5 hour imaging window, discussed below.
- This protocol should permit imaging cells for 2.5 hours after the dyes are removed without significant loss of fluorescent signal intensities due to slow leaching/washing out of the dyes from the cells. However, the rate at which the dyes leach out is affected by colony size, occurring faster in more loosely packed cells with smaller colonies than in larger, tightly packed colonies. Similarly the degree of penetration of the dye into the cells can vary based on colony size and tightness of cell packing within the colonies.
- We tested the chemical toxicity of CellMask Deep Red and NucBlue in WTC cells at the concentrations described in this protocol. We compared the mitotic index of stained and unstained cells after three hours. No significant dye effect could be determined within this time frame. We do not recommend imaging cells beyond three hours or at higher concentrations without performing appropriate additional toxicity testing. We also recommend limiting the total number of z-stacks taken within a well to reduce phototoxic-induced cell death along the edges of colonies with the well. The appropriate maximum number of z-stacks taken per well should be evaluated empirically and may be dependent on the well size, cell confluency, colony maturity, microscope objective, laser intensity used and number of acquired z-slices per z-stack. We usually avoid imaging more than 10 z-positions per well, with approximately 70 slices per z-stack, with our imaging conditions at 100x magnification using 96-well plates.

Preparations of CellMask Deep Red Plasma membrane stain and NucBlue Live Ready Probe Reagent for staining of WTC cells

(1) Pre-equilibrate phenol red-free mTeSR in the tissue culture incubator (37 ºC, 5% CO_2) one hour to overnight prior to staining the cells with CellMask Deep Red Plasma membrane stain (referred to as CellMask Deep Red) and NucBlue Live Ready Probe Reagent (referred to as NucBlue).
(2) Add 60 µl of NucBlue to 1 ml of pre-equilibrated complete, phenol red-free mTeSR in 1.5 ml Eppendorf tubes and mix by completely pipetting the mixture up and down 3-5

times. Depending on the plate format and amount of wells required (see sections below for amounts needed to stain one well of a 24-well or 96-well imaging plate), multiple tubes of NucBlue solutions have to be prepared. Spin the solution for 60 minutes at 20,000 *g* at 37 ºC*. Transfer supernatant into a fresh tube. In case multiple tubes were prepared, we recommend combining the supernatants into one tube (1.5 ml Eppendorf tube or 15 ml conical tube depending on final volume). The NucBlue solution should be stored in the tissue culture incubator protected from light until needed and should be used the same day. This diluted NucBlue solution is from now on referred to as the "NucBlue working solution".

**NucBlue has the tendency to form fluorescent aggregates in WTC cells, which may be caused by trapping of the dye within these tightly packed cell colonies (see Figure 2). Centrifuging the dye beforehand reduces the amount of fluorescent aggregates within the cells.*

(3) Dilute CellMask Deep Red (stock solution is 1000X in DMSO) with the NucBlue working solution to make a "CellMask Deep Red working solution". The working solution may differ by the dye lot. See Table 1 for lot specific dilution instructions of CellMask Deep Red lots that have been used in the optimization of this SOP and refer to section *"CellMask Deep Red lot concentration optimization procedure"* on how to optimize dye lots not tested in this SOP. Mix by completely pipetting the mixture up and down 3-5 times. The CellMask Deep Red working solution should be stored in the tissue culture incubator protected from light until needed and should be used the same day.

Table 1. Lot specific concentration of CellMask Deep Red working concentration and final concentration.

lot number	concentration of CellMask Deep Red working solution	final concentration of CellMask Deep Red in well
1730970	2x	1x
1813792	10x	5x
1853335	6x	3x
1900978	6x	3x

Staining WTC cells with CellMask Deep Red and NucBlue

(A) 24-well imaging plate

(1) Gently remove media from the well with cells of interest using a pipette instead of an aspiration needle and add 400 µl of NucBlue working solution to the well. Work quickly to avoid drying times of the well longer than 30 seconds.
(2) Incubate cells for 20 minutes in the tissue culture incubator (37 ºC, 5% CO_2).
(3) Do not remove the NucBlue working solution from the well. Add 400 µl of CellMask Deep Red working solution (as specified in Table 1) to the well and gently mix by pipetting up and down 2-3 times. The final concentrations of the dyes should be now 1x NucBlue and 1x, 3x, or 5x of CellMask Deep Red depending on the lot used (see Table 1) in a final well volume of 800 µl.
(4) Incubate cells for 10 minutes in the tissue culture incubator (37 ºC, 5% CO_2).
(5) Gently remove media from the well using a pipette instead of an aspiration needle and gently wash the cells once with 800 µl of pre-equilibrated, phenol red-free mTeSR. Avoid drying times of the well longer than 30 seconds.
(6) Add 500-1000 µl of pre-equilibrated phenol red-free mTeSR to the well. Cells can be imaged immediately and up to 2.5 hours after this final rinse. Our specific microscope imaging settings are: C Apo 100x 1.25W objective; 405 nm at 0.28 mW, 200 ms exposure; 638 nm at 2.4 mW, 200 ms exposure; acquiring each channel at each z-step (rather than collecting entire stacks before switching channels). Figure 1 and 2 show example images for WTC cells stained with CellMask Deep Red and NucBlue acquired with our specific microscope imaging settings.

(B) 96-well imaging plate

(1) Gently remove media from the well with cells of interest using a pipette instead of an aspiration needle and add 100 µl of NucBlue solution to the well. Work quickly to avoid drying times of the well longer than 30 seconds.
(2) Incubate cells for 20 minutes in the tissue culture incubator (37 ºC, 5% CO_2).
(3) Do not remove the NucBlue working solution from the well. Add 100 µl of CellMask Deep Red working solution (as specified in Table 1) to the well and gently mix by pipetting up and down 2-3 times. The final concentrations of the dyes should be now 1x NucBlue and 1x, 3x, or 5x of CellMask Deep Red depending on the lot used (see Table 1) in a final well volume of 200 µl.
(4) Incubate cells for 10 minutes in the tissue culture incubator (37 ºC, 5% CO_2).

(5) Gently remove media from the well using a pipette instead of an aspiration needle and gently wash the cells once with 200 µl of pre-equilibrated, phenol red-free mTeSR. Avoid drying times of the well longer than 30 seconds.
Add 150-200 µl of pre-equilibrated phenol red-free mTeSR to the well. Cells can be imaged immediately and up to 2.5 hours after this final rinse. Our specific microscope imaging settings are: C Apo 100x 1.25W objective; 405 nm at 0.28 mW, 200 ms exposure; 638 nm at 2.4 mW, 200 ms exposure; acquiring each channel at each z-step (rather than collecting entire stacks before switching channels). Figure 1 and 2 show example images for WTC cells stained with CellMask Deep Red and NucBlue acquired with our specific microscope imaging settings.

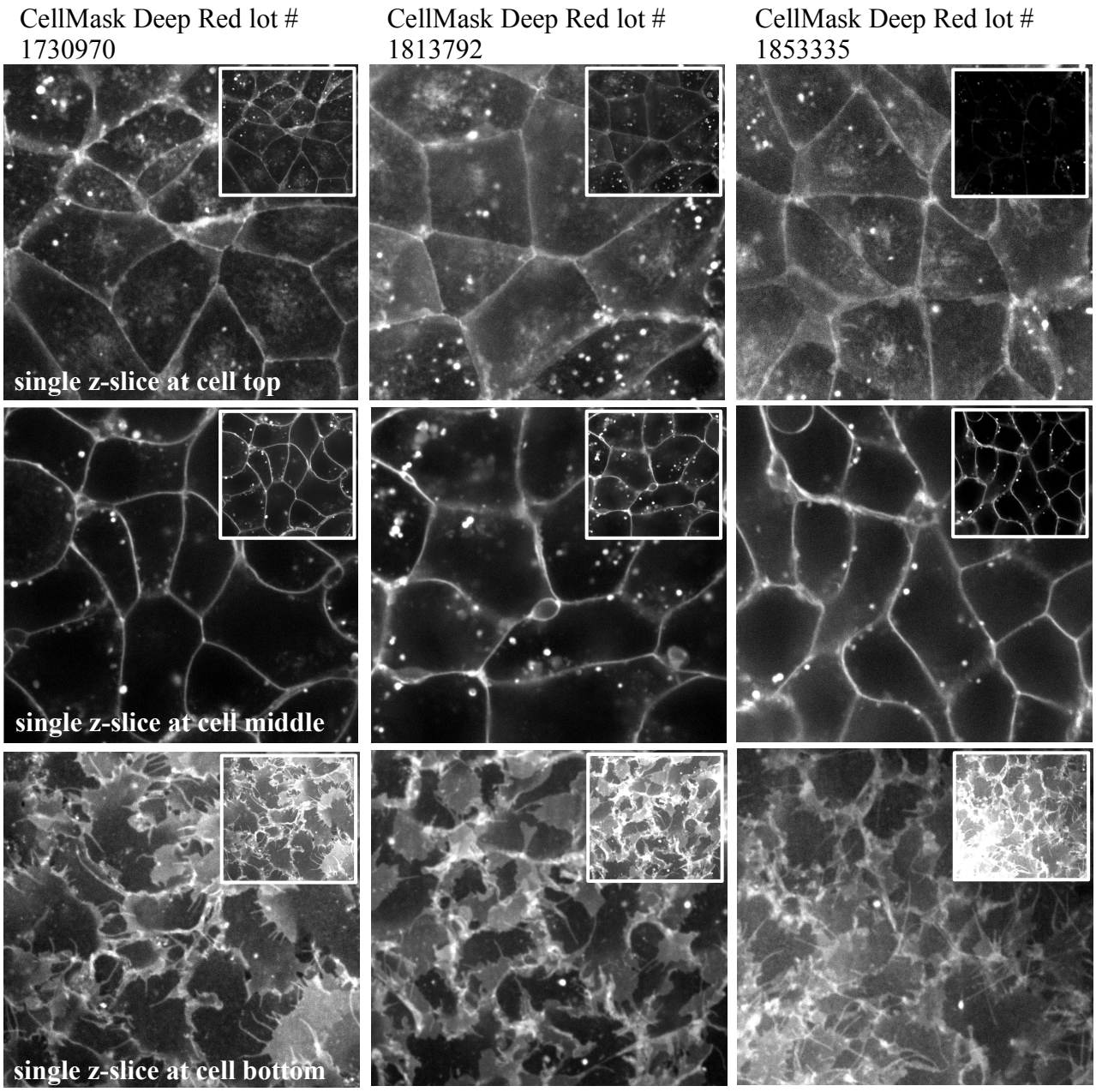

Figure 1: Images show WTC cells stained with CellMask Deep Red from different dye lots at their respective optimal concentrations. Images are single slices at the top (top row), middle (middle row) and bottom (bottom row) of the cell. Larger images are scaled to optimal brightness and contrast settings for each individual image to highlight membrane morphologies throughout the cell. Insets are scaled to the same brightness and contrast settings as the z-slice in the middle (~center) of the cells within each dye lot to highlight dye intensity differences between the top, center, and bottom of the cells.

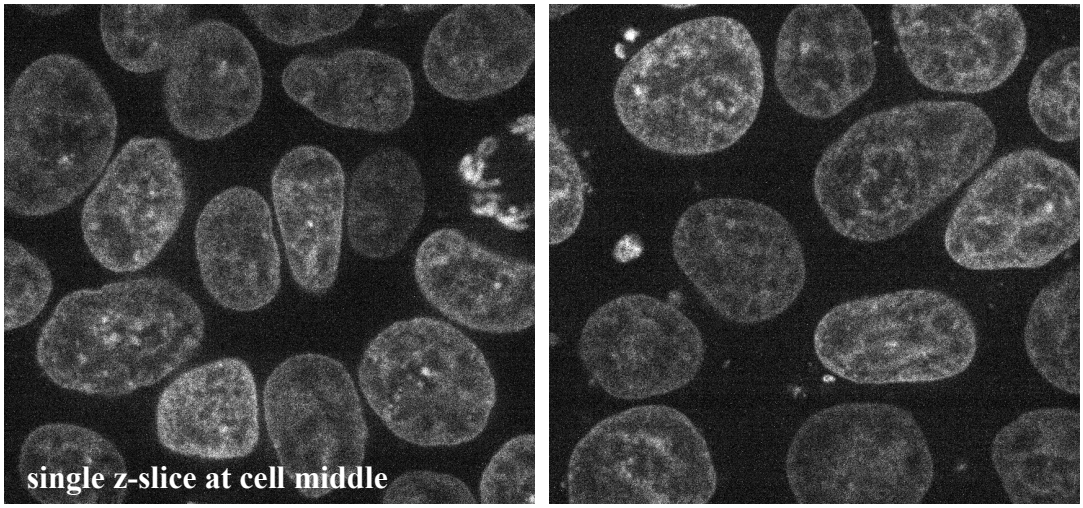

Figure 2: Images show WTC cells stained with 1x NucBlue. Images are single z-slices at the middle of the cell. The right image shows examples of NucBlue fluorescent aggregates which can form within cells of tightly packed colonies.

CellMask Deep Red lot concentration optimization procedure

We test each new lot of CellMask Deep Red to determine the lowest concentration of CellMask DeepRed that is useable with our standard labeling, imaging, and image processing parameters. "Useable" means having the least effect on cell health while still permitting segmentation of the cell boundary and the DNA/nucleus with similar accuracy in our post-imaging analysis pipeline.

We first stain cells with a wide concentration range (from 1x to 5x) of CellMask Deep Red from the new lot as well as with the already optimized concentration of the current lot. Colonies of equivalent health and maturity are imaged using our standard imaging parameters for each CellMask Deep Red lot and concentration at 30 min, 60 min, 120 min and 150 min after the final rinse (step 5 in section *"Staining WTC cells with CellMask Deep Red and NucBlue"*). Images are evaluated and compared for a) differences in dye signal intensity between the bottom and top of cells, b) differences in dye signal intensity over time (due to uptake and washout), c) amount of endocytosis (CellMask Deep Red spots within the cell) over time, and d) cell morphology, especially the extent of any cell blebbing. Images are also processed with our post-imaging analysis pipeline to evaluate cell and DNA/nucleus segmentation performance. The overall colony health with an emphasis on cell morphology, cell death along colony edges and appearance of imaging scars* in the colony is also evaluated with a tissue culture microscope 3 hours after the final rinse. Next, we repeat this procedure for a smaller concentration range of the new CellMask Deep Red stain lot until an optimal new working concentration has been identified. For example, for the primary test we might use 1x, 3x, and 5x, with 3x being a

promising concentration after analysis of the data then for the secondary test we might narrow it down to 2x and 3x to verify that 3x is truly the lowest usable concentration.
We define imaging scars as the appearance of small holes in areas of the colonies in which imaging occurred. These holes are most likely due to imaging-associated cell death.