CHAPTER

2

Generation of aneuploid cells and assessment of their ability to survive in presence of chemotherapeutic agents

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Chapter outline

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Abstract

Aneuploidy is a condition in which cells have an abnormal number of chromosomes that is not a multiple of the haploid complement. It is known that aneuploidy has detrimental consequences on cell physiology, such as genome instability, metabolic and proteotoxic stress and decreased cellular fitness. Importantly, aneuploidy is a hallmark of tumors and it is associated with resistance to chemotherapeutic agents and poor clinical outcome. To shed light into how aneuploidy contributes to chemoresistance, we induced chromosome mis-segregation in human cancer cell lines, then treated them with several chemotherapeutic agents and evaluated the emergence of chemoresistance. By doing so, we found that elevation of chromosome mis-segregation promotes resistance to chemotherapeutic agents through the expansion of aneuploid karyotypes and subsequent selection of specific aneuploidies essential for cellular viability under those stressful conditions. Here, we describe a method to generate aneuploid cell populations and to evaluate their resistance to anti-cancer agents. This protocol has been already successfully employed and can be further utilized to accelerate the exploration of the role of aneuploidy in chemoresistance.

1 Introduction

Aneuploidy is a condition in which cells have a chromosome number that is not a whole multiple of the haploid complement. This state of chromosome imbalance is the product of chromosome segregation errors during mitosis (Bakhoum & Cantley, 2018; Santaguida & Amon, 2015). Importantly, chromosome mis-segregation and the ensuing aneuploidy are frequent in tumor but very rare in untransformed cells (McGranahan, Burrell, Endesfelder, Novelli, & Swanton, 2012). Several detrimental consequences are associated with aneuploidy, including genome instability (Ohashi et al., 2015; Passerini et al., 2016; Santaguida et al., 2017; Sheltzer et al., 2011), alteration in protein production, folding and turnover (Oromendia, Dodgson, & Amon, 2012; Santaguida, Vasile, White, & Amon, 2015; Stingele et al., 2012) and metabolic stress (Williams et al., 2008). Further, stress response genes (Sheltzer et al., 2017) and cell-cycle genes are often deregulated in aneuploid cells, which in turn might lead to cell-cycle arrest and senescence (Durrbaum et al., 2014; Santaguida et al., 2017; Sheltzer, 2013). Interestingly, it has been shown in yeast that aneuploidy is associated with decreased vulnerability to several chemotherapeutic agents and antifungal drugs (Chen, Bradford, Seidel, & Li, 2012; Chen et al., 2015; Pavelka et al., 2010; Selmecki, Dulmage, Cowen, Anderson, & Berman, 2009; Yang et al., 2019). In particular, when yeast *Candida albicans* was cultured under selective pressure given by fluconazole treatment, the gain of a single chromosome arose as an adaptive survival mechanism in response to the drug. This resistance can be reproduced through the overexpression of genes encoded on such chromosome (Selmecki, Gerami-Nejad, Paulson, Forche, & Berman, 2008). Likewise, in humans, aneuploidy and the resulting chromosome

instability (CIN) are associated with chemoresistance (Ippolito et al., 2021; Kuznetsova et al., 2015; Lee et al., 2011; Lukow et al., 2021; Swanton et al., 2009). Since chemotherapy is the primary strategy for therapeutic intervention in several tumors, it is of paramount importance to study and understand the origins and consequences of aneuploidy-induced chemoresistance. As a matter of fact, a direct relationship between aneuploidy and chemoresistance is only starting to emerge. To better understand this connection, we hypothesized that aneuploidy and genome instability might increase genome plasticity, empowering cancer cells to successfully adapt under stressful conditions, such as chemotherapy. To test this hypothesis, we elevated chromosome mis-segregation rate in cancer cell lines from different tissues of origin and then exposed them to a selective pressure, by treatment with chemotherapeutic agents commonly used in clinical settings. This approach led us to discover conditions under which chromosome mis-segregation had beneficial effects during chemotherapeutic treatment. Our data further suggest that aneuploidy might induce chemoresistance through the induction of genome instability, that in turn lead to the generation of cell populations with high karyotypic heterogeneity. By doing so, cancer cells are increasing the likelihood to have within their population a specific and favorable karyotype that gives proliferative advantage under a specific stressful condition (Fig. 1). This chapter describes a protocol we have developed that employ crystal violet staining to identify conditions under which aneuploidy induction is beneficial during treatment with chemotherapeutic agents. We also describe an immunofluorescence protocol for the quantification of the degree of genome instability necessary to generate a heterogeneous karyotypic cell population that favors chemoresistance. We hope that these methods will be helpful for the characterization of resistant aneuploid clones and for the characterization of how aneuploidy impacts on chemotherapy response.

2 Materials

2.1 Disposables

1. 12-well plates for cell culture.
2. 10 cm plates for cell culture.
3. Bürker chamber for cell counting.
4. Coverslips for immunofluorescence staining.

2.2 Reagents

1. NCI-H1975 (Cat# CRL-5908), A549 (Cat# CCL-185) and A375 (Cat# BS TCL 88) cell lines used in these experiments were obtained from the American Type Culture Collection (ATCC). (see Notes 3.1 and 3.5).

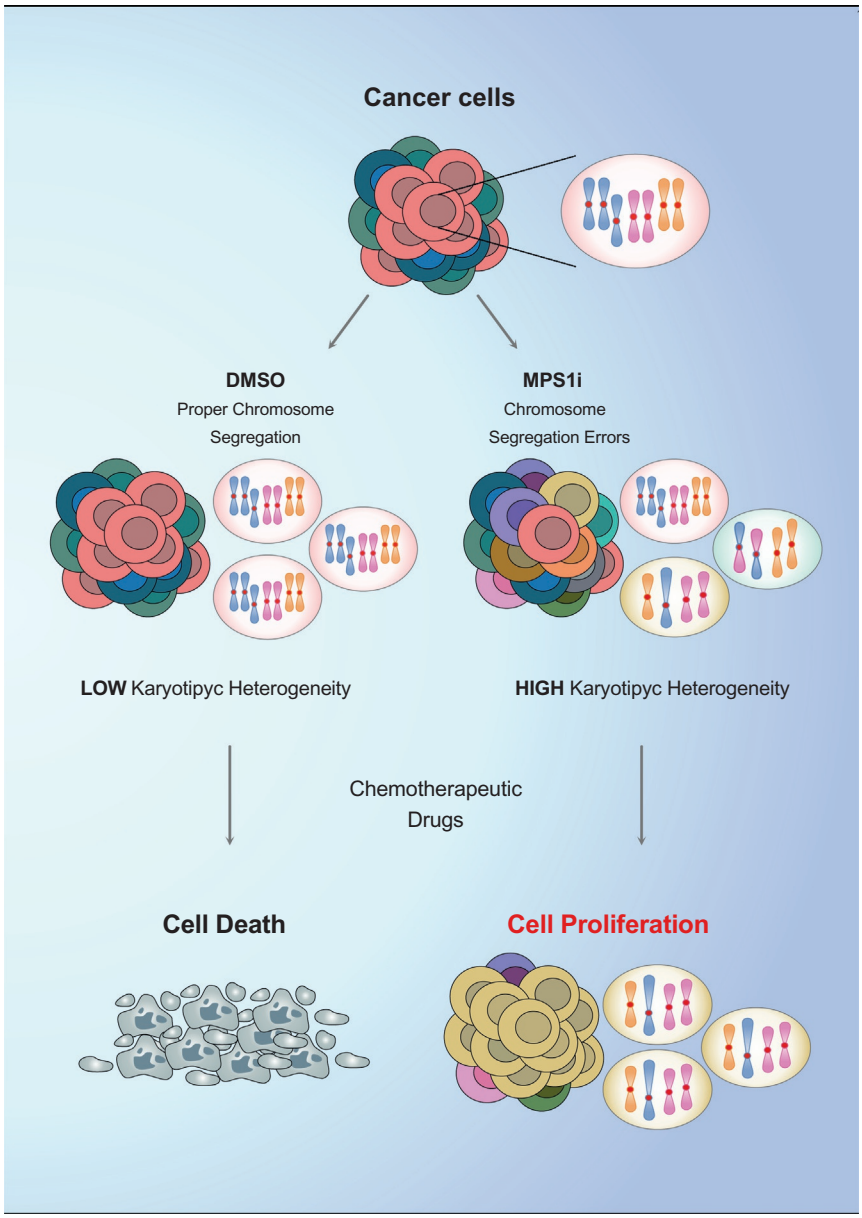

FIG. 1

Overview of chemoresistance mechanism developed after aneuploid induction. Schematic representation of beneficial aneuploidies selection following Mps1i treatment.

Figure adapted from Ippolito, M. R., V. Martis, S. Martin, A. E. Tijhuis, C. Hong, R. Wardenaar, M. Dumont, J. Zerbib, D. C. J. Spierings, D. Fachinetti, U. Ben-David, F. Foijer, and S. Santaguida. (2021) Gene copy-number

2. Dulbecco's Modified Eagle Medium (DMEM) (Euroclone, CAT# ECM0103L) was stored at 4 °C.
3. RPMI 1640 (Euroclone, CAT# ECM2001lL) was stored at 4 °C.
4. Trypsin-EDTA reagent (Euroclone, CAT# ECB3052D) was stored at 4 °C.
5. Dulbecco's Phosphate Buffered Saline 1× (PBS) (Microgem, Cat # TL1006-500ML), was stored at room temperature (RT) (see Notes 3.1, 3.2, 3.3, and 3.5).
6. Fibronectin (Sigma Aldrich, Cat # F1141), was used at a working concentration of 5 μg/mL. Stored at −20 °C (see Note 3.1.3)
7. Mowiol-dabco (mounting Medium, 5x1ml Boston Bioproducts Inc., Cat# MM-125). Stored at −20 °C (see Note 3.3.8).
8. Paraformaldehyde (PFA) solution 4% in PBS (ChemCruz, Cat #sc-281,692). Stored at 4 °C (see Notes 3.2.6 and 3.5.7).
9. RO-3306 (Sigma-Aldrich, Cat #SML0569), was used at a working concentration of 7.5 μM. Stored at −20 °C (see Note 3.2).
10. MG-132 (Tocris, Cat #1748), was used at a working concentration of 10 μM. Stored at −20 °C (see Note 3.2).
11. Reversine (Cayman Chemical, Cat# 10004412), was used at a working concentration of 0.125 μM, 0.25 μM or 0.5 μM. Stored at −20 °C (see Notes 3.2 and 3.5).
12. Topotecan (Tocris, Cat# 4562), was used at working concentration of 0.11 μM. Stored at −20 °C (see Note 3.5).
13. Vemurafenib (Selleckchem, Cat# S1267), was used at working concentration of 1 μM. Stored at −20 °C (see Note 3.5).
14. Crystal violet solution (Sigma Aldrich, Cat# V5265), was used at working concentration of 1% and was stored at RT (see Notes 3.5 and 3.6).
15. Triton X-100 (VWR, prolarbo, Cat# 9002-93-1), was used at working concentration of 0.5% and was stored RT (see Note 3.3).

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16. Bovine Serum Albumin (BSA) (Merck Life Science S.R.L., Cat# A4503-100G) was used at working concentration of 5% and was stored at 4 °C (see Note 3.3.2).
17. Dimethyl Sulfoxide (Sigma Aldrich, Cat# 67-68-5) was stored at room temperature (RT). (see Notes 3.2.2 and 3.5.1).
18. Acetic Acid glacial (Carlo Erba, Cat# 64-19-7), was stored at room temperature. (see Note 3.6).

2.3 Software

1. ImageJ (https://imagej.net/Fiji/Downloads)

2.4 Antibodies for immunostaining

See Table 1

Table 1 List of antibodies used in this study.

Primary antibody	Source organism	Company	Catalogue number
Anti-Centromere antibody	Human	Antibodies incorporated	Cat# 15-234-0001
Anti-phospho-Histone H3 (Ser10)	Mouse	Sigma Aldrich	Cat# 06-570
Anti-a-Tubulin	Mouse	Sigma-Aldrich	Cat# T9026
Secondary antibody	**Source organism**	**Company**	**Catalogue number**
Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG	Rabbit	Jackson ImmunoResearch	Cat# 715-545-152
Cy™3 AffiniPure Donkey Anti-Mouse IgG	Mouse	Jackson ImmunoResearch	Cat# 715-165-150
Alexa Fluor® 647 AffiniPure Donkey Anti-Human IgG	Human	Jackson ImmunoResearch	Cat# 709-606-149

3 Methods

3.1 Cell culture

1. Non-small cell lung cancer (NSCLC) NCI-H1975 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Lung cancer cell line A549 and

melanoma cancer cell line A375 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. All cell lines were maintained at 37 °C with 5% CO2.

2. Cells were passaged when reaching 80–90% confluence. For this, cells were washed with PBS, trypsinized and split in to 1:3/1:4 ratio. This provided cells for maintenance culture as well as for experimental procedures.
3. For the induction of chromosome misalignment, 0.5×10^5 NCI-H1975 and A549 cells, 1×10^5 A375 cells were seeded in 12-well plates containing coverslip coated with 5 mg/mL fibronectin. Coverslips were prepared by incubating them with 5 mg/mL fibronectin for 30 min at 37 °C. Excess of fibronectin was removed by rinsing coverslips three times with 1× PBS and then it was left in the incubator for use within 2 weeks. (See Notes 3.2.1 and 3.3).
4. For the generation of cell populations resistant to chemotherapeutic agents, cells were seeded at 3×10^4 or 6×10^4 in a 12-well plate. Every 4 days, controls cells were splitted and growth medium with compounds was changed to drug-treated cells. (See Notes 3.5).

3.2 Generation of chromosome mis-alignment

1. 24 h after seeding on coverslips, cells were synchronized in G2 phase using CDK1 inhibitor (RO-3306) (Fig. 2A). RO-3306 inhibits CDK1 activity reversibly and arrests cell cycle progression at the border between G2 and M phase. Thus, in order to make sure the vast majority of cells are arrested in the G2 phase of the cell cycle, the length of treatment with RO-3306 should extend for, at least, half of the doubling time of the cell line of interest.
2. After drug removal, synchronized cells undergo mitosis and within about 30/40 min, according to their mitosis duration, almost half of the cells will be in prometaphase. Therefore, RO-3306 was washed out three times by using 1× PBS and cells were released in DMSO as a vehicle control or Mps1 inhibitor reversine at several concentrations (0.125, 0.250, or 0.500 µM) for 40 min, to induce chromosome mis-alignment (Fig. 2A). Faithful chromosome segregation relies on the accuracy of chromosome alignment on metaphase plate during mitosis. Errors in this process leads to chromosome mis-segregation and the generation of aneuploid daughter cells. Several studies have shown a direct correlation between the number of mis-aligned chromosomes and the degree of aneuploidy in daughter cells (e.g., Ippolito et al., 2021) so employing micronuclei formation as a proxy of actual chromosome mis-segregation. In this respect, reversine is widely used to induce aneuploidy, since it displays a dose-dependent effect on chromosome mis-segregation and reduces correct chromosome segregation in the about 85% of treated cells.
3. To arrest cells in metaphase, the transition from metaphase to anaphase needs to be blocked. For this, it is necessary to inhibit the proteasome-mediated

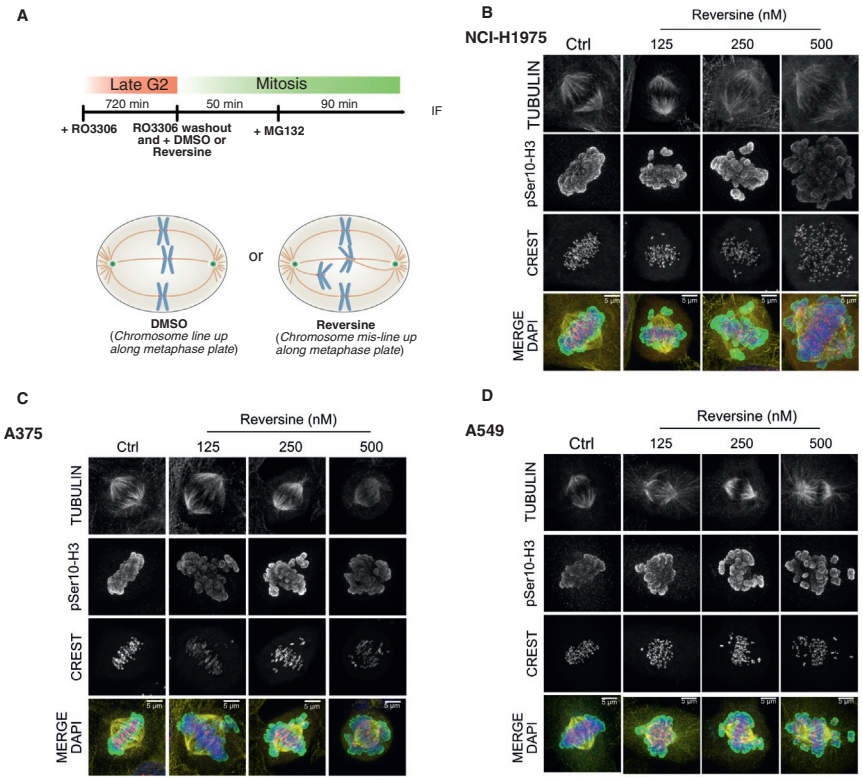

FIG. 2

(A) Schematic of metaphase plates generation. (B–D) Immunofluorescence staining of chromosome alignment of NCI-H1975, A549 and A375 cell lines treated with 0,125, 0,250, or 0,500 μM reversine or DMSO. Tubulin, CREST and Phospho-H3Ser10 proteins were stained.

degradation of cyclin B1 and securin, which induces synchronous chromosome separation in anaphase. Therefore, after DMSO or reversine treatment, cells were treated for 90 min with the proteasome inhibitor MG132 (10 μM—Fig. 2A) (Santaguida, Tighe, D'Alise, Taylor, & Musacchio, 2010).

4. Then, cells were treated with the proteasome inhibitor MG132 (10 μM for 90 min) to arrest them in metaphase (Fig. 2A).
5. Medium was then removed and cells were fixed with 4% PFA for 15 min at room temperature.
6. PFA was removed by washing cells three times with PBS and then were stored at 4 °C.

3.3 Immunofluorescence staining

1. Cells were permeabilized with 0.5% Triton X-100 diluted in PBS for 10 min at room temperature.
2. After removing permeabilization solution, cells were blocked with 5% BSA for 30 min at room temperature to reduce background signals.
3. Afterwards, incubation with primary antibodies (Tubulin 1:1500; pH3Ser10 1:400 and CREST 1:100) was performed for 90 min at room temperature. (Fig. 2B–D).
4. To remove excess antibody, cells were washed three times with PBS.
5. Then, cells were incubated at RT for 45 min with secondary antibodies (working dilution 1:400) and DAPI (1:5000) diluted in 5% BSA.
6. To remove excess secondary antibodies, cells were washed three times with PBS and then in ddH_2O for 5 min in order to remove PBS.
7. Next, water was removed and coverslips were mounted on microscope slides by using 8 μL Mowiol mounting solution.
8. Mowiol was left to polymerize over night at room temperature and then coverslips were stored at 4 °C.
9. Cells were acquired by confocal microscopy at 63× magnification.

3.4 Chromosome segregation fidelity analysis

1. To score for chromosome alignment defects, mitotic cells were obtained as outlined above and stained with antibodies against Tubulin, pH3-Ser10 and CREST (Fig. 2B–D).
2. Chromosomes were considered misaligned if their centromeric signals, as shown by CREST staining, were outside of the middle third. Cells with no alignment defects were those in which all chromosome were positioned in the middle third of the spindle (Fig. 2B–D).
3. Based on CREST signals outside of the middle third, cells were classified as showing no defects (0 chromosomes outside of middle third), mild defects (between 1 and 5 CREST signals outside of middle third), severe defects (more than 5 CREST signals outside of middle third) (Fig. 2B–D).

3.5 Crystal violet assay

1. In the absence of synchronization steps, it is advisable to treat cells with either DMSO (as a vehicle control) or Mps1i for about their cell cycle duration, in order to allow all cells to be transiting through mitosis in the presence of the drug. For this, cells at 50% of confluence were exposed to either DMSO or 0.5 μM reversine, for 30 h. (See notes 3.1.4) (Fig. 1).

2. *After wash-out*, 3×10^4 cells/well for NCI-H1975 and A549 or 6×10^4 for A375 were plated in a 12well plate support.
3. 12h later, when cells were adapted and recovered their standard proliferation, chemotherapeutic agents (Topotecan or Vemurafenib) or vehicle control (DMSO) were added for 6 weeks.
4. Every 4 days, confluent wells were split at 1:3 ratio. Growth medium containing drugs or vehicle control was replaced in the remainder of the wells.
5. At the end of the treatment, dead cells were removed by gently aspirating the supernatant. Cell monolayer was then rinsed twice with 1mL ice-cold PBS.
6. To fix the cell monolayer, ice-cold 4% PFA was gently added in each well, for 15min at room temperature.
7. Afterwards, PFA was discarded and 1% crystal violet solution was added to the wells and incubated at room temperature for 15min.
8. Plates were then washed with distilled H_2O, until the unbound crystal violet was removed and plates were dried at RT. (Fig. 3A–C).

3.6 Crystal violet intensity quantification

1. To quantify stained cells, crystal violet dye was solubilized by incubating cells with 10% Acetic Acid for 30min on an orbital shaker, until the color of the solution turned uniform.
2. As a background, a well without cells was processed in the same way as the ones with cells.
3. Later, the absorbance of the solubilized crystal violet suspension derived from each well was measured by absorbance at 600nm. The amount of Crystal Violet staining was directly proportional to the cell number contained in each well.
4. An analysis was performed to subtract the absorbance value of the wells without cells to the values of each well containing cells.

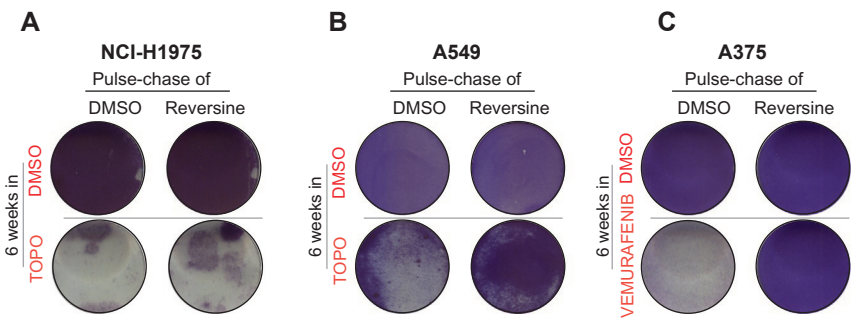

FIG. 3

(A–C) Crystal violet assay of NCI-H1975 (A), A549 (B) and A375 (C) pre-treated with DMSO or 0,500 μM reversine for 30h and then treated with 0,1 μM Topotecan or 1 μM Vemurafenib, for 6 weeks.

5. For the colony assay, every plate was scanned to obtain digital images of the colonies by using a scanning device.
6. Colonies could be manually counted by using FIJI software.

4 Notes

1. All cell lines used in this method are p53 wt. However, the p53 status of the cells should not affect the ability to induce chromosome mis-segregation. Furthermore, another study have identified that chromosomal instability accelerates chemoresistance also in p53 mutated cell lines (Lukow et al., 2021).
2. Before seeding cells, coverslips should be coated with fibronectin in order to allow attachment of cells, correct proliferation, and thus have an efficient synchronization.
3. In order to obtain a reversible arrest in late G2, RO3306 concentration needs to be set for each cell line utilized, due to intrinsic differences of cell cycle of each cell type.
4. Since each cell line recovers from RO3306 arrest with different kinetics, the number of washes to remove RO3306 needs to be correctly optimized for each cell line.
5. We found that ~50 min after RO3306 release is the appropriate time needed to enrich for metaphase plates in NCI-H1975, A549, and A375 cancer cell lines.
6. During the immunostaining, the fluorescent species must be stored carefully at the recommended temperature and kept in the dark throughout the procedure to protect their integrity.
7. To determine chromosome mis-alignment, multiple focal planes were acquired. Manual inspection of each single focal plane was used to determine and count the number of mis-aligned chromosomes per cell.
8. An optimal degree of chromosomal instability is required to successfully gain chemoresistance, therefore reversine concentration needs to be optimized for each cell type and its effects possibly tested by karyotype analysis.
9. In order to obtain all cells undergoing chromosome mis-segregation, the treatment time of reversine needs to be set according to the doubling time of each cell line.
10. The concentration of chemotherapeutic agents used for the chemoresistance assay must be sublethal. Therefore, it is important to calculate the half-maximal concentration (EC50) of each specific drug for each cell line.
11. Crystal violet as well as chemotherapeutic agents are toxic. They both can cause permanent damage if they come into direct contact with eyes or skin. Thus, they must be used with gloves under an appropriate hood.
12. PFA used to fix cells must be carefully employed under fume hood to minimize inhalation of formaldehyde vapor.
13. Be careful when handling the cells during the washes with PBS and fixation with PFA before the crystal violet staining. The cell monolayer could easily detach.

Acknowledgments

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