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Abstract	Cell synchronization is crucial when studying events that take place at specific points of the cell cycle. Several chemical agents can be used to achieve the cell culture synchronization but not all type of cells respond equally to a given concentration of these drugs. Here we describe a simple optimization method to select concentrations and timings for nocodazole or thymidine treatments using fluorescence staining. In addition, we provide detailed protocols to arrest an asynchronous culture of either suspension or adherent cells in G_1/S or in G_2/M.

Keywords (separated by '-')	Cell cycle - Synchronization - Nocodazole - Thymidine - Suspension cells - G_1/S - G_2/M

Chapter 9

Optimizing Cell Synchronization Using Nocodazole or Double Thymidine Block

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Abstract

Cell synchronization is crucial when studying events that take place at specific points of the cell cycle. Several chemical agents can be used to achieve the cell culture synchronization but not all type of cells respond equally to a given concentration of these drugs. Here we describe a simple optimization method to select concentrations and timings for nocodazole or thymidine treatments using fluorescence staining. In addition, we provide detailed protocols to arrest an asynchronous culture of either suspension or adherent cells in G_1/S or in G_2/M.

Key words Cell cycle, Synchronization, Nocodazole, Thymidine, Suspension cells, G_1/S, G_2/M

1 Introduction

Cell proliferation is a highly regulated process, key for the growth and development of any organism. Cell proliferation involves the division of one cell into two identical daughter cells, in a process termed "mitosis" and the stages a cell undergoes for this to happen are called "phases of the cell cycle." During the cell cycle, the phases involving duplication of the genetic material (S phase) and segregation into two daughter cells (M phase) are intermitted by three gap phases (G_0, G_1, and G_2). The sequential transition through these five phases ($[G_0] \rightarrow G_1 \rightarrow S \rightarrow G_2 \rightarrow M \rightarrow [G_0]$) defines the eukaryotic cell cycle [1], and a complex transcriptional and post-translational regulatory system coordinates critical molecular and biochemical events for the progression through cell division.

There are many reasons to study the cell cycle and having an interest in gene and protein expression is one of them: a periodic gene expression pattern has been confirmed in different eukaryotic cells including yeast [2], primary human fibroblast [3], and immortal human HeLa cells [4]. Furthermore, chemotherapy has been

Amanda S. Coutts and Louise Weston (eds.), *Cell Cycle Oscillators: Methods and Protocols*, Methods in Molecular Biology, vol. 2329, https://doi.org/10.1007/978-1-0716-1538-6_9,

shown to modulate the global gene expression profile and cell cycle kinetics [5]. But in order to detect and study these temporal changes in the transcriptomic and proteomic cells' profile, it is imperative to use a synchronous population of cells.

Cell synchronization is a method that brings cultured cells at different phases of the cell cycle to the same stage. The synchronized cells progress through the phases of the cell cycle as a relatively uniform cohort, aiding in the understanding of the changes occurring during a particular phase.

Different approaches have been used for batch synchronization of a cell culture and could be broadly classified into physical and chemical methods. Physical methods separate the cells in a particular phase of the cell cycle based on cell size, density, fluorescent labeling, light scattering, and/or attachment to the growth matrix (e.g., flask). Commonly used physical methods for cell synchronization include centrifugal elutriation, fluorescent-activated cell sorting, mitotic detachment, and contact inhibition. These physical methods, unlike chemical methods, have minimal effects on cell metabolism and allow cells to progress through different cell cycle phases without any perturbations. However, these methods are not universally applicable to different cell lines and synchronising the cells based on size (centrifugal elutriation) is limited by variability in cell synchrony, expensive instrumentation and technical complexities [6, 7].

Chemical methods involve the use of pharmacological agents which block the progression of the cells at a specific phase. Common drugs that arrest the cells in S phase include excess thymidine, aphidicolin, and methotrexate. These drugs affect the synthesis of DNA and inhibit DNA replication [8]. Excess thymidine has been shown to allosterically inhibit ribonucleotide reductase, altering the deoxyribonucleotide pool and halting DNA replication [9]. It arrests the cells at the G_1–S boundary and synchronizes the cell cycle at early S phase following release [10]. Consecutive exposure to thymidine increases the population of synchronous cells [8]. The initial exposure to excess thymidine for 24 h halts the cells at the S phase of cell cycle. Following the release, the cells arrested in G_1/S and early S phase would progress through G_2/M phase and cells blocked in late S phase would reenter G_1 phase. The repeated exposure to thymidine would collect most of the cell population at G_1–S interface and the release would result in cells progressing through the cell cycle synchronously.

Another category of chemical blockade is mediated by drugs that disrupt polymerization of microtubules that form the mitotic spindle, and is comprised of nocodazole, colcemid, and colchicine. These agents prevent cytokinesis, arresting the cells in G_2/M phase [11, 12]. Cells could be also arrested at G_0/G_1 phase by serum starvation, depletion of isoleucine in the medium or using lovastatin (HMG-CoA reductase inhibitor) [13].

It has been argued that the use of chemical agents for synchronization is associated with undesirable side effects such as cytotoxicity, growth imbalance, disruption of metabolic processes and deregulated expression of cyclin proteins [14–16]. It is therefore of upmost importance, that cell survival is asserted when choosing any of these chemical agents, assessing their concentration and time of exposure.

Here, we describe the steps necessary to optimize cell cycle synchronization using nocodazole or double thymidine block in a concise manner so the readers can adapt these to their particular cells of interest. We use the chronic myeloid leukemia cell line TCC-S that grows in suspension, and later provide modifications to the protocol necessary when using adherent cells, using the prostate cancer cell line DU145 and the epithelial HeLa S3 cells as examples.

2 Materials

2.1 Cell Cycle Drugs

1. 100 mM Thymidine: Dissolve 242.2 mg of thymidine in 100 mL of autoclaved double-distilled water, place in a water bath at 37 °C to ensure complete dissolution of thymidine, sterilize the solution by passing through a 0.2 μm filter and store in aliquots at −20 °C.
2. 1 mg/mL Nocodazole: Dissolve 10 mg of nocodazole in 10 mL cell-culture grade DMSO. Aliquot and freeze (−20 °C) for up to 1 year.

2.2 DNA Staining for Viability or FACS Analysis

1. ReadyProbes® Cell Viability Imaging Kit, NucBlue/NucGreen (ThermoFisher Cat. No. R37609).
2. 70% (v/v) ethanol: Take 70 mL of absolute ethanol (*see* **Note 1**) and add distilled water to bring the volume to 100 mL. Store at −20 °C.
3. 1 mg/mL RNase A solution: Dissolve 10 mg of RNase A (DNase-free RNase A) in 10 mL of nuclease-free water. Aliquot in 1.5 mL microcentrifuge tubes and store at −20 °C.
4. 1 mg/mL Propidium Iodide (PI, *see* **Note 2**): Prepared in double-distilled water, store in the dark at 4 °C.
5. PI staining solution: 250 μL of staining solution is required per sample. It is made by mixing 17.5 μL of PI solution, 35 μL of RNase A solution and 197.5 μL of PBS. The solution should be prepared fresh and used immediately. The final concentration of PI and RNase A used per sample are 50 and 100 μg/mL, respectively.
6. FACS tubes (Sarstedt, catalog number: 55.1578).
7. Beckman Coulter™ ISOTON™ II Diluent (Fischer Scientific, product code: 12754878).

2.3 Cell Culture

Cells are routinely grown in a humidified atmosphere at 37 °C with 5% CO_2.

1. Cell lines: TCC-S, suspension cell line grown in complete RPMI medium; DU145 and Hela S3, adherent cell lines grown in complete DMEM medium.
2. Complete RPMI medium: RPMI 1640 with 25 mM HEPES and L-glutamine (e.g., Lonza Cat. No. LZBE12-115F) supplemented with 10% FBS.
3. Complete DMEM medium: Dulbecco's Modified Eagle's Medium (DMEM) with high glucose and GlutaMAX™ Supplement (Thermo Fischer Scientific Cat. No. 10566016), supplemented with 10% FBS.
4. Sterile 1× Phosphate-Buffered Saline (PBS).
5. Trypsin-EDTA (0.25%).

3 Methods

Whilst the thymidine concentration used for cell cycle arrest is quite standard (2 mM) for a variety of cells, the range of concentrations for nocodazole is wider (40–200 ng/mL) as nocodazole exposure can affect cell viability. Therefore, it is essential that suitable concentrations are determined on each cell line of interest prior to attempting cell synchronization.

3.1 Preliminary Assessment of Nocodazole Concentrations

1. Seed TCC-S cells in a 24-well plate at a concentration of 0.5×10^6 cells/mL, using 500 µL per well.
2. Leave cells incubating overnight.
3. Treat three wells with nocodazole to a final concentration of 100 ng/mL and another three wells to a final concentration of 200 ng/mL (*see* **Note 3**). Add also the equivalent volume of DMSO to three wells (controls). This will be the 24 h treatment time.
4. Four hours later, treat another six wells in the same manner. This will be the 20 h point.
5. Four hours later, treat another six wells in the same manner. This will be the 16 h point.
6. The next day (24 h after the first treatments started) add 1 drop of each NucBlue® Live and NucGreen® Dead dyes to individual wells.
7. Incubate for 30 min at room temperature, covering the plate with foil to protect from light.
8. Assess cell staining under a fluorescence microscope using standard DAPI (live cells) and GFP (dead cells) filters (Fig. 1). Choose the concentration with the least effects on viability (lower proportion of green signal).

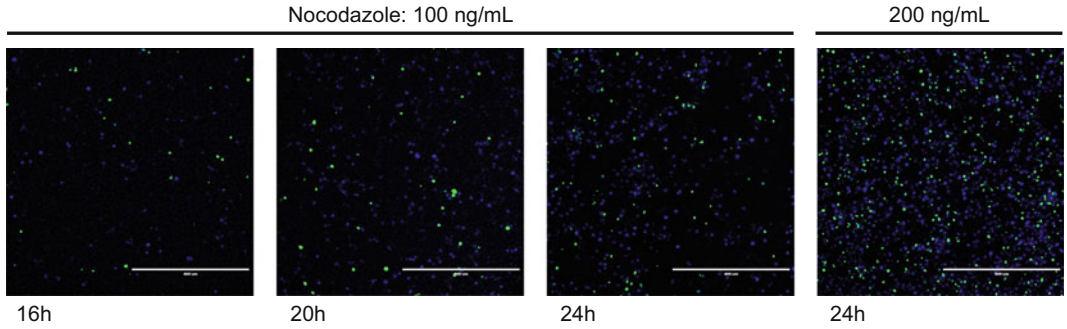

Fig. 1 Nocodazole treatments. TCC-S were treated with nocodazole at 100 or 200 ng/mL for 16–24 h and the cell viability was assessed with the ReadyProbes™ Cell Viability Imaging Kit. Green cells represent cells with a compromised cell membrane (dead) whilst live cells are stained blue. Cell viability clearly decreases with prolonged times of exposure and with the higher nocodazole concentration

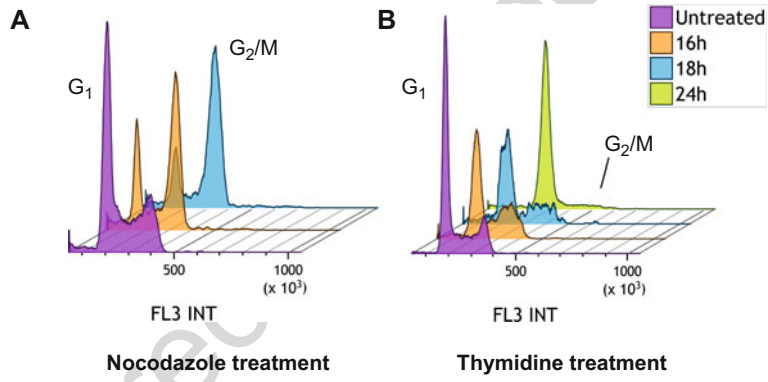

Fig. 2 Optimizing (**a**) nocodazole and (**b**) thymidine treatment time. In TCC-S cells, treating the cells with nocodazole (100 ng/mL) for 18 h was the option that produced a lower G1 peak and arrested the highest number of cells in the G_2/M phase. In the case of thymidine, the treatment time of 24 h was the most successful at arresting the cells at the G_1–S boundary AU1

3.2 Selection of Nocodazole and Thymidine Timings

All centrifugations are performed at room temperature unless otherwise stated. The steps described correspond to one set of experiments (*see* Fig. 2)—be sure to perform in duplicate or triplicate, as needed.

1. Seed TCC-S cells at a concentration of 1×10^6 cells/mL in four T25 flasks with 8 mL of complete RPMI medium and incubate overnight (*see* **Note 4**).
2. In two of the flasks, add either 8 μL of DMSO (control, asynchronous culture) or 8 μL of 1 mg/mL nocodazole to achieve a final concentration of 100 ng/mL in the flask, and return to incubator. Remove 2 mL of the cell suspension from each flask 14, 16 and 18 h after treatment, transferring cells to individual 15 mL tubes and continuing the process at **step 9**.

3. To the remaining two flasks, add either 160 µL of autoclaved water (control, asynchronous cells) or 160 µL of the thymidine stock (100 mM) to achieve a 2 mM thymidine concentration in the culture (first block), and incubate for 24 h.
4. After 24 h, collect the cell suspension from the flask and centrifuge at 200 × *g* for 5 min to pellet cells. Discard supernatant.
5. Wash the cells with sterile PBS and centrifuge at 200 × *g* for 5 min to pellet cells. Discard supernatant.
6. Reseed the entire cell pellet in the same volume of fresh media (8 mL) without thymidine (block release) and incubate for 12 h.
7. Add 160 µL of the thymidine stock (100 mM) to the synchronized flask (second block) and 160 µL autoclaved water to the control flask and incubate for 24 h.
8. Remove 2 mL of the cell suspension from control and treated cell culture flasks and transfer to individual 15 mL tubes.
9. Centrifuge at 200 × *g* for 5 min to pellet the cells then decant supernatant and flick to resuspend cells.
10. Add 2 mL of PBS, mix cells, centrifuge at 200 × *g* for 5 min, decant supernatant and flick to resuspend cells.
11. Add 500 µL of ice-cold 70% ethanol to all tubes to fix the cells and store at −20 °C for a minimum of 30 min (*see* **Notes 5** and **6**).
12. Centrifuge to pellet the cells at 300 × *g* for 5 min. Then carefully remove the ethanol supernatant with a pipette.
13. Wash with 2 mL PBS then centrifuge at 300 × *g* for 5 min, decant the supernatant and flick to resuspend cells.
14. Repeat wash with 2 mL PBS, then centrifuge (300 × *g*, 5 min) decant supernatant and flick to resuspend cells.
15. Add 100 µL PBS, resuspend the cells and transfer 100 µL to a labeled FACS tube.
16. Add 250 µL of PI staining solution to the cell suspension in the FACS tube and incubate for 30 min at room temperature in the dark (*see* **Notes 7** and **8**).
17. Transfer the cells to FACS tubes.
18. Add 100 µL ISOTON™ diluent and analyze on a flow cytometer (in our case, Beckman Coulter Gallios).
19. Optimize flow cytometer acquisition settings using unstained cells and asynchronous PI stained cells. The flow rate should be set to low and the acquisition rate should not exceed 500 cells/s.

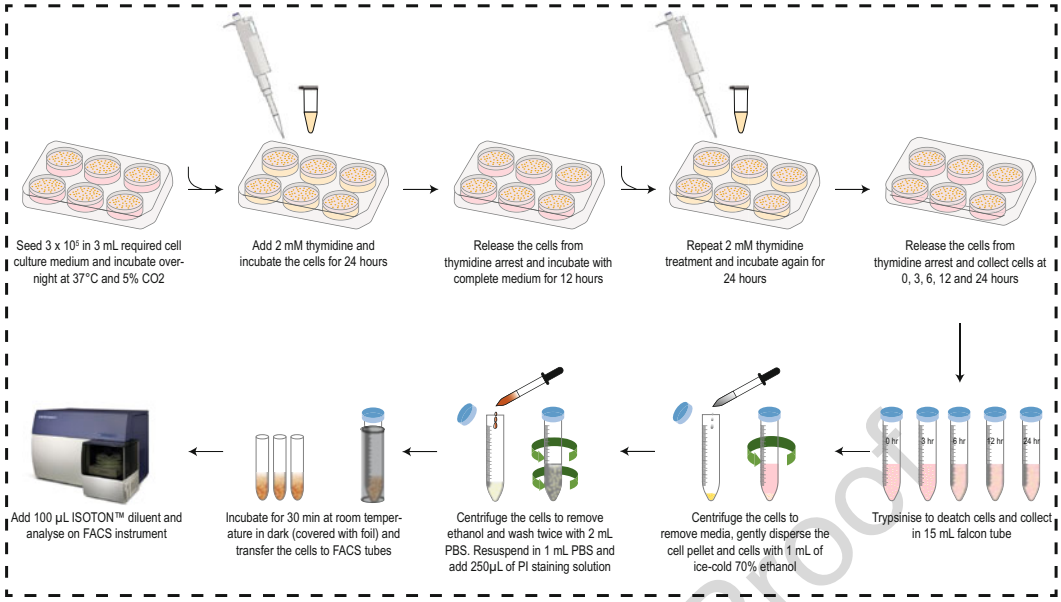

Fig. 3 Graphical representation of the double thymidine block cell synchronization process for adherent cell line DU145

3.3 Collection of Cells After Release

Once the cell culture is synchronized and the chemical removed (release step), cells can be collected every 2–3 h, or as often as desired, and analyzed following **steps 8–19** in the protocol above to assess the progression of the cells through the cell cycle or to collect RNA or protein samples. Here, we provide an adapted protocol for adherent cells synchronization (*see* Fig. 3) and we assess the outcome of the cell cycle progression in Fig. 4 (after double thymidine block) and in Fig. 5 (after nocodazole treatment).

1. Prepare the required cell culture medium and warm it up to 37 °C before use.
2. For DU145, plate the cells at 3×10^5 cells per well in 3 mL of complete prewarmed medium. This should result in approximately 30–40% confluency the following day. The seeding density needs to be optimized for the cell line under investigation.
3. Leave the cells in the incubator overnight.
4. Add 2 mM thymidine (6 µL from 100 mM stock) in each sample well. Add 6 µL of autoclaved double distilled water in the control well as vehicle control. Incubate the cells for 24 h.
5. After incubation, release the cells from thymidine by washing twice with 2 mL of prewarmed PBS. Incubate with complete medium for 12 h.

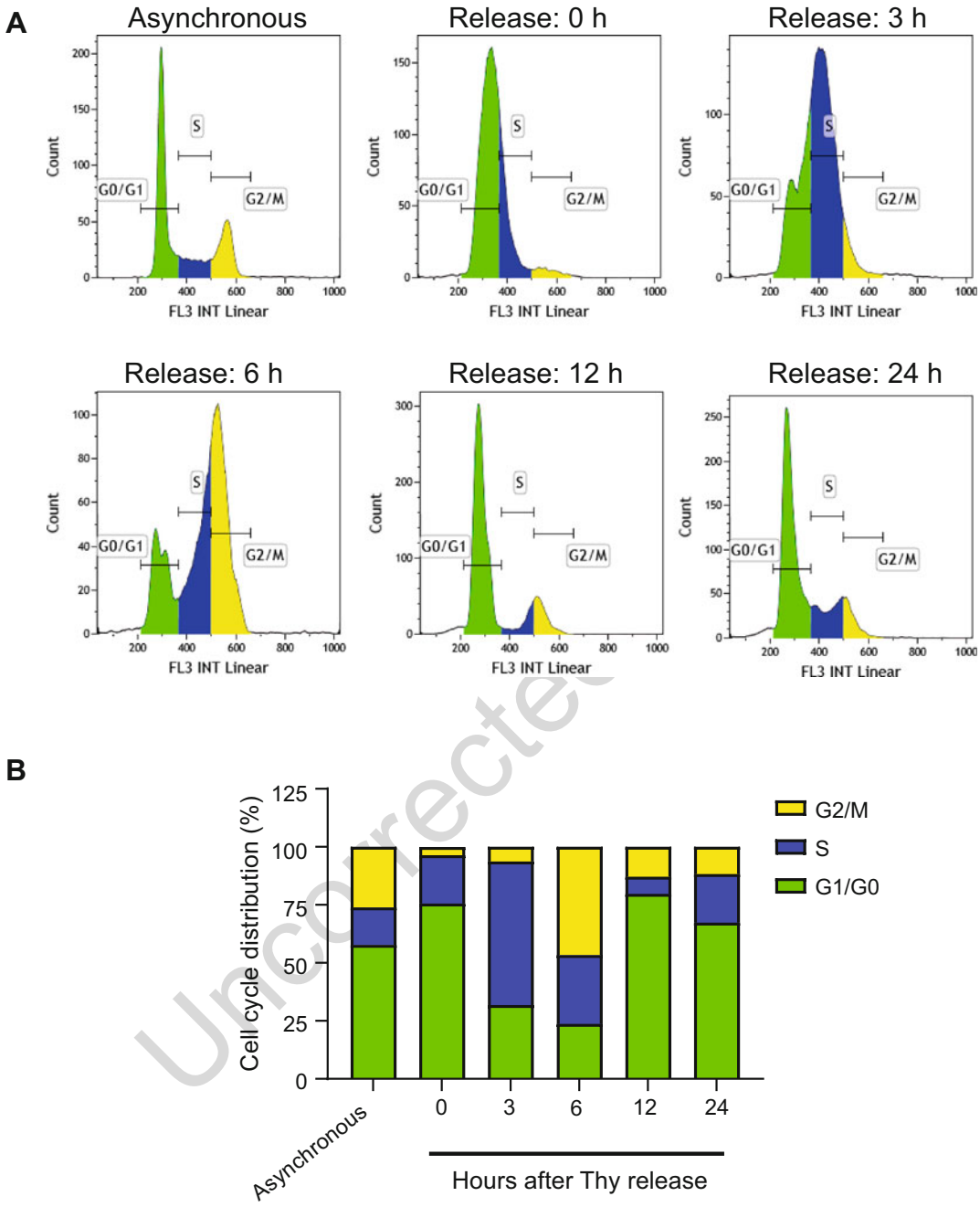

Fig. 4 Synchronization of DU145 cells after double thymidine block. DU145 were arrested at the G1–S border after a double thymidine block and their progression through the different cycle phases after the release was determined using the fluorescence intensity and can be observed in (**a**). The percentage of cells in each phase of the cell cycle at different time points is presented in (**b**). Sample data was acquired on a Beckman Coulter Gallios and subsequent analysis was performed using Kaluza analysis software. *Thy* thymidine

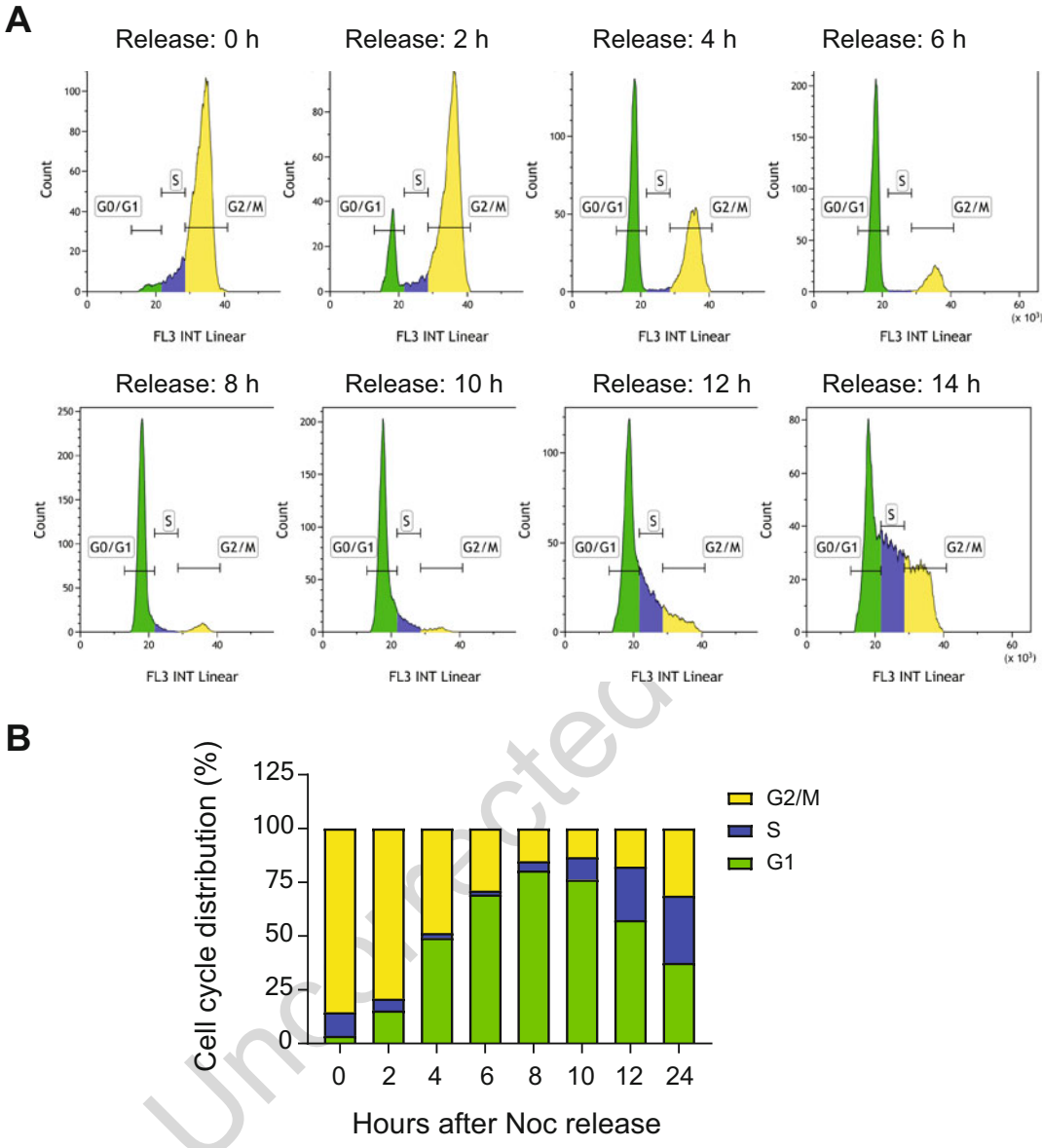

Fig. 5 HeLa S3 cell cycle arrest with nocodazole. HeLa S3 cells were treated following the protocol described in this chapter, with the modification of maintaining the 100 ng/mL nocodazole treatment for 14 h only [17]. After release, it can be observed in (**a**) how these cells transitioned through the cell cycle, with the percentages of cells in each phase of the cycle represented in (**b**). *Noc* nocodazole

6. Following incubation, repeat second thymidine treatment by adding 2 mM thymidine to each well and incubate again for 24 h.
7. After incubation, release the cells by washing twice with 2 mL of prewarmed PBS and incubate in prewarmed fresh complete medium.

8. Collect the cells at selected times, for example at 0, 3, 6, 12 and 24 h after release: remove the complete medium and rinse the well with 2 mL of prewarmed PBS. Add prewarmed Trypsin-EDTA (0.2 mL/well) and incubate at 37 °C for 5 min to detach the cells. Neutralize Trypsin-EDTA by adding 1 mL of complete medium and collect the detached cells in a 15 mL tube.
9. Centrifuge the cells at 300 × *g* for 5 min at room temperature, discard the supernatant and fix the cells by resuspending the pellet in 1 mL of ice-cold 70% ethanol.
10. Follow from **step 12** in Subheading 3.2.

4 Notes

1. There is no need to use molecular biology grade ethanol for fixation.
2. As PI causes irritation, a good option is to buy an already-made PI solution (for example, Sigma-Aldrich, catalog number: P4864-10ML).
3. Depending on the information available in the literature, this range of concentrations can be varied or expanded.
4. This initial overnight incubation in complete media after splitting the cells has the purpose to get the culture in the exponential phase, with cells dividing. The length of this incubation can be anything between 16 and 24 h and it is worth considering the timing of the treatments given afterward. For example, if an 18 h nocodazole treatment is going to be given, cells could be split in the late afternoon (not the morning) and the treatment given the day after at 3 pm, to be collected at 9 am the following day.
5. The incubation with ethanol can be prolonged overnight or over the weekend. It is a good stop in the protocol to collect all different tubes in the experiment together and then continue with the protocol.
6. To avoid precipitation and clumping, it is important to add ethanol gradually (dropwise) whilst vortexing the cells.
7. Cover the tubes with foil. Propidium iodide is a red-fluorescent counterstain that is photosensitive.
8. The PI staining solution contains the RNase. Reduced times of incubation with RNase will make your peaks less differentiated when plotting fluorescence intensity against cell count. In our hands, 30 min is the optimal time for clearly defined peaks.

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