Cell proliferation and cytokine secretion

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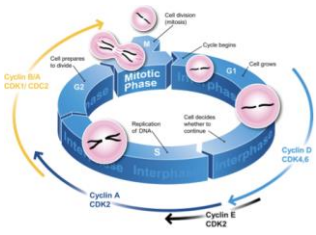
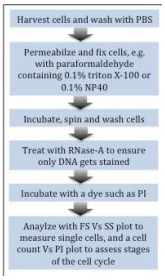
The cell cycle and cellular proliferation

- Process by which eukaryotic cells duplicate and divide.
- Interphase, consisting of G1 (Gap 1), S (synthesis), and G2 (Gap 2)
- Mitotic phase; M (mitosis and cytokinesis).
- During interphase, the cell grows (G1), accumulates the energy necessary for duplication, replicates cellular DNA (S), and prepares to divide (G2).
- At this point, the cell enters M phase, which is divided into two tightly regulated stages: mitosis and cytokinesis. During mitosis, a parent cell's chromosomes are divided between two sister cells. In cytokinesis, division of the cytoplasm occurs, leading to the formation of two distinct daughter cells.
- Each phase tightly regulated. Checkpoints detect potential DNA damage: repair or apoptosis.
- Cell quiescence or senescence state; G0.
- Checkpoint 1=End of G1
- Checkpoint 2= at the end of G2,

CELL CYCLE

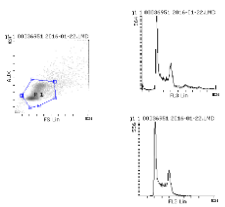




CELL CYCLE using flow cytometry

- Most accurate
- Based on measurement of cellular DNA content.
- A fluorescent dye binds to DNA is incubated with a single cell suspension of permeabilized or fixed cells. Since the dye binds to DNA stoichiometrically, the amount of fluorescent signal is directly proportional to the amount of DNA. Because of the alterations that occur during the cell cycle, analysis of DNA content **allows discrimination between G1, S, G2 and M phases**.
- Don't forget the **RNAse. Singlets** (area vs width)!

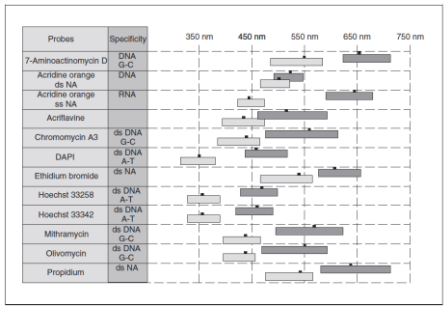
CELL CYCLE using flow cytometry



If doublets (when the DNA content of two cells in G1 are recorded as a single G2/M event) are allowed in to the analysis, it can lead to over-representation of G2/M.
Cellular aggregates and flow rates below 1000 cells/second should also be avoided to allow a low sample pressure differential to be used, which leads to an optimal coefficient of variance (CV).
Reference samples containing normal diploid DNA should be included as an additional control.

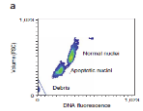
CELL CYCLE using flow cytometry

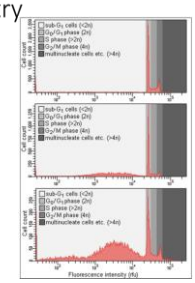
- Most common dyes: propidium iodide (PI), 7-amino actinomycin-D (7-AAD), Hoechst 33342 and 33258, and 4'6'-diamidino-2-phenylindole (DAPI).
- However, most FACS machines commonly used contain only single argon-ion lasers, and as such dyes requiring UV activation such as DAPI and Hoescht 33342 are less frequently used. A derivative of Hoechst dye, SiR-Hoechst, has excitation at 640 nm, and thus may find widespread use



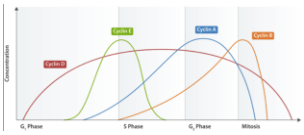
CELL CYCLE using flow cytometry

- The Nicoletti assay (Nature Protocols 1, 1458–1461 (2006)): modified form of cell cycle analysis that allows apoptosis to be assessed. Measures cells with a low intact DNA content, and high fragmented DNA content (the pre-G1 peak).
- Discriminate apoptotic nuclei from cell debris!





CELL CYCLE using flow cytometry

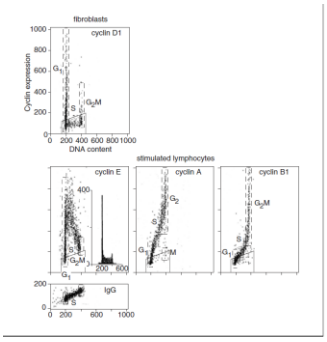


Cyclins

- The cyclins are key regulatory components of the cell cycle machinery. The cyclin family comprises the classical cyclins, cyclin-dependent kinases (CDKs) and Cdk inhibitors (CKIs). Although there is much redundancy between the individual cyclins and CDKs, the activity and expression of the individual proteins fluctuates during each distinct phase of the cell cycle, playing an important regulatory role

Cyclin	Peak Phase Expressed	Cdk Binding Partners
D	G1	Cdk4, Ckd6
E	G1/S	Cdk2
A	S/G2	Cdk1, Cdk2
B	M	Cdk1

Combining cyclin staining with flow cytometry methods examining DNA content provides a powerful and quantitative tool to accurately analyze the cell cycle.



CELL CYCLE using flow cytometry

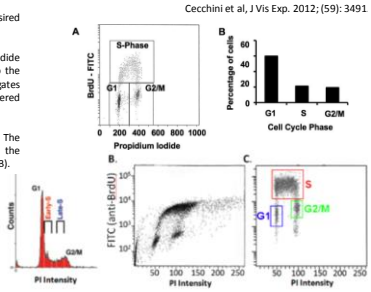
5 000 to 10 000 single cell events with the desired range of DNA content should be collected

Once cells have been measured for propidium iodide and BrdU content they need to be assigned to the G1, S, or G2/M phases. Do this by drawing gates around the two BrdU negative populations centered at 200 and 400 (G1 and G2/M respectively).

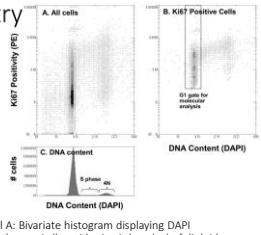
Everything above these boxes is S-phase (Fig. A). The percentage of cells in each gate represents the relative number of cells in G1, S, and G2/M (Fig. B).

BrdU log scale
PI linear scale

Cecchini et al, J Vis Exp. 2012; (59): 3491



CELL CYCLE using flow cytometry



Ki67-Phycoerthyrin (PE) and DNA content flow cytometry. Panel A: Bivariate histogram displaying DAPI fluorescence on the x-axis (linear) and Ki67-PE fluorescence on the y-axis (logarithmic; 4 decades) of diploid cells in a Barrett's esophagus biopsy. Panel B: Ki67-positive proliferating diploid cells, as determined using the bivariate subtraction algorithm (Multicycle). The total Ki67-positive fraction of this sample was calculated to be 35.5%. A representation of the diploid G1 sorting gate is shown. Panel C: A single parameter histogram of DNA content, with S and 4N (G2/M) cells indicated. The S phase fraction is 6.5% and the 4N fraction is 9.9%. There was no evidence of a tetraploid cell cycle.

CELL CYCLE APPLICATIONS

DNA
DAMAGE

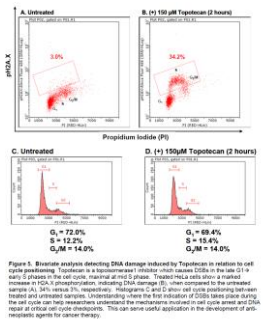
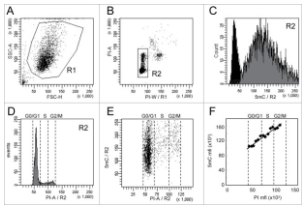


Figure 5. Bivariate analysis detecting DNA damage induced by Topotecan in relation to cell cycle positioning. Topotecan is a topoisomerase I inhibitor which causes DSBs in the late G1 → early S phases in the cell cycle, maximal at mid S phase. Treated HeLa cells show a marked increase in H2A.X phosphorylation, indicating DNA damage (B), when compared to the untreated sample (A), 34% versus 3%, respectively. Histograms C and D show cell cycle positioning between treated and untreated samples. Understanding where the first indication of DSBs takes place during the cell cycle can help researchers understand the mechanisms involved in cell cycle arrest and DNA repair at critical cell cycle checkpoints. This can serve useful application in the development of anti-neoplastic agents for cancer therapy.

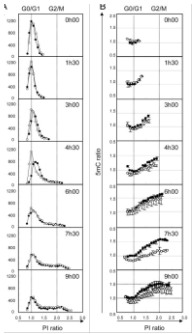
CELL CYCLE APPLICATIONS

DNA METHYLATION



Analysis of 5-methylcytosine (5mC) content in WM266–4 cel by flow cytometry. Asynchronous WM266–4 melanoma cells were labeled with anti-5mC monoclonal antibody prior to DI staining with propidium iodide (PI). For flow cytometry analysis, cells were selected according to their FSC and SSC parameters (R1 region) (A) and then gated on their PI conter (R2 region) (B). 5mC labeling of the R2 cells (gray histogram) and its isotypic control (black histogram) are displayed on a fluorescence histogram with a linear scale (C). Analysis of cel cycle (D) is combined with analysis of DNA methylation, as shown on the dot plot in (E). The graph (F) reports the mean fluorescence intensities of 5mC (anti-5mC antibody mfi minu isotype control mfi) and PI measured in contiguous intervals (5000 mfi units) on the PI scale.

Epigenetics. 2015 Jan; 10(1): 82–91. doi:10.1080/15592294.2014.995542

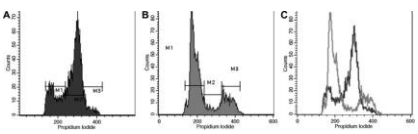


CELL CYCLE APPLICATIONS

Kinetics of DNA demethylation after 5AzadC treatment in WM266–4 cells. Synchronized WM266–4 cells were treated (white circles) or not (black circles) with 5AzadC (0.32 µM) and collected repetitively at intervals of 90 min. For each sample analysis, the number of cells (column A) and their 5mC labeling (column B) were measured taking 20 contiguous regions according to their PI labeling (intervals of 5000 mfi units). PI and 5mC ratios were calculated using normalized data, as the means of sample fluorescence intensities (mfi) reported to the mfi measured in G0/G1 cells (peak point) in untreated cells at t0. Each circle represents mfis of at least 50 cells. 5mC ratios are reported as means of 2 independent experiments.

Cell cycle applications

- Flow cytometry immunophenotyping has become a standard practice for the diagnosis, classification, staging, and monitoring of patients with hematologic neoplasms and it has been proven to be superior to immunohistochemistry.
- Flow cytometry provides a powerful tool to assess cells in G0/G1 phase versus S-phase, G2 or polyploidy. Cell cycle analysis could differentiate low from high-grade gliomas and benign from atypical/anaplastic meningiomas.
- A **prognostic significance** was found in glioma patients.
- This could permit an intraoperative cell cycle analysis of the surgical specimen. Given that intraoperative pathologic analysis of frozen sections of tissue obtained during surgery is the gold standard for intraoperative diagnosis, we set out to evaluate the role of rapid cell cycle analysis for the intraoperative characterization of intracranial lesions and tumor margins. (Alexiou et al, 2015)

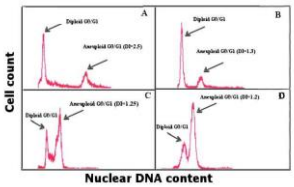


Glioblastoma tissue analysed by flow cytometry. (A) Cell cycle analysis from the tumor core. (B) Cell cycle analysis from the perilesional tissue revealing the transition of G0/G1 phase (M1), S-phase (M2) and mitosis fraction (M3) towards more benign pathology. (C) Overlay of the two histograms.

Low-grade tumors could be accurately differentiated from high-grade tumors by cell cycle analysis.

Glioma margins can be readily identified and the surgeon can be accordingly informed.

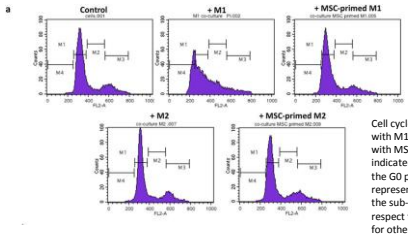
Vartholomatos et al, 2014; Alexiou et al, 2015



Different ploidy histograms of liver biopsies from pateints with HCC. Figures A,C from tumor part and B,D from residual part (non tumor part) of HCC liver biospes.

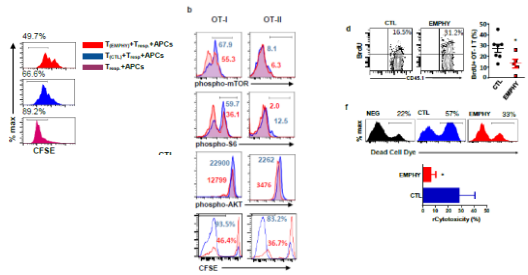
Cytometric to Cytogenetic analyses:

- Ten to thousands cells or nuclei.
- Does not rely on metaphase cells, all phases of the cell cycle are generally included in the analysis.
- cytometric analysis can not provide information on chromosome structure. Incapable of detecting balance chromosomal translocation. The limitation imposed on cytometric analysis even using high precision instruments and the most stiometric DNA binding fluorescence dye; DAPI (4, 6 diamidino 2-phenylindole), prevent cytometric from detecting chromosomal gains or losses involving more than about 5 % of the total DNA. In practice smallest change detected is the gain or loss of equivalent of one large (e.g. A-group) chromosome.

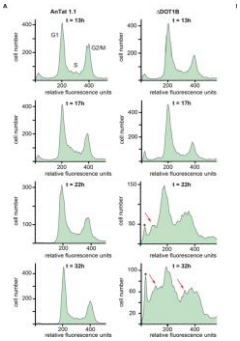


Cell cycle analysis of HUVEC cells co-cultured with M1 or M2 macrophages or alternatively with MSC primed M1 and M2 macrophages as indicated above each histogram. M1 represents the G0 phase, M2 represents the S phase, M3 represents the G2/M phase and M4 represents the sub-apoptotic phase. Gates were set with respect to control HUVEC cells and duplicated for other co-culture conditions

Vasandan et al, 2016. Scientific Reports 6, (2016) doi:10.1038/srep38308



Kerdidani et al, under revision J immunol

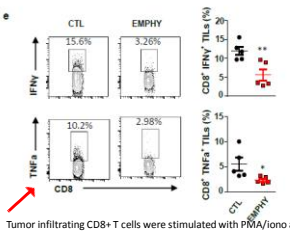


Differentiation defect of ΔDOT1B trypanosomes. (A) Cell cycle profiles of propidium iodide stained differentiating trypanosomes (flow cytometry). ΔDOT1B cells re-enter the cell cycle normally (t = 13h). Later in differentiation (t = 22h; t = 32h) the profiles show peak broadening indicating the appearance of cells with abnormal DNA content (red arrows) and enucleated " zoids " (asterisks)

Dejung et al 2016, PLOS pathogens,

Cytokine production measurement

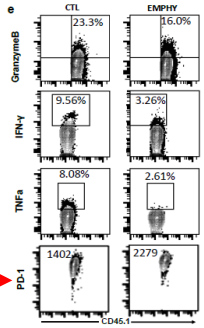
- Protein or mRNA
- All cell types (white blood cells, tumor, ECs)
- Intracellular staining (permeabilization)
- Cytometric bead arrays (intracellular or secreted)
- mRNA fluorescent probes
- Sorting option

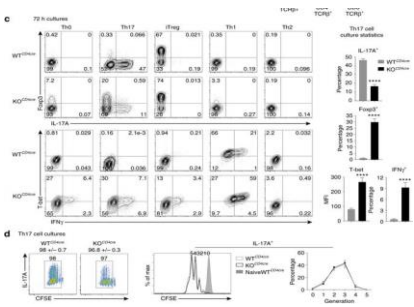


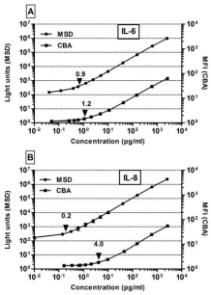
Tumor infiltrating CD8+ T cells were stimulated with PMA/iono and analyzed for IFNγ and TNFa expression.

FACS analysis of intratumoral OTI T cells (d204) showing expression of cytotoxic molecules (granzyme), effector cytokines (IFNγ, TNFa) and suppressive molecules (PD-1).

Kerdidani et al, under revision J immunol







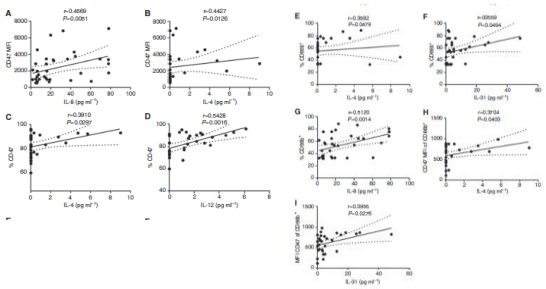
Comparison of MSD and CBA standard curves

Standard curves for IL-6 (A) and IL-8 (B) obtained using the MSD (●) and CBA (■) assays. Concentration for each standard is shown on the X-axis. The output signal is indicated as Light Units for MSD (left Y-axis) and Mean Fluorescence Intensity (MFI, right Y-axis) for CBA. Arrows on each curve indicate the lower limit of quantification.

Sensitivity of multiplex assays according to standard curves.

Cytokine	LLdetection		LQuantification	
	MSD	CBA	MSD	CBA
IL-6	0.3 ± 0.1	0.5 ± 0.3	0.7 ± 0.2	1.2 ± 0.7
IL-8	0.1 ± 0.0	1.5 ± 0.5	0.2 ± 0.0	4.0 ± 4.8
IL-10	0.4 ± 0.2	0.4 ± 0.1	0.8 ± 0.3	1.2 ± 0.1
TNF-α	0.5 ± 0.4	0.5 ± 0.4	1.0 ± 0.5	1.4 ± 0.5
IL-12p70	0.9 ± 0.3	1.2 ± 0.2	1.7 ± 1.0	2.8 ± 1.4
IL-1β	1.0 ± 1.3	1.2 ± 0.2	2.4 ± 2.3	2.4 ± 1.3

Dabitao et al. J Immunol Methods. 2011 Sep 30; 372(1-2): 71–77.



Berrera et al, 2017. British Journal of Cancer **117**, 385-397

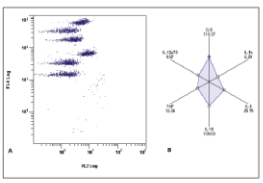


Fig. 2. An exemplary result of cytokine concentration measurement in blood plasma obtained from patients diagnosed for HLH. The FL2 vs FL4 cytogram (A) shows localization of six populations of cytokine binding beads while the Radar Plot (B) shows concentrations of particular cytokines (pg/ml)

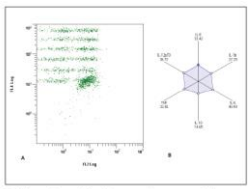


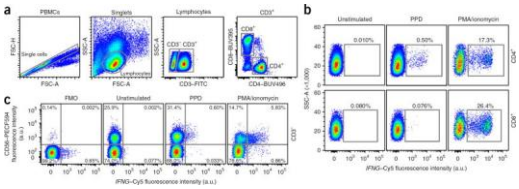
Fig. 3. An exemplary result of cytokine concentration measurement in bone marrow plasma in a patient diagnosed with ALL (CBA Inflammatory Kit). The FL2 vs FL4 cytogram (A) shows localization of six populations of cytokine binding beads while the Radar Plot (B) shows increased concentrations of the studied cytokines (pg/ml)

Stelmaszczyk-Emmel et al. 2013; 67: 879-886

FISH-Flow, a protocol for the concurrent detection of mRNA and protein in single cells

- **In situ hybridization assay.**
- **Combines branched-DNA technology with single-cell resolution of FC**
- **Simultaneous detection of up to four RNA targets + cell surface and intracellular proteins.**
- **Pairs of target-specific probes to amplify a RNA transcript, achieving 8,000- to 16,000-fold signal amplification from a desired RNA transcript.**
- **Detects mRNA, long non-coding RNA, viral RNA, telomere DNA. miRNA**
- **multiple types of cells in suspended or attached, tissues.**
- **large variety of RNA targets, including viral RNAs, are detectable within cells.**
- **Limitations: fewer fluorochromes have been tested for nucleic acid probe conjugation than for antibody labeling. requirement for ~50 (at least 30) 17- to 20-nt-long probes per set limits the selection of target mRNAs to those that are at least 500-nt long. the RNA-based assay yields signals having lower intensity than those associated with protein detection.**

FISH FLOW



Multiplex five-color analysis of IFNG mRNA expression in PBMC subsets.

Arrigucci et al. Nat Protoc. 2017 Jun; 12(6): 1245–1260.

VIABLE Cells can now be sorted according to their cytokine secretion profile!