Surface Staining with H33342 Live Cell Cycle Analysis

Reagents:

- Antibodies
- Fc Block
- PBS/HBSS
- PBS + 3% FBS + 0.01% Sodium Azide
- 5 mg/mL Hoechst (H33342/33258) stock solution.
- PI/7-AAD or Live/Dead Fixable stains

Protocol:

Prepare Cells:

- Collect cells and transfer approximately 1 x 10^6 cells/mL into a 5mL (12 x 75 mm tube)
- Wash 2 x with ice cold PBS, by centrifuging at 1000 rpm, 10 mins, 4 degrees in a swinging bucket rotor centrifuge.
- Remove supernatant
    - Avoid disturbing the pellet during aspiration and take care not to aspirate to dryness
- Disrupt the pellets by flicking the tubes and resuspend in 1mL of protein free buffer (PBS/HBSS)

Live/Dead Fixable Stain (optional)**:

- Stain cells with a live/dead fixable stain compatible with chosen surface markers and H33342
    - Note that Live-Dead violet/aqua and DAPI are not compatible with H33342
    - Alternatively, PI or 7-AAD can be used with live cells at the ***end*** of the staining procedure.
- Add 1µL of reconstituted dye to the cells and incubate for 30 minutes on ice for 30 mins.
- Wash the cells with PBS and resuspend in 100 µL of PBS

** see manufacturer's product data sheet for more details.

Surface Staining:

Block surface Fc receptors for 10 minutes at room temperature (or 15-20 minutes at 4 degrees) – DO NOT WASH

Table 1: Fc Receptor Block Reagents

Cell Type	Fc Block Source Reagents
Human	Polyclonal human IgG 10% normal human serum diluted into PBS
Mouse	Purified antibodies directed against FcγII/III receptor
Rat	Purified antibodies against FcγIII receptor
Note: Most FCM Antibody reagent companies sell blocking reagents	

- Stain cells with directly conjugated antibodies at optimized concentrations (see antibody titration) for 15 – 20 minutes at RT or 30 minutes at 4 degrees, in the dark.

  - Prepare single stain compensation controls for each fluorochrome you intend to analyze (beads or cells)
  - Include an unstained sample
  - Prepare fluorescent minus one gating control samples
  - Prepare matched isotype controls for each antibody-fluorophore combination (if necessary)
- Wash cells 2x with PBS and resuspend in 1mL of PBS + 2% FBS.

Nuclear Staining:

- Add 5uL of H33342 stock solution to the appropriate tubes
  - Do not wash.
  - The amount of H33342 needed to achieve nuclear saturation may differ between cell lines and cultures – this may require titration.
  - Note: Primary cultures with stem cell-like properties will efflux the dye resulting in poor saturation. In this circumstance, fixation and permeabilization is necessary to achieve good DNA content resolution.

Live/Dead Staining with PI or 7-AAD (optional - include, if you did not use a Live/Dead Fixable dead stain):

- Add a dead cell marker (PI or 7-AAD), incubate 10-15 minutes at RT (do not wash) to all analysis tubes except compensation controls and unstained sample.
  - PI and 7-AAD concentrations are usually on the order of 1ug/mL

Data Acquisition:

- Acquire data on an instrument equipped with a UV excitation source (355/375 nm) for H33342 excitation with detection at 450 nm.

References:

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Belloc, F., Dumain, P., Boisseau, M. R., Jalloustre, C., Reiffers, J., Bernard, P. and Lacombe, F. (1994), A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. Cytometry, 17: 59–65. doi:10.1002/cyto.990170108

Schmid, I., Krall, W. J., Uittenbogaart, C. H., Braun, J. and Giorgi, J. V. (1992), Dead cell discrimination with 7-amino-actinomcin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry, 13: 204–208. doi:10.1002/cyto.990130216