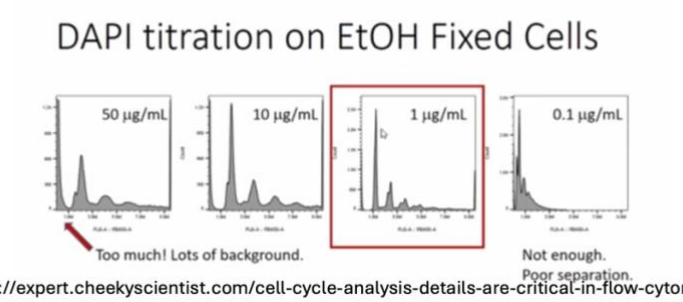
Cell Cycle Analysis using Flow Cytometry

Written by: Anja B Bohn and Anette Thomsen
Date: May 2020
Modified May 2026

IMPORTANT BEFORE AND DURING ANALYSIS

Pre-analytic considerations – *for more specific cell cycle analysis see page 3-5.*

- If you need to stain live cells you must use a cell permeant dye (See table 1).
- Optimize the Dye-to-cell ratio, incubation time, and temperature.
  - DNA saturation is needed for consistent MFIs.
  - Some dyes are fluorescent when unbound and must be titrated to minimize background (see Table 1 and Figure 1).
  - Most cell cycle dyes have low binding affinity and therefore excess dye must be present to ensure all binding sites are saturated (see Table 1). If you wash before analysis, bound dye re-equilibrates with unbound dye causing loss of saturation and thus change in shape and intensity of your cell cycle histogram.



https://expert.cheekyscientist.com/cell-cycle-analysis-details-are-critical-in-flow-cytometry/

Figure 1: Titration example.

  - If your cell concentration varies between samples, the MFI value for your G1, and G2 may vary as well. To adjust for varying staining intensities, you can use chicken or rainbow trout red blood cells as an in-tube staining control or a commercially available DNA control. Both have a constant DNA content and can be used for normalization.
- The above-mentioned controls may also be essential when analyzing **aneuploid** cells, where it may be difficult to determine which populations are aneuploid and which are diploid, normal human diploid lymphocytes may also be used for this purpose.
- Include a live dead dye, if possible, e.g. a fixable viability dye (protein- binding) before fixation.
- You may need to synchronize your cells. If so, it is best to synchronize your cells just prior to the phase of interest.
- A more direct measure of DNA synthesis (S-phase) can be achieved using Thymidine analogous (See below).
- If analyzing cells from tumor tissue samples, be aware that the tissue dissociation

method may affect the quality of the DNA histogram.

Acquisition and analysis

- Analyze your sample at low speed to ensure low CV and a better separation of G1, S, and G2.
- A minimum of 10.000 events, excluding background, aggregates, and debris, must be collected.
- 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 (1). Bear in mind that the height parameter can saturate (Figure 2).

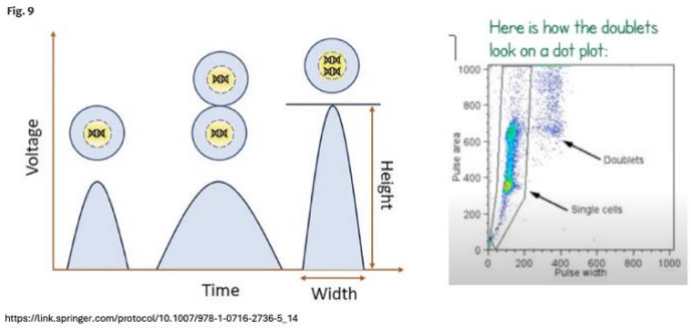


Figure 2: Doublet exclusion.

- Remove dead cells if possible.
- Use **linear** scaling in a histogram plot for the DNA dye to determine G0/G1, G2/M, and S-phase. Different algorithms for calculating the percentage of cells in G0/G1, S and G2 phase for single parameter DNA content exists (e.g. Dean-Jett-Fox or Watson Pragmatic).
- Diploid cells G0/G1 CV should be less than 8%.
- When analyzing **aneuploid** cells (Figure 3), it may be relevant to report the DNA index as the ratio between the MFI of the Aneuploid G_0G_1 peak and the MFI of Diploid G_0/G_1 peak (2, 3, 4).
  For aneuploid cells you need a more complex algorithm (modelling) for data analysis (Non-Linear Least Squares Analysis). In such cases, it is important not to gate away debris or set a threshold that will remove most debris.

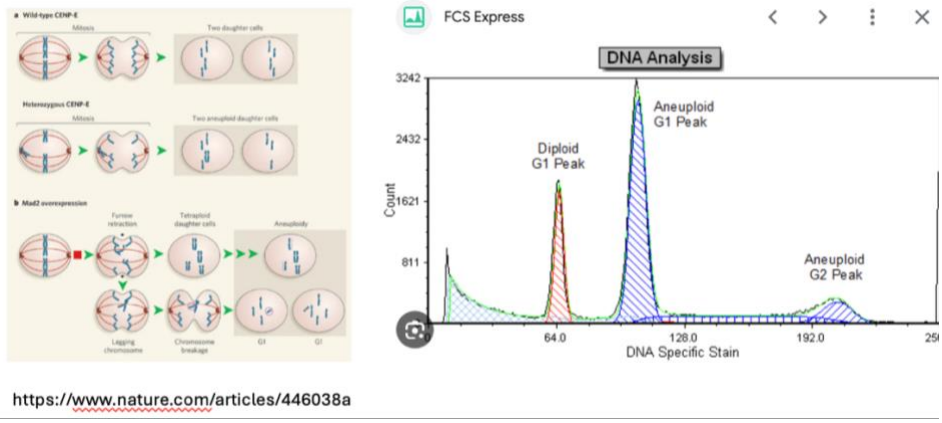


Figure 3: Cell cycle plot from aneuploid cells

DIRECT MEASUREMENT OF DNA SYNTHESIS WITH EdU OR OTHER THYMIDINE ANALOGUES

A more precise 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 be detected using a "click-it" reaction and allows simultaneous detection of surface or intracellular markers as well as other DNA binding dyes like e.g. PI or DAPI (figure 4).

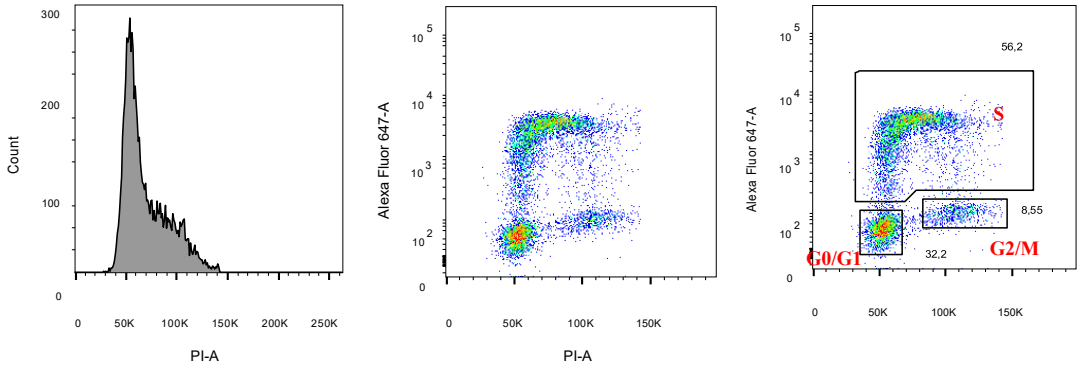


Figure 4: In this figure, EdU is visualized using Alexa Flour 647. A 2- dimensional plot with a PI pattern reflecting DNA content in one direction versus incorporated EdU in the second direction resulting in a U-shape.

- When using Thymidine analogues, be aware that
  - The Click-It technology requires a high copper concentration which is not compatible with fluorescent proteins.
  - If you include PE, PE-tandems, or Qdots in your analyses, these must be used after completing the Click reaction, consider using Click-It Plus instead.
  - EdU at higher concentrations (20µM) may alter cell progression through S phase and may induce DNA-damage-signalling.
  - Pulse-labelleling of cells with EdU may lead to cells accumulation in G2.
  - For long term experiments BrdU is recommended over EdU because of fewer cytotoxic/cytostatic effect.

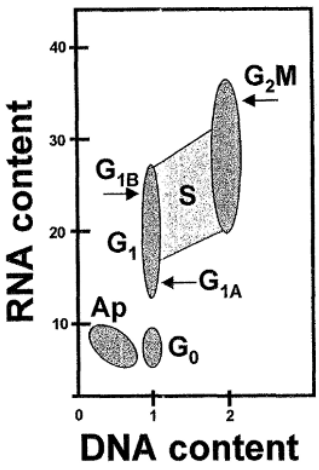
SEPARATING SPECIFIC PHASES OF THE CELL CYCLE

G_0 and G_1

Cell RNA content is low in G_0 enabling separation of G_0 and G_1 using Acridin orange (AO). At the correct concentration, ion strength and pH, AO is excited by the 488 nm laser. DNA-AO has an emission max. at 530 nm, whereas AO-RNA has an emission max above 640 nm (Fig A below).

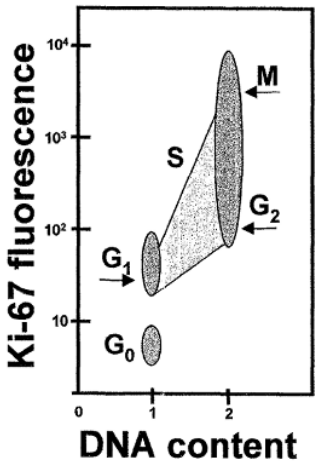
Alternatively, a combination of Hoechst 33242 (: **Ex**. 352 nm, **Em**. 454 nm) and pyronin Y (**Ex** 547 nm, **Em.** peak at 566 nm) can be used. Or anti-KI-67 (Fig B below).

A



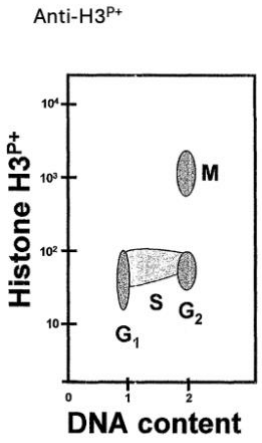
G1A: Early G1, cells in the growth phase are accumulating RNA and proteins
G1B: Late G1: Cells ready to continue to the S-phase
AP: apoptotoc cells

B



SEPARATING G_2 AND M PHASE

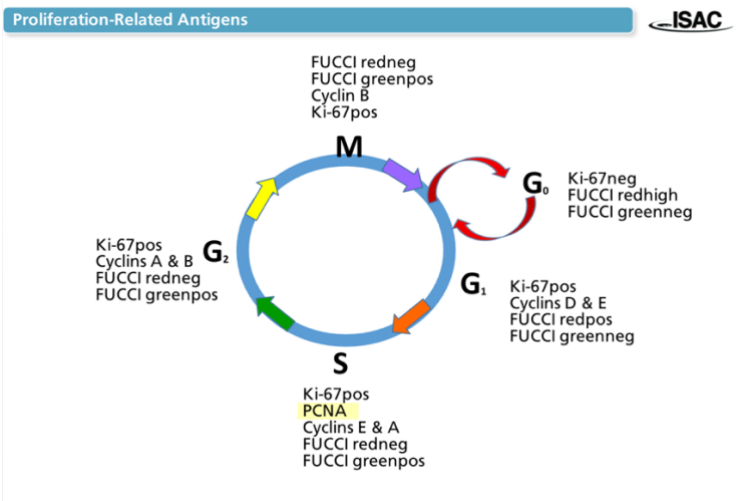
Almost all Histone H3 are phosphorylated on Serine 10 from prophase to telophase. During the remaining cell cycle only a few H3 are phosphorylated, therefore anti-$H3^{p+}$ can be used to separate G2 from mitotic cells.



Proliferation related antigens

It is possible to determine where in the cell cycle cells are based on the presence or absence of proliferation-related antigens. These techniques provide only a snapshot of where the cells are in the cell cycle and can typically not provide information about kinetics of progression through the cell cycle.
e.g. PCNA, Ki-67 (see above), or specific Cyclins (4, 5).
FUCCI: Fluorescence Ubiquitination-based cell cycle indicator for more information see Sakaue-Sawano (6).

- FUCCI cannot be used for cells that are DNA aneuploid.



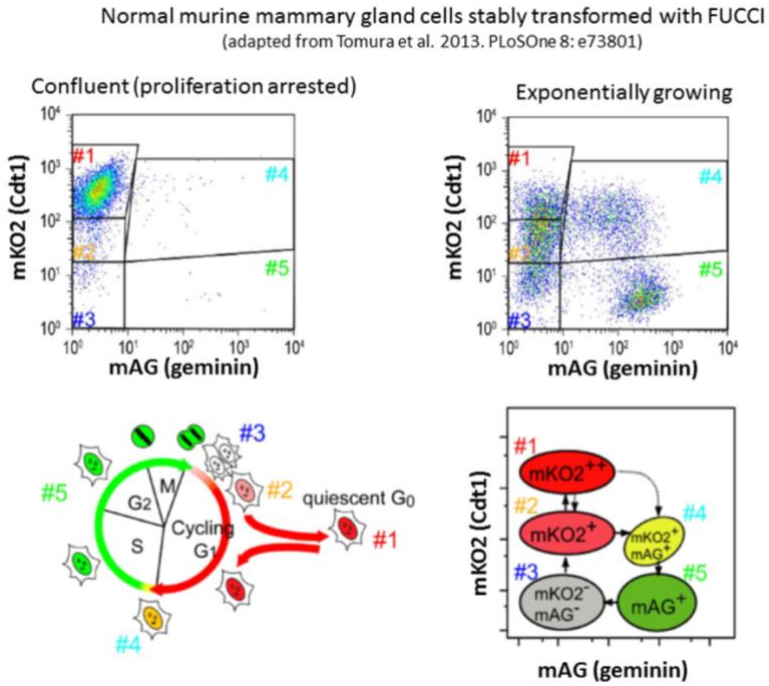


Table 1: DNA BINDING REAGENTS

Dye	Binding Mode	Equilibrium Binding	Fluorescence (Unbound)	RNA Binding	Live Cell Compatible	Key Strength	Key Limitation
Propidium iodide	Intercalation	Yes	Moderate	Yes (requires RNase)	No	Gold standard CV, strong stoichiometry	Needs fixation + RNase, background if excess dye
7-AAD	Intercalation (GC preference)	Yes	Low–moderate	Minimal	No (membrane impermeant)	Combines viability + DNA	Slightly less linear than PI
DRAQ5	Intercalation / groove	Yes	Moderate	Some	Yes	Far-red, no fixation needed	Higher background, lower resolution
Hoechst 33342	Minor groove (AT-rich)	Yes (dynamic)	Moderate	No	Yes	Live cell cycle, UV excitation	Efflux sensitive, higher background
DAPI	Minor groove	Yes	Moderate	No	Limited (mostly fixed)	Simple, robust for fixed cells	Not ideal CV vs PI
SYBR Green I	Groove/external	Yes	Very low	Yes (some)	Limited	Very high SNR ("turn-on")	Less standardized for cell cycle
Vybrant DyeCycle Green	Groove/external	Yes (dynamic)	Low	Minimal	Yes	Live-cell, low background	Efflux, condition-sensitive
Vybrant DyeCycle Violet	Groove/external	Yes (dynamic)	Low	Minimal	Yes	Sorting-compatible, UV/405	Efflux + instrument dependence

- *Be aware that Hoechst may be toxic to cells in s-phase*
- *Intercalating dyes: binds in between base pairs*

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 below for details (7).

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 fixation denatures proteins and permeabilizes the cell membrane, allowing some intracellular proteins to diffuse out. Additionally, it can compromise or abolish the fluorescence of fluorescent proteins.

Crosslinking agents - recommended when detecting surface and/or intracellular antigens

Crosslinking agents like formaldehyde are in general not recommended for cell cycle analysis because of reduced DNA-dye binding due to histone crosslinking but is preferred if you also need to examine surface or internal 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 phase, see below for details (8).

THE FOLLOWING ARE 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 show up as double 4N, 6N or 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, 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.
    b. Incubate in the dark 15-30 min, RT.

ZINK-SALT FIXATION (7)

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 (8)

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

Specific Cell Cycle references:

1) Flow Cytometry: First Principles. AL Givan
2) Darzynkiewicz *et al.* Adv Exp Med Biol. 2010;676, 137–147
3) Pellman *et al.* Nature 2007; 446, 38–39
4) Cyto U – 3 hours of E-Learning Course on Cells Cycle analysis
5) Kim HK & Sederstrom JM Curr Protoc Mol Biol. 2015 1;111: 28.6.1–28.6.11.
6) Sakaue-Sawano A *et al,* Cell 2008; 132, 487-498
7) Jensen UB *et al*. Cytometry Part A 2010; 77A: 798804
8) Vindeløv et al. Cytometry 1983; 3(5), 323-327

General Cell Cycle references:

Cossarizza *et al*. Eur J Immunol 2021; 51(12): 2708-3145
Nunez R Curr.Issues Mol Biol. 2001; 3(3): 67-70
Expertcytometry.com
Many company sites provide guidelines for temperature and incubation time