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Assessment of Cell Cycle Inhibitors by Flow Cytometry

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1. Introduction

The main approach used by large pharmaceutical companies for the design of a new drug is based on identifying an initial specific biological target that has been adequately validated. Typically biochemical or cellular assays are used by applying high-throughput screening methods (HTS) to identify hits (HitID) from large chemical libraries in the case of new chemical entities (NCEs) in order to identifying viable parent compounds with pharmacological characteristics.

These compounds are starting points for chemical expansions which result in structure activities relationships (SARs). In the next phase (H2L) identification of a lead compound is the endpoint. Leads are already more drug-like and are expected to have certain activities in a number of biological assays, including cellular assay and hints of in vivo efficacy linked to pharmadynamic biomarker modulation. The optimization of a lead compound (LO phase) are typically properties such as potency and selectivity or activity in animal models, pharmacokinetics (e.g. oral bioavailability). In this phase an initial assessment of toxicological findings and a more systematic investigation in different animal models (e.g. xenograft or transgenic animal models) for demonstrating the therapeutic efficacy performed and ideally result in the selection of the final candidate for potential clinical development (Bleicher et al., 2003).

In the complex process of drug discovery, particularly for inhibitors of the cell cycle, there are two phases in which flow cytometry (FCM) gives major contributions. Firstly, in target validation, in order to demonstrate that inhibition of a specific target determines alteration of the cell cycle, secondly in the usage of cell-based assays in order to characterize compounds to demonstrate that the modulations observed are in line with the expected mechanism of action.

FCM is a major readout the key analysis for studying mechanism of action of drugs that affect proliferation since it is rapid, precise, can be automated with adherent or non-adherent cells.

In this review we want to point out technical and strategic aspects in the cytometric field important for new targeted therapies. In the case where the modulation of a target or treatment with a compound produced, a disturbance in the cell cycle is needed, mono-parametric DNA analysis is recommended, focusing on speed and throughput of samples by automation. Where, instead, the mechanism of action of a drug is the focus of the study, two-parametric analysis such as 5-bromo-deoxyuridine (BrdU) or 5-ethyl-deoxyuridine

(EdU) incorporation is applied (detected by "click chemistry") during the synthesis of DNA can be used.

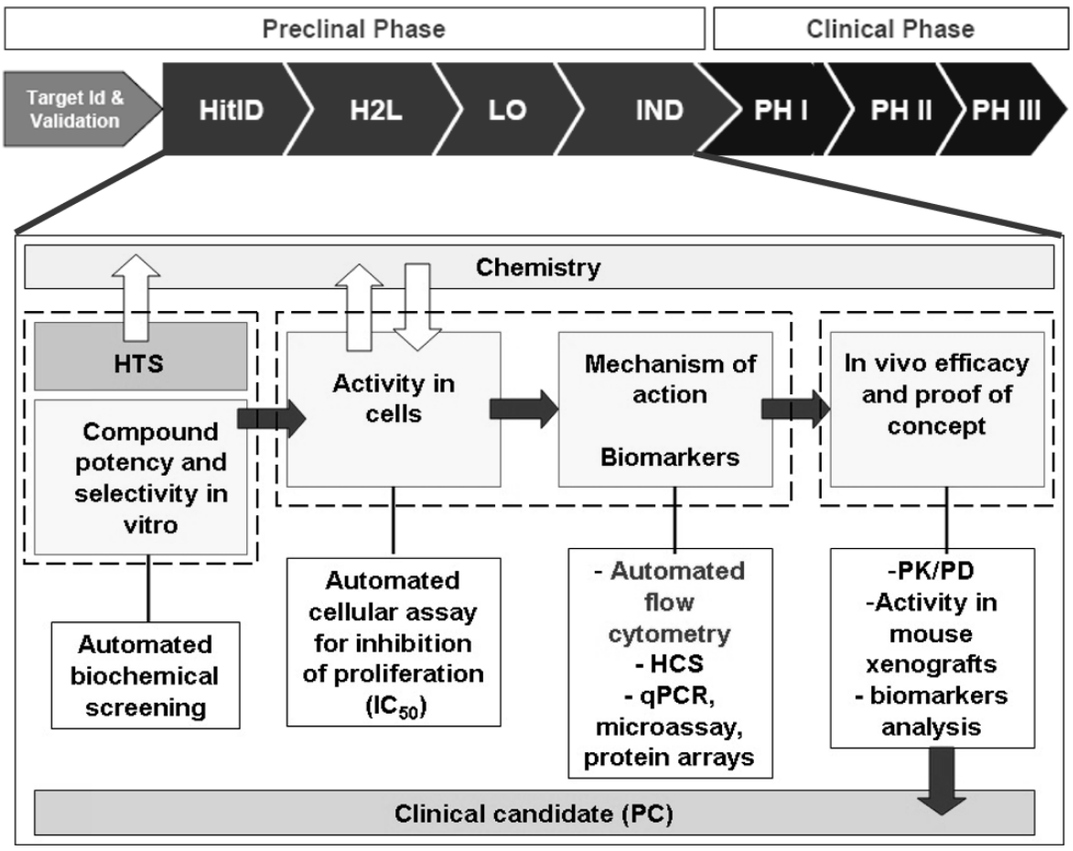

Fig. 1. A typical screening approach used for the identification of small molecule cell cycle inhibitor.

2. Flow cytometry applications for cell cycle analysis

2.1 Mono-parametric analysis of DNA content

Through the use of fluorescent dyes that bind stoichiometrically to DNA such as propidium iodide (PI) the content of cellular DNA can be quantified and the distribution of the cell cycle in a population of asynchronous cells can be determined. After treatment with drugs or SiRNA changes in specific cell cycle stages can be followed.

The profile of DNA content (x-axis, fluorescence of the dye bound to DNA, which is representative of the DNA content and the y-axis, which represents the number of cells) is analyzed using a mathematical model (e.g. Modfit) that determines the percentage of cells in the different phases of the cell cycle. Proliferating cells cycle through three major compartments name by as G1, S-phase and G2/M. The G2 and M phase includes mitosis which contains twice the DNA content before dividing to newborn G1 cells. Fraction of cells with a DNA content below G1 phase, often called “Sub G1” fraction consist of debris and fragmented cells. The degree of polyploidy of cells meaning cells with DNA content higher than G2/M, usually as a result of failure in mitosis.

A typical example is shown in Fig. 2. Human ovarian cancer A2780 cells in exponential growth were analyzed accordingly for their DNA content. Different mechanism of action upon compound treatment can be followed. For example treating A2780 cells in exponential growth with different drugs affecting the cells cycle such as kinase inhibitors show different cell cycle profiles associated with a block before (e.g. CDKs) or after (eg. PLK or Aurora) DNA synthesis (table 1 and Fig. 3).

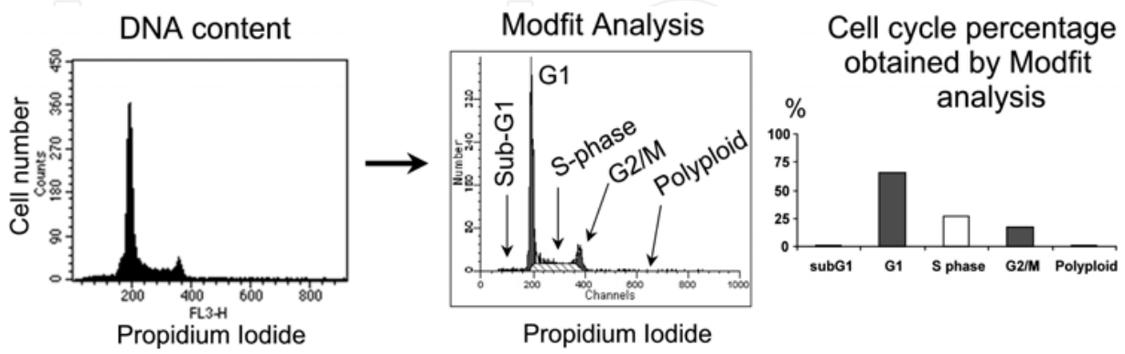

Fig. 2. A2780 cells in exponential growth were analyzed for their DNA content and the percentage of cells in specific stages of the cell cycle percentage was analyzed (X-axis, fluorescence of the dye bound to DNA, representative of the DNA content and y-axis, number of cells to DNA content).

Cell cycle	Small molecule	Major cell cycle phenotype	Reference
G1 and S	CDC-7 kinase inhibitor (PHA-767491), anthracyclins Nemorubicin	Massive cells death detected by sub-G1 due to inhibition of DNA synthesis process	(Albanese et al., 2006; Montagnoli et al., 2008)
	CDKs inhibitor (PHA-848125)	Clear G1 arrest due to CDK inhibition in G1/S transition	(Albanese et al., 2010)
	Camptothecin (irinotecan™, SN-38)	S-phase arrest and cell death	(Cappella et al., 2004)
G2 and M	MPS-1 kinase inhibitor (NMS-P715)	Broad DNA content due to asymmetrical mitosis, cell death by mitotic catastrophe	(Colombo et al., 2010)
	PLK-1 kinase inhibitor (NMS-P937), Eg-5 kinesin spindle protein, Paclitaxel	G2/M arrest due to PLK1 inhibition in mitosis followed by cell death	(Beria et al., 2010; Purcell et al., 2010; Sena et al., 1999)
	Aurora kinase inhibitor (Danusertib™)	Mitotic slippage after G2/M arrest and polyploidy	(Carpinelli et al., 2007; Fancelli et al., 2006)

Table 1. Different cellular phenotypes observed after treatment listed as exemplified for small molecule kinase inhibitors targeting cell cycle kinase or cytotoxic agents.

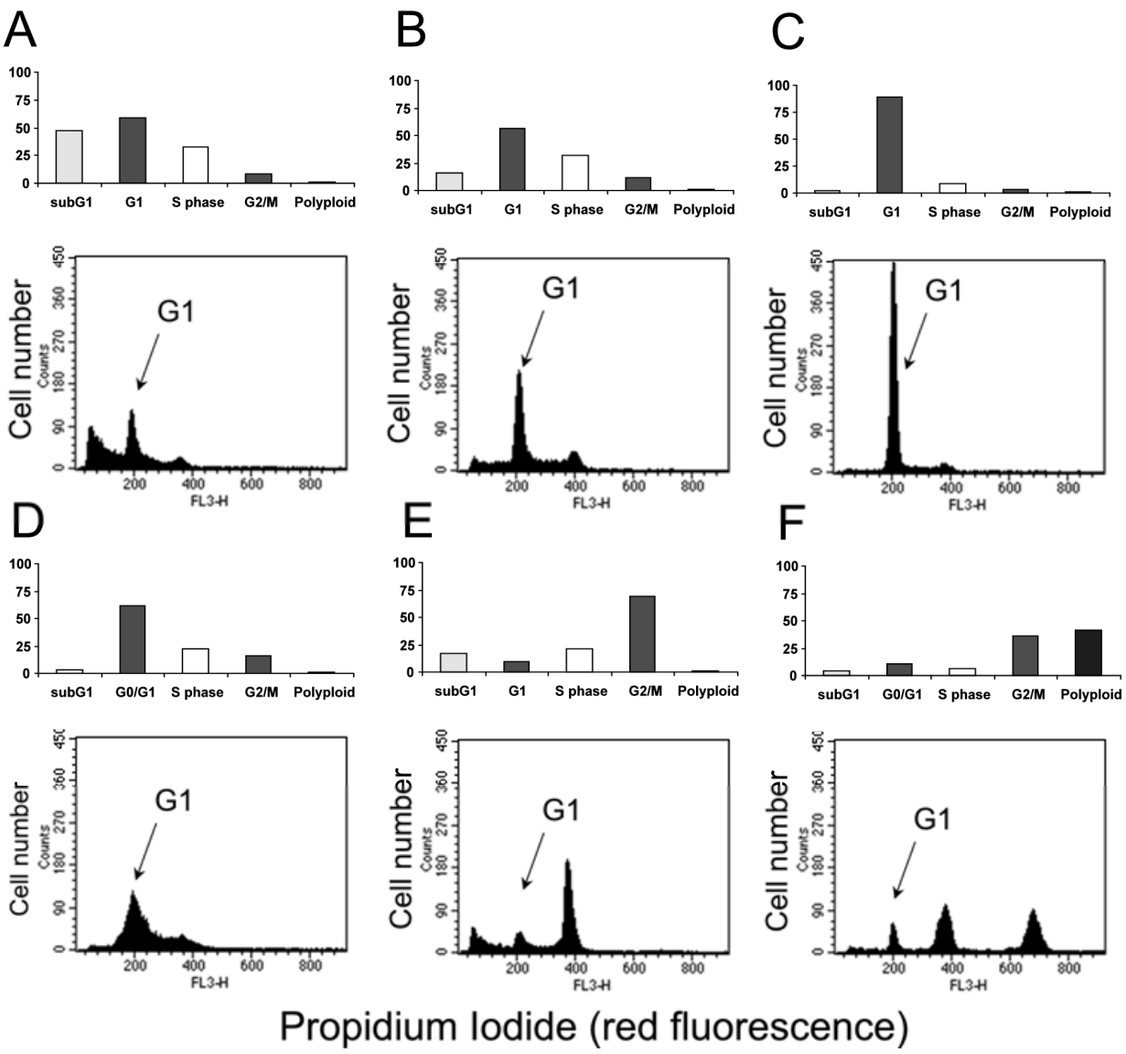

Fig. 3. DNA content and Modfit analysis of A2780 human ovarian cancer cells treated with cell cycle inhibitors at 1μM. A) CDC7 inhibitor NMS-354; B) Nemorubicin; C) CDKs inhibitor PHA-848125; D) MPS1 inhibitor NMS-P715; E) PLK1 inhibitor NMS-P937 and F) Aurora inhibitor Danusertib . DNA content of untreated cells is shown in Fig.2.

For example, Aurora kinase inhibitors such as Danusertib™ results in a specific cell cycle profile due to mitotic slippage that leads to polyploidy. This characteristic cell cycle profile could be used to analyze compound potency and mechanism of action in cells as shown in Fig. 4. Since Aurora kinase inhibitor Danusertib™ was specific on selected kinases panel and not affected G1/S checkpoint (Carpinelli et al., 2007), phenotype changes was related to Aurora A and B inhibition at observed concentrations. In this case, polyploidization process was predominant and the amount of G2/M plus polyploidy cells increased by increasing drug concentration allowing evaluation of ED_{50} by sigmoid model.

Combining ED_{50} by FCM to other mechanism of action bioassay such as antiproliferative and Aurora A and B biochemical assay, these data get compound potency during lead optimization of a putative compound as indicated below.

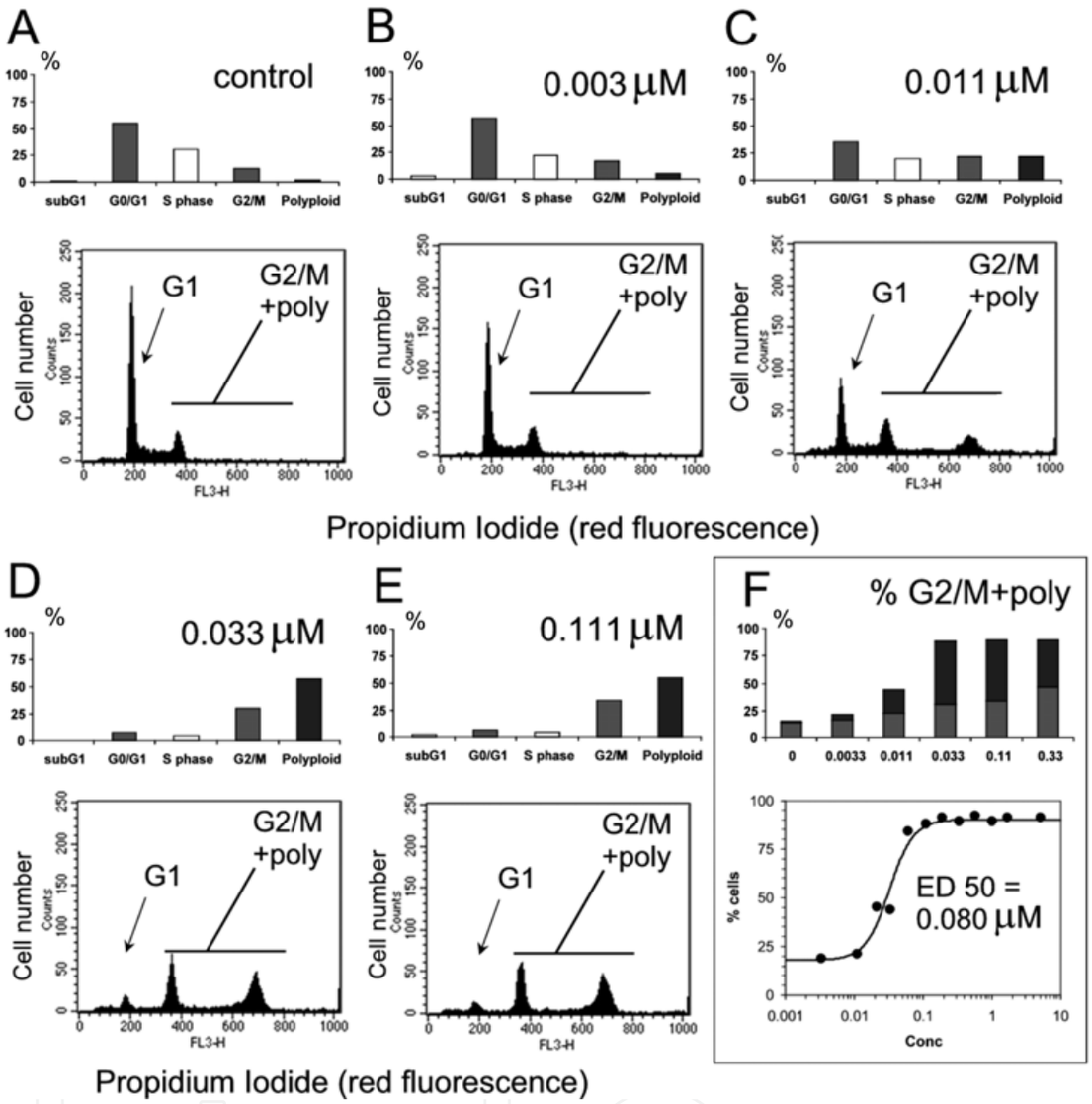

Fig. 4. Human colon cancer HCT-116 cells treated with increasing doses of an Aurora kinase inhibitor. Measurement of cellular potency by counting the number of cells in G2/M or in polyploidy measured by Modfit analysis are shown.

In Fig. 5 we have shown the prototype of classical SAR for cell cycle inhibitor as Aurora inhibitors, pyrrolopyrazole class. Based on this results, compounds endowed with high potency in biochemical and cellular assays as well as acceptable aqueous solubility as showed by compound 5 were selected. Beyond drug discovery, DNA content analysis could be used during target validation process before hit identification.

One of the techniques used for target validation and well established in mammalian cells is to turn off the genes is RNA interference using small interfering RNA (SiRNA) which consist typically of double stranded (21-25mer) oligonucleotides (Colombo & Moll, 2008).

In the outlined experiment, cells were transfected with siRNA and analyzed for up to 72 hours with respect to cell number, colony forming capabilities, DNA content analysis and changes in signal transduction pathways or gene expression pathways.

Cmpd	R′	R″	Aur-A IC_{50}[a]	HCT-116 IC_{50} [b]	HCT-116 FACS ED_{50} [c]
1	H	H	0.13	0.22	0.50
2	F	H	0.009	0.050	0.110
3	OH	H	0.006	0.097	>0.2
4	Me	H	0.024	0.021	0.100
5	**OMe**	**H**	**0.013**	**0.031**	**0.080**

Fig. 5. Structure and Aurora-A Inhibition of substituted-phenylacetyl pyrrolopyrazole.a, Enzyme inhibition IC_{50} μM. b, Antiproliferation IC50 μM. c, FACS ED_{50} evaluated on amount of cells in G2/M and polyploidy. Adapted from (Fancelli et al., 2006) .

In Fig. 6A an example is shown for Eg5 (Purcell et al., 2010) using Eg5 and the corresponding controls to exclude off-target effects.
Since cell lines in culture exhibit different genetic backgrounds, this is reflected in the DNA cell cycle profile after Eg5 siRNA treatment (Fig. 6B). A549 cells are prone to go directly to cell death, while U2-OS cells were blocked in G2/M and H1299 cells became polyploidy.
One dimensional DNA content analysis by FCM is a powerful tool to study the cell cycle as shown for mitotic checkpoints. However it also has its limitations, with respect to resolution and separation of cell cycle stages, in particular for S-phase, if there is a block in DNA synthesis. For this purpose, a second dimension is needed by using BrdU incorporation as readout.

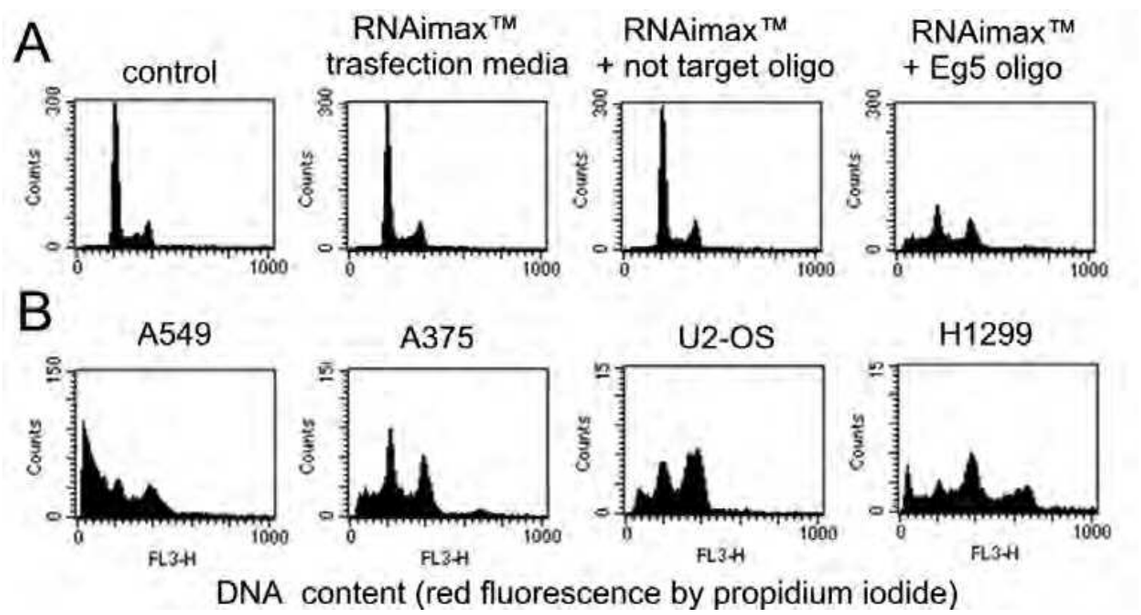

Fig. 6. A. Small RNA interference experiment in A549 cells treated for 72 hrs with 20nM Eg5 oligo, in comparison to not transfected or transfected with a non target oligo. B. Cellular phenotypes observed after RNAi experiments in different cell type;

2.2 DNA CONTENT and Bromo-deoxyuridine (BrdU)

The visualization and quantification of cells actively synthesizing DNA is important for studying the cellular response to drug treatments.

Historically for cell proliferation, incorporation of [$_3$H]-thymidine analogue was used to follow the DNA synthesis (labelling index). The use of radioisotopes ($_3$H β-emitter with a half-life of 12 years) and β-counters is a drawback of this method and technically difficult to analyze.

In 1982, an antibody against 5-bromo-deoxyuridine, BrdU was introduced, capable of binding the labelled DNA as a result of partial denaturation and use of [$_3$H]- labelled thymidine was gradually abandoned in the late 80's after the introduction of BrdU in FCM.

Several methods have been applied to measure the incorporation of BrdU with a need to denature DNA, since only upon denaturising of the double helix, or at least the introduction of specific DNA breaks, where BrdU had been introduced during the synthesis, made recognition by monoclonal anti-BrdU possible (Leif et al., 2004).

This step is the most critical and is achieved by different methods:

1. Heat treatment from 90-100 ° C for 10 min in low ionic strength solutions after partial extraction of histones. This method is less used nowadays because it needs optimization in different cell lines and is particularly destructive to the morphology and cellular constituents. However since DNA denaturation can be performed in 96 wells plate by programmable heater such as PCR apparatus, this method is particular used for HTS applications (Cappella et al., 2010) .
2. Acid or alkali treatments, followed by a neutralization step (Leif et al., 2004). The denaturation of DNA can be varied with time, only partially allowing the use of DNA probes staining such as propidium iodide (PI) , 7-aminoactynomycin D, TOPRO-3, which interacts DNA requiring double helix conformation. The use of acid denaturation may not be the most appropriate method if it is necessary to maintain cellular (scatters) morphology, surface antigenicity or cell constituents such as cyclins (Faretta et al., 1998) or phosphorylation of signaling proteins (Gasparri et al., 2006) during multiparameter analysis.
3. Enzymatic treatment with DNase I / exonuclease III against A-T hypersensitive enzyme sites by digestion at 37 ° C of generate single stranded DNA to exposure incorporated BrdU to monoclonal antibody. This method has proved to be of particular interest for its ability to maintain antigenicity and morphology, allowing to follow additional cellular parameter such as cell signalling events (Gasparri et al., 2006).

Additional methods were reported in the literature using treatment with high-energy radiation for the generation of DNA breaks by photolysis followed by anti-BrdU antibody (Leif et al., 2006).

Regardless of the denaturation method, BrdU incorporation and DNA staining is an accurate method to determinate the cell cycle phase as showed in Fig. 6. Cell cycle analysis by mathematic modelling (Jourdan et al., 2002) by fitting software (e.g,. Modfit™) usually underestimates percentage of cells in S-phase, since G1 and G2/M peaks are fitted by a gaussian model and early and late S-phase are included inside fitted peaks.

An example demonstrating this difference is shown in Fig.7 using the different methods with HCT-116 colon cancer cells, which show 30% vs. 43% of cells in S-phase, depended from the method used.

As explained for Fig. 8A and B, BrdU analysis allows to distinguish and quantify if there is an arrest in DNA synthesis, in which part of S phase and it is possible to separate early S-phase from G1 or late S-phase from G2/M (Cappella et al., 2001).

This advantage is demonstrated in Fig.8A, where HCT116 cells were treated with SN-38, a topoisomerase inhibitor which affects DNA replication. Only by analyzing BrdU incorporation was it possible to detect delays in early (gate E) or late (gate L) S-phase upon SN-38 treatment at 7h (Fig.8B).

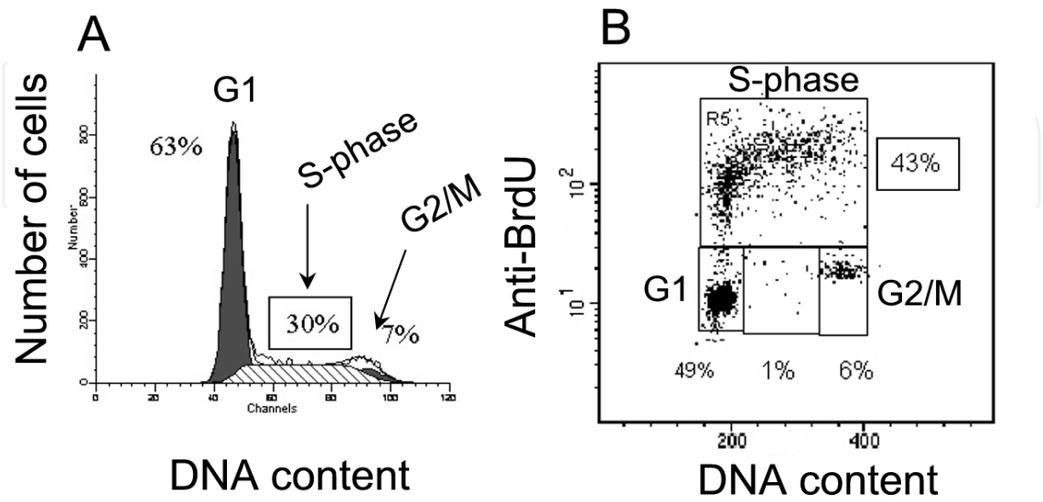

Fig. 7. Profile of DNA content and BrdU incorporation in the same sample. A, DNA content was analyzed by Modfit analysis or B, DNA content by PI (x-axis) and BrdU incorporation (y-axis)

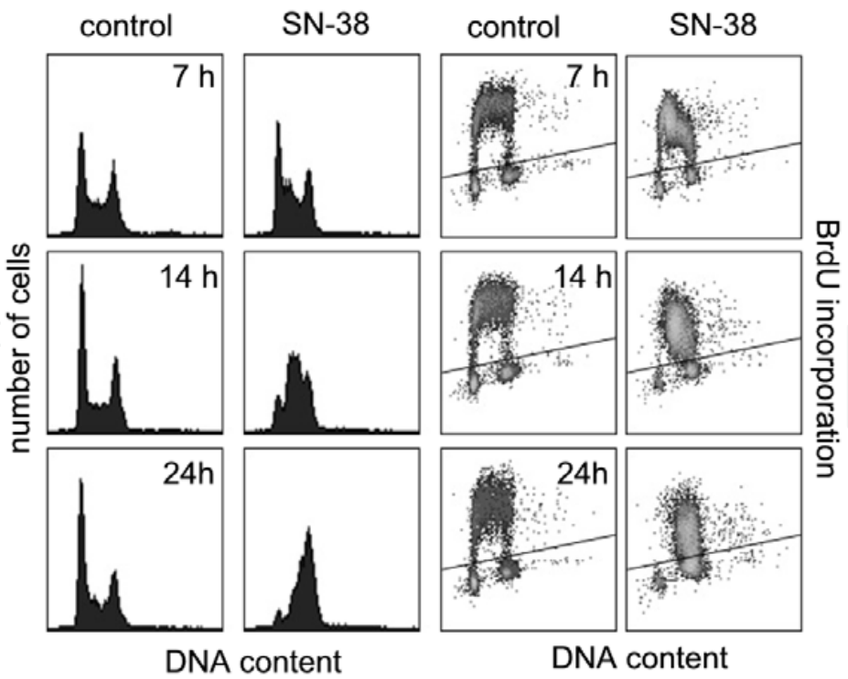

Fig. 8A. Profile of DNA content and BrdU incorporation of HCT-116 cells treated with SN-38 at 10nM for 7, 16 and 24hrs. DNA content by PI (x-axis) and BrdU incorporation (y-axis) are shown. Cells above the line define BrdU positive cells.

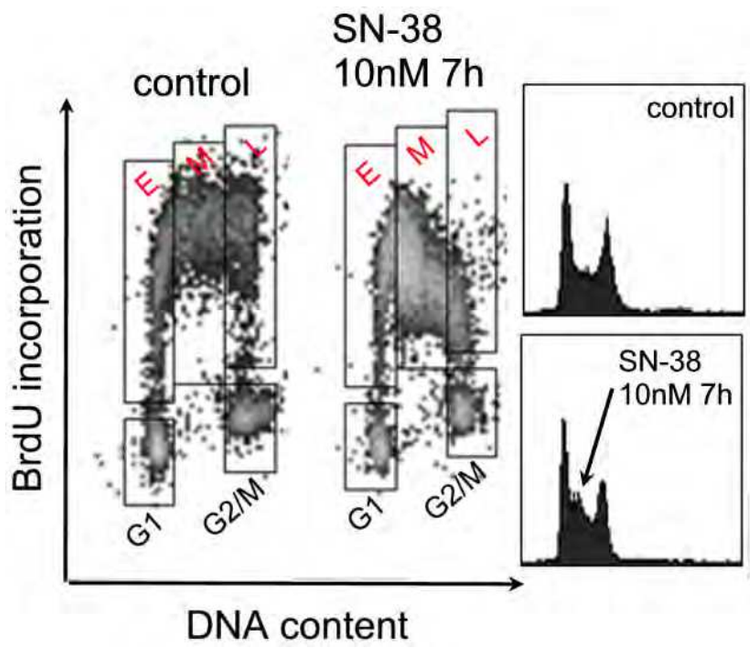

Fig. 8B. BrdU incorporation of HCT-116 cells treated with SN-38 at 10nM for 7h. Gates were set at E, early S-phase; M, middle S-phase; and L, late S-phase. The corresponding DNA content profiles are shown. Arrow indicated impossibility to detect early effects on DNA synthesis. DNA content is quantified by PI (x-axis) and BrdU incorporation (y-axis) by BrdU antibodies staining.

Another advantage is that BrdU incorporation can be performed either *in vitro* or *in vivo*. In Fig. 9 we show an *ex-vivo* analysis of mice bearing HCT-116 xenograft tumors which received intravenous injections of a single dose of irinotecan (60 mg/kg) (Ciomei et al., 2007). After drug administration, BrdU was injected intraperitoneally 2 hours before, and mice were sacrificed. Tumors were removed and disaggregated by pepsin (Terry & White, 2001). By this approach, it was possible to detect cell cycle perturbations of the drug.

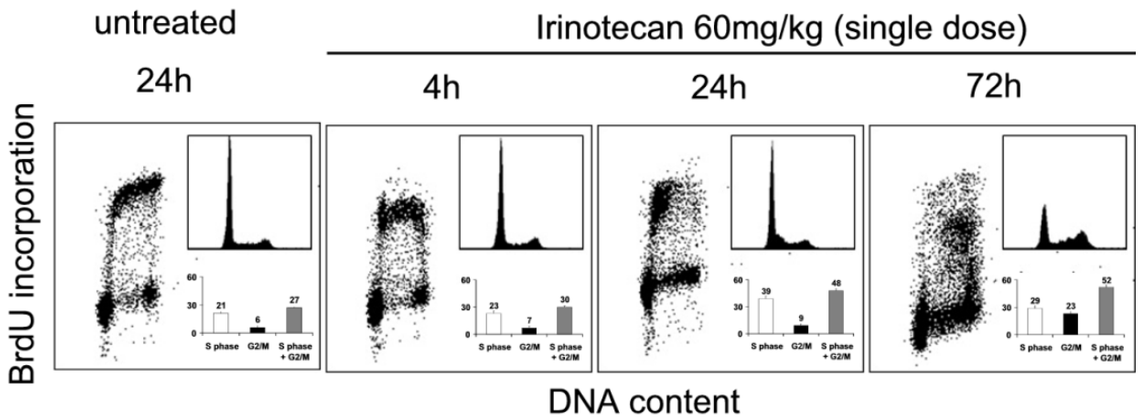

Fig. 9. BrdU incorporation (dot plots) in enzymatically disaggregated HCT-116 tumor cells treated with irinotecan. Cell-cycle profiles (DNA histograms) and percentages of cells in S-phase and/or G2/M are shown as inlets.

Since this drug acted on early DNA replication, initially a delay of G1/S phase (till 24h) was observed whereas at later time points an arrest in S phase and G2/M was more evident. Moreover since DNA synthesis is blocked and cells started to die, BrdU incorporation was abrogated (Cappella et al., 2004).
More recently BrdU incorporation was used for cell sorting of mammalian cells during DNA replication for CGH microarray analysis and for genome-scale analysis of replication timing (Ryba et al., 2011).

2.3 DNA CONTENT and BrdU by click chemistry

Although there is a plethora of methods for the analysis of BrdU incorporation, the major drawbacks are the difficulties related to their full use in the analysis of multi-color FCM and traditional imaging. Thermal denaturation destroys almost all of the antigens and denaturation with "chemicals" such as acids or bases makes the analysis of several antigens impossible (Frank et al., 1995) and therefore alternative methods are needed. In 2001, the term "click chemistry" was coined by Nobel Prize Sharpless to describe reactions with defined criteria and of the most popular reaction that fully meets these criteria is the 1,3-dipolar cycloaddition, also known as the Cu-Catalyzed Azide Alkyne Cycloaddition (CuAAC) between an azide and a terminal alkyne forming a triazole by copper (I) as catalyst (Fig.10) . These systems are very rare in nature and are inert in biological systems and thus particularly important in the "bio-orthogonal" approach where a substrate containing a chemical reporter is introduced in a target (i.e. proteins, sugars, DNA) *in vivo* and then identified by a covalent reaction with a fluorescent probe (Prescher & Bertozzi, 2005).

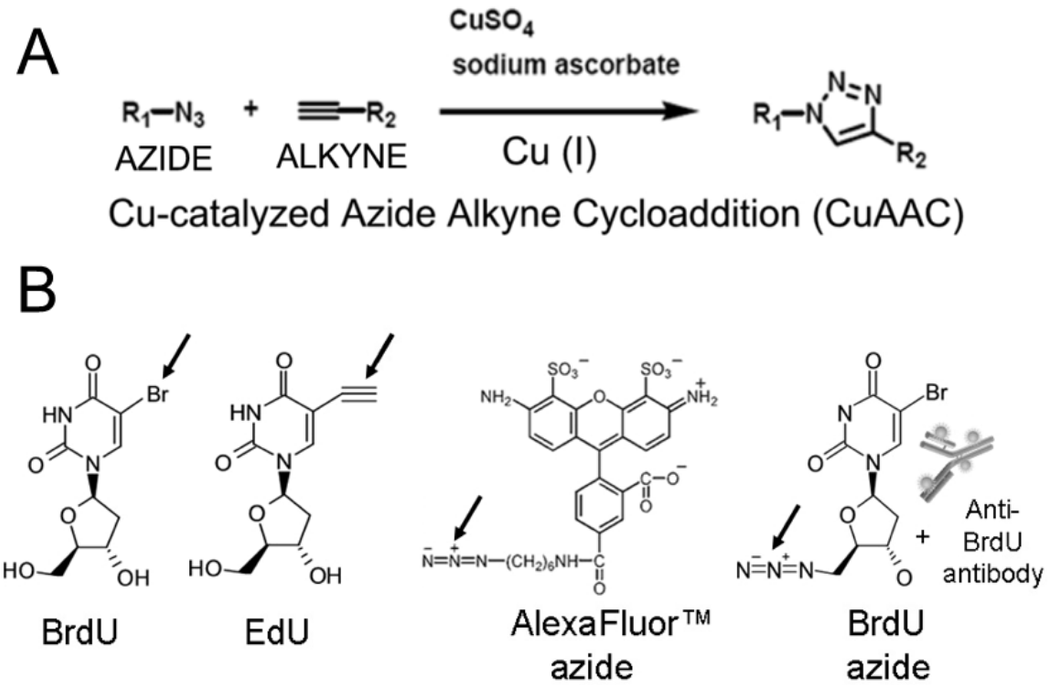

Fig. 10. A) Generic schema for Cu-Catalyzed Azide Alkyne Cycloaddition (CuAAC) between an azide and a terminal alkyne forming a triazole by copper (I), B) Players of bio-orthogonal approach for cell cycle analysis.

It is the case of EdU (5-ethynyl-2′-deoxyuridine) used instead of BrdU for DNA synthesis analysis in FCM. EdU is incorporated into the replication forks of new DNA during S-phase; exposed alkynyl residues can be identified with "click chemistry" reaction using fluorescent azides (AlexaFluor™ 488 Click-IT assay supply by Invitrogen Corp) in presence of copper (I) and cells could be visualized (Darzynkiewicz et al., 2011). One modification of this assay is by replacing dye azide, with a BrdU azide and to detect by BrdU antibody (Fig. 11). As with BrdU, EdU incorporation can also be used *in vivo*. Application and use of this approach is particularly useful in screening compounds that alter the cell cycle when multiparametric readout are needed such as HCS. In this case mild condition for cell treatment is a must in order to conserve antigenic properties and cellular integrity (Cappella et al., 2008).

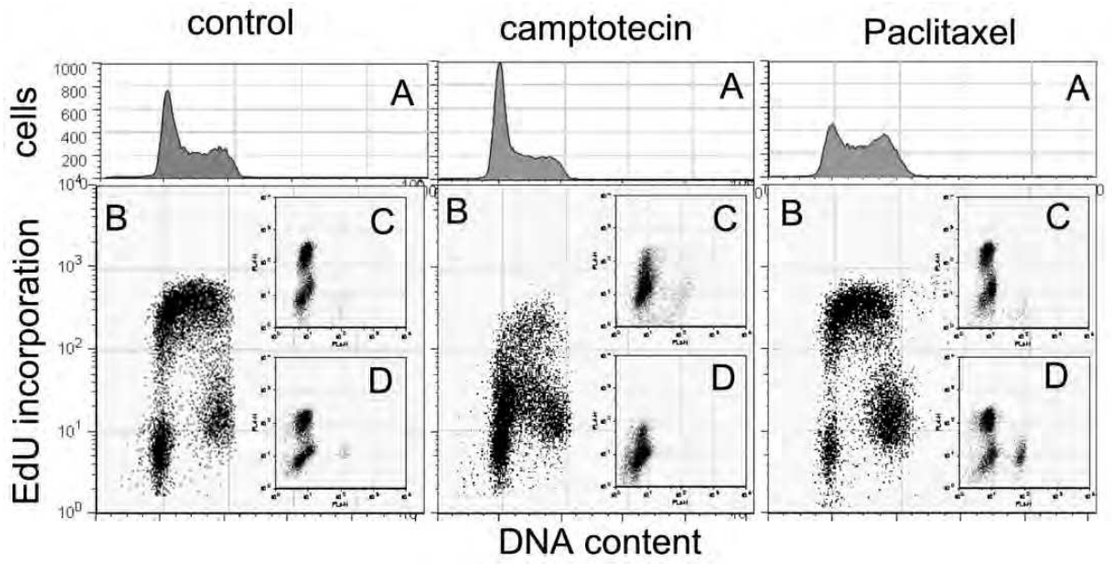

Fig. 11. EdU incorporation as example of multi parametric analysis. Cells were treated with camptotecin or paclitaxel and stained with PI or with corresponding antibodies. A, cell-cycle profiles; B, EdU incorporation and DNA content; C, EdU and cleaved caspase 3; D, EdU and phospho-histone H3 (Cappella et al., 2008).

2.4 DNA CONTENT and sample throughput

Cell cycle analysis by FCM and BrdU incorporation is one of the most powerful techniques to quantitatively distinguish cell cycle phase after treatments, however, high throughput is limited, since most mammalian cells in tissue culture require cell detachment from culture disks. Traditionally, FCM is limited to small-scale laboratory and clinical studies and high throughput methods have recently been developed for drug discovery. Hand-free automations and the introduction of 96 well plate autosamplers have increased throughput capabilities in mammalian or plant cell (Cappella et al., 2010) (Cousin et al., 2009).

Advancements in high throughput FCM have been implemented following the introduction of BD "Multiwell Autosampler" MAS™ and HTS™ , or efficient micro fluidic devices such as "plug-FCM" systems HyperCyt™ have been introduced and whereas traditional autosamplers load samples as single entities, automated sampling systems for FCM allowed individual samples to be assayed sequentially (Black et al., 2010).

Percentages of cells are usually analyzed using appropriate gates. When large numbers of data are generated, in order to visualize the results for an easy readout (Lugli et al., 2010), data can be displayed with cluster software (e.g.Spotfire™) generating heat maps. An example is the profiling of compounds in BrdU experiments regarding percentage of BrdU positive cells, percentage of S-Phase cells and G2/M cells (Fig.12) as derived by Modfit™ analysis from DNA content analysis after drug exposures (Cappella et al., 2010).
Moreover, very recently additional analyses are borrowed from the proteomics and bioinformatics approach. These involve subject classification by principal component analysis (PCA) (Lugli et al., 2007), hierarchical clustering for discovering novel building blocks for imaging probes (Shedden & Rosania, 2010) or scalable analysis (Klinke & Brundage, 2009) using appropriate bioinformatics tools.

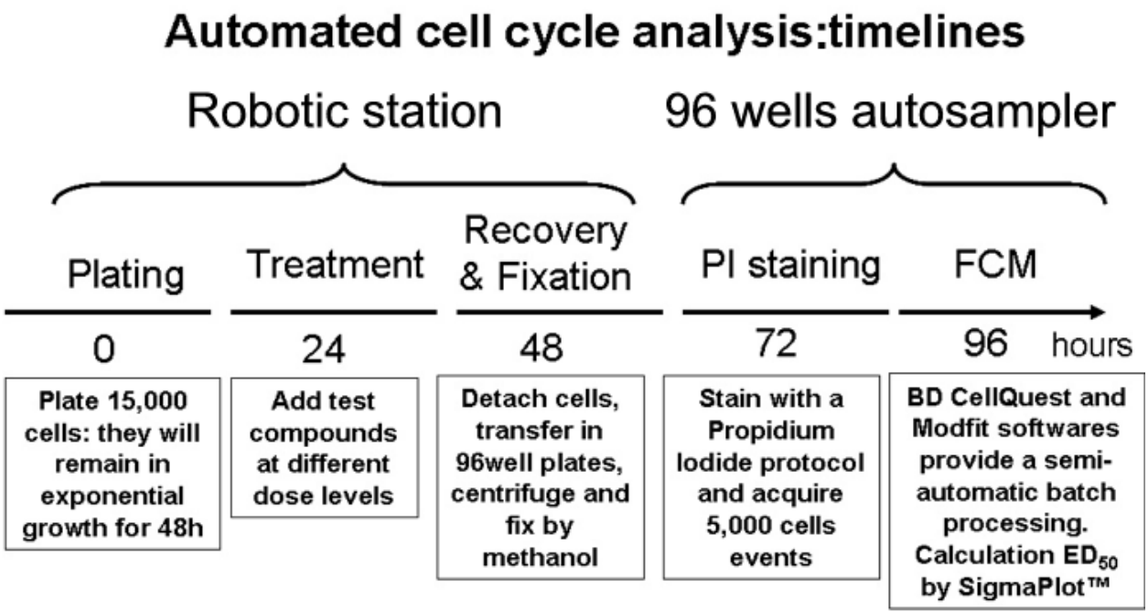

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Fig. 12. Example of cell cycle analysis timelines using lab automation.

3. Conclusion

Numerous drugs in oncology affect the cell cycle and therefore cell cycle analysis by FCM is the primary method of choice for measuring compound potency, selectivity or mechanism of action. Most anticancer drugs directly affect cellular proliferation, and their inhibitory effects usually depend on dose and treatment time. Research activities in drugs affecting mitosis, gave characteristic cellular phenotype, and FCM allowed to monitor apoptosis, mitotic arrest, polyploidization or aneuploidy , generating "compound activity fingerprints" useful for mechanism of action studies. The introduction of halogenated nucleotides such as BrdU, or "click chemistry" by EdU has revolutionized the study of cell proliferation.

Moving from tube-based to plate based readout and the recent development of semi automated techniques for staining and analyzing FCM samples has created new challenges. Finally advanced data visualization and analysis such as heat maps has boosted analytical capabilities, necessary for high throughput FCM which can generate very complex datasets.

In this paper we reviewed state-of-the-art DNA analysis for cell cycle studies *in vitro* as *in vivo* and new technologies are emerging ("new-flow methods") which will further facilitate and optimize future analysis.

4. Acknowledgment

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Drug Discovery and Development - Present and Future

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Drug discovery and development process aims to make available medications that are safe and effective in improving the length and quality of life and relieving pain and suffering. However, the process is very complex, time consuming, resource intensive, requiring multi-disciplinary expertise and innovative approaches. There is a growing urgency to identify and develop more effective, efficient, and expedient ways to bring safe and effective products to the market. The drug discovery and development process relies on the utilization of relevant and robust tools, methods, models, and validated biomarkers that are predictive of clinical effects in terms of diagnosis, prevention, therapy, and prognosis. There is a growing emphasis on translational research, a bidirectional bench to the bedside approach, in an effort to improve the process efficiency and the need for further innovations. The authors in the book discuss the current and evolving state of drug discovery and development.

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