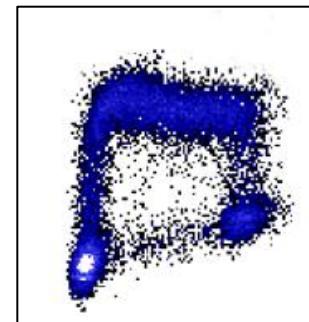
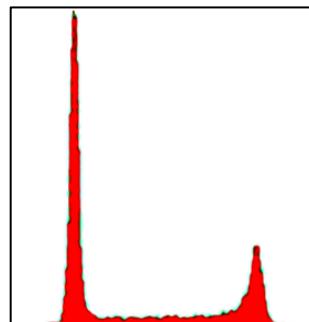


Application de la cytométrie en flux à l'analyse du cycle cellulaire



Montpellier 26 Janvier 2012



Xavier RONOT

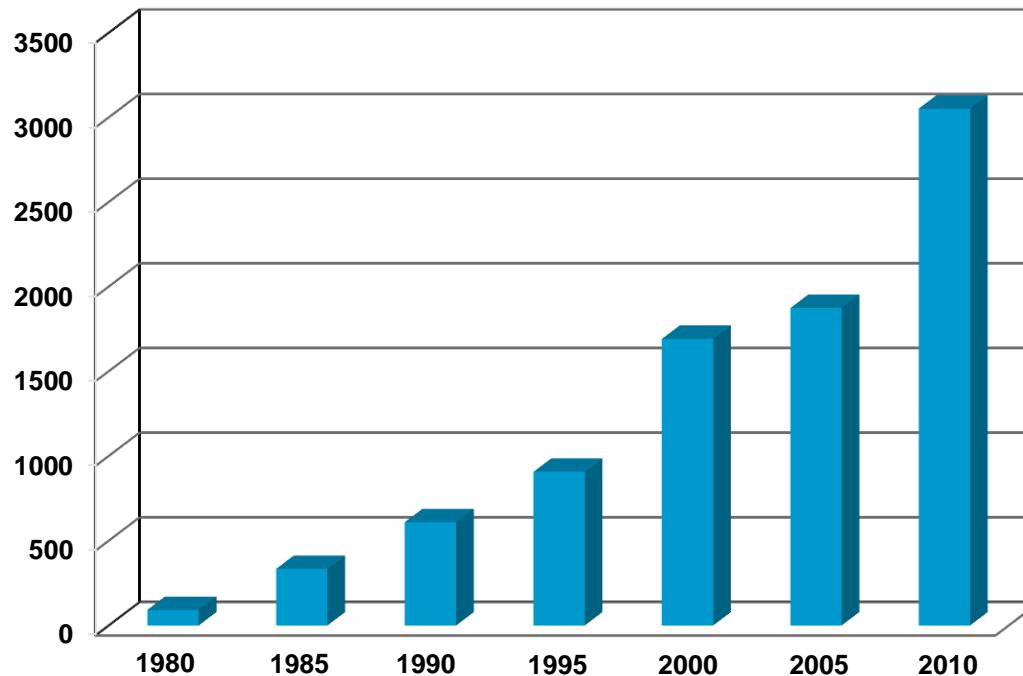
Laboratoire CaCyS
Cancer, Cycle cellulaire et Sénescence
EPHE, Grenoble

xavier.ronot@ujf-grenoble.fr



Evolution du nombre de publications

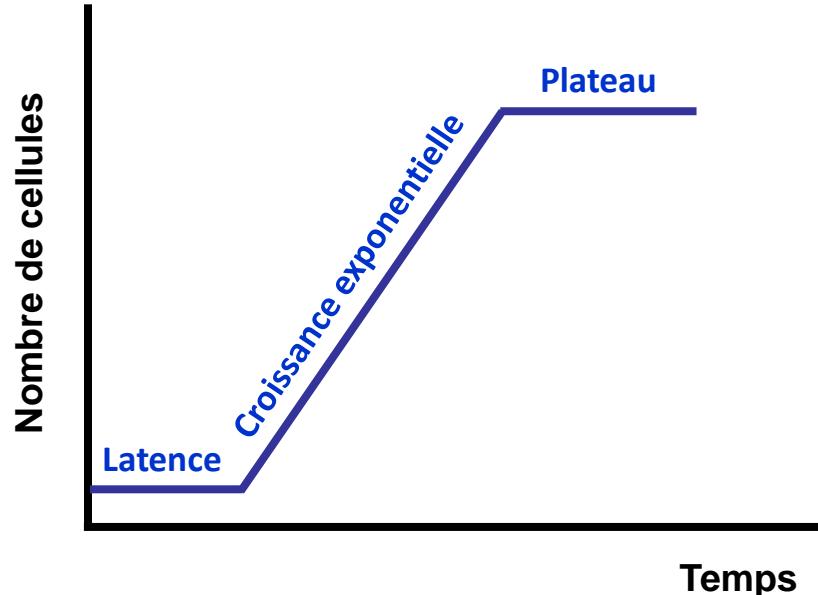
Cycle cellulaire / cytométrie en flux



Mesure de la prolifération cellulaire

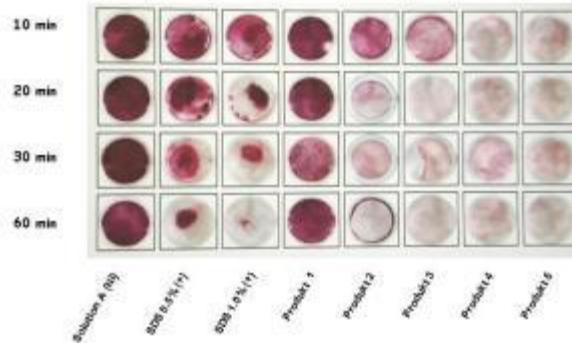
Méthodes « globales »

Courbe de croissance



Calcul du temps de doublement : T_d

Test au MTT



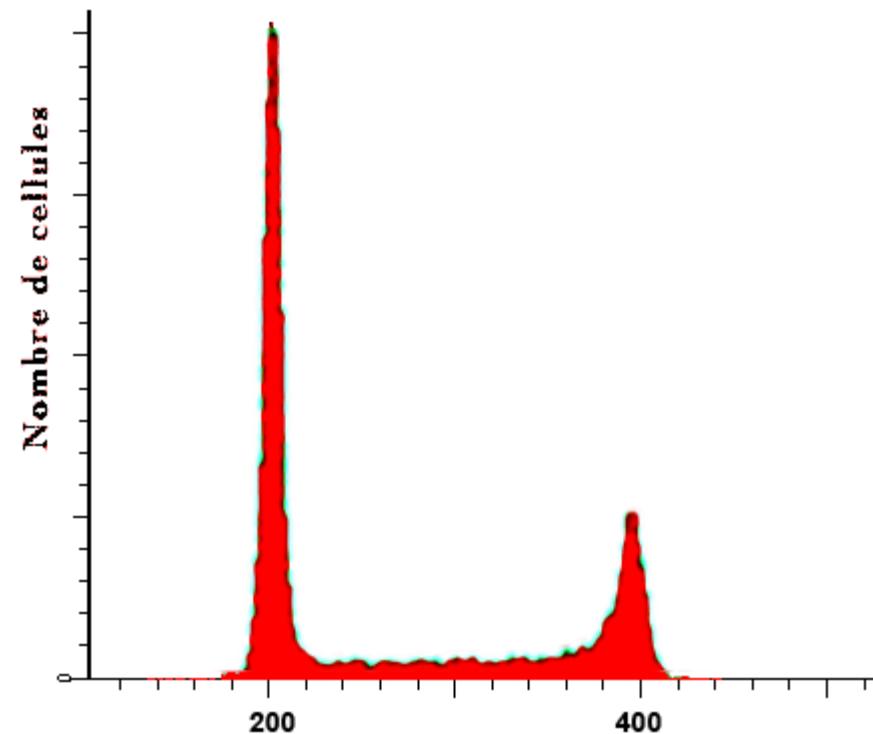
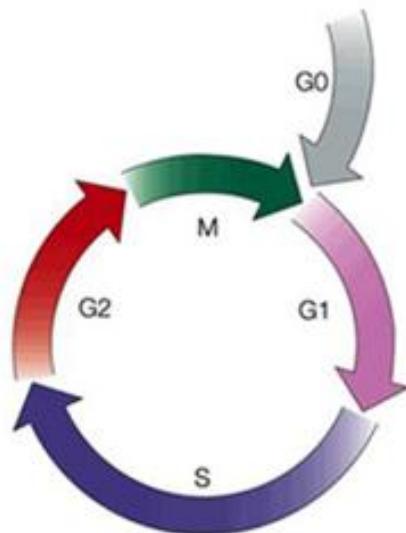
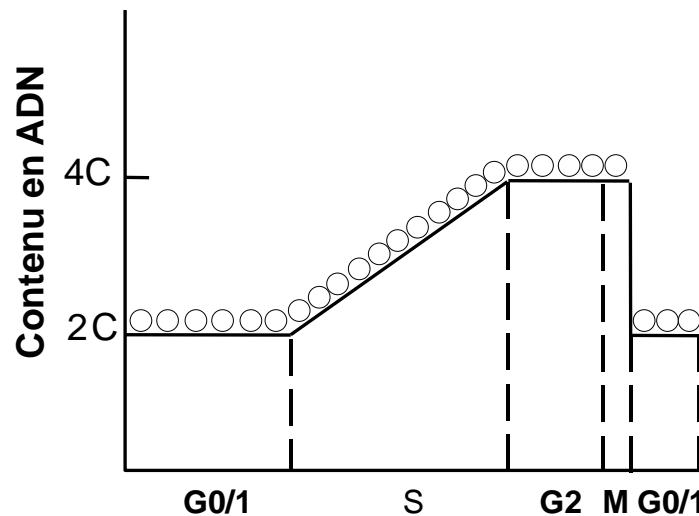
Sel de tétrazolium MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

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

Détermination de la CI 50

Analyse monoparamétrique

Méthode par cytométrie en flux



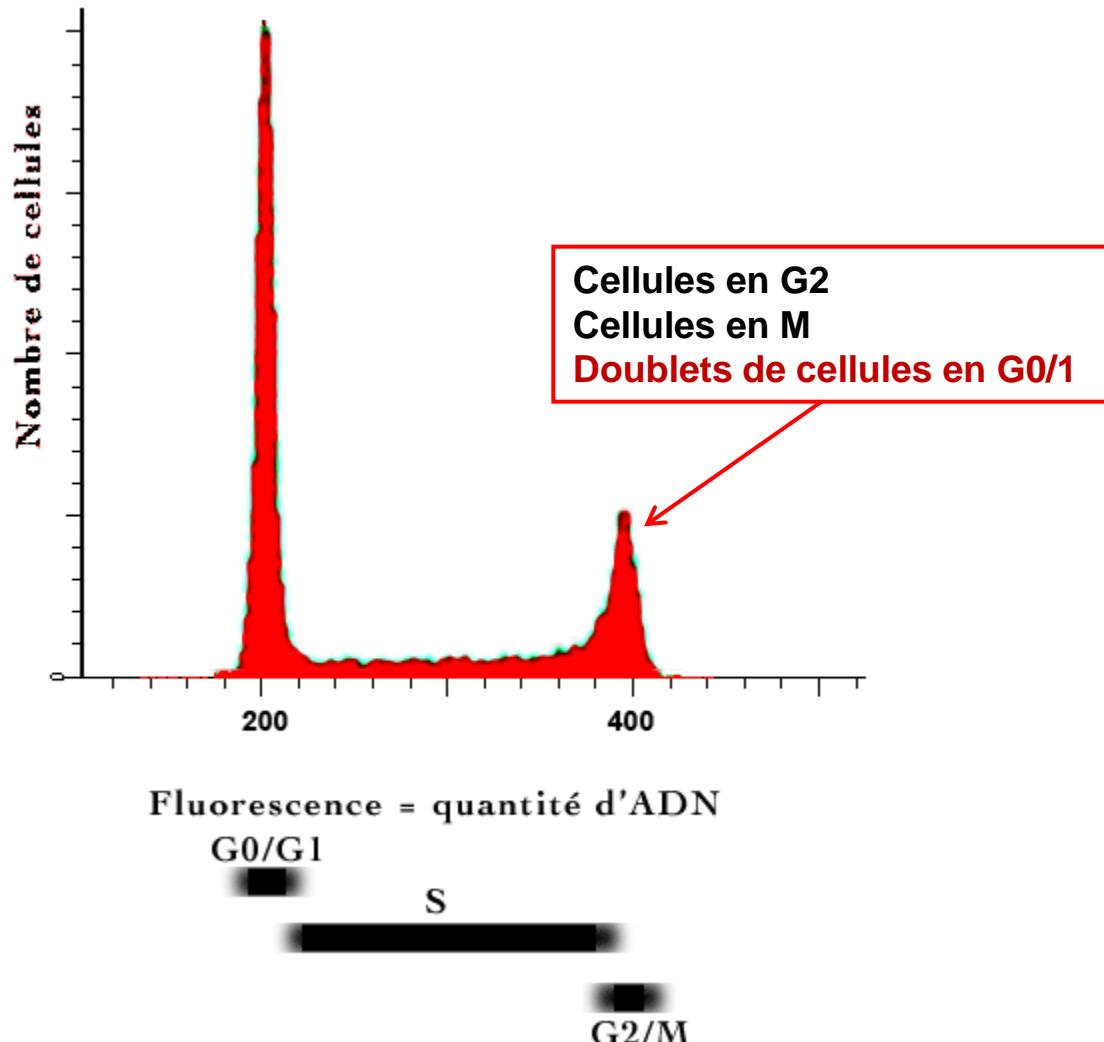
Fluorescence = quantité d'ADN (u.a.)

G0/G1

S

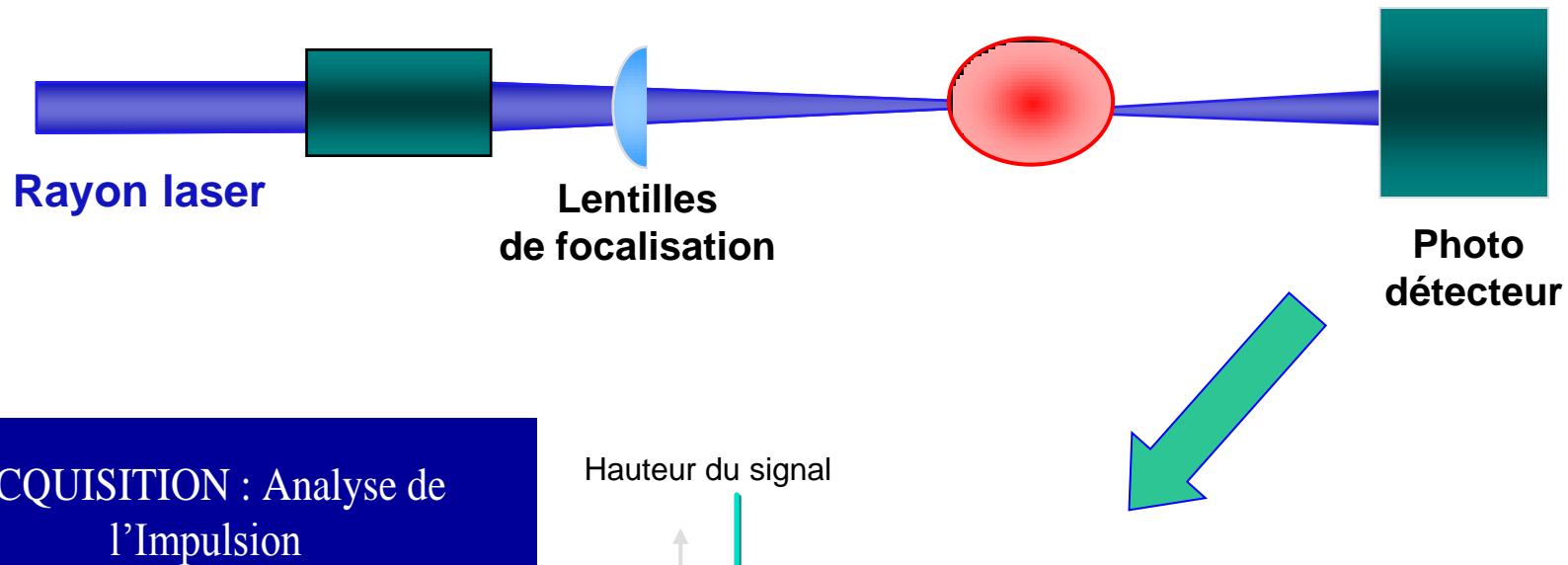
G2/M

L'histogramme monoparamétrique



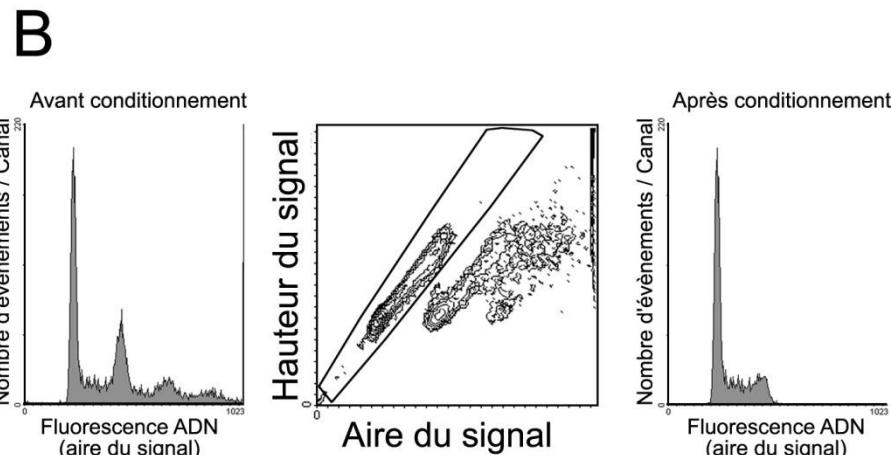
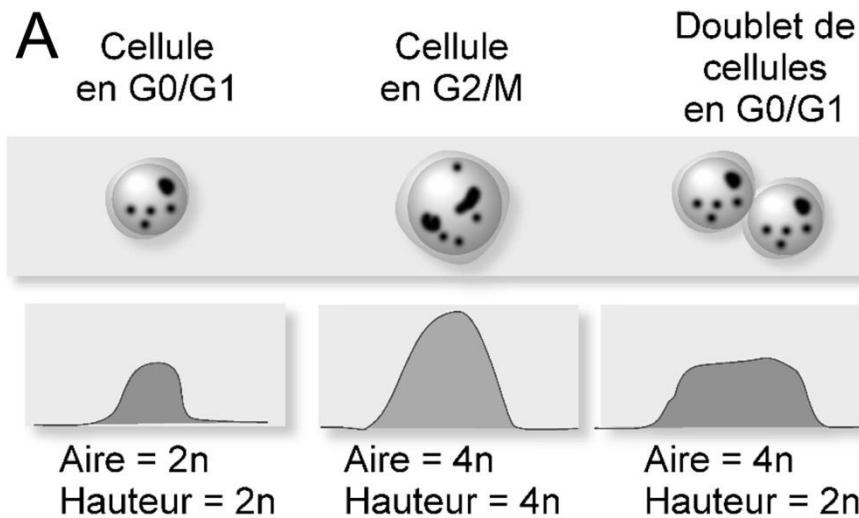
Instantané (cliché) d'une population à un moment donné
contenant des informations multiples

Elimination des doublets



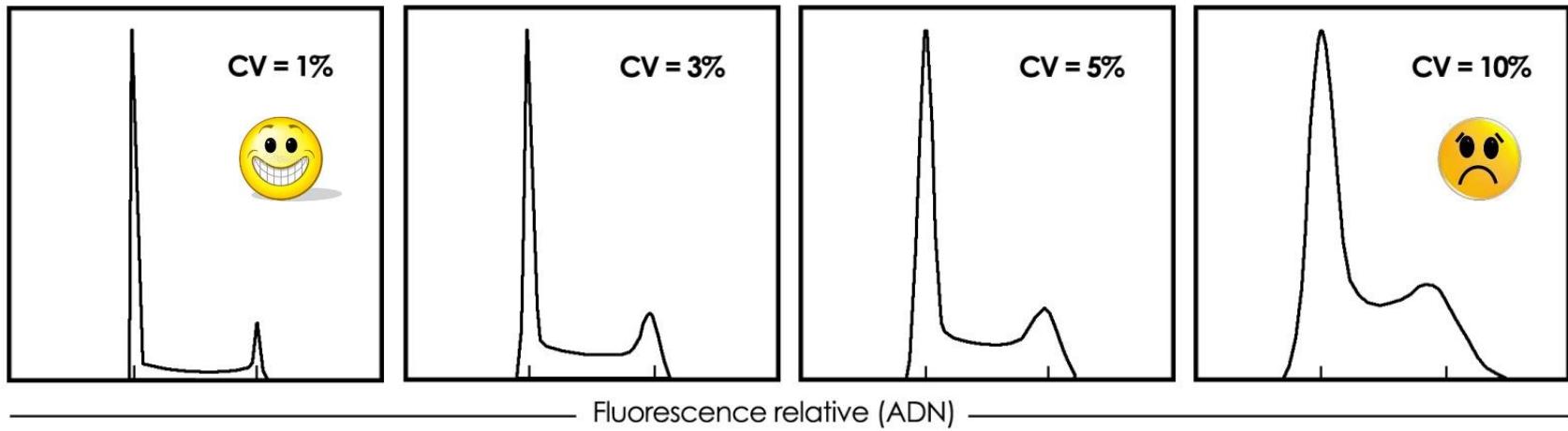
Analyse du signal de fluorescence

Elimination des doublets



Coefficient de variation (CV)

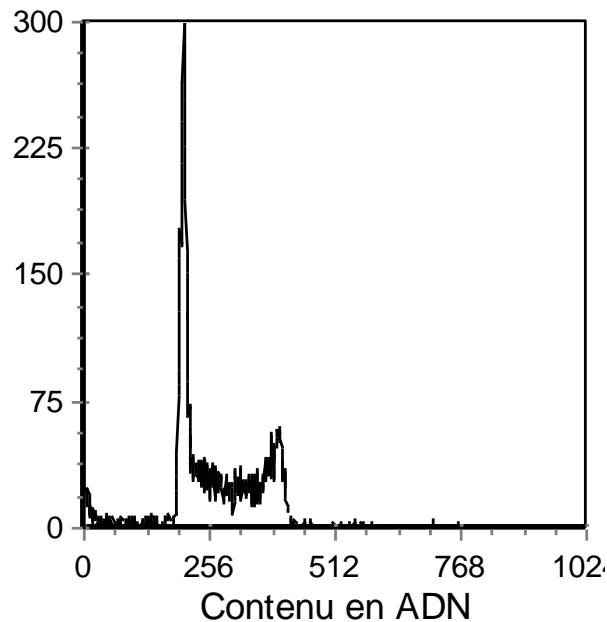
-Nombre de cellules / canal -



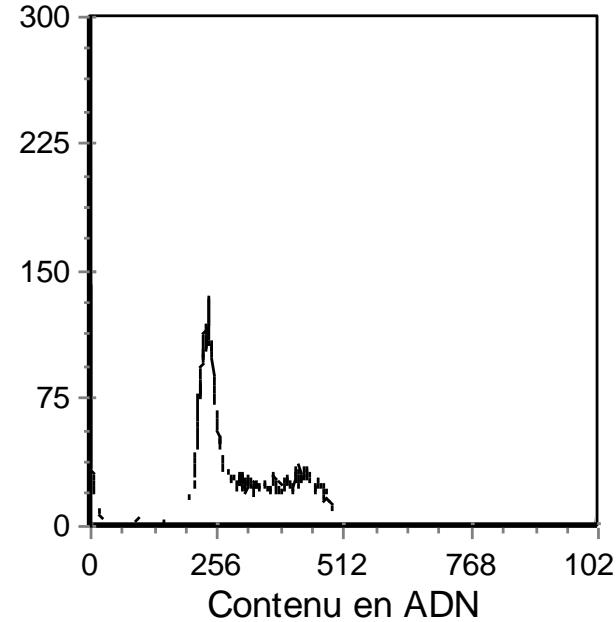
La résolution diminue avec l'augmentation du CV

CV : influence du marquage

Marquage stœchiométrique

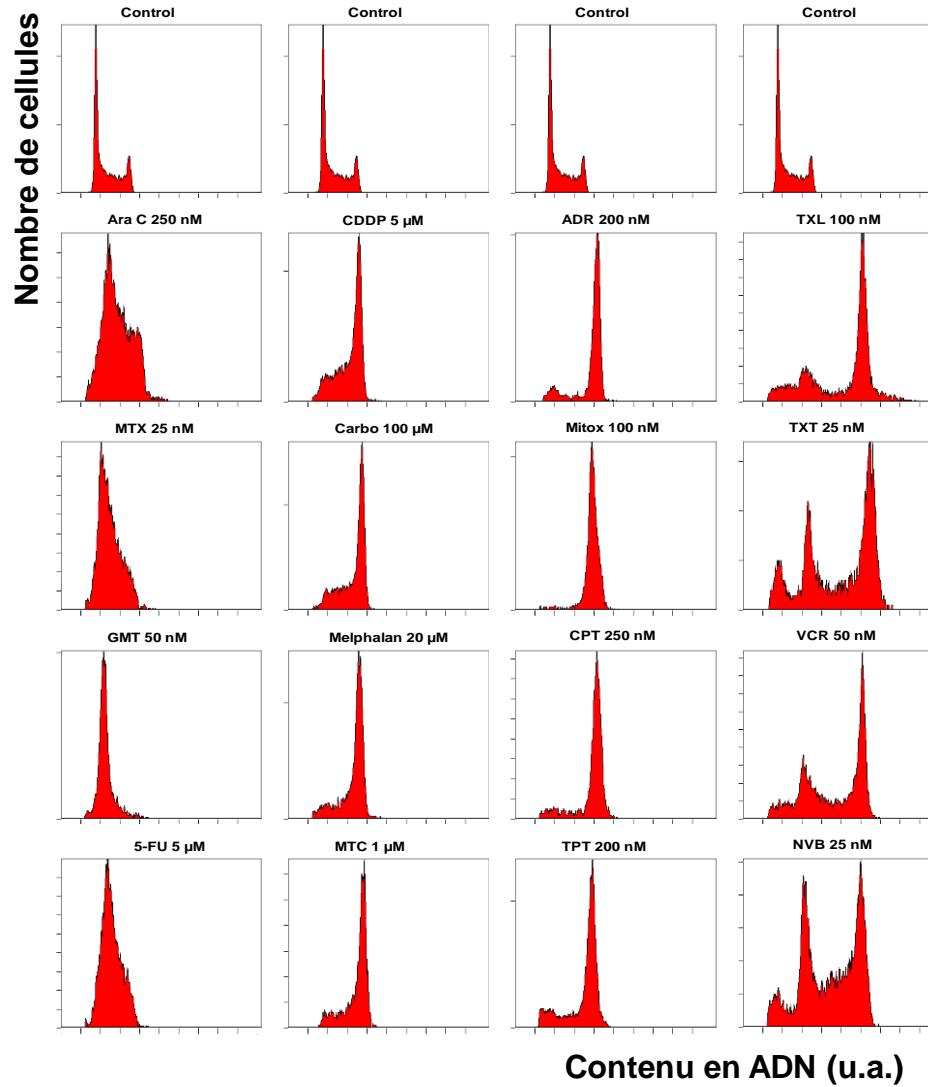


Marquage non stœchiométrique



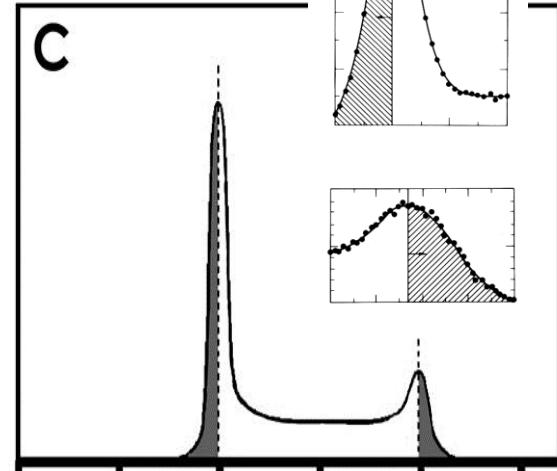
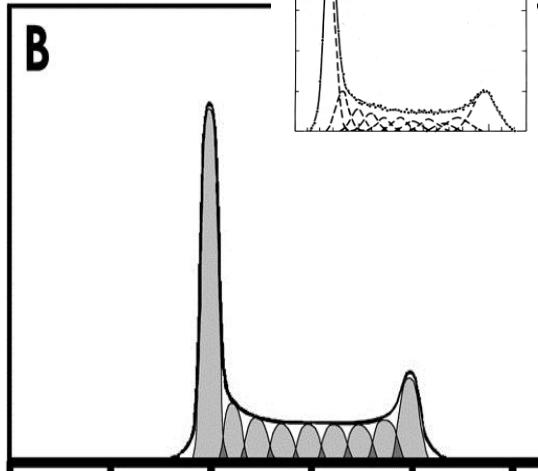
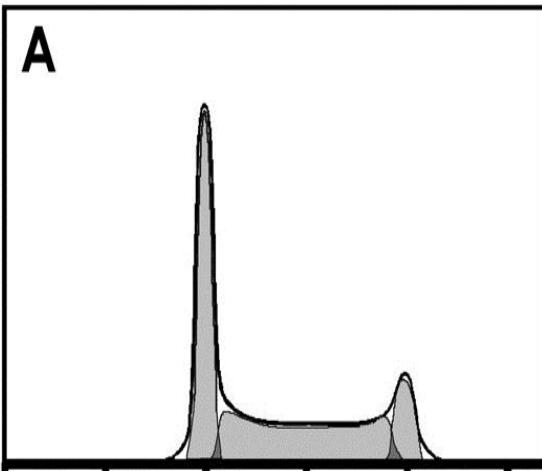
L'interprétation se fragilise avec l'augmentation du CV

Exemples de perturbations du cycle cellulaire induites par des molécules antiprolifératives



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

Nombre d'événements / canal



Fluorescence relative (ADN)

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

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

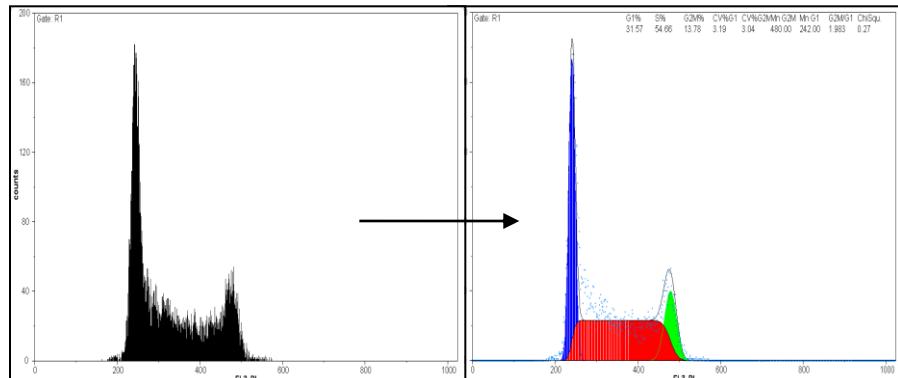
C : Méthode du miroir

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

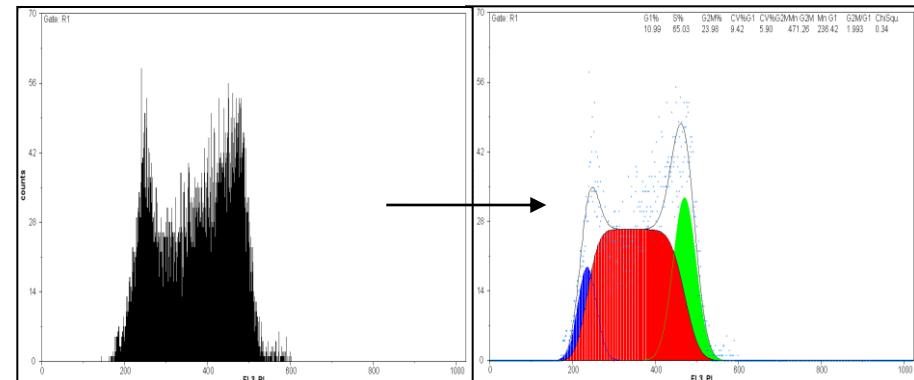
Mathematical analysis of DNA distributions derived from flow microfluorometry. Dean PN, Jett JH. *J Cell Biol.* 1974;60(2):523-527. PMID:4855906

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

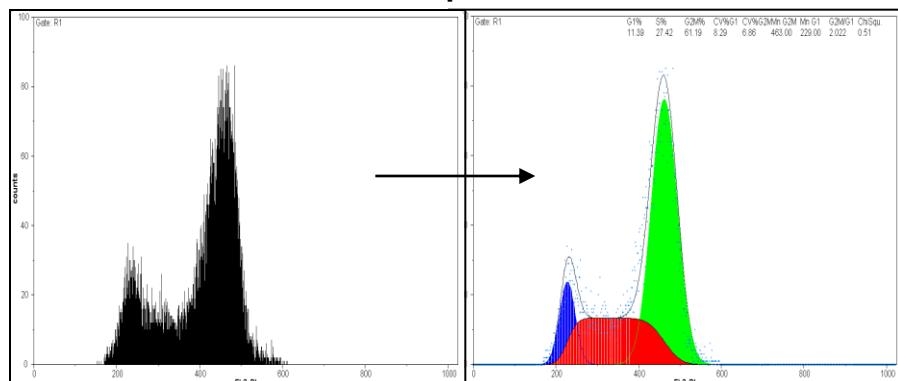
Témoin



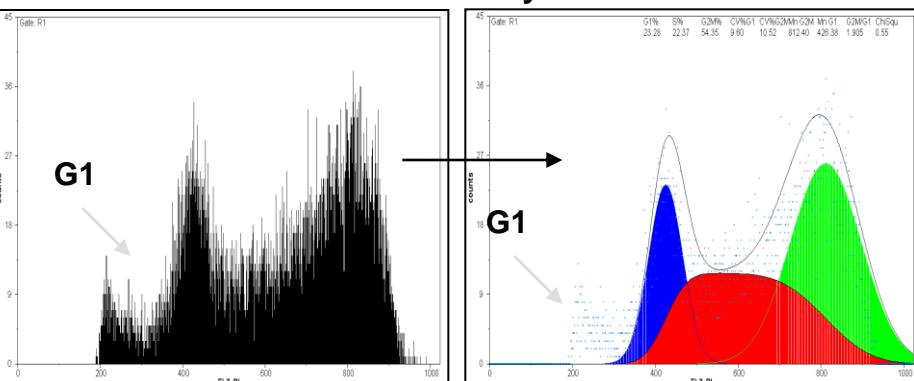
Accumulation en G1 et G2M



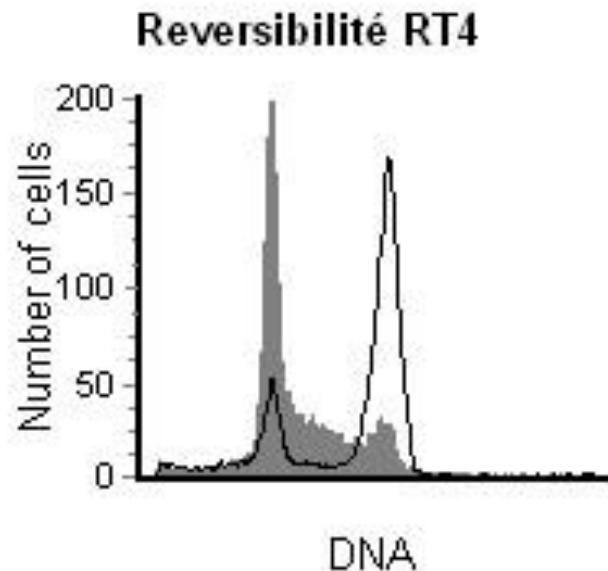
Accumulation partielle en G2M



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 : **blockage**

Cytostatique

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

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

Limites de l'analyse monoparamétrique

Des informations multiples.....mais limitées :

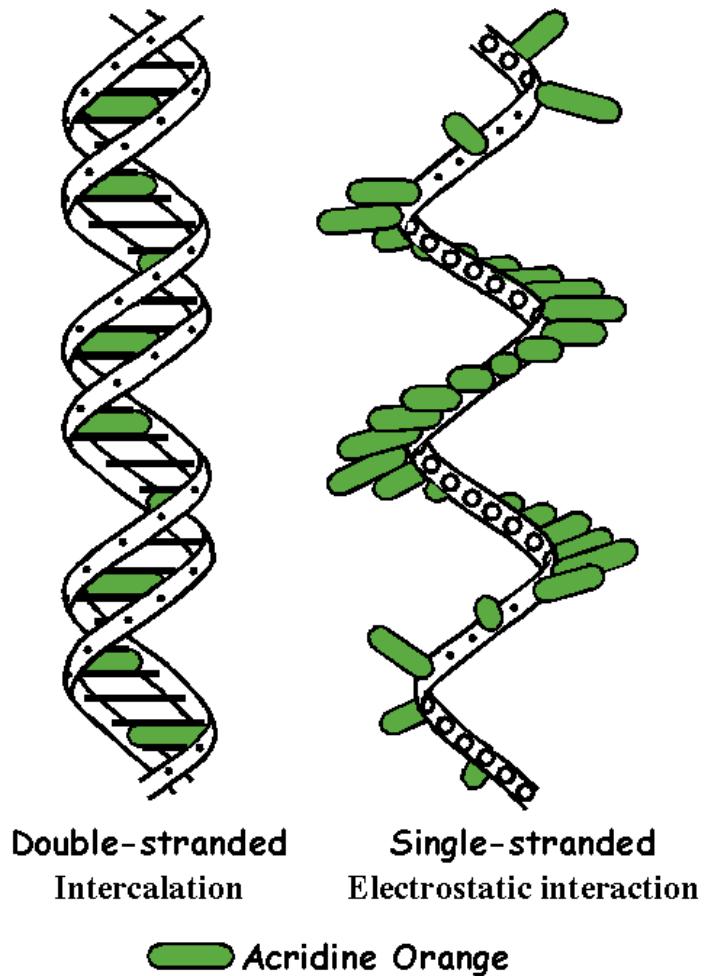
Cellules en G0 et G1 confondues

Cellules en G2 et M confondues

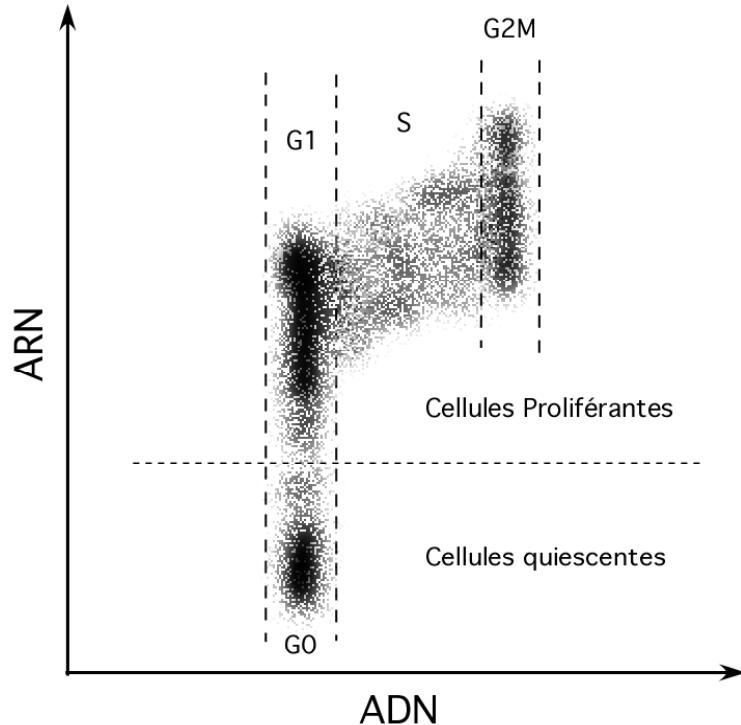
Quid des cellules en S ?

Acridine Orange

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



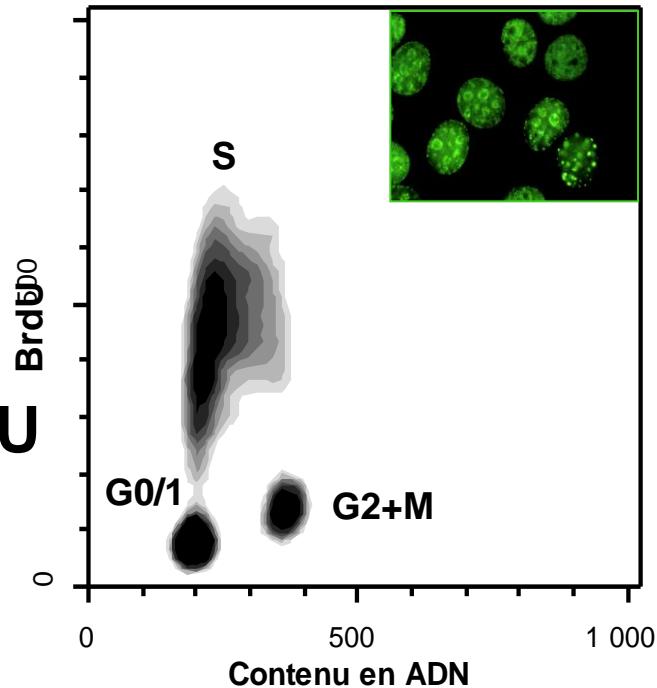
Acridine Orange



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

Incorporation de BrdU

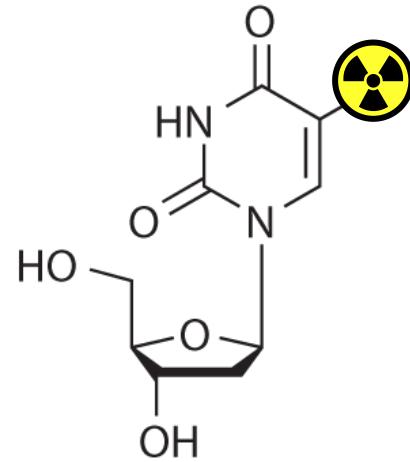
- Incubation en présence de BrdU (5-bromo-2-deoxyuridine)
- Fixation des cellules
- Dénaturation acide (élimination des histones)
- Marquage par un anticorps anti-BrdU
- Révélation par un anticorps secondaire fluorescent
- Marquage de l'ADN



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

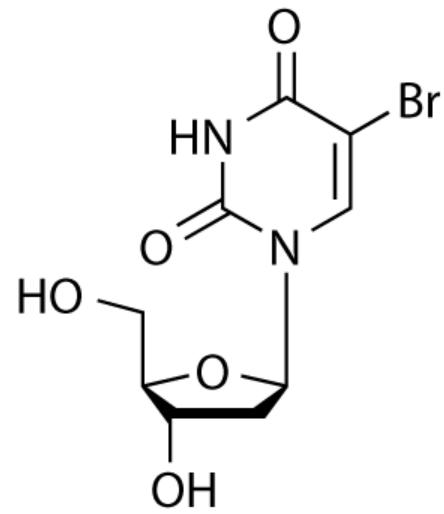
Analysis of cell proliferation using the bromodeoxyuridine/Hoechst-ethidium bromide method. Ormerod MG. Methods Mol Biol. 1997;75:357-365. PMID:9276285

³H-Thymidine



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

BrdU

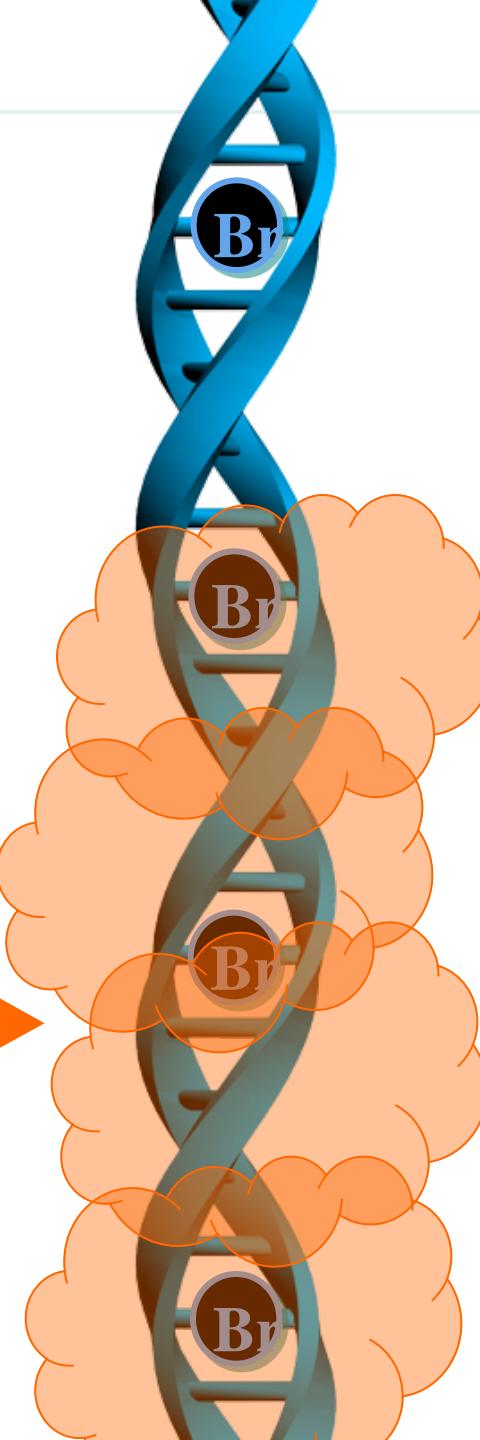


BrdU (5-bromo-2'-désoxyuridine)

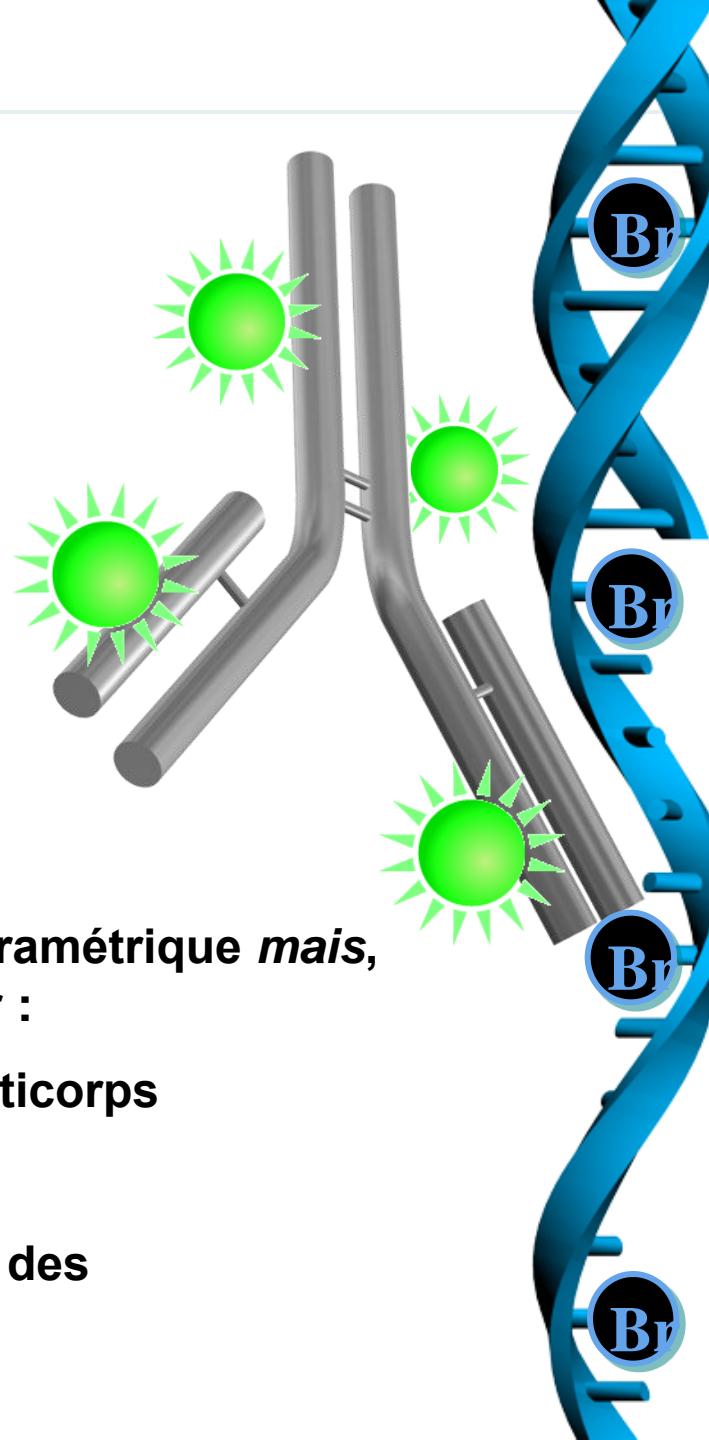


BrdU

Acide ou DNase

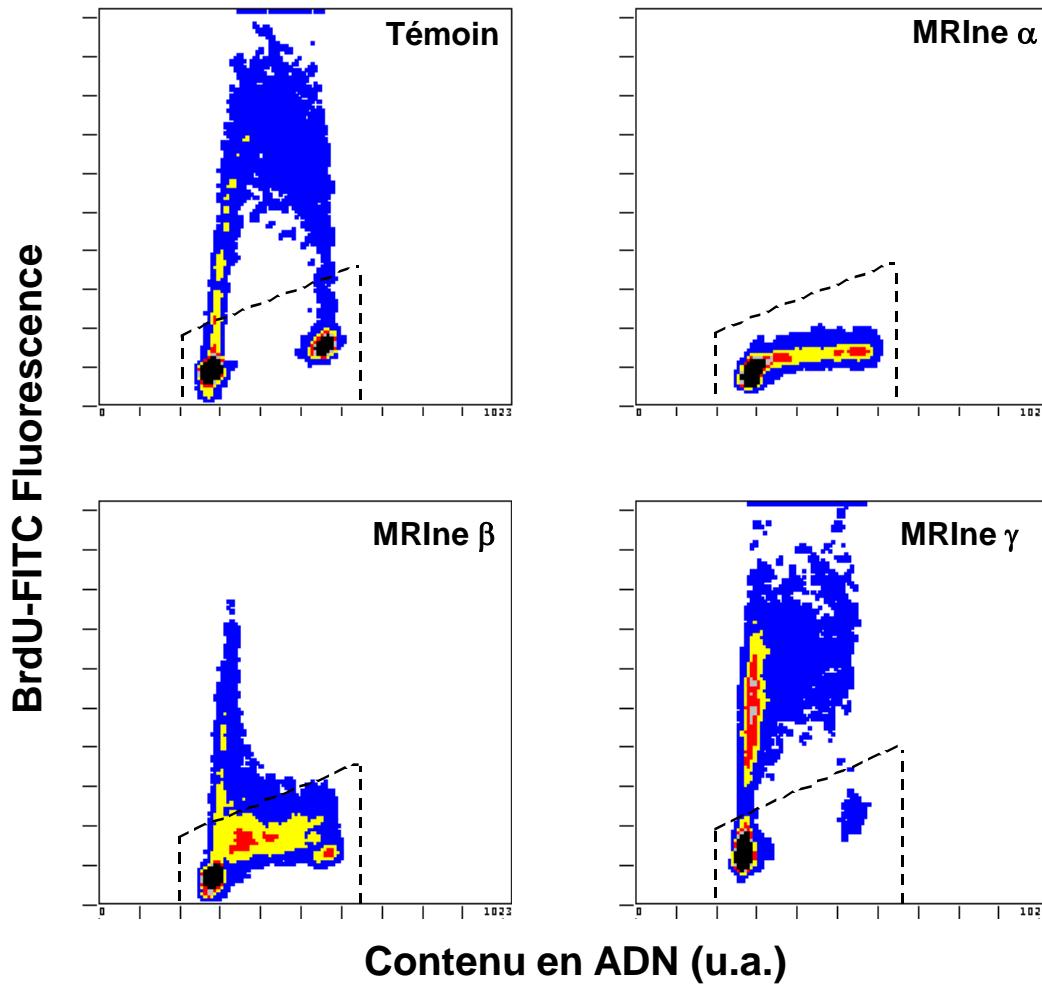


BrdU

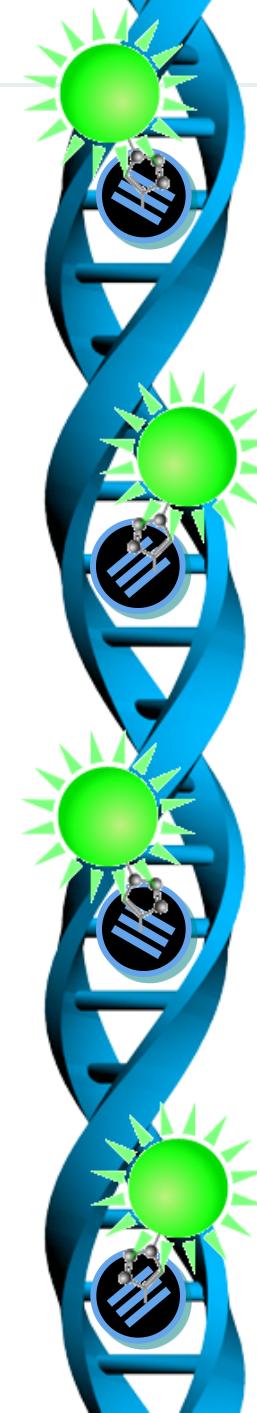


- ✓ Non radioactif
- ✓ Possibilité d'analyse biparamétrique *mais*, la dénaturation peut affecter :
 - ✓ la fixation d'autres anticorps
 - ✓ la morphologie
 - ✓ l'efficacité de fixation des fluorochromes de l'ADN

Incroporation de BrdU : exemples



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



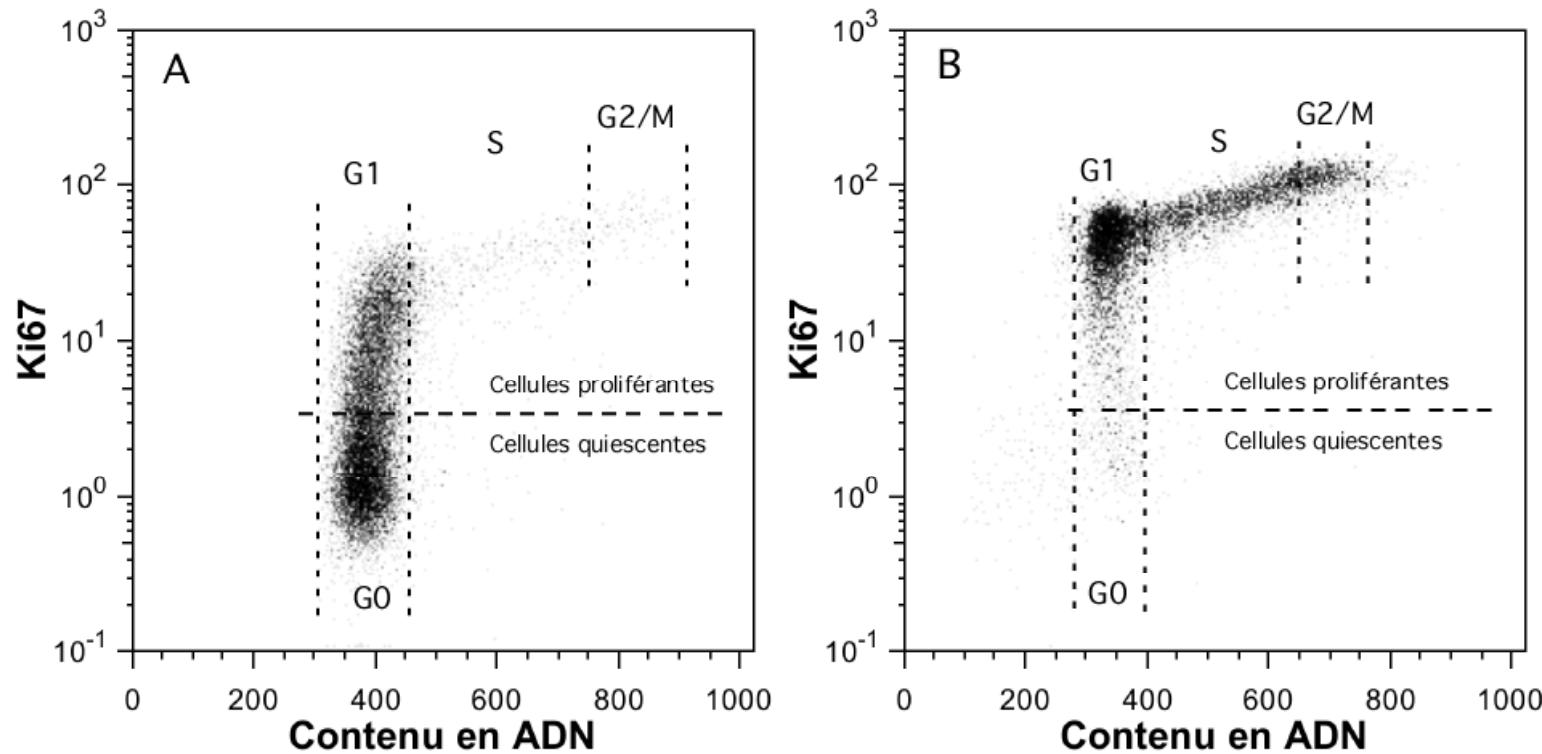
Analyse biparamétrique

....et marqueurs du cycle cellulaire

Marqueurs du cycle cellulaire

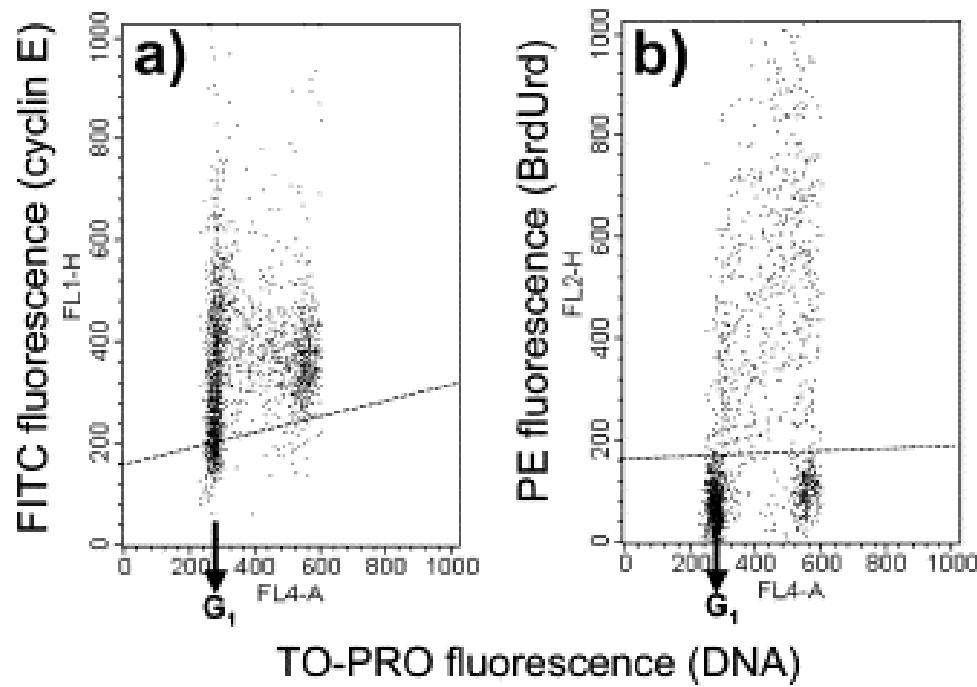


Ki 67



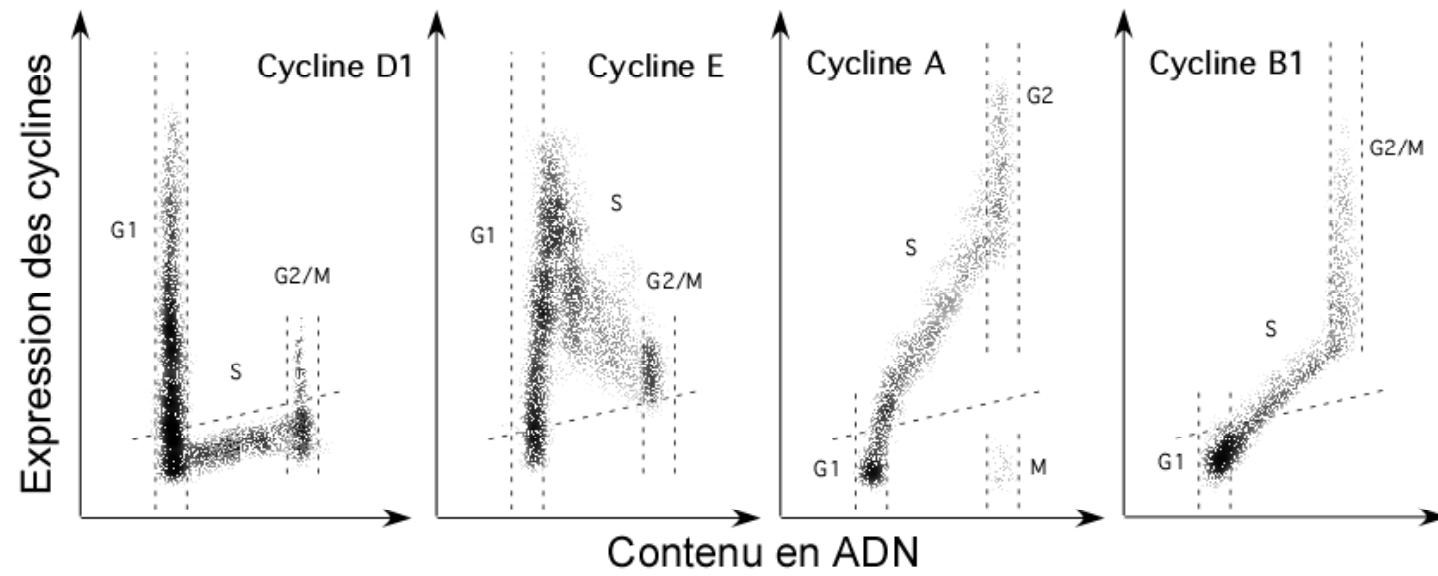
Jordan CT, Yamasaki G, Minamoto D (1996). High-resolution cell cycle analysis of defined phenotypic subsets within primitive human hematopoietic cell populations. *Exp Hematol* 24:1347-1355. PMID:8862447

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, Ardelt B, Traganos F. *Cytometry*. 1996 Sep 1;25(1):1-13. PMID:8875049

Détection des cellules en mitose : principe

Mitose

Phosphorylation de la sérine 10 de l'histone H3



Détection en immunofluorescence

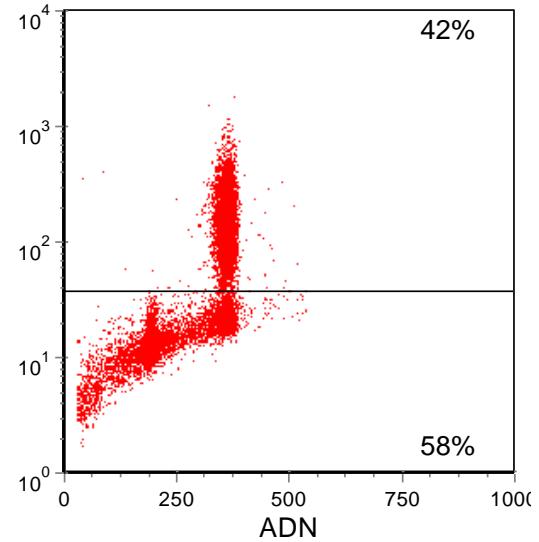
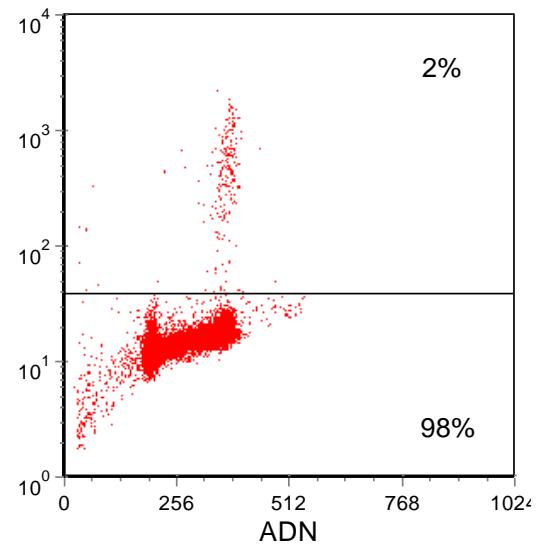
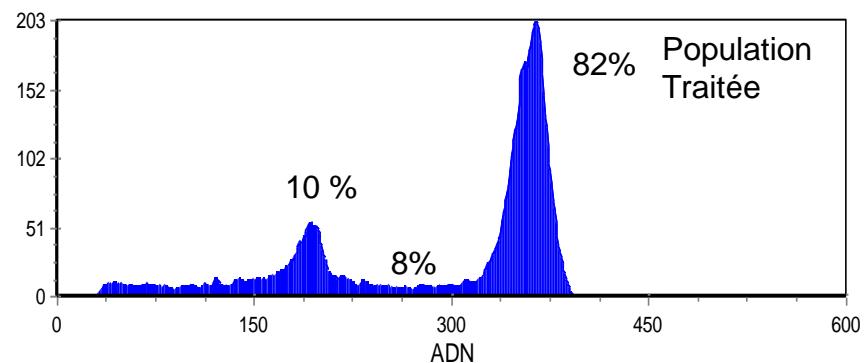
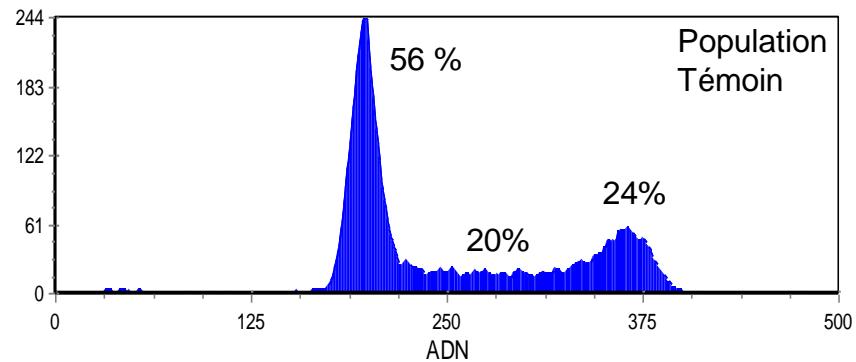


Marquage de l'ADN

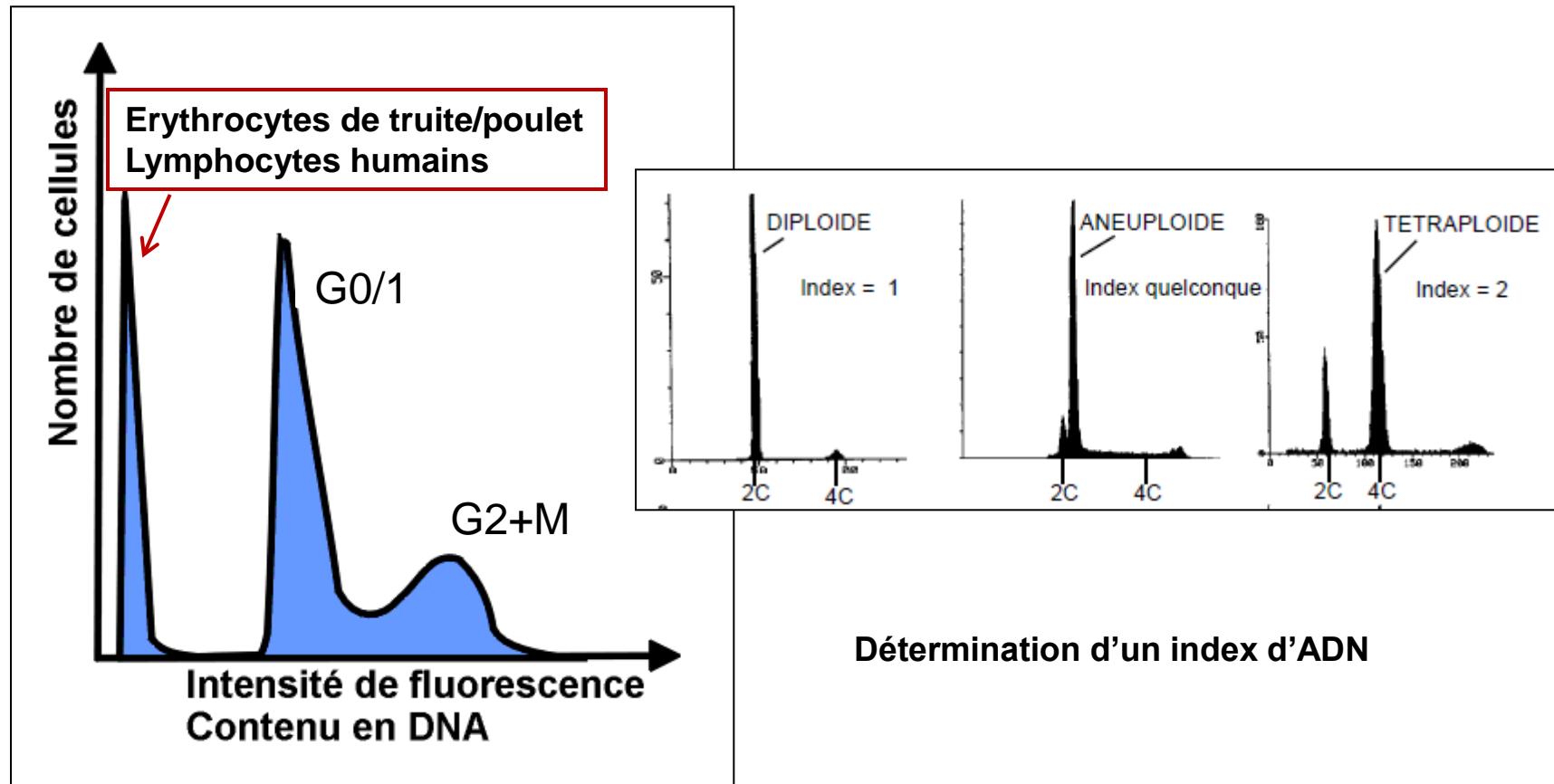
Histone H3 phosphorylation in human monocytes and during HL-60 cell differentiation. Juan G, Traganos F, Darzynkiewicz Z. *Exp Cell Res.* 1999;246(1):212-220. PMID:9882530

Autre marqueur : MPM2 (*Mitotic phosphoprotein 2*)

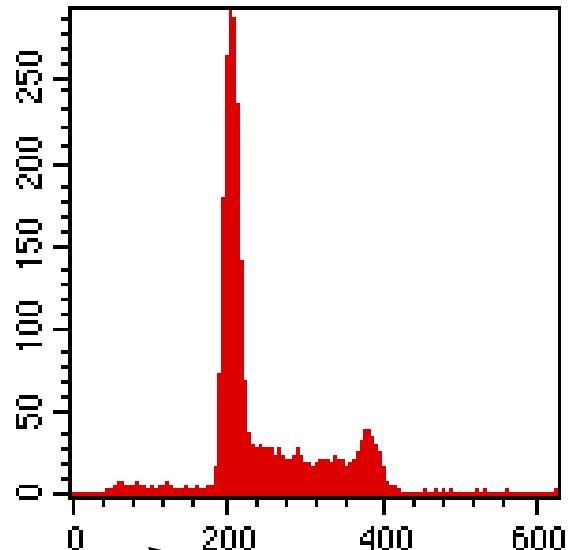
Quantification des cellules en mitose



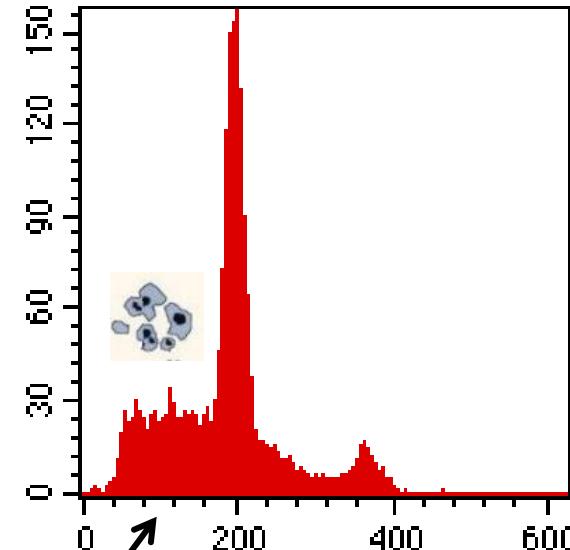
Contenu en ADN et ploidie



Contenu en ADN et apoptose

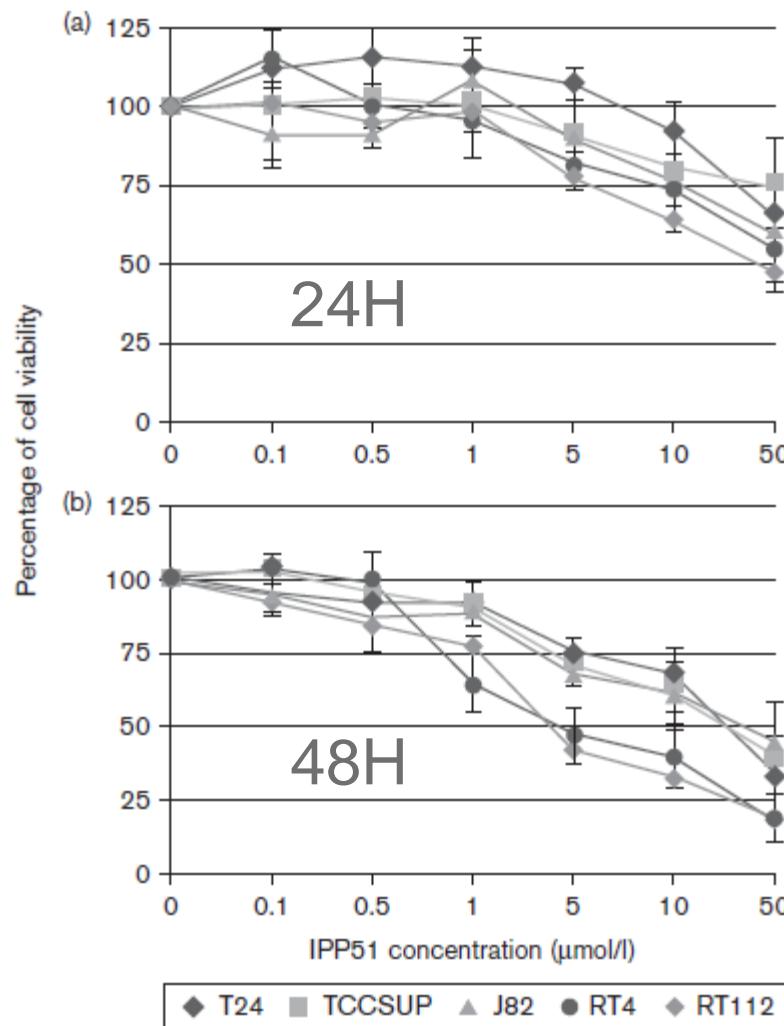


Pic subG1



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

Effet sur la prolifération cellulaire

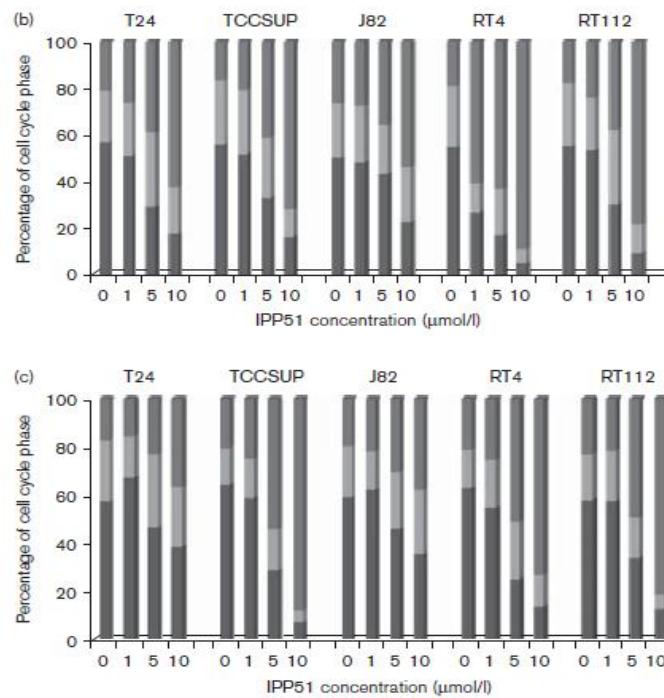
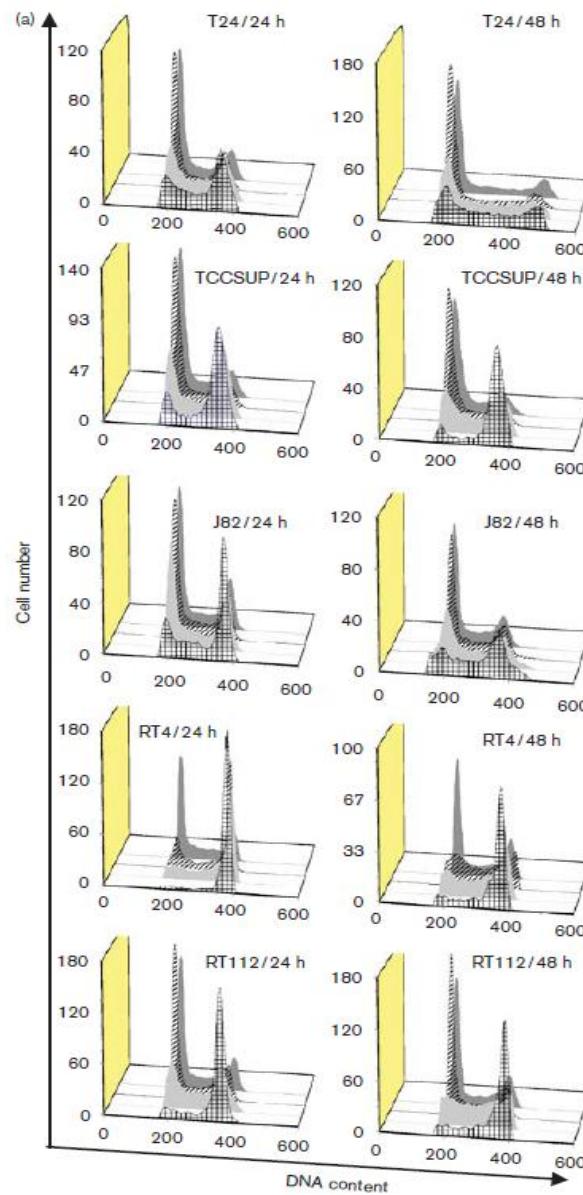


CI 50

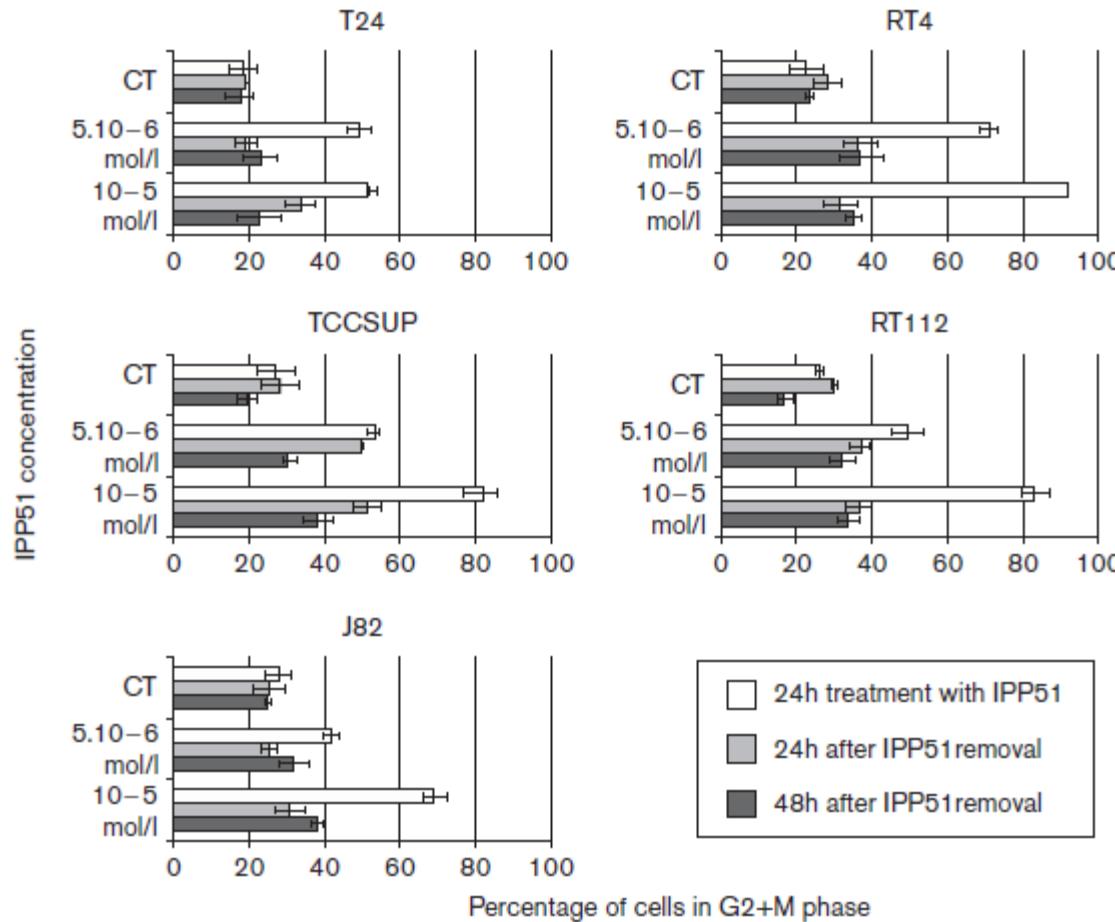
Cellules de bas grade : (RT4 and RT112) = 5.10^{-6} mol/l

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

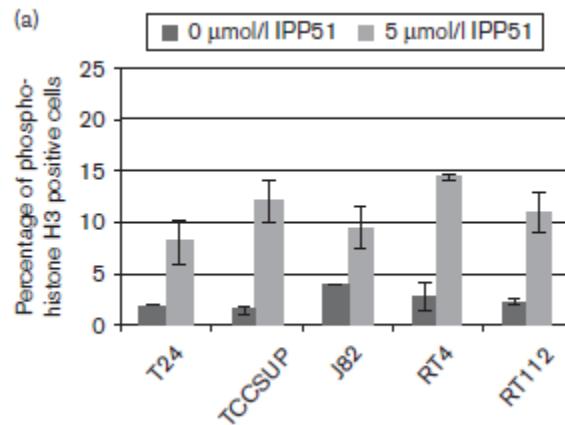
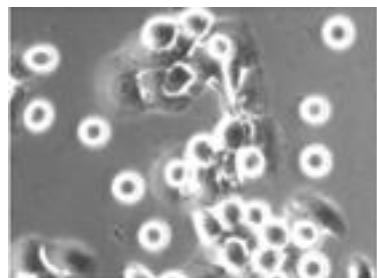
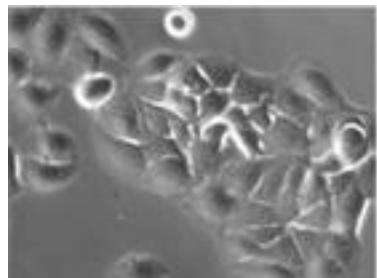
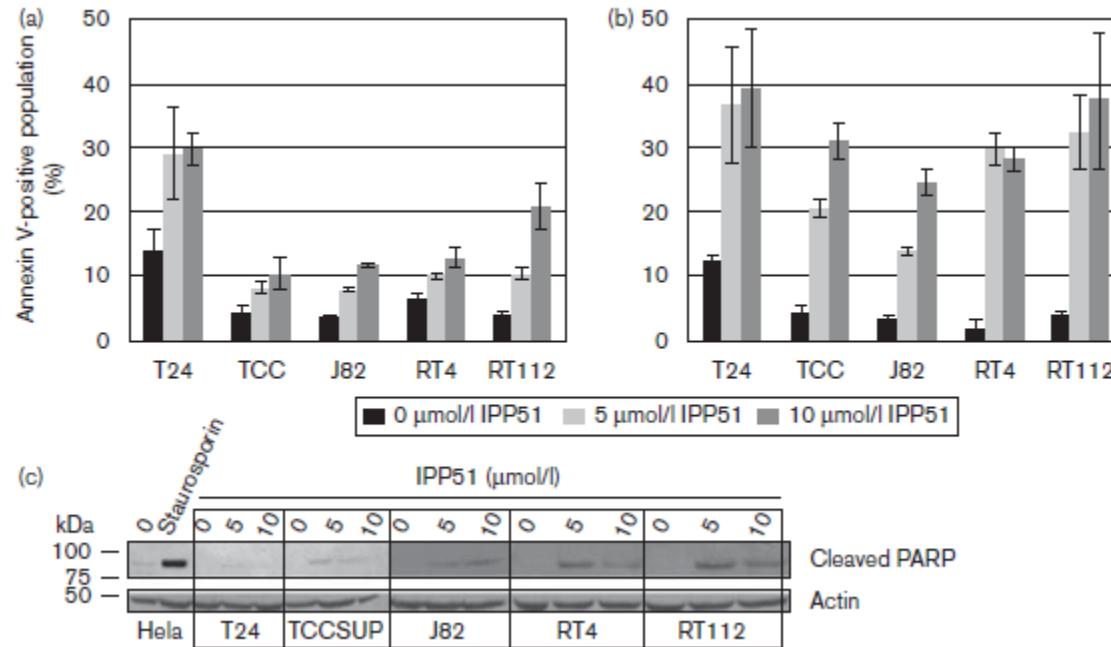


Table 1 Relative percentage of mitotic cells in the different stages of mitosis

		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



Effet sur l'induction de l'apoptose



Des références utiles !



NIH Public Access Author Manuscript

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

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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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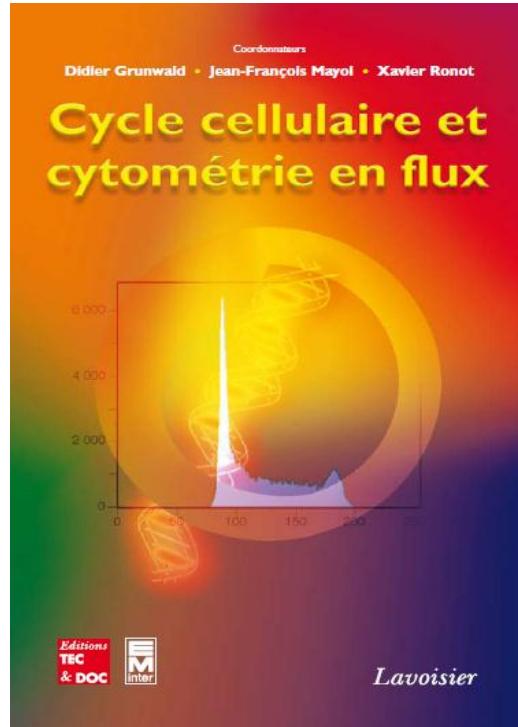
Abstract

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 utile !



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