HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2016 November 02.

Published in final edited form as:

Methods Mol Biol. 2016 ; 1413: 393–401. doi:10.1007/978-1-4939-3542-0_24.

Generation and Purification of Tetraploid Cells

Elizabeth M. Shenk and **Neil J. Ganem**[1,*]

[1]Department of Pharmacology & Experimental Therapeutics, Department of Medicine, Division of Hematology and Oncology, Boston University School of Medicine, Boston, MA 02118, USA

Abstract

Tetraploid cells are genetically unstable and have the capacity to promote the development and/or progression of human malignancies. It is now estimated that ~40% of all solid tumors have passed through a tetraploid intermediate stage at some point during their development. Understanding the biological characteristics of tetraploid cells that impart oncogenic properties is therefore a highly relevant and fundamentally important aspect of cancer biology. Here, we describe strategies to efficiently generate and purify tetraploid cells for use in cell biological studies.

Keywords

Cytokinesis; mitotic slippage; polyploid; Hippo; FUCCI; centrosome

1 Introduction

The vast majority of non-transformed human cells contain two copies of each chromosome and are termed diploid. In preparation for cell division, all chromosomes are replicated so that they may be evenly distributed to two daughter cells during mitosis. However, catastrophic failures in mitosis or cytokinesis can give rise to tetraploid cells, which have a doubled DNA content (4 copies of each chromosome). The generation of tetraploid cells through non-programmed mechanisms can have significant consequences, as spontaneously arising tetraploid cells are chromosomally unstable and have the capacity to promote tumorigenesis (1–9). It is now recognized that tetraploidization events are common in solid tumors, and correlate with poor prognosis (10, 11).

Studying the biology of tetraploid cells can allow us to more fully understand how they promote tumor progression. However, given their rarity in mixed populations of cells, it requires a rapid and efficient method to generate and purify them. Several methods currently exist to generate tetraploid cells. These include fusing diploid cells using polyethylene glycol (12); inhibiting cytokinesis using cell permeable small molecules that prevent furrow ingression and cleavage (4, 12–15); and promoting mitotic slippage by using small molecules that prevent satisfaction of the spindle assembly checkpoint (16). Of these methods, inhibiting cytokinesis is the simplest and most effective way to generate tetraploid cells. In contrast to cell fusion experiments, which are highly inefficient and time

[*]Correspondence: Neil J. Ganem, The Cancer Center, Boston University School of Medicine, 72 E. Concord St. K-702, Boston, MA 02118, Phone: (617) 638-5382, nganem@bu.edu.

consuming, or induction of mitotic slippage, which first requires a dramatically prolonged mitosis that produces extensive DNA damage and even cell death (17, 18), inhibiting cytokinesis with small molecules can be achieved on large populations of cells without inducing mitotic abnormalities or DNA damage (15, 19, 20). The most commonly used small molecules used to inhibit cytokinesis include blebbistatin, a myosin II inhibitor, and the cytochalasins, which disrupt actin polymerization (14, 21). These compounds prevent cytokinetic furrow ingression and cell cleavage following anaphase, and thus lead to the formation of binucleated tetraploid cells.

While several simple methods exist to generate tetraploid cells, purifying tetraploids from a predominantly diploid population of cells poses a significant technical challenge. One approach is size separation. However, although tetraploid cells are larger than diploid cells, purification strategies based on cell size are inefficient (our unpublished data). Moreover, DNA content alone cannot distinguish diploid from tetraploid cells because diploid cells in G_2/M phase of the cell cycle (which have replicated their chromosomes) have the same amount of DNA as tetraploid cells in G_1 phase of the cell cycle (both contain 4C DNA content). While tetraploid cells that progress to G_2/M and possess 8C DNA content can be readily distinguished from diploids, these cells are relatively rare because non-transformed tetraploids exhibit markedly reduced proliferation (13, 15).

Here, we describe an approach to generate and purify tetraploid cells with high efficiency using the fluorescent, ubiquitin-based, cell cycle indicator (FUCCI) system (22). FUCCI consists of two fluorescently labeled proteins, truncated forms of hCdt1 (consisting of amino acids 30 to 120) and hGeminin (consisting of amino acids 1 to 110), whose expression alternates based on cell cycle progression. hCdt1 (fused to a red-fluorescent protein) is a DNA replication licensing factor that is present during G_1 phase but is ubiquitinated by SCF^{Skp2} and degraded during S/G_2/M phases. hGeminin (fused to a green-fluorescent protein) is a negative regulator of DNA licensing that is present during S/G_2/M phases but is ubiquitinated by APC^{Cdh1} and degraded at the end of mitosis and throughout G_1 (*see* Note [1]). Thus, FUCCI provides a simple, fluorescence readout of cell cycle position. This system provides a critical tool in overcoming the technical barrier of isolating tetraploids from diploids: FUCCI can be used to discriminate G_1 tetraploids from G_2/M diploids, both of which possess 4C DNA content, because G_1 tetraploids emit red fluorescence while G_2/M diploids emit green fluorescence.

2 Materials

The following materials and equipment are used in this protocol:

2.1 Cell Culture

1. 10 cm^2 polystyrene tissue culture plates.
2. 15 cm^2 polystyrene tissue culture plates.

[1]The FUCCI system was developed by Dr. Atushi Miyawaki's group at the laboratory for Cell Function and Dynamics at the Riken Brain Science Institute and is commercially available through many sources. Information about the constructs can be found at: http://cfds.brain.riken.jp/Fucci.html.

3. Phenol red-free DMEM:F12 media supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (*see* Note 2).
4. 0.25% Trypsin/EDTA.
5. Sterile phosphate-buffered saline (PBS).
6. Cell line of interest expressing the FUCCI reporter system (here we use the telomerase-immortalized human retinal pigment epithelial cell line RPE-1, from ATCC). It is also important to have unlabeled cells, cells expressing hCdt1-RFP alone, cells expressing hGem-GFP alone, and cells expressing the complete FUCCI system (both hCdt1-RFP and hGem-GFP, which we will refer to as RPE-1 FUCCI)(*see* Note 3).
7. Dihydrocytochalasin B (10 mM stock in DMSO).

2.2 Fluorescence Activated Cell Sorting

1. Hoechst 33342 (10 mg/ml in water).
2. Polystyrene round bottom tubes with cell strainer caps (35 μM nylon mesh).
3. Sterile 15 ml conical tubes.
4. Sterile 50 ml conical tubes.
5. 0.05% Trypsin/EDTA.
6. Aluminum foil.

2.3 Equipment

1. Tissue culture incubator set at 37°C with 5% humidified CO_2.
2. Tissue culture hood.
3. Hemocytometer.
4. High-speed centrifuge.
5. Phase contrast microscope equipped with a 10× objective for cell counting.
6. Standard epifluorescence microscope equipped with excitation and emission filters necessary to visualize fluorescence in three colors (e.g. DAPI/FITC/TRITC).

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[2]The use of phenol-red free medium is not required, however, the absence of phenol red decreases background fluorescence and improves the efficiency of FACS sorting.

[3]Both lentiviral and retroviral vectors expressing components of the FUCCI system can be used to generate stable cell lines. Live-cell imaging should be used to confirm that the reporter constructs are cycling properly. Both hCdt1-mCherry and hGem-AzamiGreen should localize exclusively to the nucleus (except during mitosis, when the nuclear membrane breaks down and hGem-AzamiGreen becomes diffuse throughout the cytoplasm).

7. FACS machine equipped with a UV laser (355 nM), a 488 nM laser, and a 561 nM laser, and a 100 μM nozzle (*see* Note 4).

3 Methods

The following protocol describes the method used to generate and purify tetraploid RPE-1 cells using the FUCCI reporter system. In addition to growing RPE-1 FUCCI cells (hCdt-mCherry and hGem-AzamiGreen), control RPE-1 cells expressing hCdt-mCherry alone, hGem-AzamiGreen alone, and unlabeled RPE-1 cells must also be carried (*see* Note 5). These will be used to calibrate the FACS machine.

3.1 Cell Culture

1. Use freshly thawed and early passage cells for all experiments. The cells should be maintained on 10 cm^2 polystyrene tissue culture plates in phenol red-free DMEM:F12 media supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Maintain the cells at 40–80% confluence. If the cells grow beyond 80% confluence, discard them and thaw fresh cells. Maintain cells for only 1–2 months before thawing fresh cells.
2. To set up a tetraploid purification experiment, expand RPE-1 FUCCI cells from 10 cm^2 tissue culture maintenance dishes into 15 cm^2 dishes. Aspirate medium from each 10 cm^2 tissue culture dish, wash with sterile PBS, and add 2.5 ml of 0.25% Trypsin/EDTA. Incubate the cells in a tissue culture incubator at 37°C for ~5 min or until most of the cells have detached.
3. Collect the cells in 10 ml of complete medium to inactivate the trypsin, and then pellet the cells for 5 min at 280 × *g* in a high-speed centrifuge.
4. Thoroughly resuspend the cells in 10 ml of complete medium. Count the cells with a hemocytometer.
5. Plate 6 × 10^6 RPE-1 FUCCI cells per 15 cm^2 dish. In general, one seeded 15 cm^2 dish will ultimately yield ~0.5–1 × 10^6 purified tetraploid cells following FACS. Scale up the number of dishes as needed. In addition, plate 2 × 10^6 unlabeled RPE-1 cells, RPE-1 cells expressing hCdt-mCherry, and RPE-1 cells expressing hGem-AzamiGreen into separate 10 cm^2 dishes. These will be used to calibrate the FACS machine.

[4]A variety of nozzle sizes are available for sorting cellular resuspensions on FACS machines. Because of the increased size of tetraploid cells, we have found that sorting with the larger 100 μM nozzle leads to far less nozzle clogging during the sorting procedure.

[5]The original FUCCI reporters consisted of hCdt1(30/120) fused to the fluorescent protein Kusabira orange-2, and hGem(1/110) fused to the fluorescent protein AzamiGreen. Kusabira orange-2 maximally absorbs light at 551 nm and emits light at 565 nm, while AzamiGreen maximally absorbs light at 492 nM and emits light at 505 nM. A second generation version of FUCCI consists of hCdt1(30/120) fused to mCherry (absorbs light at 587 nm and emits at 610 nm), and hGem(1/110) fused to mVenus (absorbs light at 515 nm and emits at 528 nm). We found that pairing hCdt1(30/120)-mCherry with hGem(1/110)-AzamiGreen is the most ideal combination, as the mCherry and AzamiGreen emission spectra are the most spatially separated and thus exhibit the least amount of bleed-through fluorescence.

3.2 Generating Tetraploid Cells

Multiple approaches have been developed to generate tetraploid cells *in vitro.* These include inhibiting cytokinesis, promoting mitotic slippage, or fusing diploid cells. This protocol will focus on use of the cell permeable mycotoxin dihydrocytochalasin B (DCB), which disrupts actin polymerization and thus causes cytokinetic cleavage furrow regression, cytokinesis failure, and tetraploidy. This approach is beneficial for a number of reasons. First, this highly potent compound can be added to an entire population of proliferating cells in order to generate a significant number of tetraploid cells. Second, this compound does not disrupt mitotic spindle assembly or the efficiency of chromosome segregation, and does not produce DNA damage. This is in contrast to drugs (*e.g.* Taxol, nocodazole), which induce tetraploidy by promoting prolonged mitotic arrest and mitotic slippage by preventing inactivation of the spindle assembly checkpoint. Finally, DCB is a reversible drug that can be washed from cells, thus enabling the inhibition of cytokinesis during only a single cell cycle. This is a major benefit over knocking out or knocking down the expression of genes essential for cytokinesis, which will induce repeated cytokinesis failures. To generate tetraploid cells:

1. Dilute DCB to 4 μM (from a 10 mM stock) in complete 20 ml growth medium and mix thoroughly. Add the growth medium containing DCB to each of the RPE-1 FUCCI cells plated on 15 cm^2 dishes the previous day (using a 10× objective on a phase contrast microscope, confirm that cells are 50–60% confluent at the time of DCB addition)(*see* Note 6).
2. Incubate the cells in DCB for 16 h in a 37°C tissue culture incubator (*see* Note 7). It should be noted that since DCB disrupts the actin cytoskeleton, the morphology of the cells will be dramatically and visibly altered: the cells will have ruffled edges and will be less spread on the tissue culture dish.
3. Aspirate the medium and gently rinse the cells with sterile PBS.
4. Wash the cells 5 times (5 min each time) in pre-warmed growth medium. These washes are essential to completely remove residual DCB from the cells. The DCB-treated cells should flatten out and assume a normal looking morphology immediately following the completion of these washes. At this point, ~40–60% of the cells should be visible as binucleated tetraploids under a phase contrast microscope.

3.3 Purifying Tetraploid Cells by FACS

1. Following the last wash, add complete growth medium containing 4 μg/ml Hoechst dye (to label the DNA) to the RPE-1 FUCCI cells. In addition, add growth medium containing 4 μg/ml Hoechst dye to the 10 cm^2 dishes

[6]The confluence of cells is critically important at the time of DCB addition. Because DCB disrupts the actin cytoskeleton, sparsely plated cells are more likely to become detached from the tissue culture dish. Conversely, if cells are too dense at the time of DCB addition, cells may reach overconfluence by 16 h.

[7]Disruption of the actin cytoskeleton by DCB will prevent cells in early G_1 phase from entering S-phase. Thus, only cells in late G_1, S, G_2, or M phases of the cell cycle will proceed through mitosis and fail cytokinesis following DCB treatment. Incubation of cells with DCB for 16 h is recommended because that is the approximate duration of the cell cycle in RPE-1 cells. Longer treatments in DCB will not produce additional tetraploid cells.

seeded with the unlabeled control RPE-1 cells, RPE-1 cells expressing hCdt-mCherry, and RPE-1 cells expressing hGem-AzamiGreen. Incubate all dishes at 37°C for 30 min.

2. Aspirate the medium from each tissue culture dish, wash with sterile PBS, and add 6 ml of 0.05% Trypsin/EDTA to each 15 cm^2 dish (2.5 ml of Trypsin to the 10 cm^2 control dishes). Incubate the cells in the tissue culture incubator at 37°C for ~5 min or until the cells have detached (*see* Note 8).

3. Collect the cells in 10 ml of complete medium to inactivate the trypsin, then pellet the cells for 5 min at 280 × *g* in a high-speed centrifuge.

4. Resuspend the pelleted cells in complete medium containing 4 μg/ml Hoechst (for cells from one 10 or 15 cm^2 dish, resuspend in 200 μl of medium; for each additional 15 cm^2 dish, add 50 μl to the resuspension volume). Resuspend the pellets thoroughly by slowly pipetting up and down with a p1000 pipette tip (~50 times).

5. To remove clumps, strain the resuspended cells through 35 μM nylon strainer caps into round bottom FACS tubes wrapped in aluminum foil. The cells are now ready to be FACS-sorted.

6. Calibrate the FACS machine with the control cell lines. First, run the unlabeled diploid RPE-1 cells stained for Hoechst in order to calibrate the UV laser (355 nm) and produce sharp 2C and 4C peaks (Figure 1A). Second, run RPE-1 cells expressing hGem-AzamiGreen to define and gate $AzamiGreen^+$ cells (use the 488 nm laser). Third, run RPE-1 cells expressing hCdt1-mCherry alone to define and gate $mCherry^+$ cells (use the 561 nm laser).

7. Once the FACS machine is properly calibrated, sort $mCherry^+$/$AzamiGreen^-$ 2C cells (these represent G_1 diploids) into one 15 ml conical tube containing 5 ml of complete growth medium (Figure 1B). Sort $mCherry^+$/$AzamiGreen^-$ 4C cells (these represent G_1 tetraploids) into a separate 15 ml conical tube containing 5 ml of complete growth medium (Figure 1B). Both collection tubes should be wrapped in aluminum foil. To ensure maximum viability, the FACS sorting should take no longer than 1 h (*see* Note 9). To limit clumping and/or settling of cells during the FACS sort, the cellular resuspension should be briefly vortexed every 5 minutes.

8. Pellet the sorted cells for 5 min at 280 × *g* in a high-speed centrifuge, resuspend them in complete growth medium, and then count/plate into tissue culture dishes as needed. To assess purity, the sorted cells can be visualized by fluorescence microscopy once they have attached to the

[8]We have observed that using 0.05% Trypsin instead of 0.25% Trypsin reduces the amount of cell clumping during FACS.
[9]1 h is generally sufficient time to FACS sort cells from as many as 16 separate 15 cm^2 dishes. However, if more cells are required, it is recommended to use two FACS machines or stagger the experiments in order to limit the amount of time cells are kept in suspension.

tissue culture dish (this takes ~1–3 h). Tetraploid cells, which are mCherry^{+} and AzamiGreen^{-}, will appear as binucleated cells exhibiting red fluorescence (Figure 1C, right panel). Because non-transformed tetraploid cells activate the Hippo tumor suppressor pathway and do not proliferate well, there will be few binucleated cells exhibiting green fluorescence (indicative of S-phase entry). By contrast, diploid cells will be mononucleated and exhibit both red and green fluorescence, as these cells resume proliferation immediately following the sorting procedure (Figure 1C, left panel). In general, the tetraploid population should be 85–95% pure.

Acknowledgments

N.J.G is a Karin Grunebaum Cancer Research Foundation Fellow in the Shamim and Ashraf Dahod Breast Cancer Research Laboratories and is supported by grants from the Richard and Susan Smith Family Foundation, the Searle Scholars Program, the Melanoma Research Alliance, the Skin Cancer Foundation, the Sarcoma Foundation of America, and the NIH/NCI (K99/R00 CA154531-01). Elizabeth Shenk is supported by a T32 grant from NIGMS (GM008541).

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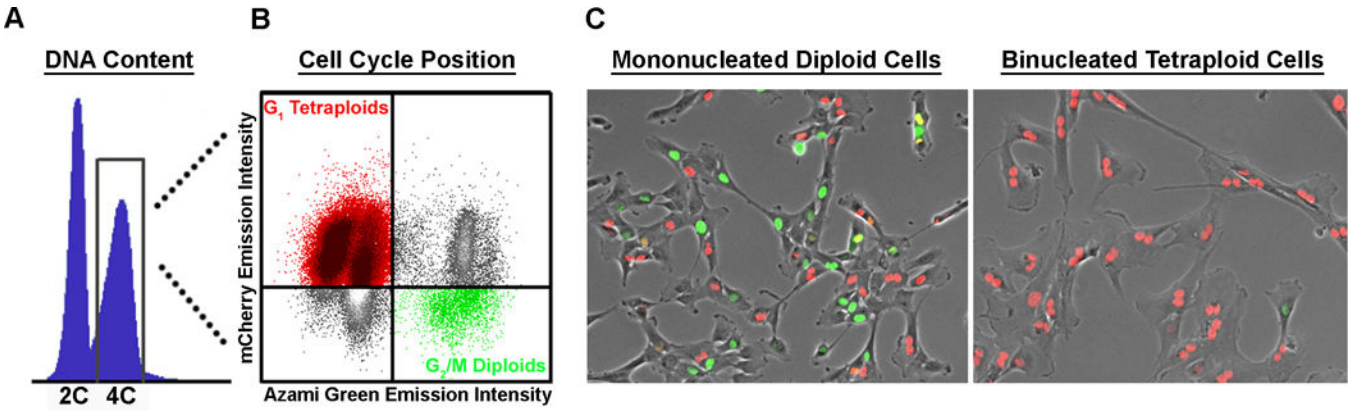

Figure 1. FACS isolation of tetraploid cells

(A) A representative FACS profile of DNA content from RPE-1 FUCCI cells following 16 h DCB treatment. The 2C peak contains G_1 diploids while the 4C peak contains both G_2/M diploids and G_1 tetraploids. **(B)** Diploid and tetraploid cells within the 4C peak are distinguished by assessing mCherry (y-axis) and AzamiGreen (x-axis) fluorescence intensity. Tetraploid cells in G_1 are $mCherry^+$ and $AzamiGreen^-$ (top left quadrant), while diploid cells in G_2/M are $mCherry^-$ and $AzamiGreen^+$ (bottom right quadrant). **(C)** Sorted tetraploid cells generated by DCB treatment are binucleated (right panel) and can be easily distinguished from mononucleated diploid cells (left panel) by phase contrast and fluorescence microscopy.