Staining Cells with Hoechst Dyes

Hoechst (bisbenzimide, HOE) is a family of cell-permeable fluorescent dyes that binds strongly to adenine-thymine-rich regions in the minor groove of double-stranded DNA. Although Hoechst dyes can bind to all nucleic acids, AT-rich dsDNA strands considerably enhance their fluorescence. Hoechst dyes are used extensively in fluorescence microscopy and flow cytometry to stain chromosomes and nuclei in live and fixed cells. They are also often used to distinguish condensed pycnotic nuclei in apoptotic cells and cell sorting.

The Hoechst family contains several dyes — Hoechst 33342, Hoechst 33258, Hoechst 34580, and Hoechst S769121 (Nuclear Yellow). The first three members are blue-emitting dyes whose spectral properties are slightly different. The excitation/emission spectra of Hoechst are given in the Table.

Spectral characteristics of Hoechst dyes (in complex with DNA)*

	Absorbtion Max.	Emission Max.
Hoechst 33342	351 nm	461 nm**
Hoechst 33258	351 nm	463 nm**
Hoechst 34580	380 nm	438 nm**
Hoechst S769121 (Nuclear Yellow)	360 nm	505 nm

*The fluorescence intensity of Hoechst dyes increases with the pH of the solvent.

**The unbound Hoechst 33342, Hoechst 33258, and Hoechst 3458 dyes fluoresce in the 510–540 nm range. The green fluorescence of unbound dye may be observed when an excessive dye concentration is used or the sample is insufficiently washed out.

Before you start

- Hoechst dyes are quenched by bromodeoxyuridine (BrdU). When BrdU is integrated into DNA, the bromine is supposed to deform the minor groove so that Hoechst dyes cannot reach their optimal binding site.
- The commonly used dye concentration to stain bacteria or eukaryote cells is 0.1–10 µg/mL. The working dilution of the Hoechst stains depends on the cell type and density and should be defined experimentally.
- The optimal cell density and staining duration for DNA content analysis may vary by cell type. The staining protocol should be optimized in preliminary experiments for best results.

Stock Solution Preparation

1. Dissolve 10 mg of Hoechst dye in 1 mL distilled water to obtain a 10 mg/mL stock solution.
2. Mix well until the dye has fully dissolved.
3. Store the stock solution in small aliquots at -20 °C or -80 °C away from light. Avoid repeated freeze-thaw cycles.

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Cell Staining

Staining of Live Cells for Nuclei Visualization

1. Grow cells on a sterile coverslip. Adherent cells can be stained directly on the coverslip.
2. Dilute Hoechst stock solution to 1-5 µg/mL in the appropriate medium immediately before use.
3. Add Hoechst solution to each sample and incubate at 37 °C for 30-60 min.
4. Aspirate staining solution and wash cells twice with 1× PBS.
5. *(Optional)* Stained cells can be fixed in 4% formaldehyde for 2 min at 4 °C. Wash fixed cells twice in PBS.
6. For fluorescent microscopy, mount cells under a coverslip using a mounting medium.

   (Optional) Cells may be analyzed without washing, but this may increase the background from unbound dye.

Staining of Fixed Cells for Nuclei Visualization

1. Grow cells on a sterile coverslip. Adherent cells can be stained directly on the coverslip.
2. Fix and permeabilize cells as desired.
3. Dilute Hoechst stock solution to 0.5-2 µg/mL in 1× PBS immediately before use.
4. Add Hoechst staining solution to each sample and incubate for at least 15 min.
5. Aspirate staining solution and wash cells twice with 1× PBS.
6. For fluorescent microscopy, mount cells under a coverslip using a mounting medium.

   (Optional) Cells may be analyzed without washing, but this may increase the background from unbound dye.

Staining of Live Cells for DNA Content Analysis by Flow Cytometry

1. Obtain a single-cell suspension.
2. Dilute Hoechst stock solution to 1-10 µg/mL in the appropriate medium immediately before use.
3. Resuspend cells at 1×10^6 cells/mL in staining solution.

   Alternatively, Hoechst dye may be added directly to the cell culture without pelleting if the cell density does not exceed 1×10^6 cells/mL.
4. Incubate at 37 °C for 15-60 min.
5. Pellet cells by centrifugation at 400 *g* for 3-4 min at room temperature; aspirate staining solution.
6. *(Optional)* If RNA analysis (e.g., RNA-seq) is to be performed after DNA analysis is completed, wash the cells 2-3 times in serum-free buffer (e.g., 1× PBS) to completely remove residual RNAse in the cell culture medium.

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7. Resuspend cells in 1× PBS and proceed to an analysis by flow cytometry. Use a low flow rate to achieve the best result. Increased flow rates may result in a higher % CV for each cell cycle compartment in the DNA histogram.

   Important! If cells are to be sorted, adding Hoechst back into the analysis buffer during acquisition prevents dye extrusion during sorting.

Staining of Fixed Cells for DNA Content Analysis by Flow Cytometry

1. Obtain a single-cell suspension at a cell density of 1-2 × 10^6 cells/mL.
2. Fix cells with 70-80 % ice-cold ethanol for 30 min on ice.
3. Wash cells once with 1× PBS.
4. Dilute Hoechst stock solution to 0.2-2 µg/mL in 1× PBS immediately before use.
5. Stain cells for 15 min at room temperature.
6. No wash is necessary before analysis.
7. Proceed to analysis by flow cytometry. Use a low flow rate to achieve the best result. Increased flow rates may result in a higher % CV for each cell cycle compartment in the DNA histogram.

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