Surface Staining with H33342 Cell Cycle Analysis on Fixed Cells

Reagents:

- Antibodies
- Fc Block
- PBS/HBSS
- PBS + 2 % FBS + 0.01% Sodium Azide
- 5 mg/mL Hoechst (H33342/33258) stock solution in PBS
- Perm Buffer: PBS + 0.5% Tween 20 + 2% FBS
- 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 2x 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**:

- 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	

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- Stain cells with directly conjugated antibodies at optimized concentrations (see antibody titration) for 15 – 20 minutes at Room temperature or 30 minutes at 4 degrees, in the dark.
    - Prepare single stain compensation controls for each fluorochrome you intend to analyze (beads or cells)
    - Reserve some unstained cells for instrument set-up.
    - 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 200 µL of PBS + 2% FBS.

Fixation and Permeabilization:

Primary cultures with stem cell-like properties will efflux H33342 resulting in poor saturation. In this circumstance, fixation and permeabilization is necessary to achieve good DNA content resolution.

- Fix cells by adding 200 uL of a 4% PFA solution to the cells for final concentration of 2% PFA
- Incubate on ice for 30 mins, in the dark.
- Wash cells 2X with PBS, pellet and resuspend in 500 uL of Perm Buffer

Nuclear Staining:

- Add 2 - 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.

Data Acquisition:

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

NOTES: Other nuclear staining dyes including DAPI the FxCycles may be used with this protocol

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