Cell cycle analyzes using flowcytometry

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STAINING LIVE CELLS
If you need to stain live cells you must use a cell permeant dye (see below). Optimize the incubation time and titrate your DNA dye. An incubation temperature of 37°C may be better than RT, but Hoechst may be kicked out of your cells at this temperature, thus, a higher Hoechst concentration is needed for live cells.
Several companies have cell cycle dyes for live cells.

FIXATIVE AND/OR DETERGENT:
Zink salt fixation
An excellent fixation method which enables analysis of both surface proteins, intracellular proteins, DNA profiles and pulse-labeling using the thymidine analog EdU in the same cell sample (see protocol below).

Dehydrating agents
If ethanol is compatible with your assay, this is a good fixative but be aware that this and other dehydrating agents may cause cell clumping due to coagulation of proteins (see the protocol below).
Ethanol or methanol will permeabilize the cell membrane (removes the lipids).
Be aware that dehydrating fixatives may affect surface epitopes and that GFP, mCherry, and cerulean can be destroyed by alcohol treatment.

Crosslinking agents
Crosslinking agents like formaldehyde reduce DNA binding as a consequence of chromatin crosslinking, but is preferred if you also need to examine surface markers.

Detergents
If you need to add detergent (e.g. triton, NP-40 or saponin), which will dissolve the cell membrane, be aware that the nuclear envelope will be broken down. Therefore, the DNA will leak out during mitosis.

If your goal is to **only analyze nuclei,** we recommend using the ***Vindeløv method*** which will give you a better CV than whole cells, making it easier to distinguish the cell cycle phases. (see below).

DNA BINDING REAGENTS
Binding site
- *Intercalating dyes* (binds in between base pairs)
  - e.g. PI, 7-AAD, and DRAQ5. These dyes bind stoichiometrically.
- *Bis-intercalators*
  - *TOTO, YOYO*

- Minor grove
  - e.g. DAPI, Hoechst, chromomycin/mithramycin.
- Major grove
  - e.g. Methyl green

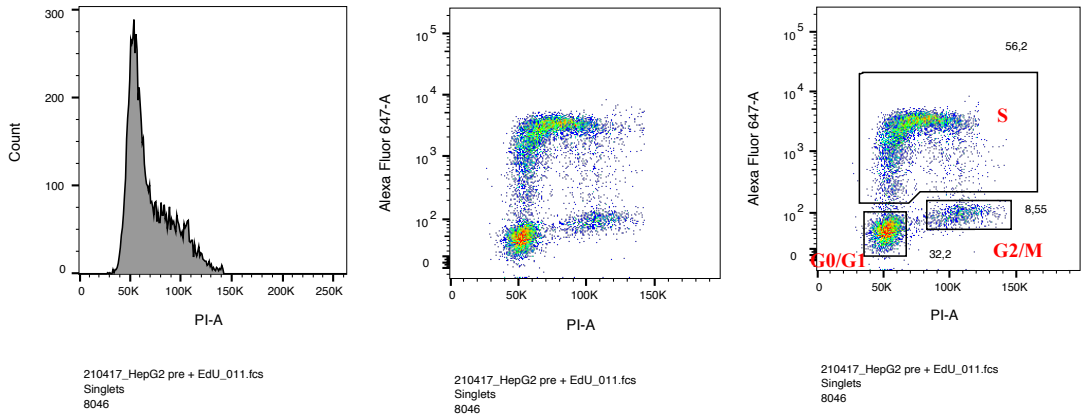
Cell permeable or not?

- *Cell permeable dyes*
  - Hoechst 33342, DRAQ5 (Be aware that DRAQ5 may be toxic to your cells)
  - Cell Cycle dyes from various companies
- *Slightly cell permeable* (will stain all cells, but dead cells will stain better)
  - DAPI, Hoechst 33258, 7-AAD
- *Cell impermeable*
  - PI, DRAQ7

Be aware that PI and DRAQ5 will bind both DNA and RNA (RNase may be needed).

EdU

A more direct measure of DNA synthesis can be achieved using EdU (5 Ethynyl 2'deoxyuridine, a thymidine analog). When pulsing cells with EdU, it will be incorporated into the cell DNA in place of thymidine. The incorporated EdU can efficiently be detected using a "click-it" reaction. This allows simultaneous detection of surface or intracellular markers as well as other DNA binding dyes like e.g. PI. It is then possible to create a 2-dimensional plot with a PI pattern reflecting DNA content in one direction versus incorporated EdU in the second direction resulting in a U-shape.



In this figure, EdU is visualized using Alexa Flour 647.

Be aware that antibody binding epitopes might change due to this method.

You can label live mice (subcutaneous or abdominal injection), cell lines, or primary cells

Adult mice: 2-4 mg EdU/mouse
Cell lines according to manufacturer's guidelines

IMPORTANT BEFORE AND DURING ANALYSIS

- Dye-to-cell ratio is very important. DNA saturation is needed.
- Titrate your DNA dye to avoid background.
- Analyze your sample at low speed (very important, to ensure low CV).
- Gate on cells of interest in a FSC versus SSC plot.
- Remove doublets in a "DNA-area" versus "DNA-width" plot or "DNA-area" versus "DNA-height" plot. Bare in mind that height can saturate.
- Remove dead cells if possible. An option is to stain with a fixable viability dye (protein-binding) before fixation. It is not possible to remove dead cells if you are analyzing nuclei e.g. using the Vindeløv method.
- Use **linear** scaling in a histogram plot for the DNA dye to determine G0/G1, G2/M and S-phase.
- Use chicken red blood cells (and rainbow trout red blood cells if possible) as controls, due to their very constant DNA content.
  Ref. Vindeløv see below.

THE FOLLOWING IS SUGGESTED PROTOCOLS WHICH NEEDS TO BE OPTIMIZED OR ADJUSTED FOR THE GIVEN EXPERIMENT

LIVE CELL STAINING

Several companies sell products for live-cell cell-cycle analysis. Start out with the company's recommended protocol.
The optimal dye concentration must be found for each cell type by titration.

If you use DRAQ5 or Hoechst 33342 the following can be used as a starting point.

1. Add the recommended amount of DNA dye to 1×10^6 cells in 1 mL.
2. Mix well and incubate at 37˚C for 30 - 90 min (cell type and species dependent), in the dark.
3. Keep cells at 37˚C until acquisition.

STAINING PROTOCOL FOR PI OR DAPI AFTER ETHANOL FIXATION

1. 1 mill cells in 0.5 mL PBS (make sure you have a single cell suspension).
2. Add 4.5 mL -20°C 70% EtOH to cells dropwise while gentle vortexing (medium speed), to avoid cell clumping. Clumps can figure as double 4N or 6N and 8N in the DNA plot.
3. Incubate at 4°C for minimum 30 min (can be left at 4°C for a week).
4. Centrifuge 10 min, 4°C at 300 x g, time and speed depends on cell type.
5. Aspirate the supernatant, be careful the pellet is a little diffuse!
6. Resuspend in 3 mL PBS and let the cells rehydrate for 30 seconds - 2 minutes.
7. Centrifuge 10 min, 4°C at 300 x g, time and speed depends on cell type.
8. Aspirate the supernatant and repeat step 6-8.
9. For a PI protocol go to step 10 for a DAPI protocol go to step 11.

10. PI:
    a. Treat cells with DNAse free RNase A: use 50µl of 100µg/mL RNase A, 30 min, RT
    b. Add PI, approximately 1 µg/mL final concentration, depending on titration results, incubate for minimum 30 min in the dark at 4°C. Analyze within 3 hours.
11. DAPI:
    a. Resuspend in 300-1000 µL DAPI staining buffer (flow buffer containing approximately 1µg/mL DAPI, depending on titration results).
    b. Incubate in the dark 15-30 min, RT.

ZINK-SALT FIXATION

ZBF-buffer:

0.1 M Tris-HCl pH 7.8
0.05 % calcium acetate $(CH_3COO)_2Ca$
0.5 % zinc acetate $(CH_3COO)_2Zn$
0.5 % zinc chloride $ZnCl_2$

1. Suspended the cells in 1 volume PBS^{--}
2. Add 10 volumes ZBF-buffer while vortexing
3. Incubate at 4°C over night
4. Choose one of the following, depending on your aim
    a) Immediately freeze cells in glycerol (1:1) (can be stored at -20°C for at least a year).
    b) Wash the cells carefully 3 times in TBS prior to use (3x15 minutes on orbital shaker not vortex)

b) Centrifuge the cells 230 x g for 3 min RT in between the washes.
c) Resuspend cells in HBSS containing 1M Hoechst 33342, 0.2 % saponin and 0.1 % BSA
d) Incubate at RT, 15-60 minutes in the dark. If you add antibodies together with Hoechst, incubate 30 min on ice and Hoechst 33342 is added once again.

THE VINDELØV METHOD

Citrate buffer:

- Dissolve the following in 800 mL distilled water
  85.50 g (250 mM) sucrose
  11.76 g (40 mM) trisodium citrate, 2 H_20
- Add 50 ml Dimethylsulfoxide zur syntese (DMSO)
- Add distilled water to a total volume of 1000 mL
- Adjust pH to 7.60.

Stock solution:

- Dissolve the following in distilled water to a total volume of 2000 mL
  2000 mg Trisodium citrate, 2 H_20, (3.4 mM)
  2 ml Nonidet P 40 (NP 40) (0.1% v/v).

1044 mg (1.5 mM) Spermine tetrahydrochloride
121 mg (0.5 mM) Tris(hydroxymethyl)-aminomethane

- Adjust pH to 7.6.

Use the stock solution as the basis for preparing the staining solutions and the sheath liquid in the flow cytometer.

Solution A:
Dissolve 15 mg trypsin in 500 mL stock solution and adjust pH to 7.6

Solution B:
Dissolve 250 mg trypsin inhibitor and 50 mg RNase A in 500 mL stock solution and adjust the pH to 7.6

Solution C:
Protect the solution from light with tinfoil during preparation, storage and the staining procedure.
Dissolve 208 mg Propidium iodide, 580 mg spermine tetrahydrochloride in 500 mL stock solution and adjust the pH to 7.6.

1. Wash and count the cells and resuspend app. 10^6 cells in 200 µL citrate buffer
2. Add 1800 µL Solution A
3. Invert the tube to mix the content gently
4. Incubate 10 min., RT, during which the tube is inverted 5-6 times
5. Add 1500 µL solution B
6. Mix by inversion of the tube for 10 min, RT
7. Add 1500 µL ice-cold 4°C solution C
8. Mix the solutions and filter through a 30-µm nylon mesh into tubes wrapped in tinfoil (to protect PI form light)
9. Keep the samples in an ice bath until analysis
10. Run the samples between 30 min and 3 hours after addition of solution C

Notes:

- Vindeløv's method creates "bare nuclei", thus the "cells" will have little forward scatter signal.
- Threshold debris using FSC threshold, then gate using PI-A vs. PI-H to eliminate doublets. Whole nuclei will have some forward scatter and little side scatter. Alternatively, threshold on PI.

Literature

General cell cyclus:
Eur J Immunol 2019.49: 1457-1973, Cossarizza et al
Curr.Issues Mol Biol. (2001) 3(3): 67-70, Rafael Nunez
https://expert.cheekyscientist.com/cell-cycle-analysis-details-are-critical-in-flow-cytometry/
and on a lot of company sites you can find guidelines to temperature and incubation time

Doublet exclusion Kylie Price: https://onlinelibrary.wiley.com/doi/pdf/10.1002/0471223948
Vindeløv: https://onlinelibrary.wiley.com/doi/epdf/10.1002/cyto.990030503
Zink fixation: https://onlinelibrary.wiley.com/doi/pdf/10.1002/cyto.a.20914