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MOLECULAR BIOLOGY PRACTICALS.

Practical 2. Hoechst or DAPI staining of DNA in eukaryotic and bacterial cells.

Compiled by Boris M. Sharga, Diana B. Pylypiv, Volodymir P. Feketa

Theoretical background. 4',6-Diamidino-2-Phenylindole (DAPI) and Hoechst dyes (HDs) are blue fluorescent, nucleus-specific dyes for live or fixed cells staining. The dyes have little fluorescence in solution, but become brightly fluorescent upon double strand DNA(dsDNA) binding. The cells can be stained without a wash step. The dyes are stable and allow live cells to survive few days or longer in specimens and because of this property these stains are called *supravital*.

DAPI was first synthesised in 1971 in O.Dann's lab as anti-trypanosomiasis compound. It was ineffective as a drug, however, able to attach to dsDNA and became more strongly fluorescent, when dsDNA bound. This findings led to its use as mitochondrial DNA identifying agent in *ultracentrifugation*. Later the DAPI was adopted as dsDNA stain in *fluorescence microscopy* (FM). Since late 1970s it is used in *flow cytometry* (FC) and for quantification of dsDNA in cells and viruses [7].

When bound to dsDNA the DAPI has maximums in absorbance at $\lambda_{Abs} = 358$ nm (UV light) and in emission at $\lambda_{Em} = 461$ nm (blue light). So, in FM the DAPI is excited by UV light and is detected through a blue/cyan filter. The emission peak is enough broad. When joined to RNA, it is less fluorescent ($\lambda_{Em} \approx 500$ nm) [12, 16].

Hoechst 33258, 33342 and 34580 (HDs) belong to a family of bisbenzimidazole blue fluorescent dyes used to stain dsDNA. They were invented by the German company Hoechst AG in the early 1970s [4, 10]. First 2 are most commonly used. In contact with dsDNA the HDs 33258 and 33342 are excited by UV light at 352 and 350 nm, respectively, and fluoresce with maximum at $\lambda_{Em} = 461$ nm. Unbound dyes emit light with maximums in the 510–540 nm range. Upon dsDNA binding, their fluorescence increases ~30-fold due to suppression of rotational relaxation and hydration [1, 5, 13].

HDs stain dsDNA in methods: FM, *immunocytochemistry*, often with other fluorophores [12]; FC, e.g., for cell sorting by phase of the cell cycle [8]; dsDNA determination and quantification in the presence of RNA [11]; automated dsDNA detection [19]; chromosomes (chrs) sorting [11].

Stem cells (hematopoietic or embryonic) are effectively efflux the HDs and can be detected via FC in the *side population*. This is done by passing the HDs-emitted fluorescence through both red and blue filters, and plotting HD red data against blue.

Live or fixed cell membranes are HDs-permeable. Cells with specific ATP-binding cassette transporter proteins can also actively efflux these stains. Comparing to DAPI, the HDs have additional C_2H_5 group that renders them more cell-permeable. HD 33342 exhibits a 10-fold greater cell permeability than HD 33258. HDs are less

toxic, than DAPI and HDs-stained cells survive better. DAPI is somewhat less cell membrane permeant than HDs, and applied at a higher concentration, usually at concentration 10 $\mu\text{g/mL}$, for live cells and at 1 $\mu\text{g/mL}$ for fixed (dead) cells. Because of more high concentration of DAPI than HDs needed for live cell staining, the DAPI is often substituted by HDs for live cells [20]. DAPI dilactate is more soluble DAPI salt, good for making stock and ready-to-use aqueous solutions of DAPI [17].

HDs are soluble in water or organic solvents such as dimethyl sulfoxide and dimethyl formamide up to 10 mg/mL. HD 33258 is more water soluble than HD 33342. The aqueous solutions are kept at 2–6°C for ≤ 6 months in light-proof containers or at –20°C or below for longterm storage.

DAPI and HDs are used also, when multiple fluorescent stains are applied in a single sample. Partial fluorescence overlap between DAPI or HDs and green-fluorescent molecules like *fluorescein* and *green fluorescent protein* (GFP) has little effect on study results. If extremely precise image analysis is necessary, spectral unmixing approach can be applied.

Both dyes penetrate cell membranes and used at 1 $\mu\text{g/mL}$ as nuclear counterstains for live or fixed cells [17]. HDs 33342 and 33258 are quenched by thymidine substitutive, the bromodeoxyuridine (BrdU), which is commonly used to detect dividing cells. When BrdU is integrated into dsDNA, it is supposed that the bromine deforms the minor groove so that HDs can't reach their optimal binding site. The HDs bind even stronger to BrdU-substituted dsDNA, however, no fluorescence observed. BrdU can be used together with HDs to monitor cell cycle progress [3,9].

High specificity towards dsDNA make HDs dyes excellent targeting moieties, which can be conjugated to various other molecules in order to tether them to dsDNA for studies of cells and medicinal treatment [4].

The DAPI and HDs bind to the minor groove of double-stranded dsDNA with affinity to A-T rich sequences and significant increase in fluorescence [16] after these dyes binding.

Joining of DAPI and HD stains to dsDNA, may interfere with DNA replication. Thus, they are potentially *mutagenic* and *carcinogenic*. HD stain 33342 as less cytotoxic is used to sperm sex sorting in animal or human. Safety of this method has been questioned [2, 14].

DAPI is used to check for contaminant *Mycoplasma* or virus dsDNAs in cell cultures or growth media [18].

In bacterial or eukaryotic cells dsDNAs are stained with DAPI solutions 0.1–12 $\mu\text{g/mL}$ for 1-30 minutes at 18-37°C, then, washed to remove unbound dye. Green fluorescence may be observed on samples overloaded by dye or washed only partially. HDs are often used instead of DAPI. HDs or DAPI at concentration 12-15 $\mu\text{g/mL}$ in phosphate buffer saline (PBS) or 150 mM NaCl solution stain live or killed bacteria during 30 minutes at 18-37°C more dimly than mammal cells. Dead cells tend to stain more brightly than live cells. In *Saccharomyces cerevisiae* at 12-15 $\mu\text{g/mL}$, DAPI and HDs preferentially stain nuclear and cytoplasmic dsDNA of dead cells. In live yeast, HDs shows dim nuclear and cytoplasmic staining, while DAPI shows dim mitochondrial staining. The fluorescence intensity of HDs is pH dependant [12].

Experiment 1. Staining of live cells

Materials and equipment. Laboratory gloves, fluorescence microscope, centrifuge, thermostatic chamber, pipette with disposable sterile noses, marker for glass, fresh bakery yeast *Saccharomyces cerevisiae* from supermarket; HeLa cells culture grown on medium DMEM (Dulbecco's Modified Eagle Medium) (Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1.375 mg penicillin/streptomycin antibiotics (Gibco/Invitrogen) in 10-cm culture dish; 125 mL of the same sterile medium for HeLa cells; 0.1% trypsin - ethylenediamine tetraacetic acid (EDTA) solution; phosphate buffer saline (PBS); Antifade mounting medium (Boster); Hoechst 33258 and DAPI dyes (ThermoFisher Scientific).

Procedure.

Medium exchange method provides the uniform exposure of live cells to stain. They must be collected by centrifugation step to exchange the medium. However, for some cell types, morphology and/or viability may be affected by this procedure. Floating dead cells may be lost during medium removal.

1. **Wear gloves!** Dyes are potentially *mutagenic* and *carcinogenic*. Take few milliliters of fresh medium and add to it Hoechst 33258 to 1 µg/mL or DAPI to 10 µg/mL content.
2. Heat 0.1% trypsin - EDTA to room temperature and incubate sterile medium to 37°C.
3. Check grown HeLa culture under microscope for cell confluency and for cell death. If the cells reached $\approx 90\%$ confluency, remove out spent medium using pipette and wash cells 2 times with PBS.
4. Cover completely cells area of the culture dish with 1 ml of 0.1% trypsin-EDTA solution and keep the cells at 37°C for 2 minutes
5. Observe cells under the microscope. If they are fully detached, remove out the 0.1% trypsin-EDTA using pipette.
6. Wash cells 2 times with PBS.
7. Add 6 ml of fresh medium to the dish, gently pipet the cells, centrifugate at 1,000 rpm for 5 minutes.
7. Remove the supernatant. Do not disturb the cell pellet.
8. Gently resuspend the cells from the pellet in 2 ml of fresh medium containing Hoechst 33258 at 1 µg/mL or DAPI at 10 µg/mL. **Note:** DAPI or Hoechst 33258 can be combined with other fluorescent dyes.
9. Incubate cells at room temperature or 37°C for 5-15 minutes.
10. Apply 20 µL drop of the stained suspension onto each of 2 glass slides, place a cover slip on one of them and allow another to air dry completely without cover slip.
11. Observe covered preparation at objectives 4×, 10× and 40×. Once good view has been located, rotate the high-dry objective out of the light pass and drop the immersion oil on the light spot. Rotate the oil-immersion objective and place its lens in contact with the oil drop.
12. Place 2 dots of antifade mounting medium on the air-dried drop of culture and cover them with cover slip. Squeeze the excess of antifade mounting medium out by gentle

pressing against cover slip surface with blunt end of the pencil eraser. Allow the mounting medium to dry during 1 hour before using an oil-immersion microscopy.

Note: Washing is not needed for specific staining, but nuclear fluorescence is stable after washing. If protected from light, mounted slides can be stored at 4°C to -20°C for 2-3 weeks [17].

13. Observe the HeLa cells with blue stained nuclei and nuclei with bright blue chrs within them (Fig.1). The culture of HeLa cells originate from cervical cancer of *Henrietta Lacks*, the patient who died in 1951 at age of 31. This tumor cells often contain more or less than 46 chrs (2n number in human). Although 50 to 70 per nucleus most frequently found, HeLa cells containing 200 chrs have been observed [15]. Count chrs in your preparations.

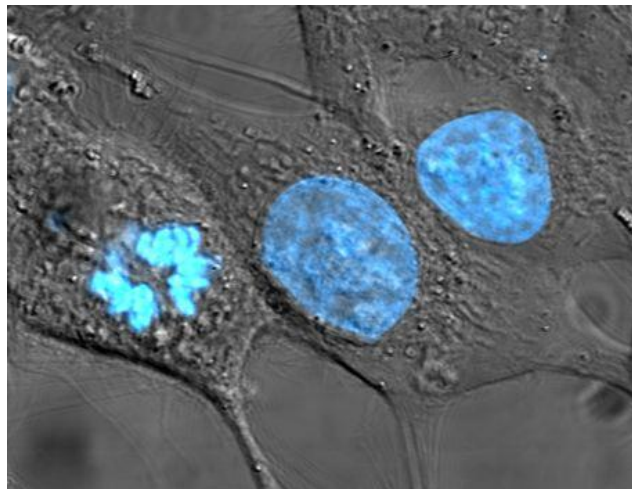


Fig. 1. The HeLa cells: Hoechst 33258 stains nuclei are blue. The leftmost cell is in mitotic prometaphase. Its chrs fluoresce brightly as they consist of highly compacted DNA. Magnification 1000 ×. By Masur, 2007 [6].

Live HeLa cells staining by direct addition of 10× dye

Direct addition of 10× dye is a suitable method that doesn't require medium exchange, but suspension must be mix immediately yet gently to avoid high transient stain concentration or disruption of cells by pipetting. Adding highly concentrated dye directly to cells in culture is not good, as this will result in local areas of high dye exposure [17].

1. The dye is included into culture medium at 10 times the final staining concentration. Dilute DAPI to 100 µg/mL, or Hoechst 33258 to 10 µg/mL. **Note:** DAPI or Hoechst 33258 can be combined with other fluorescent dyes.
2. Add 1/10 volume of 10× dye directly to the well without removing the medium from the cells.
3. Provide thorough mixing the medium with dye by immediate gentle pipetting up and down. The plates with larger well sizes (e.g., 24-well to 6-well plates), can be gently swirled to mix.
4. Cells are incubated at 18-37°C for 5-15 minutes, than prepared onto slides as described above. **Note:** Washing can be omitted for specific staining, but nuclear staining is stable after washing [17].

Experiment 2. Staining of fixed tissue sections and fixed or non-fixed microbes

Materials and equipment. Fluorescent microscope, centrifuge, marker for glass, fresh bakery yeasts *Saccharomyces cerevisiae* from the supermarket, liquid cultures of *Escherichia coli* and *Bacillus subtilis* on defined media; phosphate buffer saline (PBS); antifade mounting medium (Boster); 150 mM NaCl solution; Antifade mounting medium (Boster); Hoechst 33258 and DAPI dyes (ThermoFisher Scientific); formaldehyde fixed tissue sections.

Procedure.

1. Add the dye to PBS at 1 $\mu\text{g/mL}$.
2. **Note:** DAPI or Hoechst 33258 can be included together with labeled antibodies or other fluorescent stains; the dyes also can be diluted in buffers with detergent or blocking agents if convenient.
3. Prepare suspension from bakery yeast in PBS. Spin this suspension and cultures of *E. coli* and *B. subtilis* in centrifuge and suspend the pellets in PBS for washing twice.
4. Place 10 μL of bacterial suspensions (*Escherichia coli* or *Bacillus subtilis*) or yeast suspension (*Saccharomyces cerevisiae*) onto each of 2 glass slides per one culture.
5. Allow drop of suspension on each slide to dry near the flame. To fix microbes on 1 slide of 2 prepared for each culture, move the slide quickly through the flame upper part 2-3 times. **Note:** Do not burn the cells! Control temperature of the slides by keeping them in your fingers. Mark fixed slides "F" with marker. As an alternative, yeast suspension (1 mL) can be fixed with 0.1 mL 36% formaldehyde.
6. Stain all the glass slides with 12-15 $\mu\text{g/mL}$ Hoechst 33258 or DAPI in phosphate buffer saline (PBS) or 150 mM NaCl solution for 30 minutes at 18-37°C.
7. Prepare slides for microscopy as described above.
8. Add the PBS with dye to fixed tissue sections, incubate at 18-37°C for at least 5 minutes.
9. Prepare slides for microscopy; washing is optional but not required. **Note:** Samples can be stored at 4°C after staining and before imaging. DAPI can be included directly in antifade mounting medium for 1-step mounting and staining. When use DAPI or Hoechst 33258 in mounting medium, longer incubation times may be required for DAPI to completely penetrate the cell nuclei. [17].
10. Observe preparations in microscope at 10 \times ; 40 \times and in immersion objectives. Compare fixed and non-fixed preparations.

It is known, that dead bacterial cells tend to stain more brightly, than live cells. In dead yeast cells, DAPI and Hoechst 33258 stain the dsDNA within nucleus and cytoplasm. In live yeast cells, Hoechst 33258 shows dim nuclear and cytoplasmic staining, while DAPI shows dim mitochondrial staining [17]. What are your results?

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