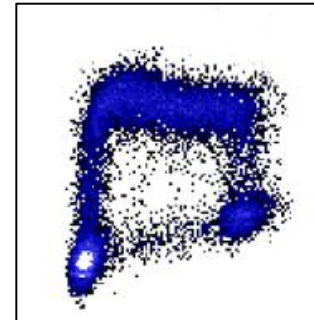
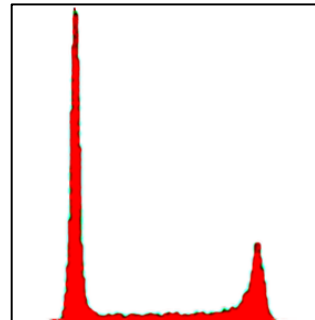


Application de la cytométrie en flux à l'analyse du cycle cellulaire



Montpellier 26 Janvier 2012

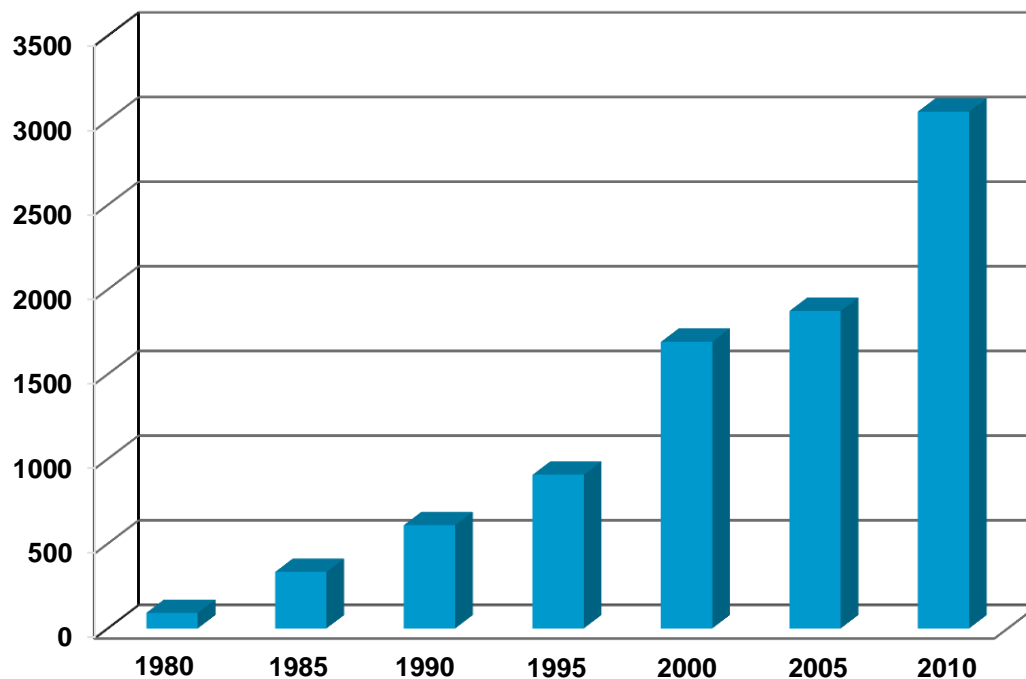
Xavier RONOT

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xavier.ronot@ujf-grenoble.fr



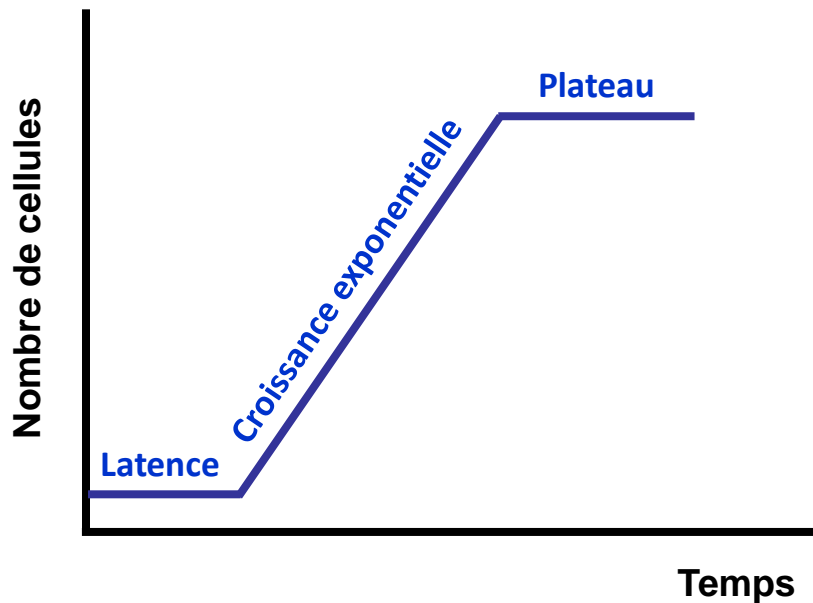
Cycle cellulaire / cytométrie en flux



Mesure de la prolifération cellulaire

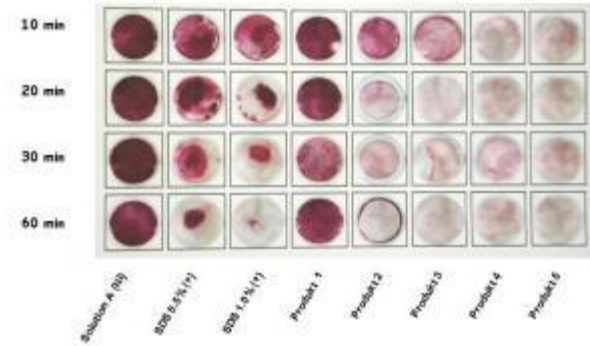
Méthodes « globales »

Courbe de croissance



Calcul du temps de doublement : Td

Test au MTT



Sel de tétrazolum MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

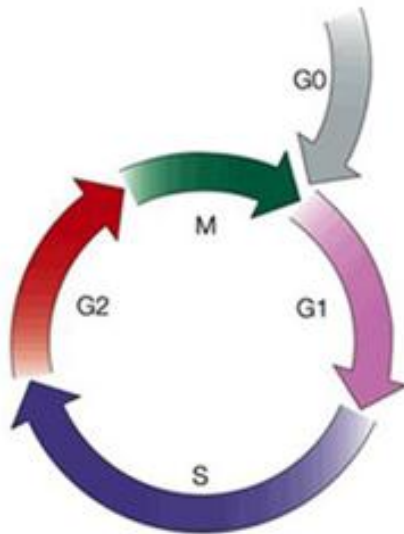
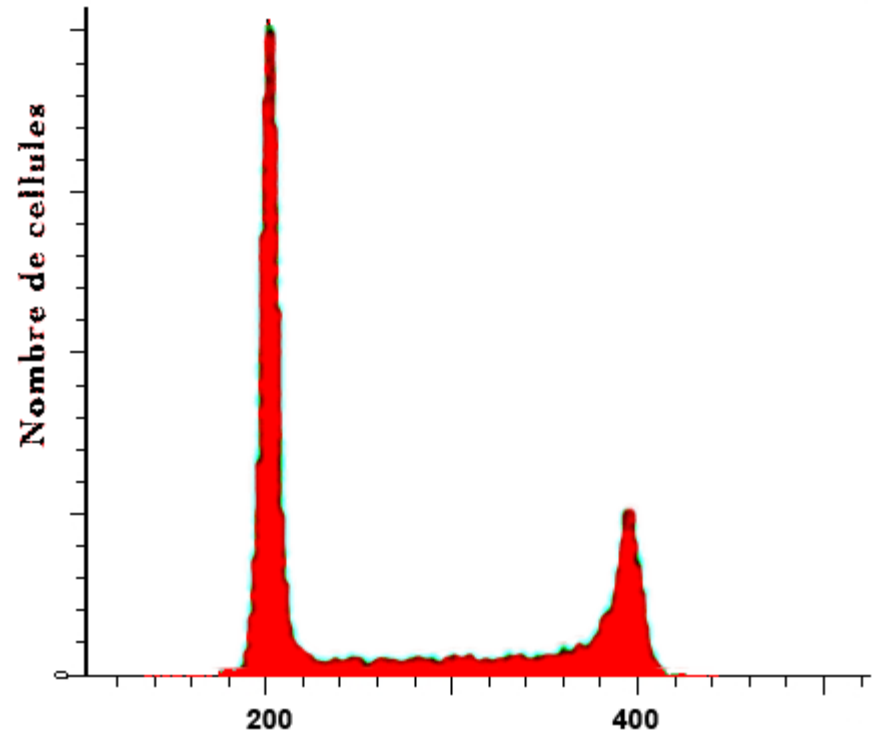
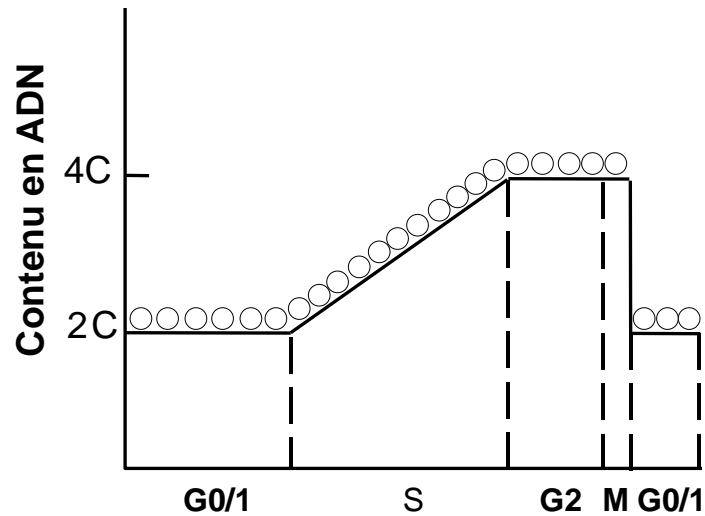


Réduction du tétrazolum par la succinate déshydrogénase mitochondriale des cellules vivantes en formazan

Détermination de la CI 50

Analyse monoparamétrique

Méthode par cytométrie en flux



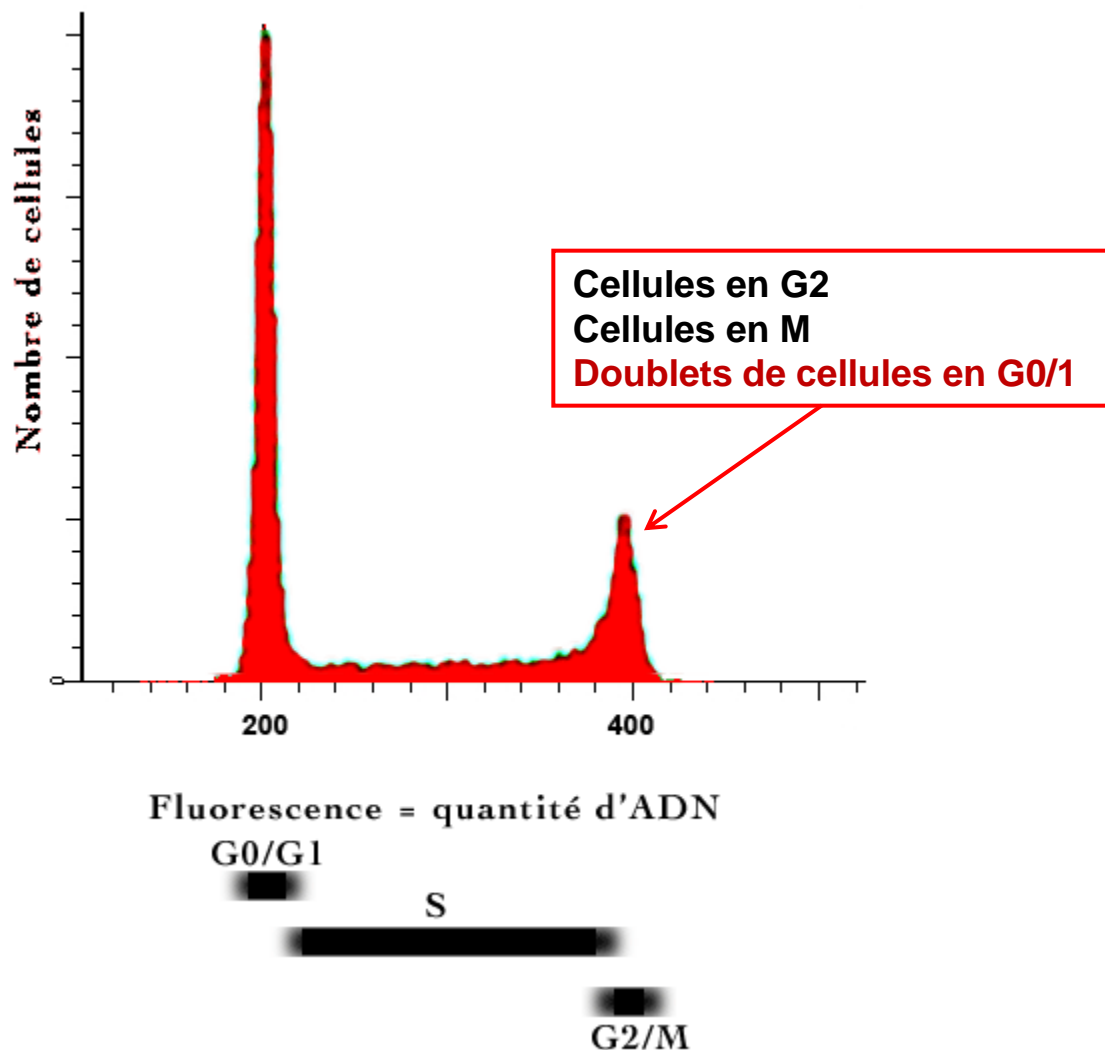
Fluorescence = quantité d'ADN (u.a.)

G0/G1

S

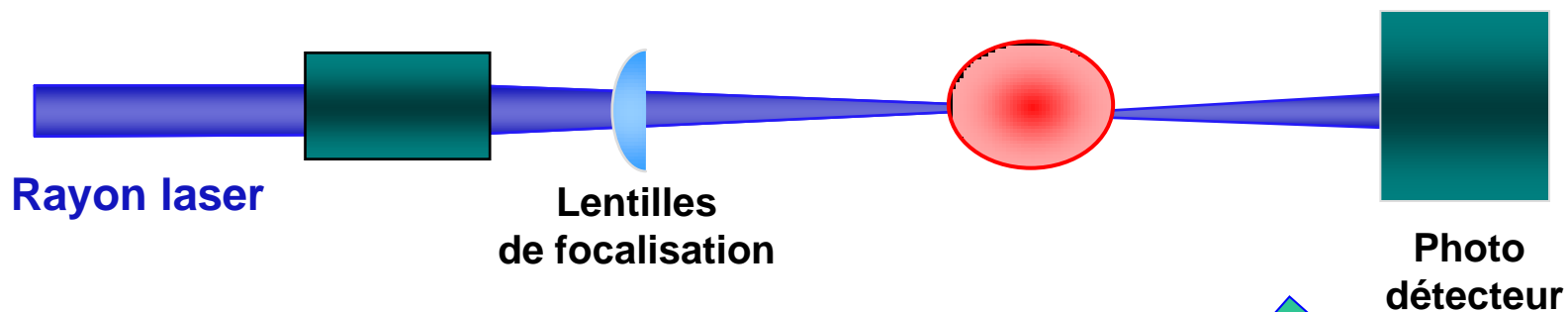
G2/M

L'histogramme monoparamétrique

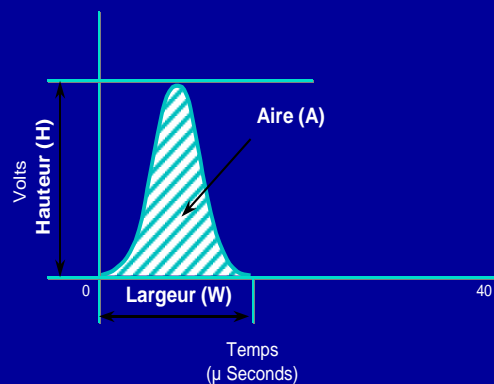


Instantané (cliché) d'une population à un moment donné
contenant des informations multiples

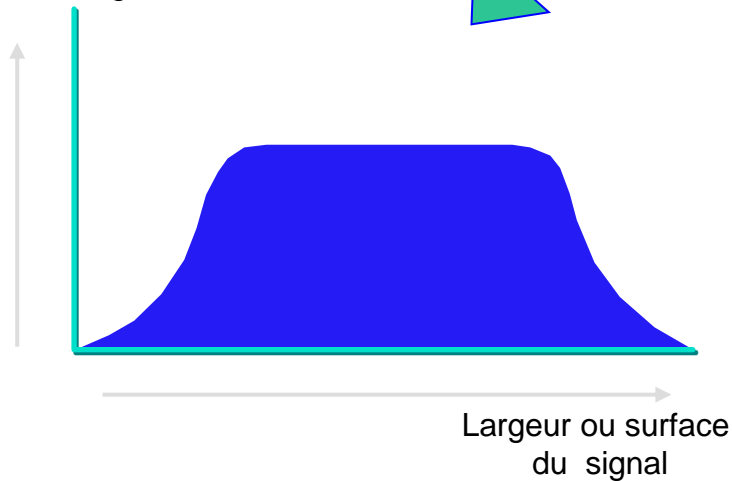
Elimination des doublets



ACQUISITION : Analyse de l'Impulsion

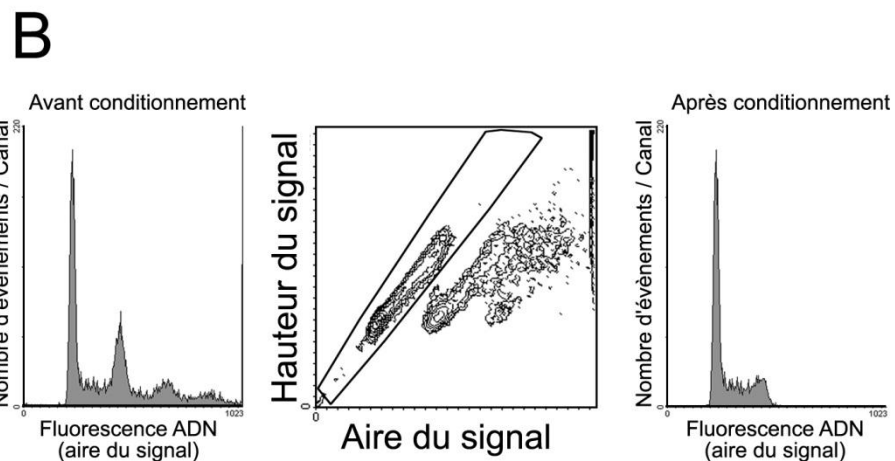
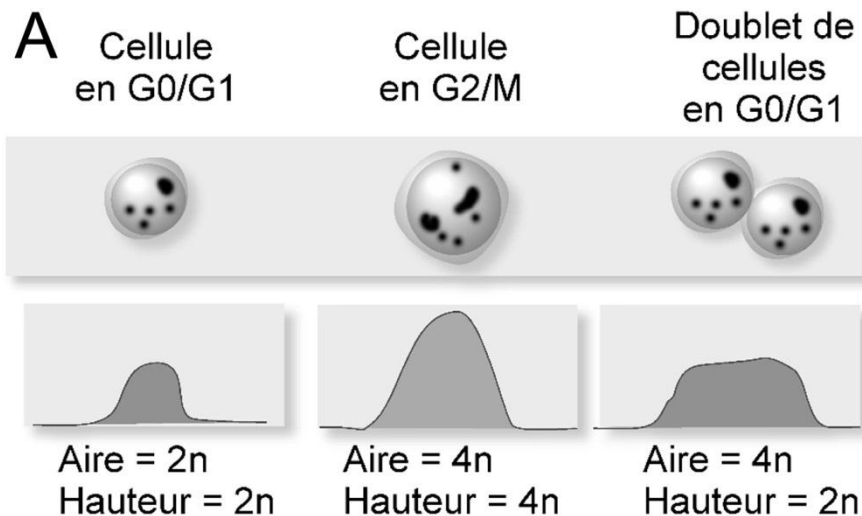


Hauteur du signal

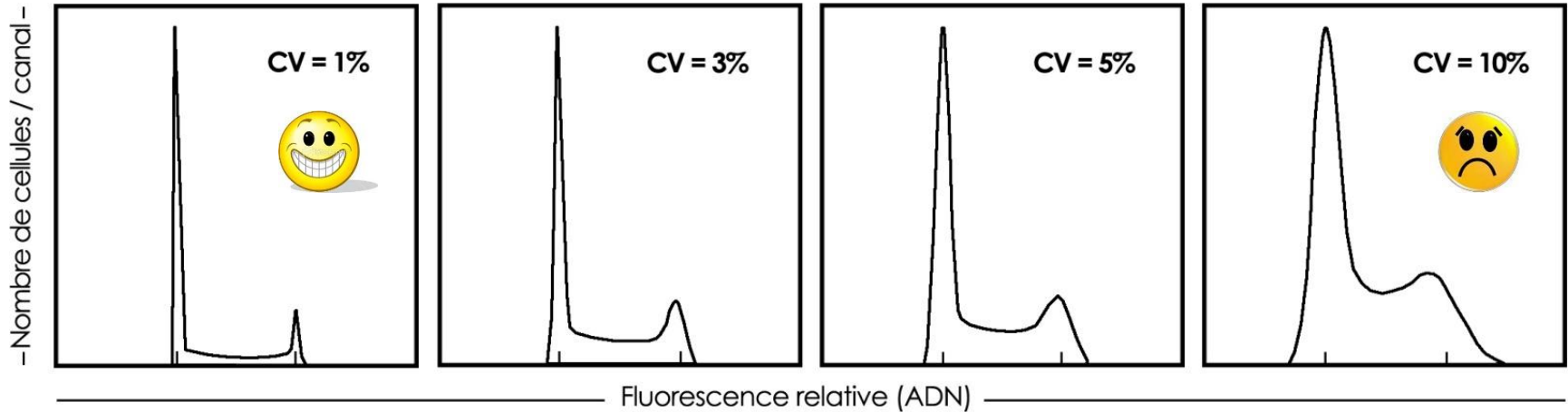


Analyse du signal de fluorescence

Elimination des doublets



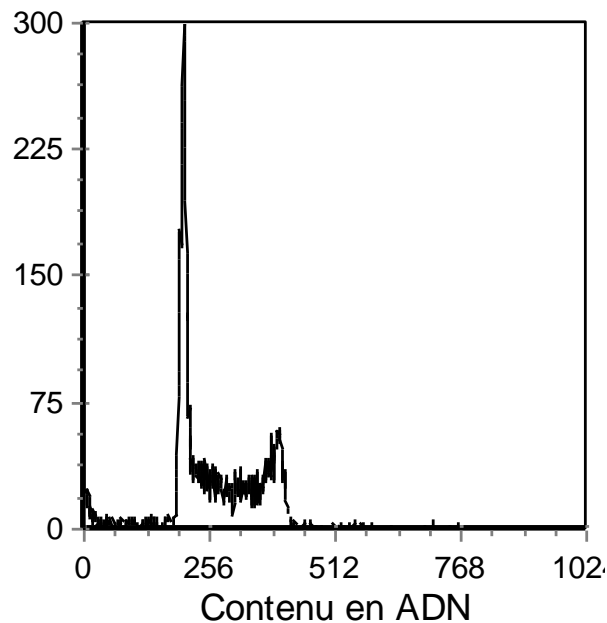
Coefficient de variation (CV)



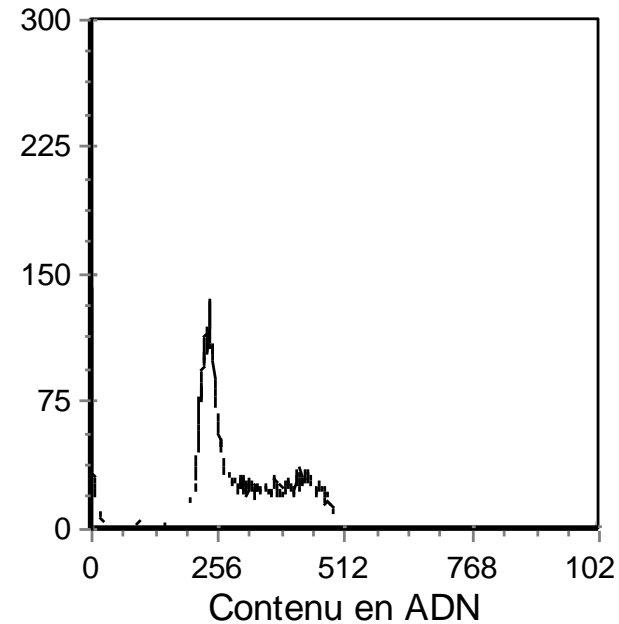
La résolution diminue avec l'augmentation du CV

CV : influence du marquage

Marquage stœchiométrique

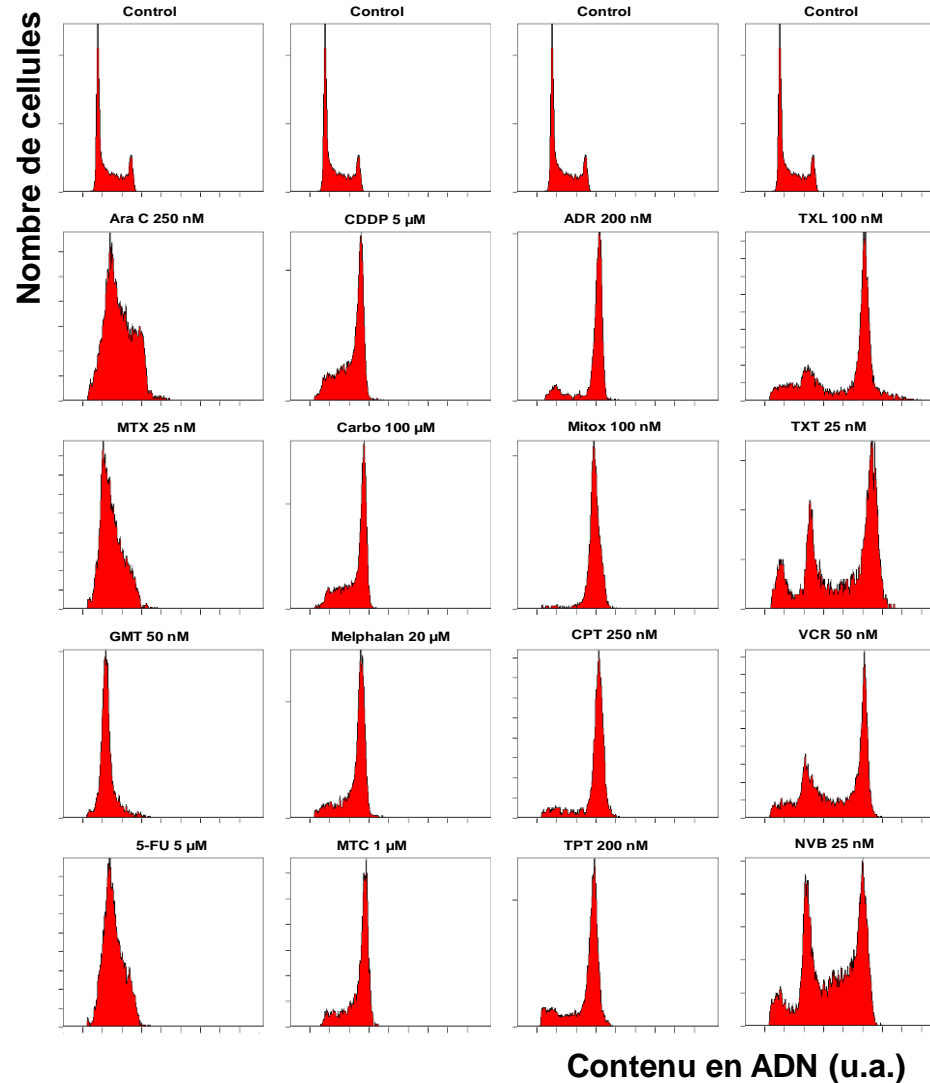


Marquage non stœchiométrique

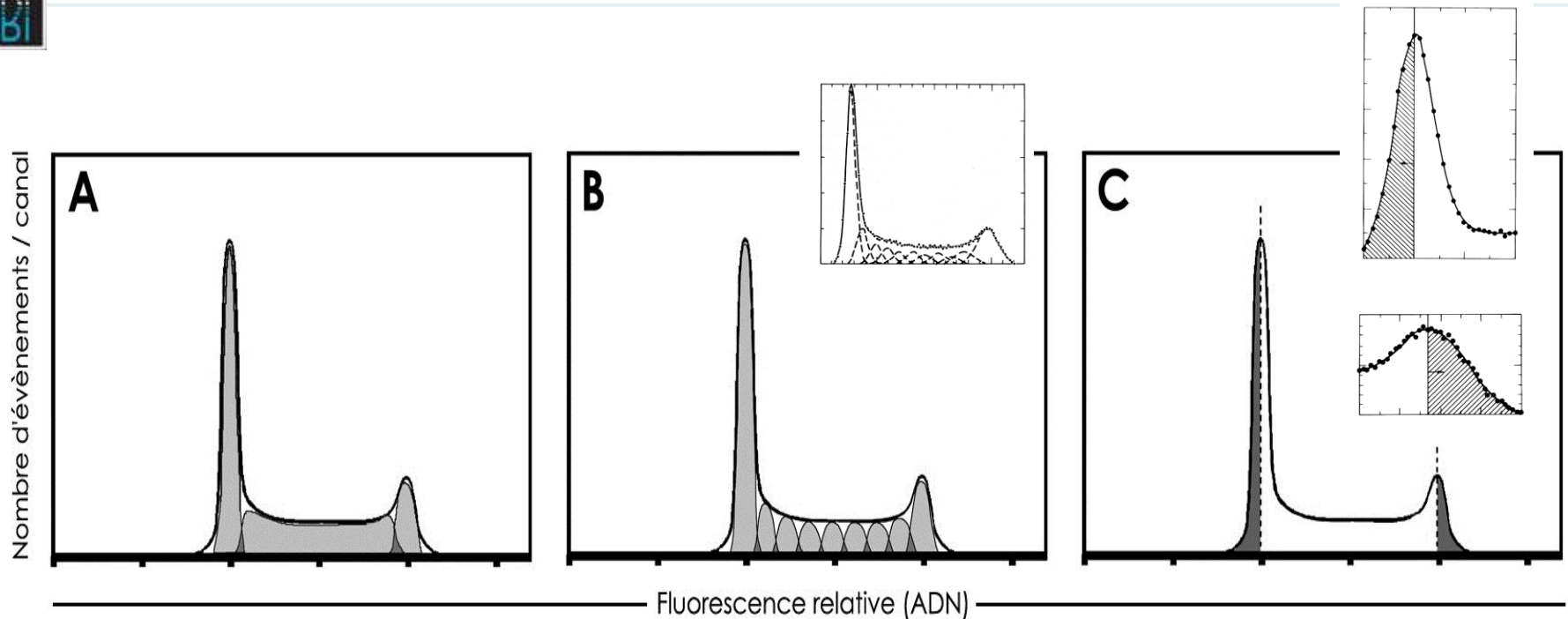


L'interprétation se fragilise avec l'augmentation du CV

Exemples de perturbations du cycle cellulaire induites par des molécules antiprolifératives



Extraction des fractions de cellules en G0/1, S et G2+M : méthodes



A : Estimation de la phase S par un polynôme du second degré

B : Estimation de la phase S par une somme de gaussiennes

C : Méthode du miroir

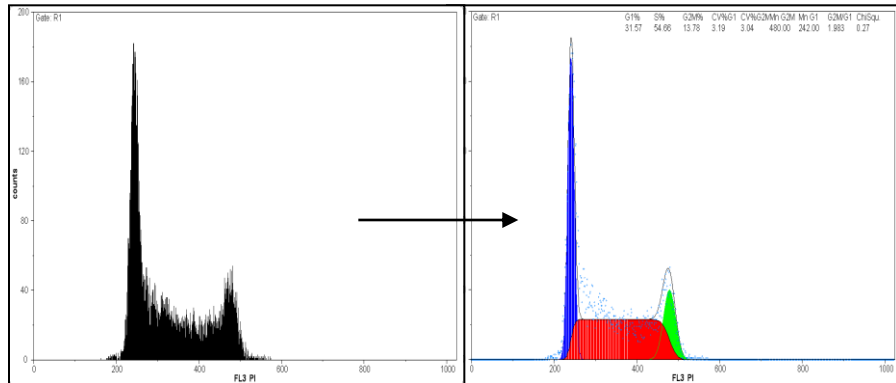
Pulse cytophotometric analysis of cell cycle perturbation with bleomycin in vitro. Barlogie B, Drewinko B, Schumann J, Freireich EJ. *Cancer Res.* 1976;36(3):1182-1187. PMID:56231

Mathematical analysis of DNA distributions derived from flow microfluorometry. Dean PN, Jett JH. *J Cell Biol.* 1974;60(2):523-527. PMID:4855906

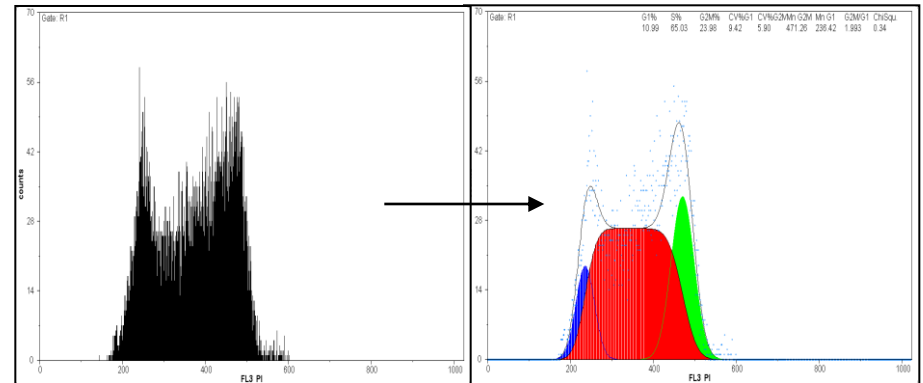
Extraction des fractions de cellules en G0/1, S et G2+M : exemples



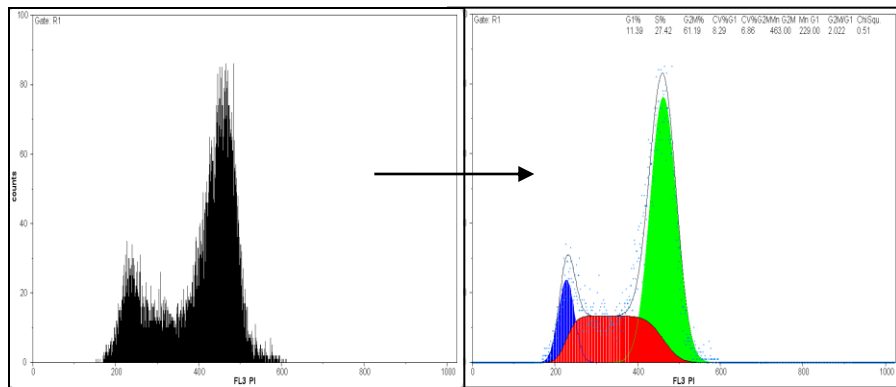
Témoin



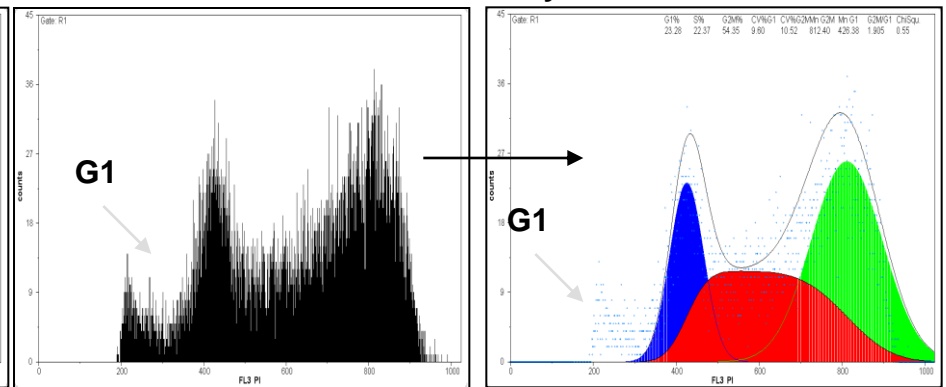
Accumulation en G1 et G2M



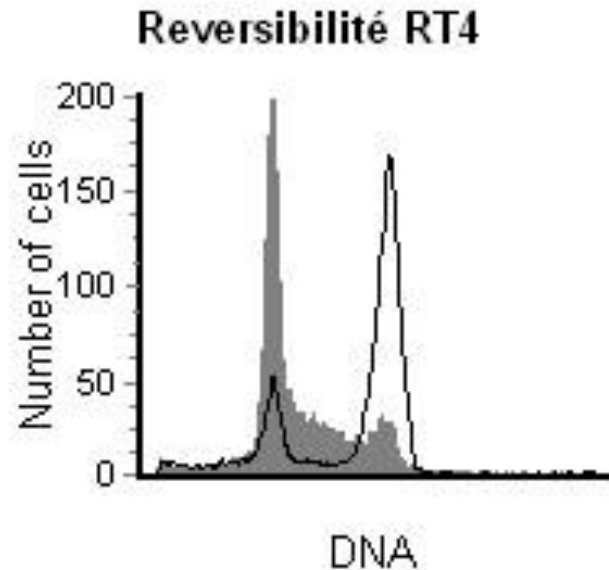
Accumulation partielle en G2M



Induction de noyaux > 4n



Blocage, arrêt ou accumulation ?



Intérêt de la réversibilité
Cytostatique ou cytotoxique ?

Cytotoxique - Cytostatique

Cytotoxique

Inhibition de la prolifération cellulaire, induction de la mort cellulaire.
Action directe ou indirecte sur la synthèse d'ADN (antimitotiques, inhibiteurs de la topo-isomérase, antimétabolites,....)

Arrêt irréversible du cycle cellulaire : **blocage**

Cytostatique

Inhibition de la prolifération (inhibiteurs de la synthèse protéique, de voies de signalisations, cyclines, kinases, récepteurs), le plus souvent par inhibition de phosphorylation.

Effet généralement réversible sur le cycle cellulaire : **accumulation**

Des informations multiples.....mais limitées :

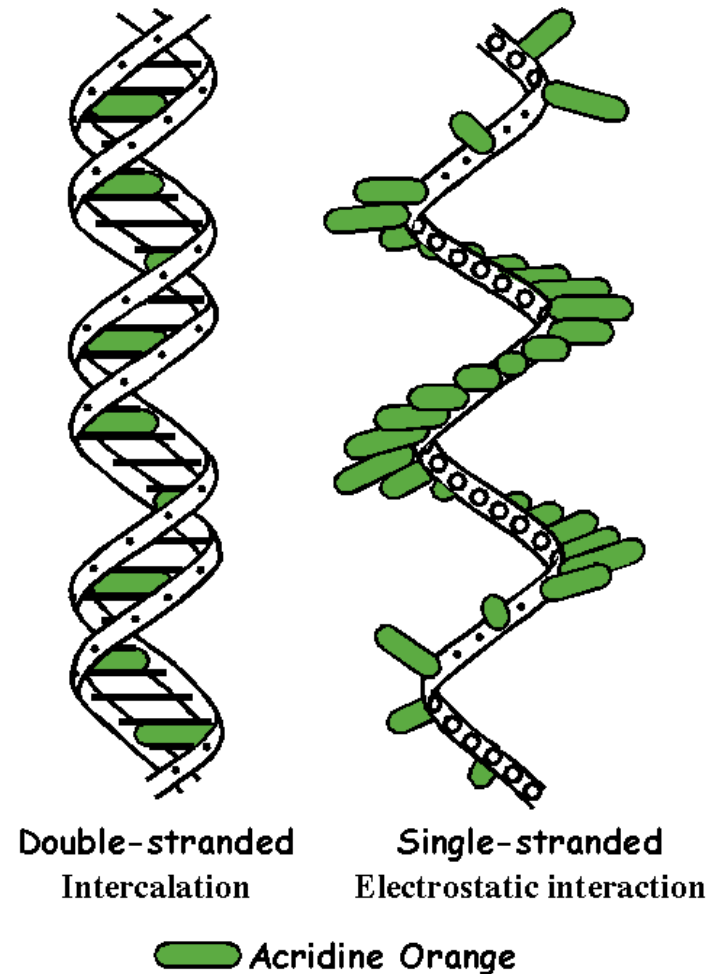
Cellules en G0 et G1 confondues

Cellules en G2 et M confondues

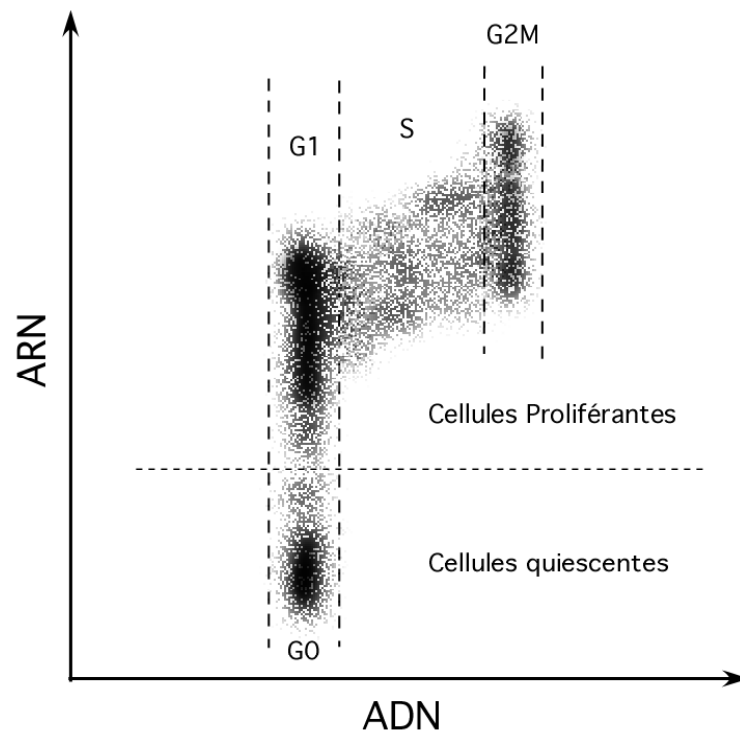
Quid des cellules en S ?

Acridine Orange

- **Acridine Orange**
(AO, 3,6-dimethylamino acridine)
- **Marqueur métachromatique**
 - ✓ fluorescence **verte**
polynucléotide double-brin (ADN)
 - ✓ fluorescence **rouge**
polynucléotide mono-brin (ARN)

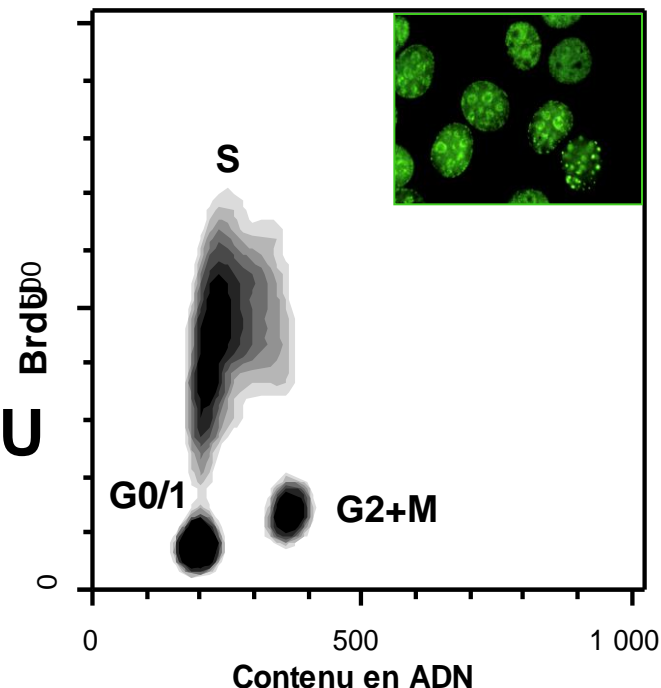


Acridine Orange



G0 ; faible contenu en ARN - G1-S-G2-M : fort contenu en ARN

- Incubation en présence de BrdU (5-bromo-2-deoxyuridine)
- Fixation des cellules
- Dénaturation acide (élimination des histones)
- Marquage par un anticorps anti-BrdU
- Révélation par un anticorps secondaire fluorescent
- Marquage de l'ADN

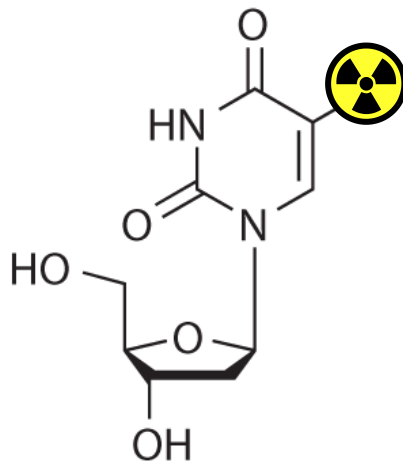


Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part I: Historical perspectives, histochemical methods and cell kinetics. Dolbeare F. Histochem J. 1995 27(5):339-369.

PMID:7657555

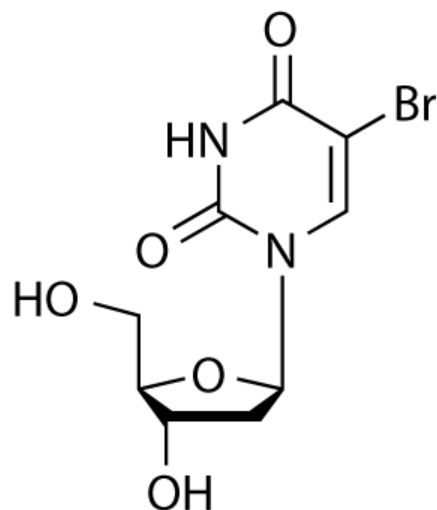
Analysis of cell proliferation using the bromodeoxyuridine/Hoechst-ethidium bromide method. Ormerod MG. Methods Mol Biol. 1997;75:357-365. PMID:9276285

³H-Thymidine



- ✓ Méthode originale de mesure de la prolifération cellulaire
- ✓ Radioactive
- ✓ Incompatible avec des analyses multiples

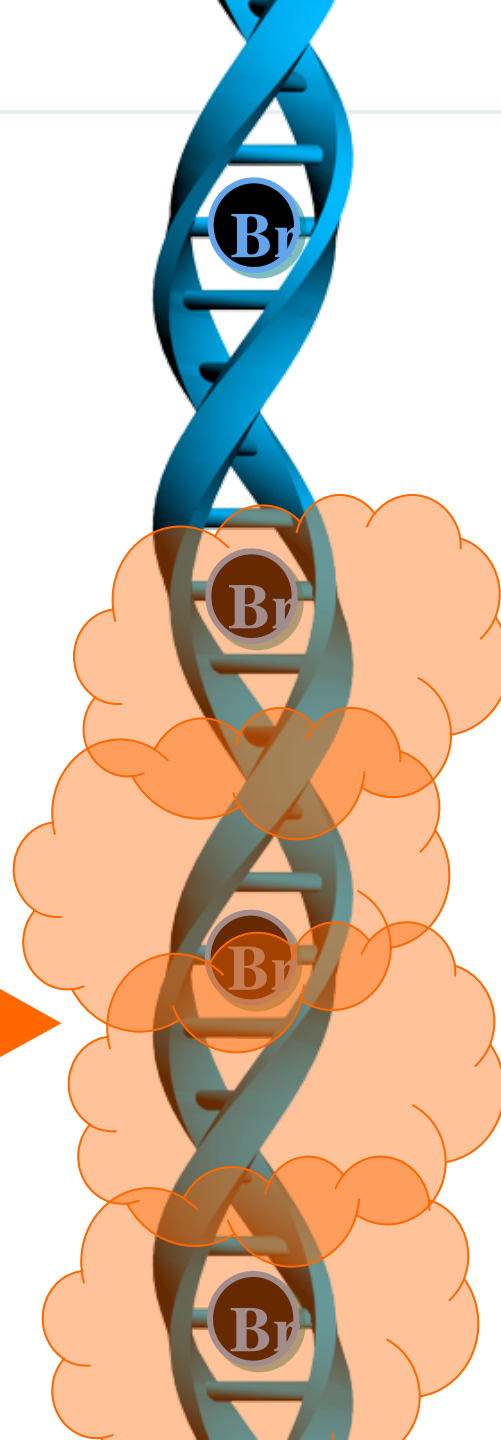


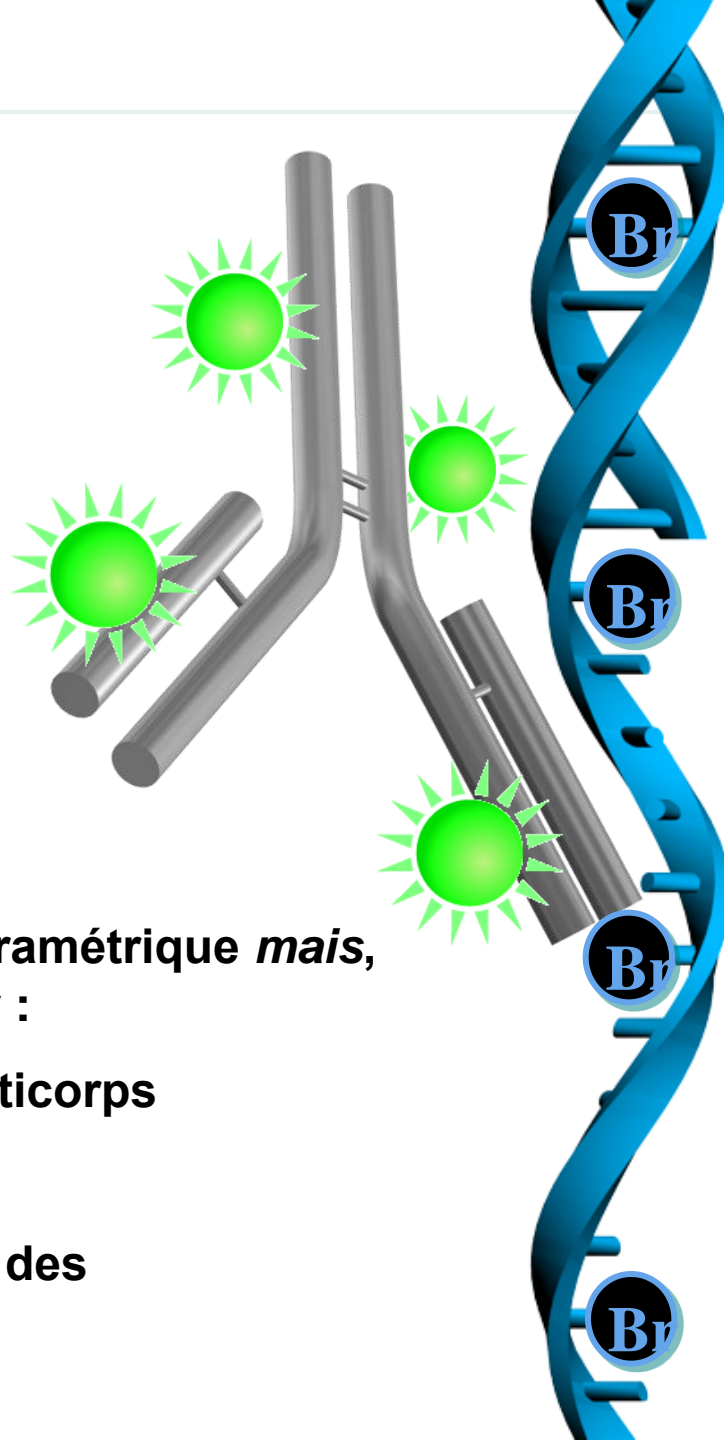


BrdU (5-bromo-2'-désoxyuridine)



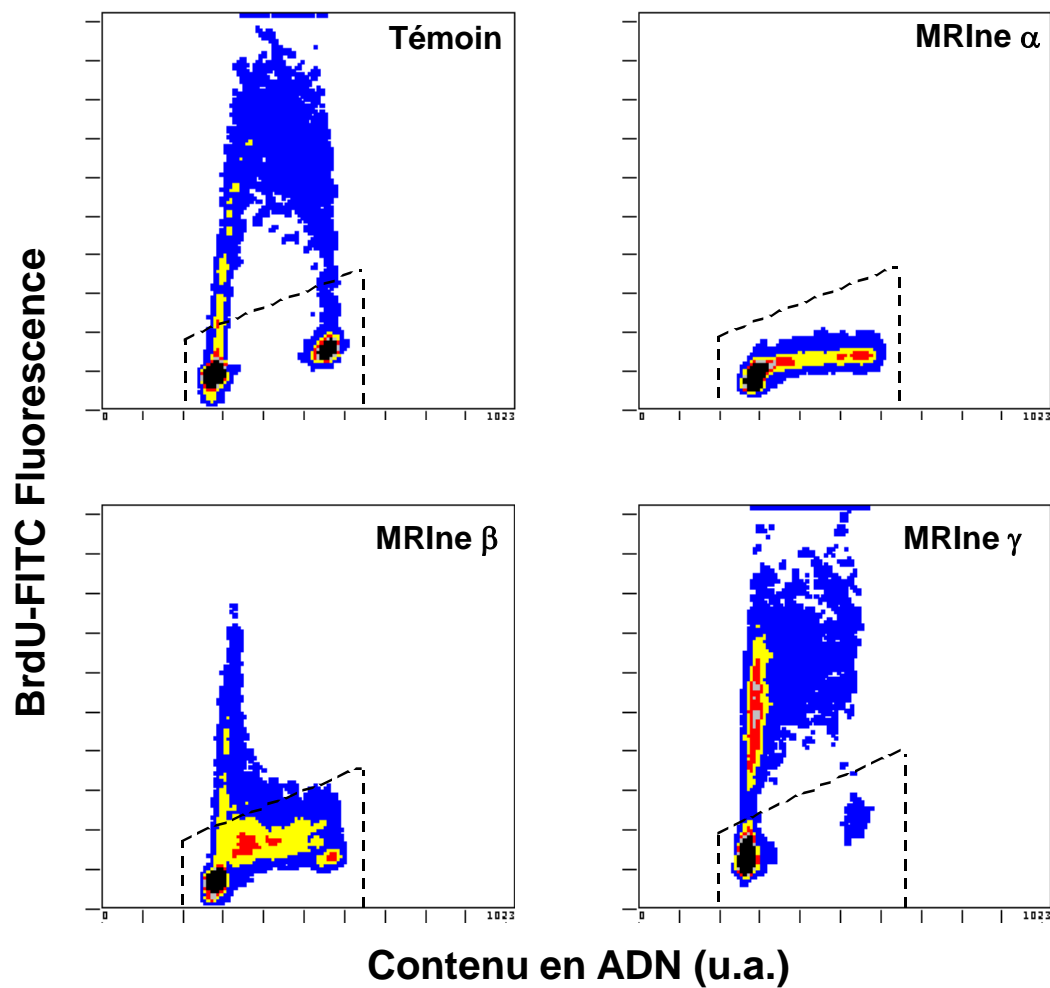
Acide ou DNase →



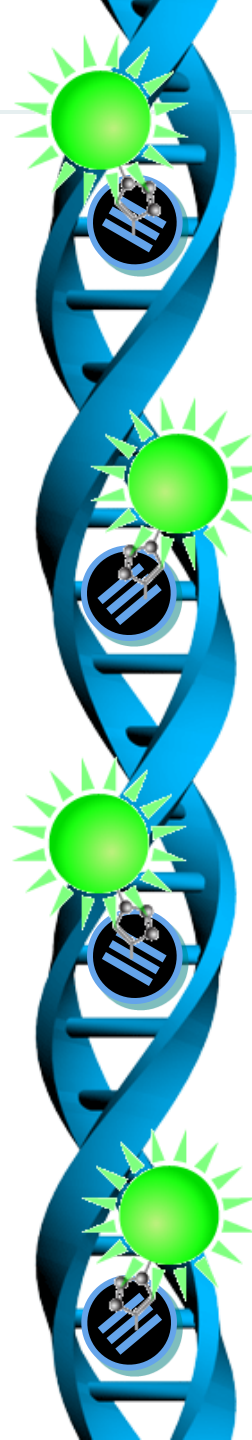


- ✓ Non radioactif
- ✓ Possibilité d'analyse biparamétrique *mais*, la dénaturation peut affecter :
 - ✓ la fixation d'autres anticorps
 - ✓ la morphologie
 - ✓ l'efficacité de fixation des fluorochromes de l'ADN

Incorporation de BrdU : exemples

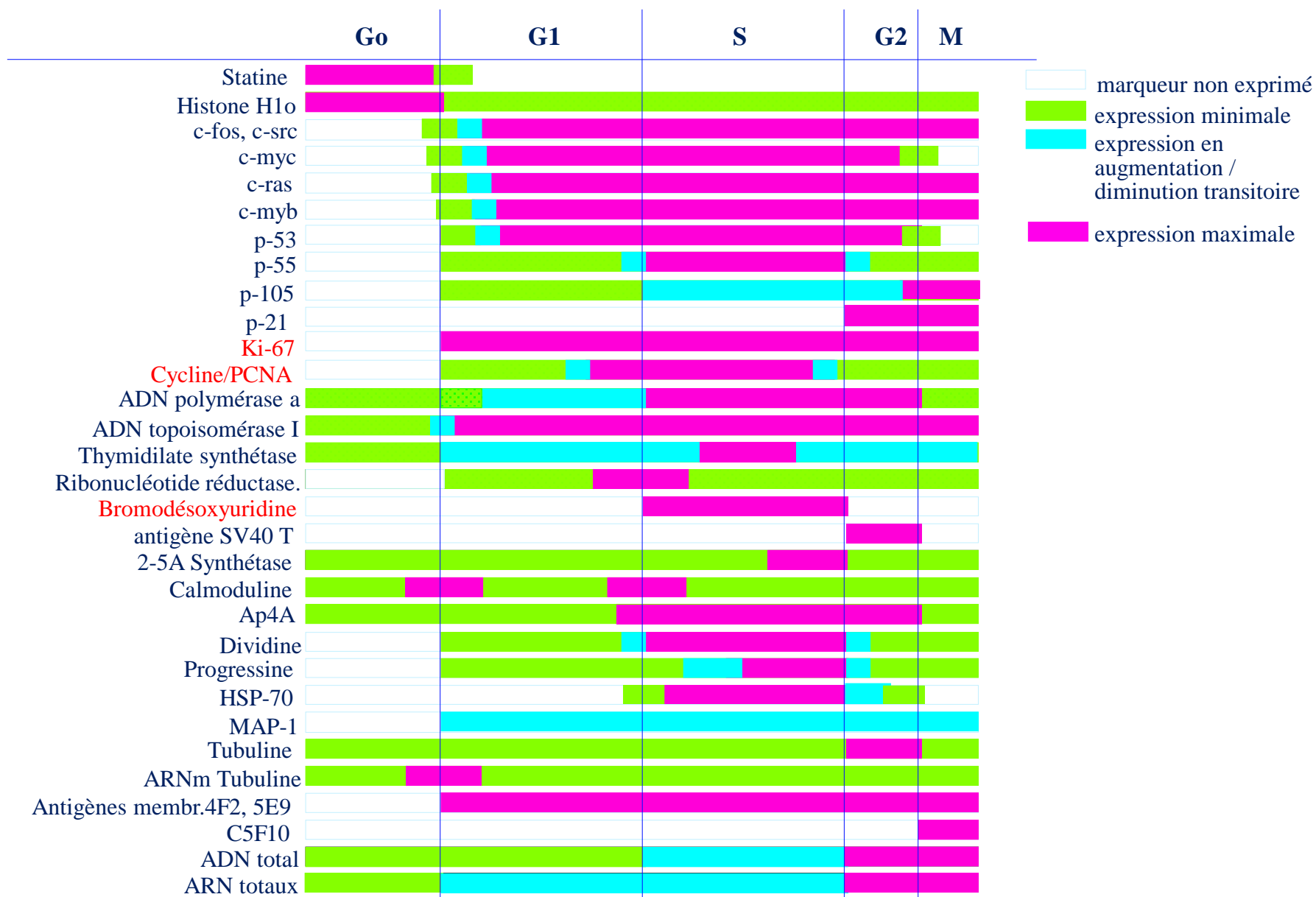


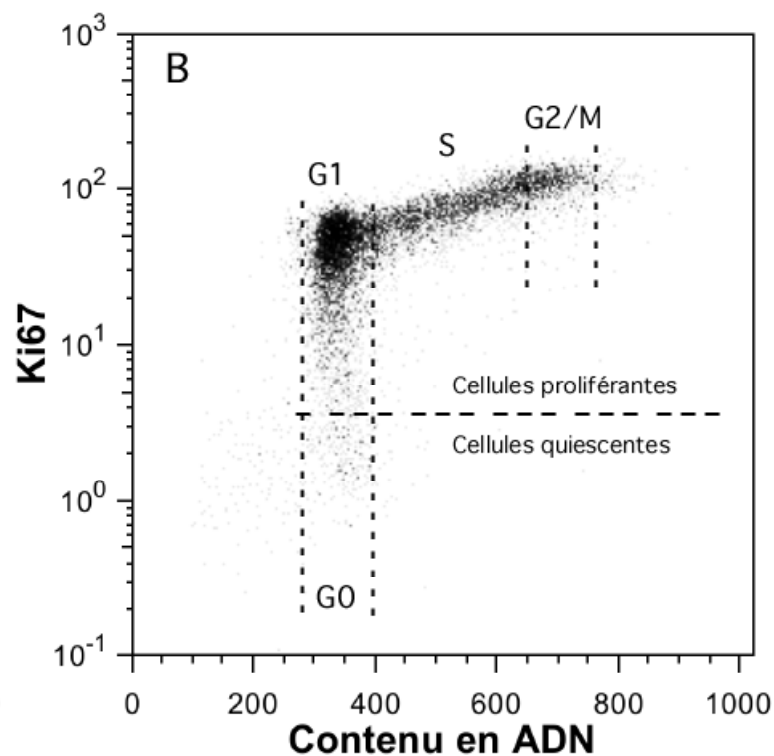
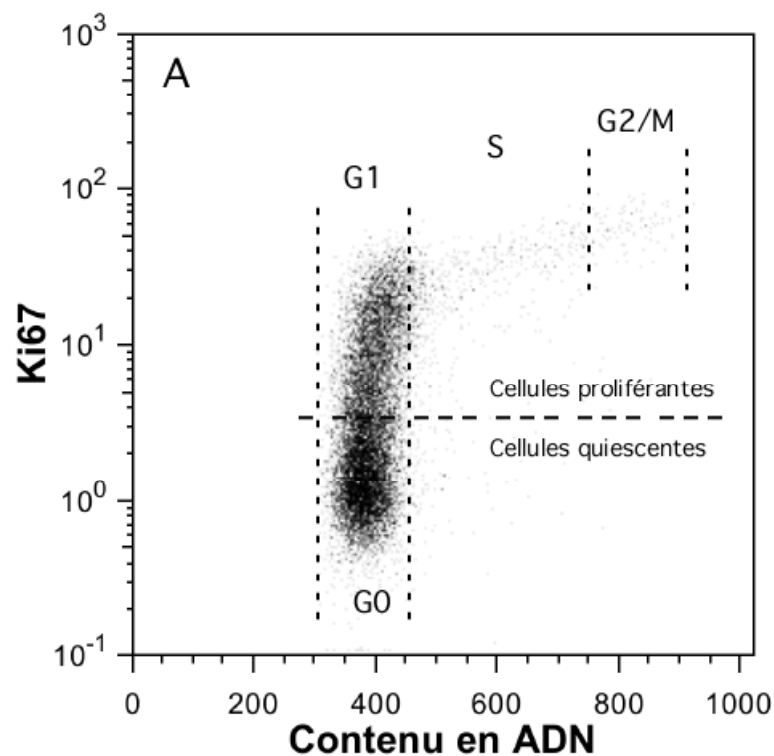
- ✓ Non radioactif
- ✓ Absence de dénaturation
- ✓ Protocole simplifié



....et marqueurs du cycle cellulaire

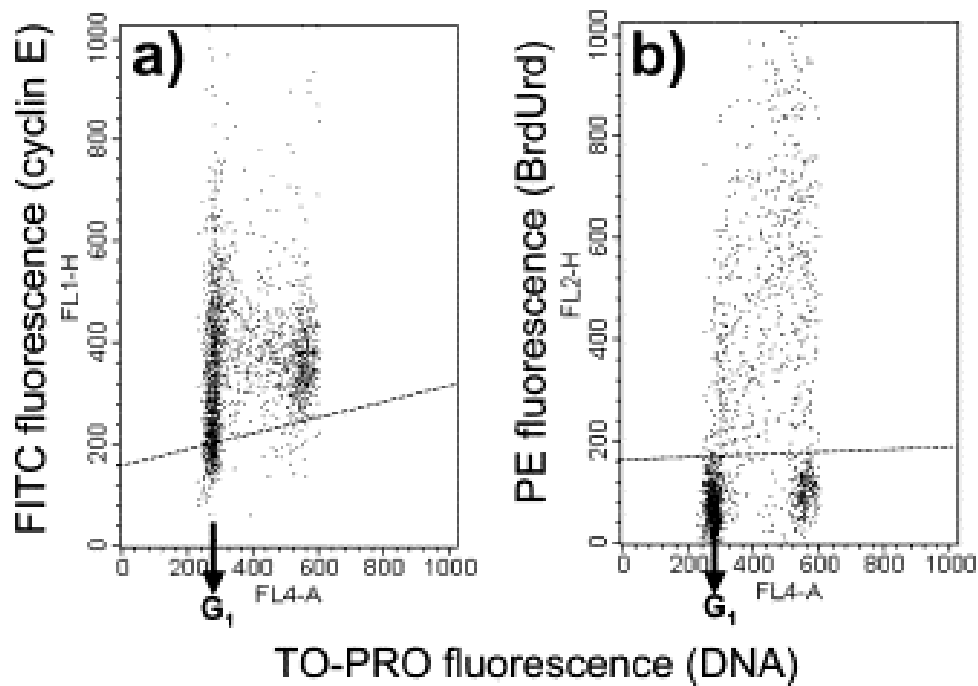
Marqueurs du cycle cellulaire





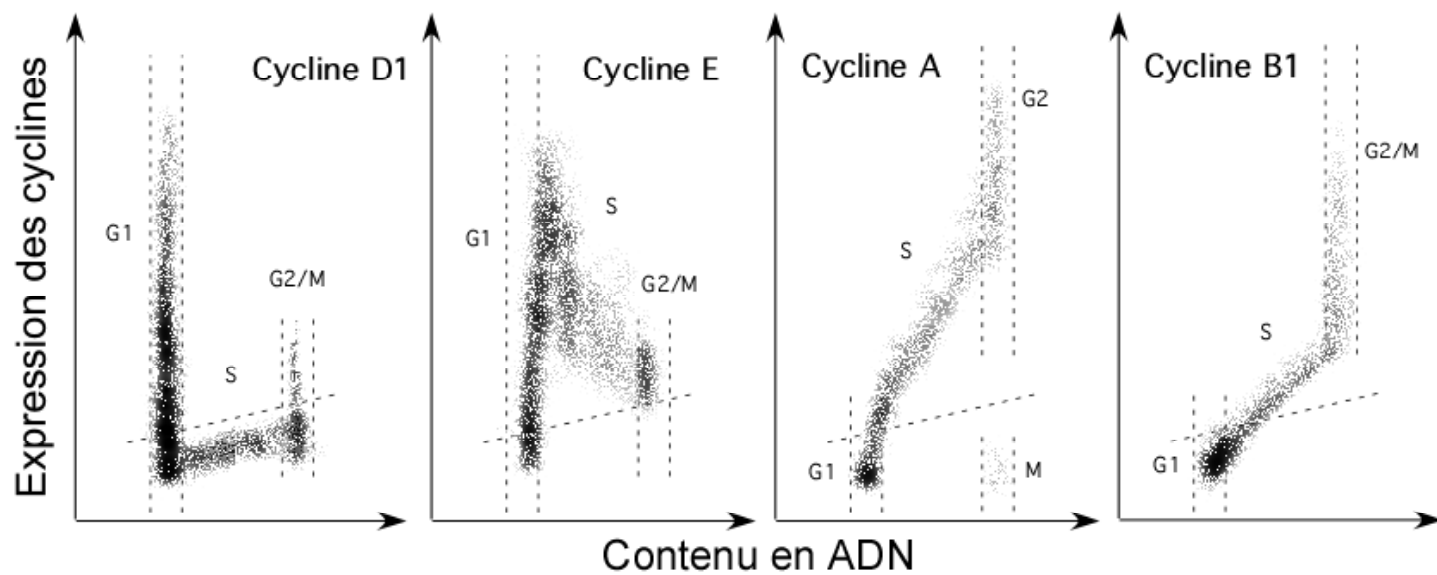
Jordan CT, Yamasaki G, Minamoto D (1996). High-resolution cell cycle analysis of defined phenotypic subsets within primitive human hematopoietic cell populations. *Exp Hematol* 24:1347-1355. PMID:8862447

Cycline E /BrdU



Cycline E : élément critique pour la progression dans la phase S

Cyclines



Cytometry of cyclin proteins. Darzynkiewicz Z, Gong J, Juan G, Ardelet B, Traganos F. *Cytometry*. 1996 Sep 1;25(1):1-13. PMID:8875049

Mitose

Phosphorylation de la sérine 10 de l'histone H3



Détection en immunofluorescence

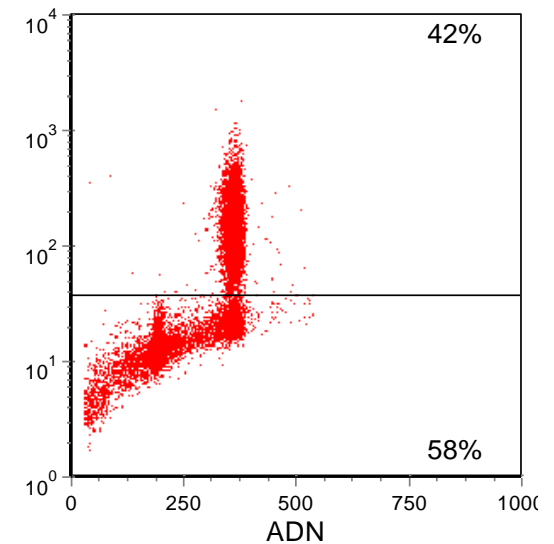
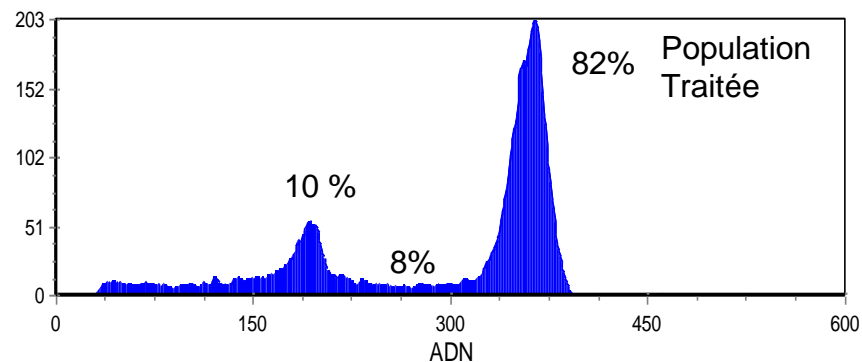
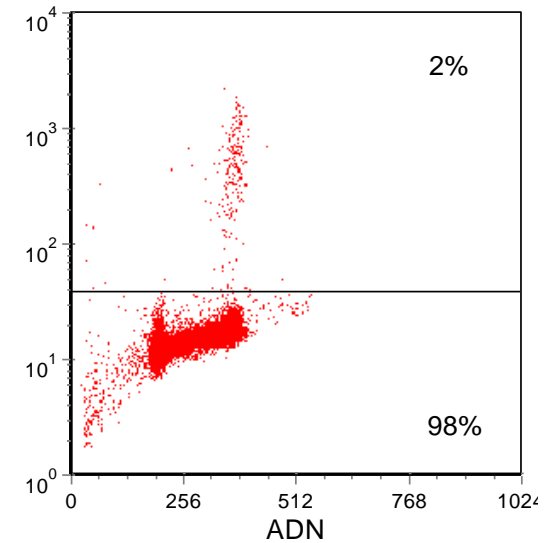
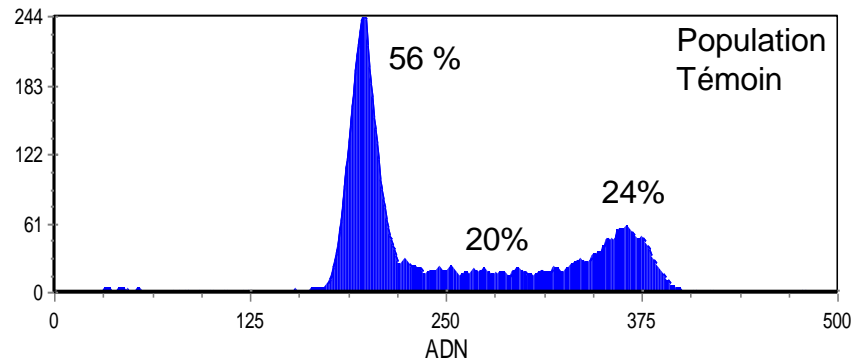


Marquage de l'ADN

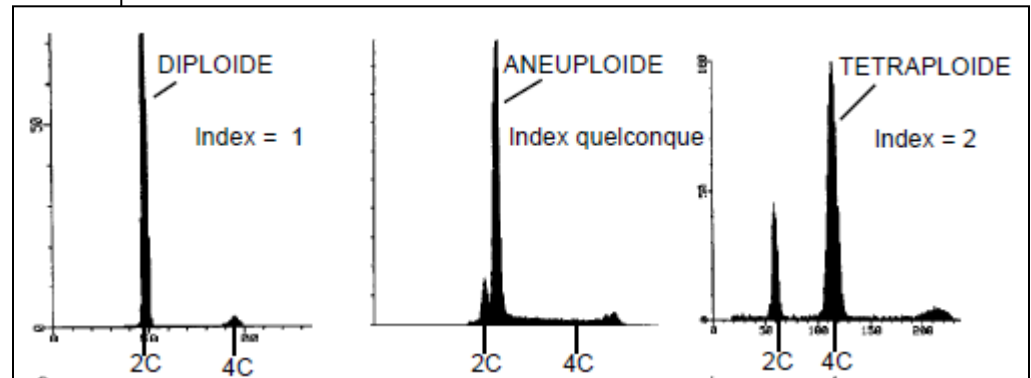
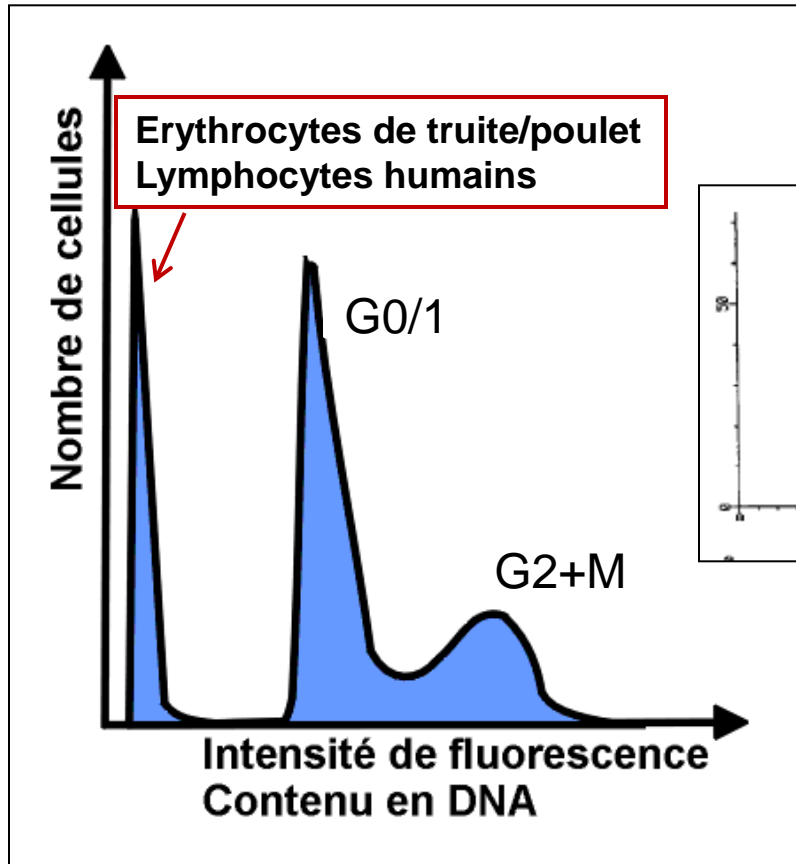
Histone H3 phosphorylation in human monocytes and during HL-60 cell differentiation. Juan G, Traganos F, Darzynkiewicz Z. *Exp Cell Res.* 1999;246(1):212-220. PMID:9882530

Autre marqueur : MPM2 (*Mitotic phosphoprotein 2*)

Quantification des cellules en mitose

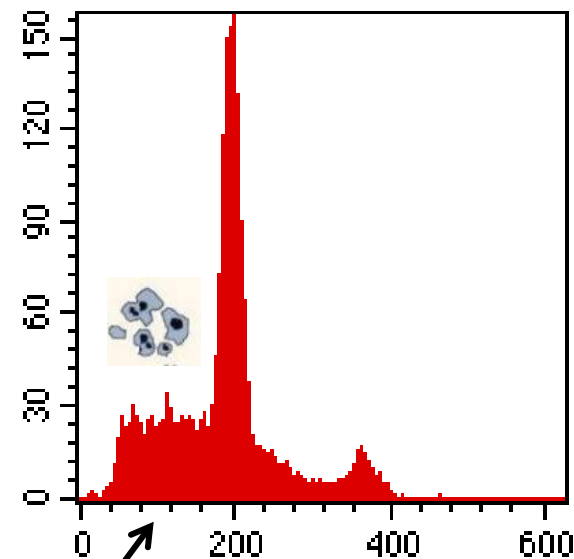
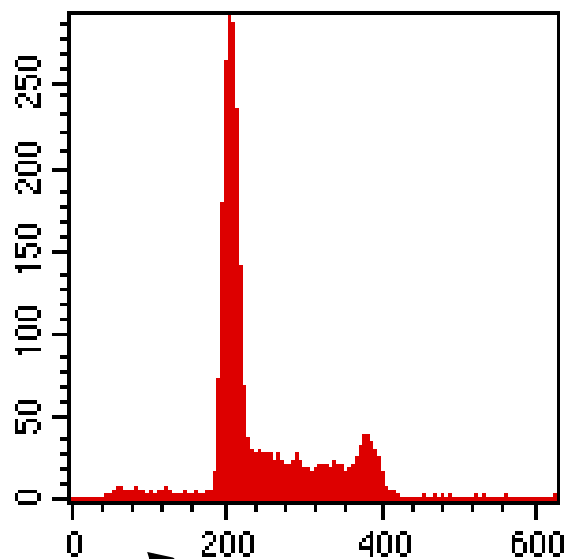


Contenu en ADN et ploïdie



Détermination d'un index d'ADN

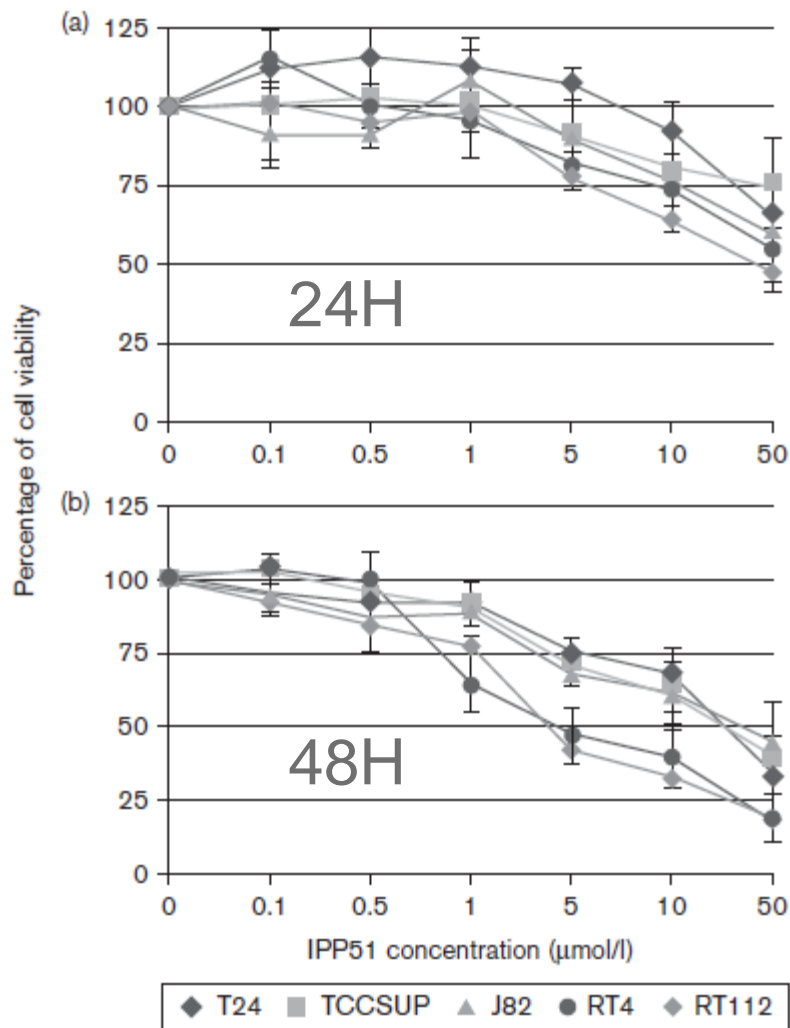
Contenu en ADN et apoptose



Pic subG1

***Exemple d'effet d'un dérivé de
flavonoïde sur le cycle cellulaire***

Effet sur la prolifération cellulaire

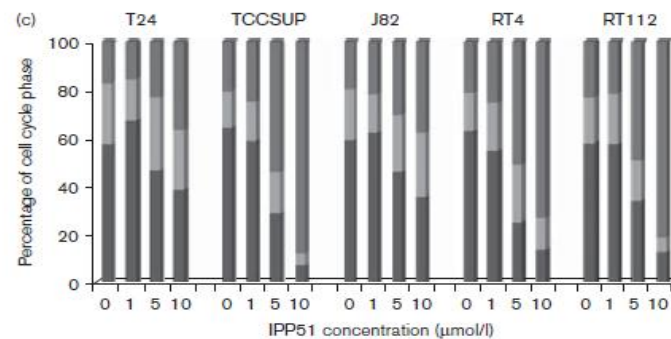
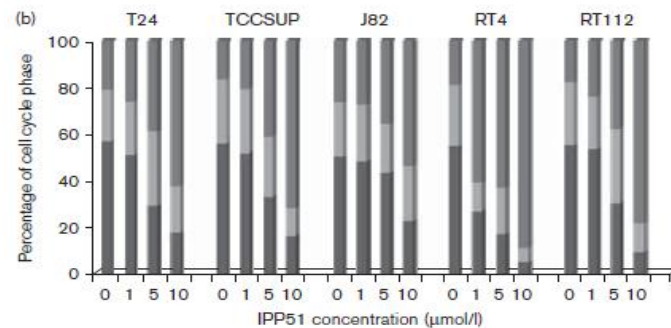
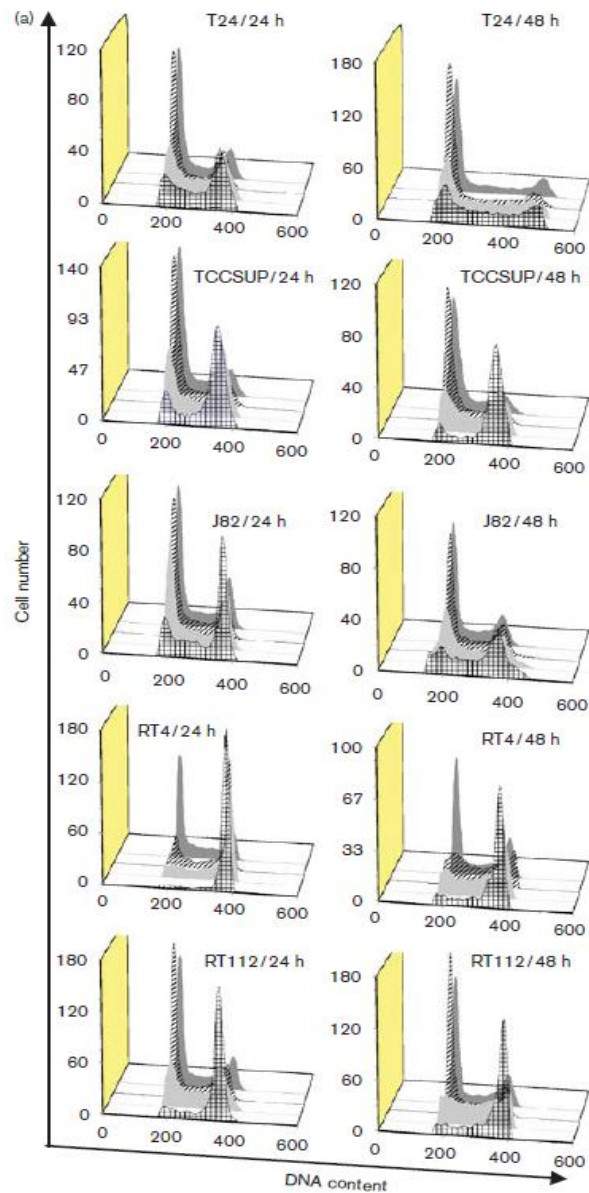


CI 50

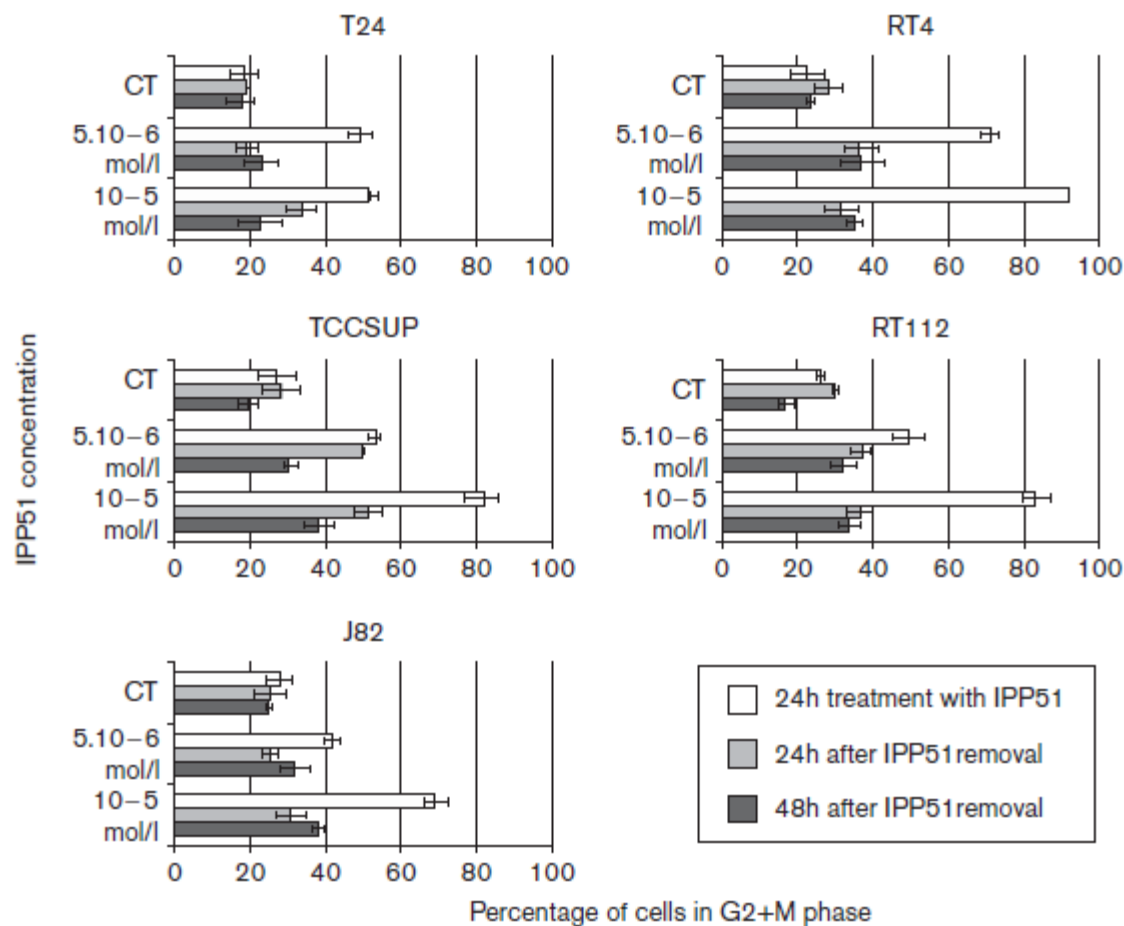
Cellules de bas grade : (RT4 and RT112) = 5.10^{-6} mol/l

Cellules de haut grade : (T24, TCCSUP, and J82) = 5.10^{-5} mol/l

Effet sur le cycle cellulaire



Réversibilité de l'effet sur le cycle cellulaire



Détection des cellules en mitoses

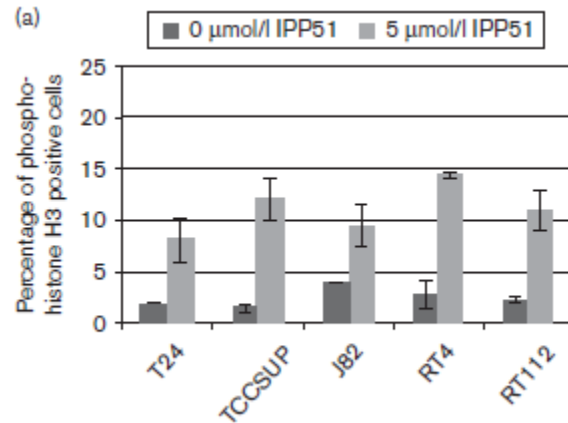
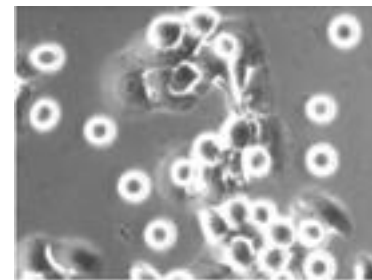
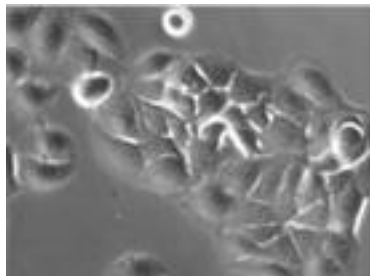
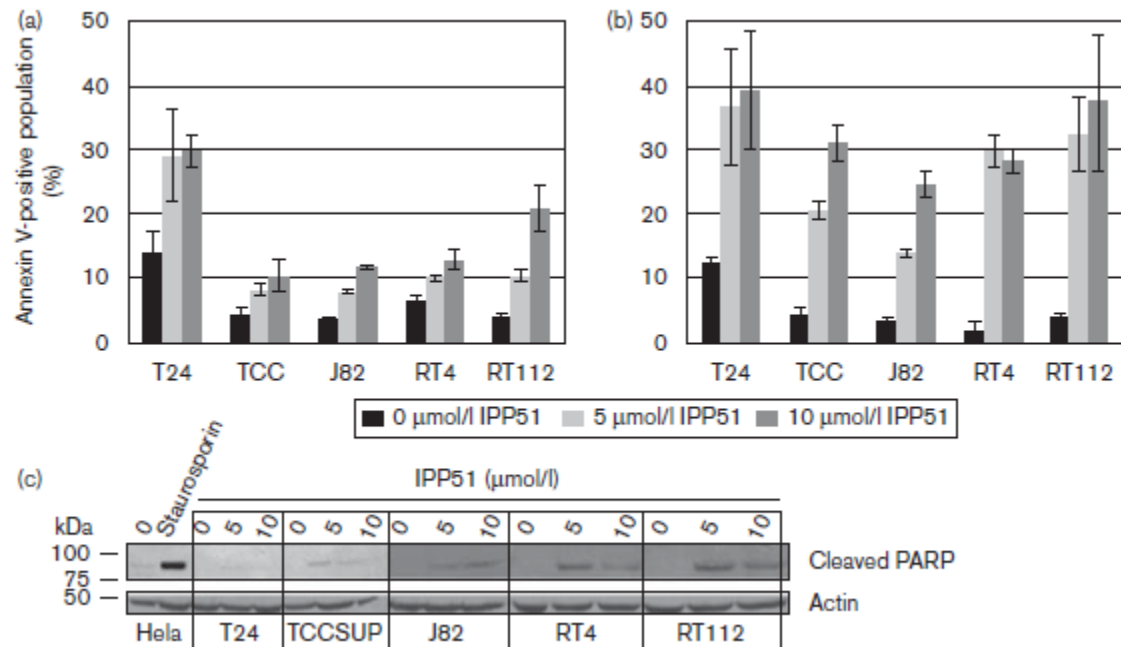


Table 1 Relative percentage of mitotic cells in the different stages of mitosis

		Prophase (%)	Prometaphase (%)	Metaphase (%)	Anaphase (%)
T24	CT	29.1	9.1	58.2	3.6
	JAI51	24.3	62.1	13.1	0.5
TCC-SUP	CT	19.0	31.0	50.0	0.0
	JAI51	9.4	74.5	15.4	0.7
J82	CT	26.25	47.50	23.75	2.50
	JAI51	6.56	55.74	37.16	0.55
RT4	CT	27.66	23.40	42.55	6.38
	JAI51	15.56	77.78	6.67	0.00
RT112	CT	46.27	34.33	17.91	1.49
	JAI51	16.95	69.49	13.56	0.00







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Curr Protoc Cytom. Author manuscript; available in PMC 2011 April 1.

Published in final edited form as:

Curr Protoc Cytom. 2010 April ; CHAPTER: Unit7.2. doi:10.1002/0471142956.cy0702s52.

Critical Aspects in Analysis of Cellular DNA Content

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¹ The Brander Cancer Research Institute and Department of Pathology, New York Medical Center, Valhalla, New York

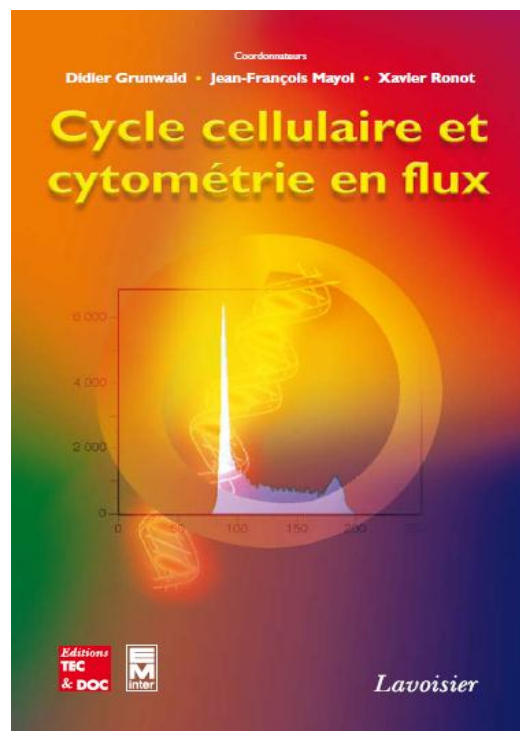
Abstract

This unit covers general aspects of DNA content analysis and provides introductory or complementary information to the specific protocols of DNA content assessment in this chapter. It describes principles of DNA content analysis and outlines difficulties and pitfalls common to these methods. It also reviews methods of DNA staining in live, permeabilized, and fixed cells, and in cell nuclei isolated from paraffin-embedded tissues, as well as the approaches to stain DNA concurrently with cell immunophenotype. This unit addresses factors affecting accuracy of DNA measurement, such as chromatin features restricting accessibility of fluorochromes to DNA, stoichiometry of interaction with DNA, and “mass action law” characterizing binding to DNA in relation to unbound fluorochrome concentration. It also describes controls to ensure accuracy and quality control of DNA content determination and principles of DNA ploidy assessment. Because many aspects of DNA content analysis are common to protocols in *UNITS* 7.3, 7.6, 7.16, 7.20, 7.23, & 7.25, certain parts of this unit provide information redundant with commentaries in these units.

Keywords

cell cycle; apoptosis; ploidy; DNA index; stoichiometry; fluorochrome; chromatin

Des références utiles !



Cycle cellulaire et cytométrie en flux
D. Grunwald, J.F. Mayol, X. Ronot (eds)
Lavoisier, Mars 2010