Chapter 1

Overview of Cell Synchronization

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Abstract

The widespread interest in cell synchronization is maintained by the studies of control mechanism involved in cell cycle regulation. During the synchronization distinct subpopulations of cells are obtained representing different stages of the cell cycle. These subpopulations are then used to study regulatory mechanisms of the cycle at the level of macromolecular biosynthesis (DNA synthesis, gene expression, protein synthesis), protein phosphorylation, development of new drugs, etc. Although several synchronization methods have been described, it is of general interest that scientists get a compilation and an updated view of these synchronization techniques. This introductory chapter summarizes: (1) the basic concepts and principal criteria of cell cycle synchronizations, (2) the most frequently used synchronization methods, such as physical fractionation (flow cytometry, dielectrophoresis, cytofluorometric purification), chemical blockade, (3) synchronization of embryonic cells, (4) synchronization at low temperature, (5) comparison of cell synchrony techniques, (6) synchronization of unicellular organisms, and (7) the effect of synchronization on transfection.

Key words Basic concepts of synchronization, Criteria of synchronization, DNA staining, DNA analysis, C-value

1 Introduction

The description of the double helical structure of DNA was and has remained the most famous research paper in biology [1]. In 1953, another important paper was published that established the model of the cell cycle as we know it today [2]. The famous statement of the reduplication of DNA recognized by Watson and Crick: "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material" initiated studies on DNA replication. Howard and Pelc used ^{32}P label and after the incorporation of the biological tracer in the root cells of *Vicia faba* (broad bean) they removed all of the non-DNA phosphorus labeled compounds with hydrochloric acid at 60 °C which allowed them to trace the cellular levels of DNA. Their autoradiographic analysis showed that DNA synthesis occurs only at one discrete period during the

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interphase between two mitoses. Microspectrometric methods confirmed that DNA replication is limited to a well-defined segment of the interphase preceded and succeeded by other periods where the DNA content was constant [3, 4]. Better spatial resolution was obtained when the highly localized tritiated thymidine was introduced as a radioactive probe and increased the precision of autoradiographic procedures [5]. The incorporation of tritiated thymidine was restricted specifically to DNA, not to RNA and only one of the double strands was labeled. This result was consistent with semiconservative model proposed by Watson and Crick. The principle of semiconservative replication was then proved by the classical experiments of Meselson and Stahl in 1958 [6].

The importance of the experiments of Howard and Pelc is that they led to the recognition that the cell cycle consists of phases known as G1, S, G2, and M. Although the examination of the kinetics of cell proliferation with labeled compounds and autoradiography remained an important technique especially when the duration of the cell cycle phases matters, flow cytometry has become a more popular way to study the cell cycle. Laser scanning cytometry (LSC) technology of flow cytometry heritage is not limited to cell cycle analysis. LSC also allows to inspect and interrogate specific cells of defined genetic, biochemical, or morphological properties.

1.1 Basic Concepts of Cell Cycle Synchronization

Synchronization of cell populations offers a unique strategy to study the molecular and structural events taking place as cells travel through the cell cycle. It allows the exact study of individual phases of the cell cycle, the regulatory mechanisms which determine cell cycle regulation at the level of gene expression and posttranscriptional protein modifications, and contributes to drug discovery. Before going into details first of all we define the basic concepts of cell cycle synchronization. In the process of synchronization cells representing different stages of the cell cycle are selected and brought to the same phase. The cell cycle is composed of the replication of genetic materials (S phase) and the successive distribution of genetic materials as well as the other components of the cell onto two daughter cells (M phase). The progression of these two processes is intermitted by two gap phases (G1 and G2) and defined as the cell cycle (Fig. 1).

Originally the cell cycle was recognized in plant cells [7], but soon it turned out that the principal mechanism of the cell cycle is common in all eukaryotic organisms. The study of molecular events of the cell cycle was taken over by animal and yeast cells [8] simply because there were no suitable cell synchronization systems in plant cells.

1.2 Principal Criteria and Shortcomings of Synchronization Methods

There are several principal criteria for synchronization that should be met: (a) both normal and tumor cells should be arrested at the same specific phase of the cell cycle, (b) synchronization must be noncytotoxic and reversible, (c) the metabolic block should be targeted to

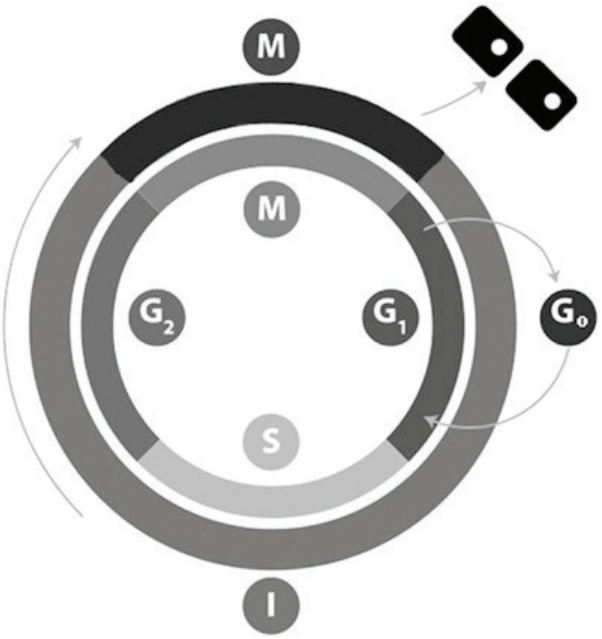

Fig. 1 Cell cycle phases of eukaryotic cells. Cells start to increase in size in Gap 1 (G1) phase. In the resting phase (G_0, Gap 0) cells have left the cycle and stopped dividing. DNA replication occurs during the synthetic (S) phase. During the Gap 2 (G2) between DNA synthesis and mitosis cells continue to grow. In mitosis (M) cell growth stops and the orderly division into two daughter cells takes place. Interphase (I) consists of three stages: G1, S, G2

a specific reaction and must be reversible, (d) large quantities of synchronous cell populations should be obtained, (e) the synchronization must be medium independent, (f) synchrony should be maintained for more than one cell cycle to study biochemical processes taking place in cycling cells, (g) synchronized cells should exhibit uniform size, (h) DNA content in the initial cell culture and during their growth should be the same. These criteria turned out to be quite stringent and resulted in heated debates that still exist among scientists. The major problem is that although many methods have been developed, none of them seems to be perfect for more reasons: (a) the proportion of synchronized cells is not sufficiently high, (b) manipulations during synchronization perturb cell physiology to an unacceptable extent, (c) most of the synchronizing methods are toxic and not applicable in vivo. For example agents that prevent DNA synthesis (excess thymidine, hydroxyurea, aphidicolin) or inhibit the formation of mitotic spindles (nocodazole) not only arrest the cell cycle at certain points [9, 10], but their toxic effect may kill important fractions of the cells [11]. The low cost and simplicity of DNA replication inhibitors is contrasted by their major disadvantage of inducing growth imbalance. Another example is nocodazole treatment. Cells subjected to this agent do not enter mitosis and cannot form metaphase spindles due to the inhibition of polymerization of microtubules. Moreover, when nocodazole was

used as a whole-culture synchronization agent, it worked poorly on bovine kidney (MDBK) cells. After 0.5 ng/ml nocodazole treatment for 4 h there was no accumulation of M phase cells, rather a massive cell death (>80%) was induced [12]. Other observations also demonstrated that populations of synchronized cells obtained by different drug treatments supposedly blocked at biochemically distinct cell cycle points were not apparent by cytometric measurement of DNA content. These results indicate that induced synchrony methods may differ with respect to their impact on cell cycle organization and from the pattern seen with nonperturbing cell selection methods [13]. It is neither the intension of this book to make strict distinctions nor to decide which method should be used. Debates on cell-synchronization methodologies are found in opinion papers [14–18].

2 Methods of Cell Cycle Synchronization

The most widely used methods of cell cycle synchronization are based on two distinct strategies:

1. Physical fractionation.
2. Chemical approach.

2.1 Physical Fractionation

The separation of cells by physical means is based on cell density, cell size, antibody binding to cell surface epitopes, fluorescent emission of labeled cells and light scatter analysis. The two most often used methods of biophysical fractionation are the centrifugal elutriation and fluorescent activated cell sorting.

2.1.1 Velocity Sedimentation

Several techniques that separate cells take advantage of their differences in sedimentation velocity. These methods belong to three classes:

1. Sedimentation at unit gravity [19–21].
2. Density gradient centrifugation [22–25].
3. Velocity sedimentation by counterstreaming centrifugation [26–32].

Major objections against the velocity sedimentation techniques are that they are relatively slow and the size of the nearby fractions may overlap substantially increasing the heterogeneity of the samples. Other major problems with the sedimentation at unit gravity and density gradient centrifugation are the reproducibility, owing to artifacts associated with sedimentation in swinging bucket rotors [33]. These two techniques are not used anymore and therefore not discussed here.

2.1.2 Centrifugal Elutriation

The sedimentation velocity that is based on cell size is operative in the technique of centrifugal elutriation also referred to as counterstreaming centrifugation [26–32]. Lindahl was the first to describe the separation of cells by velocity sedimentation utilizing counterstreaming centrifugation. His method was later modified and renamed centrifugal elutriation. The Beckman elutriation system is an advanced centrifugation device that uses an increasing sedimentation rate to yield a better separation of cells in a specially designed centrifuge and a rotor containing the elutriation chamber. The advantages of centrifugal elutriation are as follows:

1. Differences in sedimentation velocity are exploited to isolate various types of cells from various inhomogeneous cell suspensions,
2. Different subpopulations representing different stages of the cell cycle of the same cell type can be separated.
3. The isolated cells or subpopulations of cells can be used in clinical experiments.
4. Centrifugal elutriation fulfills the three principal criteria for synchronization.

Autoradiographic data indicated that fractions containing ≥ 97% G1 cells, > 80% S cells, and 70–75% G2 cells could be routinely recovered with centrifugal elutriation [34]. This distribution indicates that the resolution of the G1 and S phases belonging to the interphase could be increased while the heterogeneity of the G2 and M phases would not allow a higher resolution. In exponentially growing cultures ≥ 20% of the cells are in G1, about ≥60% in S phase and only 16–18% in G2/M phase. This distribution makes it clear that in animal cells the resolution of the interphase G1 and S phases would be possible. As a result of the improved resolution of the centrifugal elutriation the two known replicative phases (early and late S phase) could be resolved to many subphases the number of which corresponded to the number of chromosomes [35, 36].

In the budding yeast G1-phase cells are unbudded and often smaller than one would expect from the cell cycle status. The budding profile serves as a basis of estimating cell synchrony. In the fission yeast there are no such distinctive features that would allow the morphological determination of the cell cycle position. The cell cycle progression of the yeast cells is size dependent similarly to the cells of budding yeasts and higher eukaryotes [37].

With centrifugal elutriation, several different cell populations synchronized throughout the cell cycle could be rapidly obtained with a purity comparable to mitotic selection and cell sorting [38]. Centrifugal elutriation will be described in Chapter 2. Chapter 14 compares the results from the elutriation protocols with other synchronization protocols.

2.1.3 Flow Cytometry and Cell Sorting

The original name of this technology was pulse cytophotometry, but flow cytometry became the most popular among others such as cytofluorometry, flow microfluorometry. To understand this technique that uses a system for the measurement on individual cells obtained from cell suspension the analogy with a conveyor belt is taken. Conveyor belts have been originally used as rubber belts in factories [39], but they are also running in supermarkets, where they carry to selected goods to the interrogation point, where the cashier picks them individually and identifies them with a laser that reads the barcode. At the interrogation point the items can then be packed without selection into shopping bags or similar items can be grouped and then packed in separate bags. A similar process takes place during flow cytometry without the separation of cells or combined with their separation named cell sorting (Fig. 2).

Flow cytometry is a technique that allows the counting and examination of small (0.2–150 μm) particles (cells, nuclei, chromosomes) suspended in a stream of fluid passed through an electronic detection apparatus. In cell biology individual cells contained in a thin stream of fluid intercept the light source. These cells scatter light and fluorochrome dyes are excited. There are several

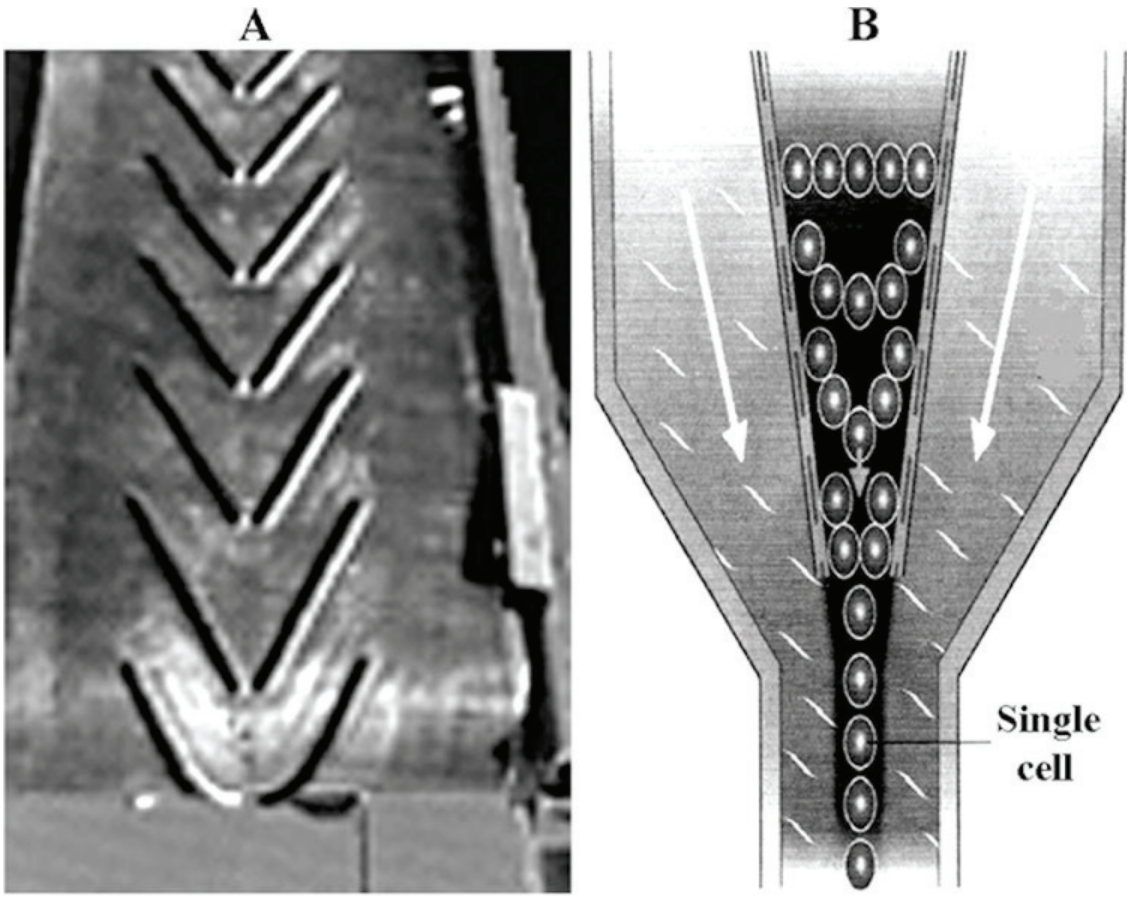

Fig. 2 Analogy between a conveyor belt and flow cytometry. (**a**) Industrial belt [39], (**b**) Hydrodynamic focusing during flow cytometry to produce a single stream of cells [40]

fluorochrome dyes that can be used for DNA staining. For quantitative DNA analysis, cells are normally fixed in ethanol. followed by staining with DNA-binding dyes, such as:

1. Propidium iodide (Phenanthridinium compound).
2. Hoeschst 33342 (Bisbenzimidazole).
3. DAPI (4′-6-diamidino-2-phenylindole).
4. 7 Aminoactinomycin D (Actinomycin).
5. Mithramicin (Chromomycinone).
6. DRAQ5 (Anthraquinone).
7. TO-PRO-3 (comprising a single cyanine dye and a cationic side chain, with only two positive charges).

Some of the dyes are used to quantitatively stain DNA and to separate live from dead cells in unfixed samples. Fluorochromes excited from a lower (300–650 nm) to a higher energy state emit this energy as a photon of light at various frequencies (450–700 nm) with spectral properties unique to different fluorochromes. Photomultipliers convert the light to electrical signals and cell data is collected. High purity (100%) cell sub-populations can be identified and sorted when flow cytometry is combined with cell sorting.

Flow cytometers and analyzers are capable to collect multiparameter cytometry data but do not separate or purify cells. Sorting is an additional process requiring sophisticated electronic components not incorporated into most benchtop instruments. In flow cytometry and fluorescent activated cell sorting (FACS analysis) the light beam (regularly laser) is directed to the stream of fluid containing the particles. Most often used light sources in flow cytometry are argon-, krypton-, helium-neon-, helium cadmium lasers and mercury lamp. Detectors are focused to the interrogation point where the light beam (regularly laser beam) passes through the fluid stream. In forward scatter analysis (FSC) detectors are in line with the light beam, while in side scatter analysis (SSC) several detectors, among them fluorescent detectors, are directed perpendicularly to the beam. Forward and side scatter are used for the preliminary identification of cells. Forward scatter depends on the cell volume and cell size, while side scatter analysis is correlated with the inner complexity of the particle (i.e., shape of the cell or nucleus, amount and type of granules or roughness of cellular membranes). Forward and side scatter were used earlier to exclude debris and dead cells. Recently it has been adapted for the detection of apoptotic cell death.

Advantages and disadvantages of flow cytometry:

1. Flow cytometry measures fluorescence per cell or particle and contrasts with spectrophotometry where the percent absorption and transmission at a certain wavelength is measured for a bulk volume. One of the fundamentals of flow cytometry is its ability to measure the properties of individual particles.

2. The sorting of particles based on their physical properties, to purify populations of interest.
3. The simultaneous physical and/or chemical analysis of thousands of particles per second.
4. Routinely used diagnostic tool in health disorders (especially cancers) and has many applications in both research and clinical practice.
5. Data of samples can be stored in computer as listmode and/or histogram files.
6. The disadvantage of cell sorting is that it exhibits limitations in sample size and time required for synchronization [40].

Listmode files contain the complete list of all events corresponding to all the parameters collected and written in the file. Listmode files contain raw cytometer data. Listmode describes flow cytometry values of the scatter and fluorescence parameters for each event, in the order it passed through the cytometer's interrogation point. Listmode files can be replayed by the computer and appropriate software. Different signals can be amplified and processed by an Analog to Digital Converter (ADC) allowing the events to be plotted on a graphical scale. These histogram files can be either single-parameter

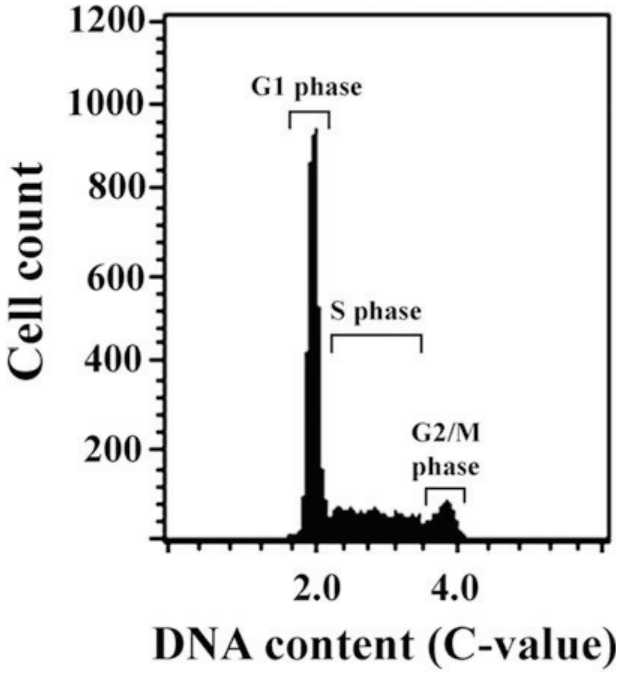

Fig. 3 Flow cytometry: single-parameter histogram. Flow cytometry measures the properties of individual particles. After injecting the sample solution into to flow cytometer, the particles are randomly distributed in three-dimensional space. Upon hydrodynamic focusing, each particle passes through one or more beams of light. Single particles can be interrogated by the machine's detection system. Histograms can be given as density plots, contour diagrams, single parameter (*see* DNA profile above) or two-parameter (dual-color fluorescence) histograms (next figure)

or two-parameter files. The single-parameter histograms have 1024 channels and consist of graphs plotting the cell count on the y-axis and the measurement parameter (e.g., C-value) on x-axis (Fig. 3). When measuring fluorescence from a DNA-binding fluorochrome we regard this to be the same as the DNA content expressed in C-value (C value of 1 corresponds to haploid DNA content per cell). A graph of a two parameter histogram represents two parameters of measurements: the DNA content on the x- and the cell count height on the y-axis similarly to a topographical map (Fig. 4).

DNA histograms yield the relative number of cells in G1/G0, S, and G2/M phases of the cell cycle. The percentage of cells being in each of these phase can be estimated. However, the static information of histograms does not provide information how many cells are progressing through these phases and what percentage of cells is trapped in these phases.

2.1.4 *Cytofluorometric Purification of Cells*

A novel procedure for the purification of cells in distinct phases of the cell cycle was developed based on the stable transfection of cells with a chimeric protein made up by histone H2B and green fluorescent protein (GFP) [11]. Cytofluorometric purification of cells by their size and their H2B-GFP-dependent fluorescence allowed the efficient separation of diploid and tetraploid cells in a fluorescence-activated cell sorter (FACS). DNA content analysis after staining with fluorochromes (propidium iodide, Hoechst 33342) serves as a basis for fluorescence-activated cell sorting (FACS) to obtain synchronous cell populations. A more detailed description and protocol for cytofluorometry for the quantification of ploidy is given in Chapter 3.

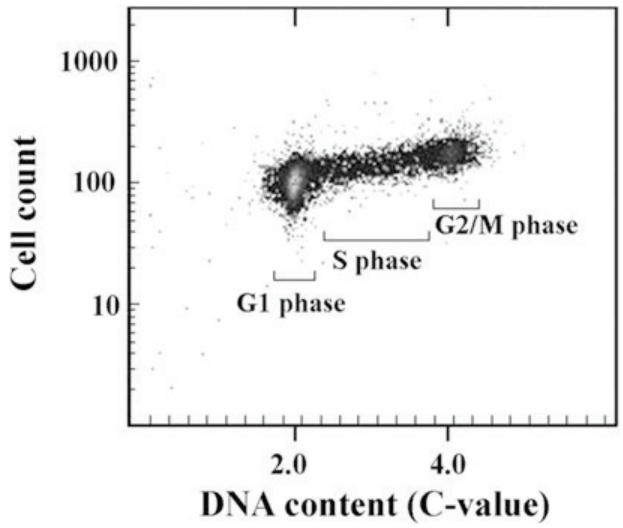

Fig. 4 Flow cytometry: two-parameter histogram. The graph displays two measurement parameters, one on the *y*- and one on the *x*-axis and the cell count as a density plot. Parameters may come from side scatter channel (SSC), forward scatter channel (FSC), or fluorescence

2.1.5 Microchip-Based Flow Cytometers

Flow cytometry is carried out for various medical purposes including the counting of blood cells, detection of pathogenic microbes etc. However, the running of commercial flow cytometers is expensive and requires skilled operators. Miniaturized microchip-based flow cytometers have been developed with built in chambers and tunnels which require much lower volumes of reagents. These devices reduce substantially the cost of diagnosis especially when the chips are disposable. In microchip-based flow cytometers the detected and sort particles are focussed hydrodynamically to pass them through a small detection region [41–46]. Particles are pressed through the device under an external hydrostatic pressure gradient, or under electro-osmotic flow by controlling the movement of the fluid.

2.1.6 Flow Cytometry to Monitor Nucleocytoplasmic Protein Expression

This new flow cytometry assay avoids cell synchronization and applies a stepwise biochemical fractionation procedure to whole cells and isolated nuclei. DNA and immunostaining techniques for the dual labeling of cells and nuclei for DNA and nuclear proteins have been combined with refined flow cytometric data processing and calculation. The combination of flow cytometry with cell size analysis established a resolution map of cell cycle progression to which protein expression in cells or nuclei was correlated [47].

2.1.7 Dielectrophoresis

This method uses laminar flow and electrokinetic forces for the efficient, noninvasive separation of living cells. The alternative current moves particles forward and backward from microelectrodes by periodically reversing the direction of the electric charge [48, 49]. A dielectrophoresis activated cell synchronizer device has been constructed that accepts an asynchronous mixture of cells, fractionates the cell populations according to the cell-cycle phase, and elutes them through different outlets. The device utilizes electric fields that are 1–2 orders of magnitude below those used in electroporation and enriches asynchronous tumor cells in the G1 phase to 96% in one round of sorting, in a continuous flow manner [50].

2.2 Cell Separation with Chemical Blockade ("Arrest and Release" Strategy)

2.2.1 Mitotic Selection

An alternative to overcome the objections of the mitosis-inhibiting (stathmokinetic) approaches involves mitotic selection [51]. Stathmokinetic approaches are discussed later. In monolayer cultures cells are spread over a relatively large area. During mitosis cell become spherical and only a small area of cell membrane remains in contact with the containing vessel. The nuclear material is divided during mitosis (Fig. 5).

The mitotic cells can be completely detached by gently shaking and isolated from the supernatant medium by centrifugation. In actively dividing cells the percentage of mitotic cells known as the mitotic index is relatively low. The mitosis is relatively short compared to the duration of the cell cycle. It can be judged by multiplying the mitotic index by the cell cycle which is normally between 1.0 and 2.5 h. Assuming that the average durations of G1, S, and

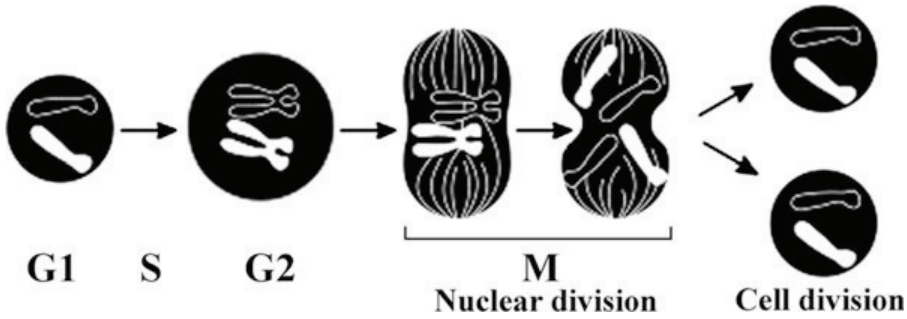

Fig. 5 Nuclear and cell division. The scheme does not show the duration of these processes which represent only a short period of time (1.5–2.5 h) of the cell cycle

G2+M are 8, 10, and 2 h, respectively, then the mitosis takes less than 10% of the cell cycle. To collect sufficient number of mitotic cells for synchronization purpose, the mitotic shake-off should be repeated after 2 h. However, the use of this method is limited to only those cell lines that are grown in a single layer on a flask or Petri dish containing the culture medium and detach during mitosis. Consequently, this method is applicable only to anchorage-dependent, monolayer cell cultures (e.g., HeLa, HaCaT, CHO). Nevertheless, either alone or in combination with blocking agents such as hydroxyurea, the mitotic shake-off turned out to be an excellent method for synchronizing cells [38]. Large scale isolation of mitotic mammalian cells is described in Chapter 4.

2.2.2 Membrane-Elution for Synchronization ("Baby Machine")

This method is analogous to the mitotic shake-off in the sense that one can harvest synchronized cells without any chemical treatment. In this method for batch synchronization of membrane-elution growing cells are bound to a membrane. Baby machines incubate "mother cells" under normal conditions and collect their "babies" similar in cell cycle phase and in age. At division, one cell remains attached to the membrane, the other small daughter cell ("baby") is released into the medium [52, 53]. These newborn cells have a G1 phase DNA content that reflects a normal cell cycle [11]. Among the several macroscale and microfluidic baby machines applied to synchronization, a new baby machine has been presented for rod-shaped cells like fission yeast (*Schizosaccharomyces pombe*). This microfluidic device fixes one end of the cell and releases the free-end daughter cell every time the cell finishes cytokinesis [54].

2.2.3 Types of Chemical Blockade

The idea behind in vitro chemical synchronization is the exposure of a random population of cells to agents that interfere with specific biosynthetic processes, such as DNA replication. The cells blocked in a specific stage of the cell cycle are then released by washing in control media or buffer, followed by the addition of exogenous substrates (e.g., nucleosides, nucleotides) to follow the wavelike rhythm of the cell cycle.

Cells separation by blocking metabolic reactions has been reviewed by Merrill in 1998 [55]. The steady state of cell growth can be altered by the addition of drugs that block or delay the progression of the cell cycle in a specific phase. Methods may be grouped depending on which cell cycle phase has been arrested. Classes of batch synchronization methods arrests cells at:

1. Mitosis by mitotic spindle poisons such as colchicine, vinca alkaloids (vincristine), and nocodazole. Razoxane specifically inhibits the enzyme topoisomerase II resulting in the inhibition of cell division in the premitotic and early mitotic phases of the cell cycle.
2. Other synchronization methods affect DNA synthesis and are proposed to block the cell cycle in S phase (double thymidine block, hydroxyurea, aphidicolin),
3. The third class of chemical synchronization arrests cells at a specific point in the G1 phase, often at the "restriction point" (serum starvation).
4. Combined administration of blocking agents.

Chemical Blockade of Mitosis

Roscovitine. This is an olomoucine-related purine flavopiridol, and is a highly potent inhibitor for the kinase activity of cyclin-dependent kinases (CDK1, CDK2, CDK5, and CDK7, but not for CDK4 and CDK6) by competing with ATP at the ATP binding site of the kinase [56]. Roscovitine is a potent inhibitor acting in G0/G1. However, roscovitine has been reported to prevent cell cycle progression of mammalian cells not only at the G_1–S but also in G_2–M checkpoints [57, 58].

Mimosine. The plant amino acid mimosine may inhibit initiation at origins of replication [59]. Theoretical considerations argue against the use of batch synchronization methods as they are likely to disturb cell growth and prevent the understanding the normal cell cycle [11].

Colchicine. One of the best known examples of chemical synchronization is the addition of colchicine (colcemid) which causes cell cycle arrest in metaphase by depolymerizing tubulin in microtubules. After treating cells with colcemid (or rasoxane, ICRF 159) cells accumulate in M phase and cannot proceed to anaphase. Sampling of cell populations at intervals after colcemid treatment showed a progressive shift of the flow cytometric profile from left to the right. Colchicine has been used to increase proportions of G2/M cells from 13 to 27–32% in pig mammary cells, and up to 37% in fibroblasts [60].

Vincristine (*Oncovin*, *leurocristine*). This vinca alkaloid from the Madagascar periwinkle *Catharanthus roseus* (*Vinca rosea*) is also a mitotic inhibitor, and is used in cancer chemotherapy. The mitodepressive effect and stathmokinetic action of cathinone from

Catha edulis [61] and pantopon, a preparation of opiates [62], were reported on the meristematic region in the root tips of *Allium cepa* (garden onion).

Major objections against these and other stathmokinetic (mitosis-inhibiting) approaches are that cell cycle blockades are likely to perturb and alter the behavior of the cell population in an unpredictable manner. Such a known example is the colcemid block of mitogenically stimulated B lymphocytes that resulted not only in G2 block but also in a G1 arrest. Moreover, tumorigenic lymphoblastoid cell lines have lost their sensitivity to growth inhibition by colcemid during early G1 [63].

Cell Separation by the Inhibition of DNA Synthesis

One method that is generally used is the exposure of randomly proliferating cells to agents that interfere with specific biosynthetic activities, e.g., DNA replication. The cells blocked in the S phase are subsequently released by washing in control media, followed in some cases by the addition of exogenous tracers such as nucleotides. The removal of the blocking agent permits cells to move into succeeding segments of the cell cycle.

Metabolic reactions of cells are most often blocked by the inhibition of DNA synthesis and by nutritional deprivation. DNA synthesis can be inhibited during S phase by inhibitors of DNA replication such as excess thymidine [64], aminopterin (4-amino folic acid) [65–67], hydroxyurea [68, 69], methotrexate [70], fluorodeoxyuridine [71], butyrate [12, 72, 73], cytosine arabinoside [74], and low temperature [75]. The cell cycle is blocked by these inhibitors primarily in S phase resulting in viable cells. Their effects are variable and often debated.

Nutrient modification or stability. Best known nutrient deprivation methods are serum starvation and statin (e.g., lovastatin) treatment. By optimizing nutrient modulation periods using a microfluidic device a relatively high level (80%) of synchronization could be achieved [76]. In this nutrient modification system the microfluidic cell culture chip was fixed on the motorized stage of an inverted microscope, which made the multipoint data acquisition possible. Two kinds of culture media were perfused with the help of two syringe pumps [76]. Alternatively, the stability of nutrient can be maintained by giving fresh medium and removing the old one from the cell culture. The addition of synchronizing agents can also be performed in such a perfusion system adapted for the synchronization of cells based on time-lapse microscopy and using syringe pumps (Chapter 10).

Double thymidine block (*early S-phase block*). A typical procedure for a double-thymidine block was described by Whitfield in 2002 [77]. Cells (e.g., HeLa) at ~30% confluency are washed with buffer (PBS) growth medium and cells are grown in the presence of 2 mM thymidine for 18 h. After the first block thymidine is removed, cells are washed and grown in fresh medium for 9 h to

release cells from the block. The release is followed by the second block by the addition of 2 mM thymidine and cultivation for 17 h. As a result of this synchronization cells progress synchronously through G2- and mitotic phase and will be arrested at the beginning of S phase. Synchrony can be monitored by flow cytometry of propidium iodide-stained cells. After release from the thymidine block, >95% of the cells entered S phase (in 0–4 h), progressed into G2 phase (5–6 h), underwent a synchronous mitosis at 7–8 h, and reentered S phase after completing one full cell cycle at 14–16 h. Typically two to three additional synchronous cell cycles were obtained [77]. Thymidine synchronization showed a constant rate of synthesis of DNA throughout S-phase, but cells synchronized with aminopterin show a slower initial rate of DNA synthesis [67].

Hydroxyurea. Hydroxyurea is used to treat certain types of cancer or blood disorders. This medication may also be useful for chronic urinary tract infections or certain cases of psoriasis. In cell biology hydroxyurea synchronization increases mitotic yield of cell lines. Large quantities of synchronized cells have been isolated in late G1 by growth in isoleucine-deficient medium followed by resuspension in fresh, complete medium containing either hydroxyurea (to 10^{-3} M) or cytosine arabinoside (to 5 μg/ml) [78].

The inhibitors that have been used most frequently for synchronization are hydroxyurea, which inhibits ribonucleotide reductase [79], and aphidicolin, an inhibitor of DNA polymerases [80]. However, none of these agents has been regarded as truly satisfactory since both are chain elongation inhibitors that would not inhibit initiation of replication at the replication forks. Thus, most of the events of interest (i.e., initiation at early-firing origins) would have already occurred prior to release from the blocking agent. Unfortunately, drugs that specifically prevent either entry into the S period or initiation at origins have not yet been identified.

Aphidicolin. This tetracyclic diterpenoid produced by the fungus *Cephalosporium aphidicola* [81] selectively inhibits DNA polymerase α [82]. This enzyme is essential for nuclear replicative DNA synthesis, without affecting nuclear DNA repair synthesis and mitochondrial DNA replication [83, 84]. Rodent and porcine fibroblasts can be reversibly synchronized at the S phase with aphidicolin treatment [85, 86]. Aphidicolin turned out to be an effective agent in the synchronization of human HeLA [87] blocking the cell cycle at the transition from G1 to S phase [80]. Cell synchronization is achieved by inhibition of DNA replication in Chapter 7.

Butyrate. A specific cell cycle synchronization was developed for a bovine kidney (MDBK) cell line. Exposure of MDBK cells to 10 mM butyrate caused inhibition of cell growth and cell cycle arrest in a reversible manner. Flow cytometric evidence was presented that butyrate affected the cell cycle at a specific point immediately after

mitosis at the early stage of G1 phase. In comparison with other inhibitors, such as serum deprivation and aphidicolin, butyrate induced similar synchrony of cell populations [12]. A protocol for cell cycle synchronization with butyrate and cell cycle analysis by flow cytometry will be given in Chapters 9 and 10.

Nutrient Deprivation

Serum starvation (*G0/G1 block*). Removal of serum from a rapidly growing cell culture for about 24 h results in the accumulation of cells in G1 phase. Synchronized cells can then be released into S phase by the addition of serum. Nutritional serum starvation has been widely used for synchronizing cells by arresting them in the G0/G1 phase of the cell cycle, but it often reduced cell survival and increased DNA fragmentation [86] which caused high-embryonic losses after NT [88]. Regularly only non-tumor cells can be synchronized in G_0/G_1 by removal of growth factors ("serum starvation", amino-acid depletion). In transformed cells proliferation is relatively independent of serum and growth factors, thus neoplastic cells may not cease proliferation upon serum deprivation, nor do they cease stringent G_0 arrest [89]. A generally applicable procedure to synchronize mammalian cells by serum deprivation is given in Chapter 6.

Treatment with statins. The mechanism by which lovastatin synchronizes cells is unknown. Lovastatin suppresses the synthesis of mevalonate by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the sterol-biosynthetic pathway. In addition to the deprivation of mevalonate by lovastatin, compactin (mevastatin), a related fungal toxin [90] and mevinolin [91], these statins also inhibit DNA replication [92–97]. Lovastatin was successfully introduced as an early G1 synchronization agent with cell lines including normal and tumor cells of mouse, hamster and human origin [98]. Statins as G_1 inhibitors prevent farnesylation and geranylation of proteins [99] and are effective CDK2 protein kinase inhibitors [100–102]. Synchronization was achieved by the use of compactin [90, 103] presumably by blocking DNA replication and mevalonate production

Other inhibitors. It has been reported that roscovitine, a specific cyclin-dependent kinase (CDK) 2 inhibitor more efficiently synchronized cells in the G0/G1 phase of the cell cycle than serum starvation, and resulted in an increase in cloning efficiency as defined in terms of survival of fetuses and calves following embryo transfer [104].

Combined Administration of Blocking Agents

Blocking agents have been often applied for synchronization due to the simple procedure consisting of cell growth in the presence of the inhibitor. Cells blocked in one of the cell cycle phases (regularly in S phase) were then subsequently released by washing away the inhibitor and thereby permitting their simultaneous movement into succeeding segments of the cell cycle. Partial synchronization

of cell division in vitro has been obtained with considerable success through the implementation of these techniques. The utilization of more than one antimetabolite was expected to improve the efficiency of synchronization.

Thymidine–nocodazole. Cells can be arrested in mitosis by blocking first in thymidine followed by release and then blocking in nocodazole. After release from the nocodazole block, most of the cells (>75%) divided synchronously within 2 h of release from the arrest, entered S phase by 10–12 h after release, and completed the next synchronous mitosis by 18–20 h, ultimately completing two full cell cycles [77].

Thymidine–colcemid. When Successive treatment with excess thymidine and colcemid was applied all in one generation a high degree of synchrony was achieved by this method [105]. The mitosis index of blastomeres increased from 9% metaphases (colchicine synchronization) to 18% (thymidine + colchicine synchronization) per embryo. The in vitro development of embryos was not affected by treatment [106].

Cytosine arabinoside–colcemid. A number of studies have demonstrated that cytosine arabinoside (ara-C) inhibits mitotic activity in mammalian cells both in vitro [107] and in vivo [108, 109]. The mitotic activity of Lieberkuhn's crypt epithelial components increased from 3 to 5% levels to peak values of 15% in rats treated with a single injection of either ara-C or colcemid. In animals treated with ara-C followed by colcemid the efficiency of synchronization was significantly improved, at least two-thirds of the epithelial cells were identified as dividing cells [110].

Methotrexate versus *serum deprivation and colcemid treatment*. Methotrexate or aphidicolin treatment induces a reversible blockade at the beginning of S phase which can be reversed upon drug removal with a consequent wave of synchronization [111]. The combination of serum deprivation and aphidicolin was compared with low nontoxic concentrations (0.04–0.08 μM) of methotrexate under standard culture conditions in cancer cell lines. Synchronization with methotrexate alone turned out to be a better choice for obtaining highly synchronous human cancer cell population than those induced by aphidicolin alone or by a combination of serum deprivation and aphidicolin [112]. These experiments indicate that the combination of synchronization techniques and/or repeated perturbation of cell physiology do not necessarily contribute to a more homogeneous pool of synchronized cells. It is thus better to use the combination of methods that have less or no effect on the cell cycle. Such a combination is centrifugal elutriation with a nutrient starvation protocol. Nutrient starvation contributes to a better size selection and improves synchrony. Moreover, less cells will be needed for elutriation.

2.3 Synchronization of Embryonic Cells

Serum deprivation of embryonic stem cells before cell transplantation was adapted to decrease the rate of cell death after transplantation [113]. To decide whether G0 or G1 cells function better as donor cells has been analyzed during the cell cycle of goat transfected fibroblasts and determined the timing of transition from G0 to G1. Northern blot analysis showed that after 4 days 4-day serum-starved quiescent cells started entering G1 and a few hours after addition of 10% serum to the medium cells were at the mid-G1 stage [114].

2.4 Synchronization at Low Temperature

Human fibroblasts which are in S phase at the time of switching to low temperature (30 °C) complete their DNA synthesis and become arrested in the G1 phase of the cell cycle. The arrested cells can continue proliferation by restoration of the optimal growth temperature (37 °C). At 30 °C DNA replication was inhibited while the excision-repair process was operative but at a slightly reduced rate in comparison. This method was recommended for the study of S-phase-dependent processes, as well as for repair studies in the absence of cell division [115]. The effect of low incubation temperature on synchronization was also studied in cultures of *Plasmodium falciparum*. Growth seemed to be arrested when parasites reached maturation, but reestablished their growth normally when returned to 37 °C [116].

2.5 Comparison of Cell Synchrony Techniques

Different methods–same cell culture. This was done by taking aliquots from the same culture of exponentially growing CHO cells, which were then synchronized using mitotic selection, mitotic selection and hydroxyurea block, centrifugal elutriation, or an EPICS V cell sorter. It was concluded that, either alone or in combination with blocking agents such as hydroxyurea, elutriation and mitotic selection were both excellent methods for synchronizing cells [38]. The use of aphidicolin or hydroxyurea resulted in highly synchronized CHO cell populations, but methotrexate yielded inadequate synchronization [117]. It was demonstrated that both aphidicolin and hydroxyurea were useful drugs for obtaining highly synchronized cell populations after an initial synchrony in mitosis. Aphidicolin was regarded as the best choice because of less toxicity to S phase cells when used in low concentrations.

Selecting inhibitors for bone marrow cultures. Methods for synchronization of bone marrow cells with fluorodeoxyuridine (FdU) and methotrexate (MTX) were compared. Fluorodeoxyuridine did not require cell washing for release of the DNA synthesis block and was found to be more beneficial for bone marrow cultures because it generally produced a higher mitotic yield and was less damaging to chromosomes than methotrexate [71]. However, after FdU treatment flow cytometric analysis showed not only the accumulation of cells in early S phase over time, but also the increased radiation sensitivity of the entire population. Early S turned out to be the most

radiosensitive phase of the cell cycle [118]. Synchrony was tested in vivo in acute leukemic patients after a single injection of l-asparaginase, hydrocortisone, cyclophosphamide, cytosine arabinoside, methotrexate, and an exchange transfusion (62% of the total blood volume). l-asparaginase and hydrocortisone were found to arrest the entry of cells into the S period. Cyclophosphamide appeared to inhibit DNA synthesis, arrested cells in mitosis, and inhibited the entry of cells into the S period. Cytosine arabinoside and methotrexate inhibited DNA synthesis. During the period of time the cells were inhibited in the S phase by these two drugs, cells continued to enter the S period. Thus partial synchronization was achieved after Cytosine arabinoside, and methotrexate treatment [74].

Selection among G1/S blocking agents. For cytogenetic replication studies the timing of cell synchronization is restricted to G1 or early S phase. To get more precise information with regard to the point of G1–S transition different blocking systems have been investigated. Human amniotic fluid cells and fibroblast were temporarily blocked by replication inhibitors: thymidine (dT) surplus, fluorodeoxyuridine (FU), hydroxyurea (HU), and methotrexate uridine (MU). Most variation was found after HU treatment. MU synchronized cells before the unset of S phase. The arresting point of dT surplus and FdU was in early S phase [119].

2.6 Synchronization of Unicellular Organisms

Synchronization of E. coli. To synchronize E. coli cells in culture, two major approaches have been routinely used. One involves temperature-sensitive alleles of the DNA replication protein DnaC [120] that interacts with DNA helicase, DnaB [121] required for the initiation of DNA replication. When the culture is shifted to nonpermissive temperature this will allow all replication forks to be completed without the initiation of new ones. Subsequent shift to permissive temperature allows initiation of the synchronized culture [122]. The other method is based on the attachment of cells to a membrane, while newborn cells are released to the medium. This technique is the adaptation of the "baby machine" [123, 124]. Although cells are hardly perturbed, large quantities of cells cannot be collected by this method. Moreover, there are significant variations in stickiness among different *E. coli* strains [124]. A new method of *E. coli* synchronization applies DL-serine hydroxamate, an amino acid analog, which arrests DNA replication at initiation [125]. Sporulation of bacteria and outgrowth of spores can be utilized for the synchronization of *B. subtilis* (Chapter 13).

The stationary phase was selected for the synchronization of *Escherichia coli* and *Proteus vulgaris*. This method utilized the early stationary phase, by harvesting bacteria quickly under minimal conditions of stress, and inoculating them into fresh medium at a dilution of about sevenfold. Repeated partial synchronization up to four-generation, cycles of high percentage of phasing were obtained [126]. Other earlier methods used filtration to synchronize bacteria

[127–129], by binding bacteria to various surfaces and elute unbound sister cells from the surface [123, 130], by means of temperature shift [131], or by amino acid starvation [132].

Synchronization of yeast. Budding yeast can utilize a wide range of carbon sources (glucose, galactose, ethanol, and glycerol. These carbon sources may serve as a means to control cell cycle times, growth rates, and cell size can be useful in the development of synchronization methods, which in turn may contribute to exploit yeasts in biotechnology.

The synchronization of yeast cells include: feeding and starving, magnesium exhaustion, repetitive heat shock, DNA division cycle block and release, α-factor mating pheromone block, cdc mutants arresting at specific cell cycle stages at a restrictive temperature, continuous synchrony by periodic feeding or dilution. These methods have been summarized by Walker in 1999 [133]. An overview of the most commonly used methods to generate synchronized yeast cultures is presented in Chapter 14.

2.7 Effect of Synchronization on Transfection

Aphidicolin cell synchronization in G2/M phase led to a slight increase in plasma membrane permeabilization, to a three-fold increase in percentage of transfected Chinese hamster cells and to an eight-fold increase in gene expression. This increase in cell transfection was specifically due to the G2/M synchronization process. Cell synchronization in G1 phase by sodium butyrate had no effect on cell permeabilization and transfection [134]. S-phase synchronized CHO cells also showed elevated transfection efficiency [135]. The hypothesis that cell cycle synchronization increases transfection was efficiency was confirmed in human trabecular meshwork cells. While efficiency of transfection increased about three–fold in synchronized cells compared to controls, it still remained low (~3 %) [136].

2.8 Assessment of Synchronization

Validation protocols. Several protocols including flow cytometry with propidium iodide or BrdU labeling, immunoblotting, detecting the levels of cyclins, fluorescent ubiquitin-based cell cycle indicators and time-lapse microscopy, for validation of synchrony of the cells and monitoring the progression of the cell cycle are provided in Chapter 12.

Chapters related to cell cycle. Fertilized eggs shift their cell cycle from mitotic to meiotic pattern for embryogenesis. The formation of chromosomes during fertilization in eggs is described in Chapter 8. Enzymes play a key role in maintaining genomic integrity during the cell cycle. The turnover of these nuclear uracil-DNA glycosylase enzymes during the cell cycle is presented in Chapter 11. Cytofluorometric and time-lapse videomicroscopy methods to detect cell cycle progression, including the assessment of cell cycle distribution, mitotic cell fraction, and single cell fate profile of living cells, can be found in Chapter 5. Time-lapse videomicroscopy

perfusion system is used to visualize individual cells synchronized by butyrate and serum starvation in Chapter 10. Protocols for the synchronization of unicellular organisms are given for bacteria (Chapter 13), yeast (Chapter 14), and protozoans (Chapter15). The synchronization of nuclear progression of porcine oocytes has been achieved by the transient inhibition of meiotic resumption (Chapter 16). Synchronization of cell division can be achieved using different plant tissues. Chapter 17 describes the methodology of the establishment, propagation and analysis of a *Medicago sativa* suspension culture.

Stem cells. Cloning of organisms by mammalian somatic cell nuclear transfer (SCNT) was successfully accomplished in farm animals initially in sheep (Dolly the Sheep), then in rodents, and more recently in primates for reproductive and therapeutic purposes. Somatic cell nuclear transplantation has become the focus in stem cell research. Chapter 18 describes how mouse somatic cell transfer is performed after the treatment and synchronization of donor cells. The next chapter deals with the generation of large numbers of hematopoietic stem cells (Chapter 19).

Clinical aspects and applications of flow cytometry are represented by novel intracellular protocols in Chapter 20.

Cell cycle control. The last chapter reviews the aspects of physiological and molecular transitions that occur in the cell cycle and discusses the role of mathematical modeling in elucidating these transitions and understanding of cell cycle synchronization (Chapter 21).

References

1. Watson JD, Crick FH (1953) Molecular structure of nucleic acids: a structure for deoxyribonucleic acid. Nature 171:737–738
2. Howard A, Pelc S (1953) Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity 6:261–273
3. Walker PM, Yates HB (1952) Nuclear components of dividing cells. Proc R Soc Lond B Biol Sci 140:274–299
4. Swift H (1953) Nucleoproteins in the mitotic cycle. Tex Rep Biol Med 11:755–774
5. Taylor JH, Woods PS, Hughes WL (1957) The organization and duplication of chromosomes as revealed by autoradiographic studies using tritium-labeled thymidine. Proc Natl Acad Sci U S A 43:122–128
6. Meselson M, Stahl FW (1958) The replication of DNA in Escherichia coli. Proc Natl Acad Sci U S A 44:671–682
7. Howard A, Pelc SR (1951) Nuclear incorporation of P^{32} as demonstrated by autoradiographs. Exp Cell Res 2:178–187
8. Baserga R (1985) The biology of cell reproduction. Harvard University Press, Cambridge, MA
9. Amon A (2002) Synchronization procedures. Methods Enzymol 351:457–467
10. Cooper S (2003) Rethinking synchronization of mammalian cells for cell cycle analysis. Cell Mol Life Sci 60:1099–1106
11. Coquelle A, Mouhamad S, Pequignot MO, Braun T, Carvalho G, Vivet S et al (2006) Enrichment of non-synchronized cells in the G1, S and G2 phases of the cell cycle for the study of apoptosis. Biochem Pharmacol 72:1396–1404
12. Li CJ, Elasser TH (2006) Specific cell cycle synchronization with butyrate and cell cycle analysis by flow cytometry for Madin Darby Bovine Kidney (MDBK) cell line. J Anim Vet Adv 5:916–923
13. Urbani L, Sherwood SW, Schimke RT (1995) Dissociation of nuclear and cytoplasmic cell cycle progression by drugs employed in cell synchronization. Exp Cell Res 219:159–168

14. Cooper S (2004) Is whole-culture synchronization biology's 'perpetual-motion machine'? Trends Biotechnol 22:266–269
15. Cooper S (2004) Rejoinder: whole-culture synchronization cannot, and does not, synchronize cells. Trends Biotechnol 22:274–276
16. Spellman PT, Sherlock G (2004) Reply: whole-culture synchronization - effective tools for cell cycle studies. Trends Biotechnol 22:270–273
17. Spellman PT, Sherlock G (2004) Final words: cell age and cell cycle are unlinked. Trends Biotechnol 22:277–278
18. Liu SV (2005) Debating cell-synchronization methodologies: further points and alternative answers. Trends Biotechnol 23:9–10
19. Macdonald HR, Miller RG (1970) Synchronization of mouse L-cells by a velocity sedimentation technique. Biophys J 10:834–842
20. Durand RE (1975) Isolation of cell subpopulations from in vitro tumor models according to sedimentation velocity. Cancer Res 35:1295–1300
21. Tulp A, Welagen JJ (1976) Fractionation of ascites tumour cells at 1 g: separation of cells in specific stages of the life cycle. Europ J Cancer 12:519–526
22. Mitchison JM, Vincent WS (1965) Preparation of synchronous cell cultures by sedimentation. Nature 205:987–989
23. Schindler R, Ramseier L, Schaer JC, Grieder A (1970) Studies on the division cycle of mammalian cells. 3. Preparation of synchronously dividing cell populations by isotonic sucrose gradient centrifugation. Exp Cell Res 59:90–96
24. Wolff DA, Pertoft H (1972) Separation of HeLa cells by colloidal silica density gradient centrifugation. I. Separation and partial synchrony of mitotic cells. J Cell Biol 55:579–585
25. Probst H, Maisenbacher J (1973) Use of zonal centrifugation for preparing synchronous cultures from Ehrlich ascites cells grown in vivo. Exp Cell Res 78:335–344
26. Lindahl PE (1956) On counter streaming centrifugation in the separation of cells and cell fragments. Biochim Biophys Acta 21:411–415
27. Sörenby L, Lindahl PE (1964) On the concentrating of ascites tumour cells in stages of pre-mitosis and mitosis by counter-streaming centrifugation. Exp Cell Res 35:214–217
28. Grabske RJ, Lake S, Gledhill BL, Meistrich ML (1975) Centrifugal elutriation: separation of spermatogenic cells on the basis of sedimentation velocity. J Cell Physiol 86:177–189
29. Meistrich ML, Meyn RE, Barlogie B (1977) Synchronization of mouse L-P59 cells by centrifugal elutriation separation. Exp Cell Res 105:169–177
30. Grdina DJ, Peters LJ, Jones S, Chan E (1978) Separation of cells from a murine fibrosarcoma on the basis of size. I. Relationship between cell size and age as modified by growth in vivo or in vitro. J Natl Cancer Inst 61:209–214
31. Gohde W, Meistrich ML, Meyn RE, Schumann J, Johnston D, Barlogie B (1979) Cell-cycle phase-dependence of drug-induced cycle progression delay. J Histochem Cytochem 27:470–473
32. Shumaker VN (1967) Zone centrifugation. Adv Biol Phys 11:245–339
33. Banfalvi G (2008) Cell cycle synchronization of animal cells and nuclei by centrifugal elutriation. Nat Protoc 3:663–673
34. Keng PC, Li CKN, Wheeler KT (1980) Synchronization of 9L rat rain tumor cells by centrifugal elutriation. Cell Biophys 2:191–206
35. Banfalvi G, Mikhailova M, Poirier LA, Chou MW (1997) Multiple subphases of DNA replication in Chinese hamster ovary (CHO-K1) cells. DNA Cell Biol 16:1493–1498
36. Rehak M, Csuka I, Szepessy E, Banfalvi G (2000) Subphases of DNA replication in Drosophila cells. DNA Cell Biol 19:607–612
37. Day A, Schneider C, Schneider BL (2004) Yeast cell synchronization. Methods Mol Biol 241:55–76, Clifton NJ
38. Grdina DJ, Meistrich ML, Meyn RE, Johnson TS, White RA (1984) Cell synchrony techniques. I. A comparison of methods. Cell Tissue Kinet 17:223–236
39. Continental conveyors Ltd http://www.ccpl.in/?gclid=CL2u7qbjxp8CFVmHzAodFzb4ew
40. Rahman M http://www.abdserotec.com/uploads./Flow-Cytometry.pdf
41. Lee G-W, Hung C-I, Ke B-J, Huang G-R, Hwei G-R, Hwei B-H et al (2001) Hydrodynamic focussing for a micromachined flow cytometer. Trans ASME 123:62–679
42. Fiedler S, Shirley SG, Schnelle T, Fuhr G (1998) Dielectrophoretic sorting of particles and cells in a microsystem. Anal Chem 70:1909–1915
43. Fu AY, Spence C, Scherer A, Arnold FH, Quake SR (1999) A microfabricated fluorescence-activated cell sorter. Nat Biotech 17:1109–1111

44. Gawad S, Schild L, Renaud P (2001) Micromachined impedance spectroscopy flow cytometer for cell analysis and particle sizing. Lab Chip 1:76–82
45. Kruger J, Singh K, O'Neill A, Jackson C, Morrison A, O'Brien P (2002) Development of a microfluidic device for fluorescence activated cell sorting. J Micromech Microeng 12:486–494
46. Schrum DP, Culbertson CT, Jacobson SC, Ramsey JM (1999) Microchip flow cytometry using electrokinetic focusing. Anal Chem 71:4173–4177
47. Rosner M, Schipany K, Hengstschläger M (2013) Merging high-quality biochemical fractionation with a refined flow cytometry approach to monitor nucleocytoplasmic protein expression throughout the unperturbed mammalian cell cycle. Nat Protoc 8:602–623
48. Morgan H, Holmes D, Green NG (2003) 3D focusing of nanoparticles in microfluidic channels. IEE Proc Nanobiotechnol 150:76–81
49. Holmes D, Sandison ME, Green NG, Morgan H (2005) On-chip high-speed sorting of micron-sized particles for high-throughput analysis. IEE Proc Nanobiotechnol 152:129–135
50. Kim U, Shu CW, Dane KY, Daugherty PS, Wang JY, Soh HT (2007) Selection of mammalian cells based on their cell-cycle phase using dielectrophoresis. Proc Natl Acad Sci U S A 104:20708–20712
51. Teresima T, Tolmach LJ (1963) Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. Exp Cell Res 30:344–362
52. Thornton M, Eward KL, Helmstetter CE (2002) Production of minimally disturbed synchronous cultures of hematopoietic cells. Biotechniques 32:1098–1100
53. Cooper S (2002) Minimally disturbed, multicycle, and reproducible synchrony using a eukaryotic "baby machine". Bioessays 24:499–501
54. Wang S, Luo C (2016) Cell cycle synchronization using a microfluidic synchronizer for fission yeast cells. Methods Mol Biol 1342:259–268
55. Merrill GF (1998) Cell synchronization. Methods Cell Biol 57:229–249
56. Mgbonyebi OP, Russo J, Russo IH (1999) Roscovitine induces cell death and morphological changes indicative of apoptosis in MDA-MB-231 breast cancer cells. Cancer Res 59:1903–1910
57. Rudolph B, Saffric JZ, Henglein B, Muller R, Ansorge W, Eilers M (1996) Activation of cyclin-dependent kinases by Myc mediates induction of cyclin A but not apoptosis. EMBO J 15:3065–3076
58. Azevedo WF, Leclerc S, Meijer L, Havlicek I, Strnad M, Kim SH (1997) Inhibition of cyclin-dependent kinases by a purine analogs: crystal structure of human cdk2 complexed with roscovitine. Eur J Biochem 243:518–526
59. Mosca PJ, Dijkwel PA, Hamlin JL (1992) The plant amino acid mimosine may inhibit initiation at origins of replication in Chinese hamster cells. Mol Cell Biol 12:4375–1483
60. Boquest AC, Day BN, Prather RS (1999) Flow cytometric cell cycle analysis of cultured porcine fetal fibroblast cells. Biol Reprod 60:1013–1019
61. Al-Meshal IA (1987) Mitodepressive effect of (–)-cathinone, from Catha edulis (khat), on the meristematic region of Allium cepa root tips. Toxicon 4:451–454
62. Kabarity A, El-Bayoumi A, Habib AA (1979) Mitodepressive effect and stathmokinetic action of pantopon hydrochloride. Mutat Res-Gen Tox En 2:143–148
63. Kenter AL, Watson JV, Azim T, Rabbitts TH (1986) Colcemid inhibits growth during early G1 in normal but not in tumorigenic lymphocytes. Exp Cell Res 167:241–251
64. Viegas-Pequiqnot E, Dutrillaux B (1970) Une methode: simple pour obtenis des prophases et des prometaphases. Ann Genet 21:122–125
65. Rueckert RR, Mueller GC (1960) Studies on unbalanced growth in tissue culture. I. Induction and consequences of thymidine deficiency. Cancer Res 20:1584–1591
66. Kishimoto S, Lieberman I (1965) Nuclear membranes of cultured mammalian cells. J Biol Chem 25:103–107
67. Adams RLP (1969) The effect of endogenous pools of thymidylate on the apparent rate of DNA synthesis. Exp Cell Res 56:55–58
68. Gallo JH, Ordomez JV, Brown GE, Testa JR (1984) Synchronization of human leukemic cells: relevance for high resolution chromosome banding. Hum Genet 66:220–224
69. Biegel JA, Leslie DS, Bigner DD, Bigner SH (1987) Hydroxyurea synchronization increases mitotic yield in human glioma cell lines. Acta Neuropathol 73:309–312
70. Yunis JJ, Bloomfield GD, Ensrud K (1981) All patients with acute nonlymphocytic leukemia may have a chromosomal defect. N Engl J Med 305:135–139
71. Webber LM, Garson OM (1983) Fluorodeoxyuridine synchronization of bone

marrow cultures. Cancer Genet Cytogenet 8:123–132

72. Wright JA (1973) Morphology and growth rate changes in Chinese hamster cells cultured in presence of sodium butyrate. Exp Cell Res 78:456–460
73. Kruh J, Defer N, Tichonicky L (1992) Molecular and cellular action of butyrate. C R Seances Soc Biol Fil 186:12–25
74. Lampkin BC, Nagao T, Mauer AM (1971) Synchronization and recruitment in acute leukemia. J Clin Invest 50:2204–2214
75. Boucher B, Norman CS (1980) Cold synchronization for the study of peripheral blood and bone marrow chromosomes in leukemia and other hematologic disease states. Hum Genet 54:207–211
76. Tian Y, Luo C, Lu Y, Tang C, Ouyang Q (2012) Cell cycle synchronization by nutrient modulation. Integr Biol (Camb) 4:328–334
77. Whitfield ML, Sherlock G, Saldanha AJ, Murray JI, Ball CA, Alexander KE et al (2002) Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell 13:1977–2000
78. Tobey RA, Crissman HA (1972) Preparation of large quantities of synchronized mammalian cells in late G1 in the pre-DNA replicative phase of the cell cycle. Exp Cell Res 75:460–464
79. Skoog L, Nordenskjold B (1971) Effect of hydroxyurea and 1-0-D-arabinofuranosylcytosine on deoxyribonucleotide pools in mouse embryo cells. Eur J Biochem 19:81–89
80. Wang TF (1991) Eukaryotic DNA polymerases. Annu Rev Biochem 60:513–552
81. Brundret KM, Dalziel W, Hesp B, Jarvis JAJ, Neidle S (1972) X-ray crystallographic determination of the structure of the antibiotic aphidicolin: a tetracyclic diterpenoid containing a new ring system. J Chem Soc Chem Commun 18:1027–1028
82. Nagano H, Ikegami S (1980) Aphidicolin: a specific inhibitor of eukaryotic DNA polymerase alpha. Seikagaku 52:1208–1216
83. Sala F, Parisi B, Burroni D, Amileni AR, Pedrali-Noy G, Spadari S (1980) Specific and reversible inhibition by aphidicolin in the alpha-like DNA polymerase of plant cells. FEBS Lett 117:93–98
84. Sala F, Galli MG, Levi M, Burroni D, Parisi B, Pedrali-Noy G, Spadari S (1981) Functional roles of the plant alpha-like and gamma-like DNA polymerases. FEBS Lett 124:112–118
85. Levenson V, Hamlin J (1993) A general protocol for evaluation the specific effects of DNA replication inhibitors. Nucleic Acids Res 21:3997–4004
86. Kues WA, Anger M, Carnwarth JW, Motlik J, Nieman H (2000) Cell cycle synchronization of porcine fibroblasts: effects of serum deprivation and reversible cell cycle inhibitors. Biol Reprod 62:412–419
87. Pedrali-Noy G, Spadari S, Miller-Faurès A, Miller AO, Kruppa J, Koch G (1980) Synchronization of HeLa cell cultures by inhibition of DNA polymerase alpha with aphidicolin. Nucleic Acids Res 8:377–387
88. Lawrence JL, Schrick FN, Hopkins FM, Welborn MG, McCracken MD, Sonstegard T et al (2005) Fetal losses and pathologic findings of clones derived from serum-starved versus serum-fed bovine ovarian granulosa cells. Reprod Biol 5:171–184
89. Scher CD, Stone ME, Stiles CD (1979) Platelet-derived growth factor prevents Go growth arrest. Nature 281:390–392
90. Endo A, Kuroda M, Tansawa K (2004) Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B fungal metabolites having hypocholesterolemic activity. Atheroscler Suppl 5:39–42
91. Langan TJ, Slater MC (2005) Isoprenoids and astroglial cell cycling: diminished mevalonate availability and inhibition of dolichol-linked glycoprotein synthesis arrest cycling through distinct mechanisms. J Cell Physiol 149:284–292
92. Larson RA, Chung J, Scanu AM, Vachnin S (1982) Neutrophils are required for the DNA synthetic response of human lymphocytes to mevalonic acid: evidence suggesting that a nonsterol product of mevalonate is involved. Proc Natl Acad Sci U S A 79:3028–3032
93. Habenicht AJ, Glomset JA, Ross R (1980) Relation of cholesterol and mevalonic acid to the cell cycle in smooth muscle and swiss 3T3 cells stimulated to divide by platelet-derived growth factor. J Biol Chem 255:5134–5140
94. Maltese WA, Sheridan KM (1987) Isoprenylated proteins in cultured cells: subcellular distribution and changes related to altered morphology and growth arrest induced by mevalonate deprivation. J Cell Physiol 133:471–481
95. Langan TJ, Volpe JJ (1987) Cell cycle-specific requirement for mevalonate, but not for cholesterol, for DNA synthesis in glial primary cultures. J Neurochem 49:513–521
96. Doyle JW, Kandutsch AA (1988) Requirement for mevalonate in cycling cells: quantitative and temporal aspects. J Cell Physiol 137:133–140

97. Sinensky M, Logel J (1985) Defective macromolecule biosynthesis and cell cycle progression in a mammalian cell starved for mevalonate. Proc Natl Acad Sci U S A 82:3257–3261
98. Keyomarsi K, Sandoval L, Band V, Pardee AB (1991) Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. Cancer Res 51:3602–3609
99. Jakóbisiak M, Bruno S, Skierski J, Darzynkiewicz Z (1991) The cell cycle specific effects of lovastatin. Proc Natl Acad Sci U S A 88:3628–3632
100. Crissman HA, Gadbois DM, Tobey RA, Bradbury EM (1991) Transformed mammalian cells are deficient in kinase-mediated progression through the G_1 phase of the cell cycle. Proc Natl Acad Sci U S A 88:7580–7585
101. Bruno S, Ardelt B, Skierski JS, Traganos F, Darzynkiewicz Z (1992) Different effects of staurosporine, an inhibitor of protein kinases, on the cell cycle and chromatin structure of normal and leukemic lymphocytes. Cancer Res 52:470–476
102. Bruno S, Traganos F, Darzynkiewicz Z (1996) Cell cycle synchronizing properties of staurosporine. Methods Cell Sci 18:99–107
103. Nagel WW, Vallee BL (1995) Cell cycle regulation of metallothionein in human colonic cancer cells. Proc Natl Acad Sci U S A 92:579–583
104. Gibbons J, Arat S, Rzucidlo J, Miyoshi K, Waltenburg RD, Respess DS et al (2002) Enhanced survivability of cloned calves derived from roscovitine-treated adult somatic cells. Biol Reprod 66:895–900
105. Doida Y, Okada S (1967) Synchronization of L5178Y cells by successive treatment with excess thymidine and colcemid. Exp Cell Res 48:540–548
106. Rottmann OJ, Arnold S (1983) Enhancing the mitotic index of blastomeres by thymidine synchronization. Anim Reprod Sci 6:239–242
107. Kim JH, Eidinoff ML (1965) Action of 1-(3-D-arabinofuranosylcytosine on the nucleic acid metabolism and viability of HeLa cells. Cancer Res 25:698–702
108. Lenaz L, Sternberg SS, Philips FS (1969) Cytotoxic effects of 1-beta-D-arabinofuranosyl-5-fluorocytosine and of 1-beta-D-arabinofuranosylcytosine in proliferating tissues in mice. Cancer Res 29:1790–1798
109. Bertalanffy FD, Gibson MHL (1971) The in vivo effects of arabinosylcytosine on the proliferation of murine B16 melanoma and Ehrlich ascites tumor. Cancer Res 31:66–71
110. Verbin RS, Diluiso G, Liang H, Farber E (1972) Synchronization of cell division in vivo through the combined use of cytosine arabinoside and colcemid. Cancer Res 32:1489–1495
111. Erba E, Sen S, Lorico A, D'Incalci M (1992) Potentiation of etoposide cytotoxicity against a human ovarian cancer cell line by pretreatment with non-toxic concentrations of methotrexate or aphidicolin. Eur J Cancer 28:66–71
112. Erba E, Sen S (1996) Synchronization of cancer cell lines with methotrexate in vitro. Meth Cell Sci 18:149–163
113. Zhang E, Li X, Zhang S, Chen L, Zheng X (2005) Cell cycle synchronization of embryonic stem cells: effect of serum deprivation on the differentiation of embryonic bodies in vitro. Biochem Biophys Res Commun 333:1171–1177
114. Memili E, Behboodi E, Overton SA, Kenney AM, O'Coin M, Zahedi A et al (2004) Synchronization of goat fibroblast cells at quiescent stage and determination of their transition from G0 to G1 by detection of cyclin D1 mRNA. Cloning Stem Cells 6:58–66
115. Enninga IC, Groenendijk RT, van Zeeland AA, Simons JW (1984) Use of low temperature for growth arrest and synchronization of human diploid fibroblasts. Mutat Res 130:343–352
116. Rojas MO, Wasserman M (2007) Effect of low temperature on the in vitro growth of plasmodium falciparum. J Eukaryot Microbiol 40:149–152
117. Fox MH, Read RA, Bedford JS (1987) Comparison of synchronized Chinese hamster ovary cells obtained by mitotic shake-off, hydroxyurea, aphidicolin, or methotrexate. Cytometry 8:315–320
118. Miller EM, Kinsella TJ (1992) Radiosensitization by fluorodeoxyuridine: effects of thymidylate synthase inhibition and cell synchronization. Cancer Res 52:1687–1694
119. Vogel W, Schempp W, Sigwarth I (1978) Comparison of thymidine, fluorodeoxyuridine, hydroxyurea, and methotrexate blocking at the G_1/S phase transition of the cell cycle, studied by replication patterns. Hum Genet 45:193–198
120. Carl PL (1970) Escherichia coli mutants with temperature-sensitive synthesis of DNA. Mol Gen Genet 109:107–122
121. Wickner S, Hurwitz J (1975) Interaction of *Escherichia coli* dnaB and dnaC(D) gene products *in vitro*. Proc Natl Acad Sci U S A 72:921–925
122. Withers HL, Bernander R (1998) Characterization of dnaC2 and dnaC28

mutants by flow cytometry. J Bacteriol 180:1624–1631

123. Helmstetter CE, Cummings DJ (1963) Bacterial synchronization by selection of cells at division. Proc Natl Acad Sci U S A 50:767–774
124. Helmstetter CE, Eenhuis C, Theisen P, Grimwade J, Leonard AC (1992) Improved bacterial baby machine: application to *Escherichia coli* K-12. J Bacteriol 174:3445–3449
125. Ferullo DJ, Cooper DL, Moore HR, Lovett ST (2009) Cell cycle synchronization of Escherichia coli using the stringent response, with fluorescence labeling assays for DNA content and replication. Methods 48:8–13
126. Cutler RG, Evans JE (1966) Synchronization of bacteria by a stationary-phase method. J Bacteriol 91:469–476
127. Maruyama Y, Yanagita T (1956) Physical methods for obtaining synchronous culture of *Escherichia coli*. J Bacteriol 71:542–546
128. Abbo FE, Pardee AB (1960) Synthesis of macromolecules in synchronously dividing bacteria. Biochim Biophys Acta 39:473–48
129. Lark KG, Lark C (1960) Changes during the division cycle in bacterial cell wall synthesis, volume, and ability to concentrate free amino acids. Biochim Biophys Acta 43:520–530
130. Helmstetter CE, Uretz RB (1963) X-ray and ultraviolet sensitivity of synchronously dividing *Escherichia coli*. Biophys J 3:35–47
131. Lark KG, Maaloe O (1954) The induction of cellular and nuclear division in *Salmonella typhimurium* by means of temperature shift. Biochem Biophys Acta 15:345–356
132. Ron EZ, Rozenhak S, Grossman N (1975) Synchronization of cell division in *Escherichia coli* by amino acid starvation: strain specificity. J Bacteriol 123:374–376
133. Walker GM (1999) Synchronization of yeast cell populations. Methods Cell Sci 21:87–93
134. Golzio M, Teissié J, Rols M-P (2002) Cell synchronization effect on mammalian cell permeabilization and gene delivery by electric field. Biochim Biophys Acta 1563:23–28
135. Grosjean F, Batard P, Jordan M, Wurm FM (2002) S-phase synchronized CHO cells show elevated transfection efficiency and expression using CaPi. Cytotechnology 38:57–62
136. Hoffman EA, Poncius A, McKay BS, Stamer WD (2004) Cell cycle synchronization and transfection efficiency of human trabecular meshwork cells. Invest Ophthalmol Vis Sci 45:4432

Part II

Physical Fractionation

Chapter 2

Synchronization of Mammalian Cells and Nuclei by Centrifugal Elutriation

Gaspar Banfalvi

Abstract

Synchronized populations of large numbers of cells can be obtained by centrifugal elutriation on the basis of sedimentation properties of small round particles, with minimal perturbation of cellular functions. The physical characteristics of cell size and sedimentation velocity are operative in the technique of centrifugal elutriation also known as counterstreaming centrifugation. The elutriator is an advanced device for increasing the sedimentation rate to yield enhanced resolution of cell separation. A random population of cells is introduced into the elutriation chamber of an elutriator rotor running in a specially designed centrifuge. By increasing step-by-step the flow rate of the elutriation fluid, successive populations of relatively homogeneous cell size can be removed from the elutriation chamber and used as synchronized subpopulations. For cell synchronization by centrifugal elutriation, early log S phase cell populations are most suitable where most of the cells are in G1 and S phase (>80 %). Apoptotic cells can be found in the early elutriation fractions belonging to the sub-Go window. Protocols for the synchronization of nuclei of murine pre-B cells and high-resolution centrifugal elutriation of CHO cells are given. The verification of purity and cell cycle positions of cells in elutriated fractions includes the measurement of DNA synthesis by [^{3}H]-thymidine incorporation and DNA content by propidium iodide flow cytometry.

Key words Counterstreaming centrifugation, Cell separation, Velocity sedimentation, Elutriator, Resolution power

1 Introduction

1.1 Definition of Terms: Elution, Elutriation, Centrifugal Elutriation

Elution is an analytical process by which chemicals are emerging from a chromatography column and normally flow into a detector. The material obtained with the carrier (eluent) is the mobile phase and known as the eluate. In liquid column chromatography, the eluent is a solvent (Fig. 1a). Elutriation is a technique to separate small particles suspended in a fluidized bed into different size groups by passing an increasing flow rate through the elutriation chamber. In elutriation, particles are suspended in a moving fluid which can be liquid or gas. In vertical elutriation, smaller particles move upward with the fluid. Large particles will not be driven out of the elutriation chamber and tend to settle out on the walls or at

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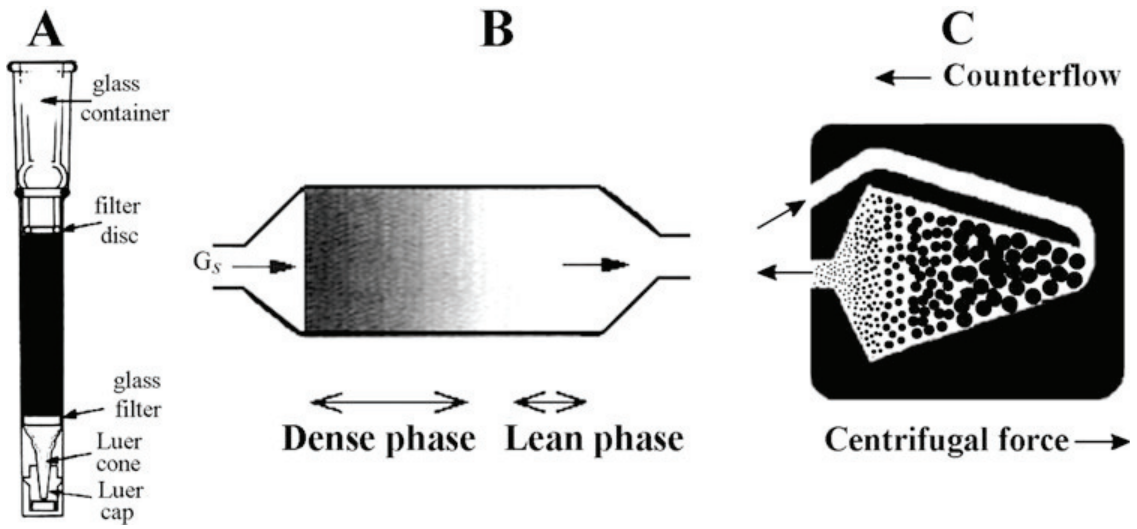

Fig. 1 Schematic views of elution, elutriation, and centrifugal elutriation. (**a**) For elution in column chromatography, a liquid eluent is used. (**b**) Horizontal elutriation in a fluidized bed of particles with flux G_s. (**c**) Fractionation of cells by centrifugal elutriation. Cells are introduced with an initial lower flow rate which is opposed be the centrifugal force. By increasing the flow rate, the floating cells in the elutriation chamber can be driven out and separated into 10–30 distinct size fractions

the bottom of the elutriator due to the higher gravity exerted on their larger size. In horizontal position, the suspended particles are passed through the elutriation chamber. By increasing stepwise the fluid velocity, the particles can be separated into fractions (Fig. 1b). The principle of centrifugal elutriation is similar to elutriation except that the fluid is pumped into a rotating separation chamber that converges into an outlet tube (Fig. 1c).

1.2 Development and Application of Centrifugal Elutriation

The first apparatus that exploited differences in velocity sedimentation for the separation of cells by counterstreaming centrifugation was described by Lindahl in 1948 [1]. The Beckman Instrument Company modified this instrument and named it elutriator and termed the process centrifugal elutriation [2]. Further refinement and a second chamber style know as the Sanderson chamber was introduced giving a better resolution especially with small cells [3]. Centrifugal elutriation has been applied to separate hemopoietic cells, mouse tumor cells, testicular cells, and a variety of other specialized cells from other cells and cells in particular phases of the cell cycle reviewed first by Pretlow in 1979 [4]. Several further applications have been described [5–9]. The construction of the commercially available centrifugal elutriator resulted in a rapid purchase of these instruments especially in the United States in the second half of the 1970s, with high expectations and significantly lower outcome than originally expected. The major reason of insufficient or lacking data is the missing technical skill necessary for the assembling and operation of these instruments and the use of such heterogeneous batch cultures that made the separation irreproducible. It is thus important to discuss first the

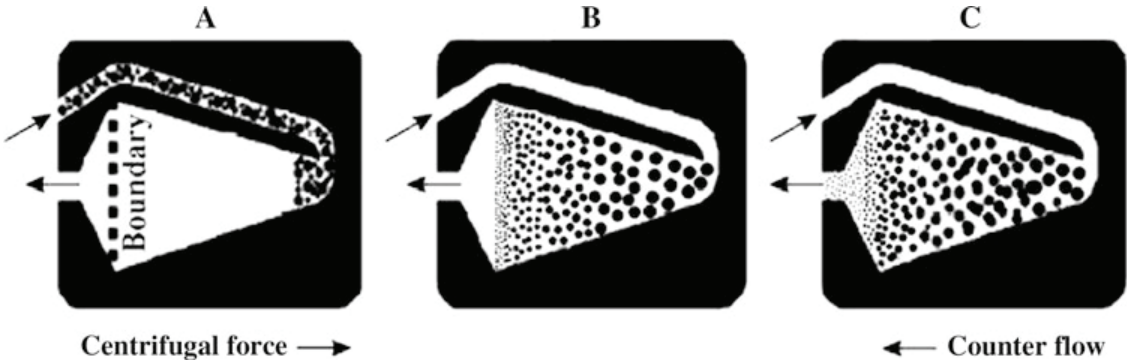

Fig. 2 Cell synchronization by centrifugal elutriation based on different cell size. The centrifugal force and the counter stream act in opposite directions in the elutriation chamber. (**a**) Early S phase cells enter the elutriation chamber. (**b**) Size gradient is balanced by centrifugal force and counterflow of elutriation keeping cells floating inside the chamber. (**c**) Increasing flow rate elutes smaller cells first followed by larger ones. Reproduced with permission of Banfalvi [11]

circumstances that may cause experimental failures during the centrifugal elutriation.

1.3 Principles of Centrifugal Elutriation

Among the methods used to monitor cell cycle changes, two distinct strategies prevailed [10]. One is the chemical "arrest-and-release" approach that arrests cells at a certain stage of the cell cycle and then by the removal of the blocking agent synchronized cells are released to the next phase. In the alternative approach, cells are separated by physical means such as mitotic shake-up, gradient centrifugation, cell sorting, and centrifugal elutriation. These methods have been overviewed in Chapter 1.

Counterflow centrifugal elutriation separates subpopulations of cells on the basis of cell volume and density. As there is only a slight change in cell density during the cell cycle, the principle of cell separation during elutriation is based on cell size (Fig. 2).

Before going to the point-by-point elutriation protocol, the following precautions are advised to avoid unnecessary mistakes and problems during the execution of this cell synchronization technique:

Culture medium. For test run and pilot experiments, saline can be used to find out the right conditions (centrifugal force, flow rate) for cell fractionation. Although any medium can be used for elutriation, it is advised that the elutriation fluid should be the physiological culture medium. To limit the cost of elutriation, the fetal calf serum concentration can be reduced to 1%. Some investigators use cost-efficient 1% bovine serum albumin. The protein content prevents cells from clumping and the cellular debris from adhering to the chamber wall. Cell aggregates normally accumulate at the entrance of the elutriation chamber and may clog it. In such case, the chamber has to be disassembled, whipped out with a soft paper towel, cleaned with a mild detergent, and reassembled

taking care of the right position of the plastic insolation. High protein concentration may cause foaming and cell lysis. The culture medium should contain only soluble materials, any particle left in the medium may contribute to aggregation or will appear in one of the elutriated fractions obscuring the cell cycle profile.

Cells. Most significant cell growth takes place in S phase; it is thus not surprising that nearly 100% of G1 cells and more than 80% of S cells could be recovered by centrifugal elutriation [12]. Best separation can be achieved by using cell populations maintained in early S phase of growth for several generations. This will reduce the number of aging and dying cells due to anoxia, the formation of artifacts, cell debris, and will prevent clumpiness. Early S phase cells contribute to the reproduction of the results causing only small shift in the elutriation profile that can be attributed to minor differences in cell distribution.

Any suspension cell line can be used for centrifugal elutriation. Cells that tend to stick together or attach to the culture substrate (e.g., Chinese hamster ovary cells) need more attention. These cells should be kept in suspension before synchronization by growing them in spinner flasks continuously stirred with a magnetic stirrer at speed high enough to prevent sedimentation and low enough to avoid cell breakage (Fig. 3). The resuspension of the cell suspension is also recommended by passing it several times through an 18-gauge needle right before loading in the elutriation chamber to ensure monodispersion. Once cells are in the chamber, the flotation of cells keeps them separated.

1.4 Components of the Centrifugal Elutriation System

The counterflow elutriation system (earlier Beckman, now Beckman-Coulter Inc.) consists of a centrifuge, elutriation rotor containing the elutriation chamber, stroboscop, peristaltic pump, manometer, sample mixing tube, and the flow system (gauge for monitoring back pressure, injection and bypass valves, T-connector, rubber stoppers, and silanistic tubing) (Fig. 4). Additionally, the pump may be interfaced with a fraction collector. The cell separation is carried out in a specially designed centrifuge hosting either the standard JE-6B rotor or the larger JE-5.0 rotor. The standard elutriation chamber is available in two sizes. The small elutriation chamber has a capacity of approximately 4.5 ml and is suitable for the fractionation of 10^7–10^9 cells. The large chamber is able to separate ten times more volume and cells. The geometry of the Sanderson chamber gives a better resolution for the separation of small cells in the range between 10^6–10^8 cells. The peristaltic pump is providing a gradually changing flow through the chamber with flow rates between 2 and 400 ml/ml depending on the size of the chamber and the cells. The elutriation system uses either a constant centrifugal force and gradually increasing counterflow rate or constant flow rate and reducing rotor speed to yield high resolution of synchronization. As the fine control of rotor speed reduction is not

Fig. 3 Spinner flasks for suspension cultures. Cell cultures (suspension or monolayer) are started in T-flasks containing 25 ml medium. After reaching confluency, the cells of two flasks are combined in a smaller (200 ml) spinner flask that is sitting in the CO_2 incubator. Cells in suspension are grown under constant stirring in 100 ml medium starting at ~10^5 ml/ml density. Logarithmic growth is maintained by scaling up daily from 100 to 200 ml medium, then switching to a larger spinner flask (2 L) and doubling the volume of the medium to 400, 800 and if necessary 1600 ml. Up to 3–4 × 10^8 cells can be grown in one week before starting elutriation

possible, constant speed and increasing flow rate are preferred. The rotor speed may vary between 1800 and 3500 rpm (~550–1700 g relative centrifugal force) depending on cell size. Although higher speed gives a better resolution, the pump speed will also go up increasing the shearing force and the amount of cell debris. The relationship between cell size and centrifugal force is demonstrated in Table 1.

1.5 Monitoring the Efficiency of Centrifugal Elutriation

Due to the close correlation between cell size and DNA content, the effectiveness of the separation can be tested by monitoring the results by cell number, cell size, and DNA content. The most simple method is to count the cells and estimate their size in a hemocytometer. This indispensible method visualizes cells and distinguishes among small and large cells as well as cell aggregates. Particle counters generally monitor changes in conductivity and help to automatize the counting procedure. Most of these devices provide information with respect to particle size and volume distribution. However, size distributions do not exclude errors generated by aggregation, coincidence, and by nonspherical particles. Coincidence can be taken into consideration by counting the same cell population several times in a Bürker chamber and by the

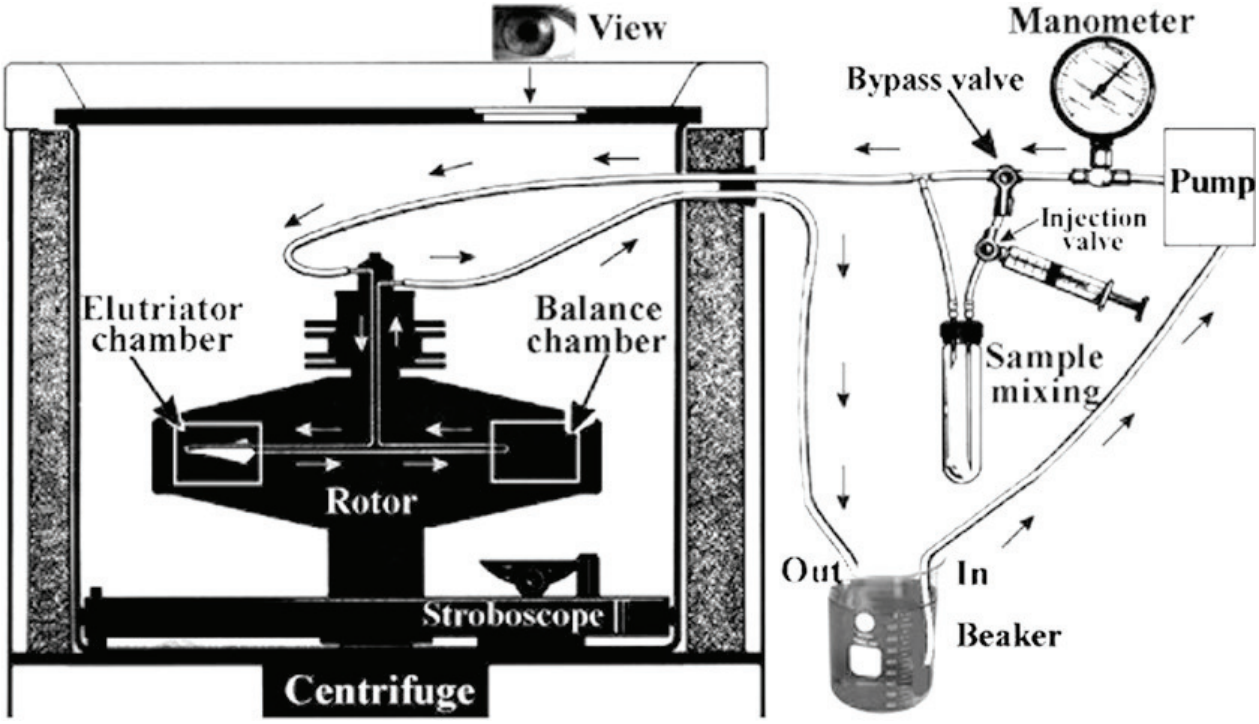

Fig. 4 Counterflow centrifugal elutriation system. The stroboscope is placed and fixed at the bottom of the centrifuge. The centrifuge is hosting the rotor that is running with constant speed. The pump is turned on and sterilization takes place by running 70 % ethanol through the system followed by physiological buffer to remove traces of ethanol. The physiological buffer is then replaced by the elutriation fluid at an initial flow rate. The preliminary run also serves to remove bubbles from the system. The loading chamber serves as a sample mixer and in its bypass position traps small remaining bubbles and compensates pump pulsation. The bypass valve helps to remove residual bubbles from the sample mixer chamber. Reproduced with permission of Banfalvi [11]

Table 1
Elutriation conditions for *Drosophila* and mammalian cells and murine preB nuclei

			Centrifugal force of elutriation		
Cell type	**Cell diameter (μm)**	**Flow rate (ml/min)**	**Rpm**	**Rcf average (g)**	**Rcf maximum (g)**
CHO	11.9	12–49	2000	569	753
CHO	11.9	19–52	2200	575	683
Indian muntjac	11.4	12–50	2200	575	683
Murine preB	10.2	11–73	2200	688	911
Drosophila S2	8.1	13–53	2500	889	1176
PreB nuclei	5.0	14–77	3500	1742	2305

Temperature was 20 °C for each elutriation
Cell diameter refers to an average value of exponentially growing cells and average size of nuclei before synchronization, measured by multiparametric particle size counting
Rotational speed is given as revolution per minute (Rpm), relative centrifugal force (Rcf) in *g*. Reproduced with permission of Banfalvi [11]

particle counter. The ratio of cell count obtained by the cell number counted by the Bürker chamber over the cell number obtained by the particle counter serves then as a factor which is used to multiply the counter number to get the real cell number.

Flow cytometry is used to asses the quality of synchronization by monitoring size distribution with forward scatter analysis and concomitantly the DNA content of each elutriated fraction after staining with propidium iodide. Flow cytometry is also generally accepted for the evaluation of cellular processes [13, 14] for calculating proliferative parameters [15] to identify apoptotic cells by nuclear staining [16–18] or by the appearance of phosphatidylserine on cell surface [19] and monitor other cellular changes [20–22].

2 Materials

2.1 Disposables

1. 1.5 ml microcentrifuge tubes.
2. 15 and 50 ml centrifuge tubes.
3. 15 and 50 ml conical centrifuge tubes.
4. Polystyrene test tubes.
5. 25 and 75 cm^2 flasks for cell culture.
6. 5 ml, 12×75 mm FACS tubes.

2.2 Media and Solutions

1. RPMI 1640 growth medium.
2. Fetal bovine serum (FBS).
3. *Ham's medium*: Measure 10.64 g l-glutamine containing F12 powder for 1 L of 1× solution. Add 1.176 g/L of $NaHCO_3$ powder. Adjust pH to 7.1–7.2, as it will rise to 0.2–0.3 pH units during filtration. Filter-sterilize it immediately using a membrane with a porosity of 0.22 μm. Aseptically dispense into 100-ml sterile bottles. Store them in a cold room or in a refrigerator under 5 °C until use. Before use, add 11 ml FBS albumin to each 100-ml F12 medium. F12 can be ordered as 1-, 5-, 10-, and 50-L liquid medium containing l-glutamine, without sodium hydrogen carbonate. Sterilized medium can be stored at 5 °C for several weeks if unopened.
4. *Elutriation medium* (for 30 elutriation fractions): Prepare fresh 3.5 L of F12 culture medium in a 4-L flask containing 1 % FBS. Omit filter sterilization, as fractionated cells are processed immediately after elutriation. Larger particles originating from F12 medium or from cell aggregates can be removed by passing the medium and cell suspension through a 100-mesh stainless steel sieve.

5. *Phosphate-buffered saline (PBS)*: Instead of culture medium plus 1% FBS, Ca^{2+}- and Mg^{2+}-free PBS with 0.01% EDTA or without EDTA is successfully used as elutriation buffer. PBS contains 2.7 mM KCl, 4.3 mM sodium phosphate dibasic (Na_2HPO4), 1.8 mM potassium phosphate monobasic (KH_2PO4), 137 mM NaCl, pH 7.2. Sterilize in an autoclave.
6. *Saline*: Dissolve 9 g of NaCl in 1 L of distilled water and sterilize in an autoclave.
7. *Trypsin/EDTA solution*: Make up from 0.25% (wt/vol) trypsin, 1 mM EDTA in phosphate-buffered saline (PBS). Filter-sterilize. Stored at –20 °C.
8. *PI solution for flow cytometry*: Dissolve 50 mg/ml of PI in 0.1 M ammonium citrate solution (not sterile).

3 Methods

3.1 Cell Growth

1. Keep the epithelial-like CHO-K1 cells (ATCC, CCL61) in suspension culture in spinner flasks using F12 Ham's medium supplemented with 10% heat-inactivated FBS at 37 °C and 5% CO_2. Grow the murine pre-B cell line 70Z/3-M8 [23] in suspension culture at 37 °C in RPMI 1640 medium supplemented with 10% FBS, 2 mg/ml mycophenolic acid, 150 mg/ml xanthine and 15 mg/ml hypoxanthine and 2×10^{-5} M β-mercaptoethanol.
2. Maintain CHO cells as either monolayers in 75 cm^2 tissue culture flasks when smaller quantities of cells (10^6–10^7) are needed or in suspension in 250 ml and in 1-L spinner flasks if many cells are required, such as in elutriation ($2–3 \times 10^8$).
3. Maintain cells in logarithmic growth and high viability by culturing at densities between 1×10^5 and 4×10^5.
4. Replace media by splitting cells in the ratio of 1:2 daily. Grow CHO cells in suspension culture in 1 L spinner flasks containing a final volume of 800 ml F12 Ham's medium supplemented with 10% FBS. The suspension culture should initially contain $1–2 \times 10^5$ cells/ml[1] at 37 °C in 5% CO_2.
5. Grow cells for 24 h to a final concentration of $2–4 \times 10^5$ cells/ml for fractionation by centrifugal elutriation. Harvest cells by centrifugation at $600 \times g$ for 5 min at 5 °C and resuspend in 10 ml of F12 medium containing 1% FBS. To avoid the presence of dead and fragmented cells in elutriated fractions, *see* **Note 1**. Remove cell aggregates by passing the cell suspension through a 100-mesh stainless steel sieve.

3.2 DNA Isolation

Isolate high molecular weight DNA from 10^6 cells of elutriated fractions, determine the amount of DNA by a DyNA Quant fluorimeter (Hofer Scientific Instruments) using Hoechst 33258 dye,

which binds to the minor groove of double-stranded DNA. Excite the bound dye with long UV light at 365 nm and measure its fluorescence at 458 nm with calf thymus as DNA standard.

3.3 Determination of DNA Content

1. Isolate DNA from elutriated fractions by phenol extraction from an equal number of cells (10^6) from each elutriated fraction.
2. Stain the DNA. The amount of DNA can be determined by specific dyes (e.g., Hoechst 33258).
3. Excite the bond dye with UV light and measure DNA content. Use calf thymus DNA as DNA standard.

3.4 Measurement of Cell Number and Cell Size

Confirm the synchrony of elutriated fractions by measuring cell size in a Coulter Channelyzer or Coulter multisizer. The measurement of gradual and simultaneous increase both in cell size and cell volume as well as the correlation between cell size and DNA content confirm that synchrony is maintained not only at low, but also at increased and higher resolution.

3.5 Flow Cytometry

1. Stain cells with 50 mg/ml of PI in 0.1 M ammonium citrate for 15 min at 0 °C.
2. Add an equal volume of 70% ethanol.
3. Perform cell cycle analysis in a fluorescence-activated cell sorter. Use the flow cytometric profiles of elutriated fractions to calculate the nuclear DNA content expressed in average C-values [24]. The C-value increases from 2C to 4C as the cell progresses through the S phase, providing quantities measure of cell growth. When many fractions (>20) have to be processed within the same day, stained and fixed cells can be stored overnight in refrigerator (4 °C) and flow cytometry can be performed next day.

3.6 Conditions Used for Centrifugal Elutriation

The elutriation system (earlier Beckman, now Beckman Coulter Inc.) is an advanced device that uses either a constant centrifugal force and gradually increasing counterflow rate or constant flow rate and reducing rotor speed to yield high resolution of synchronization. The cell separation is carried out in a specially designed centrifuge and rotor. This elutriator system was designed exclusively for research, not for diagnostic or therapeutic use.

3.6.1 Hardware

The hardware of the JE-6 elutriator system (JE-6B Rotor, elutriator, 6000 rpm, 5080 × *g*, recoverable cells per run 10^9) consists of the following subassemblies: centrifuge, rotor assembly, stroboscope, flow system, modified door for the Model J-21/J-21B or J2-21 centrifuges as well as various tools and lubricants. In our elutriation system, we used a J2-21 centrifuge and a JE-6B separation chamber (Beckman Coulter Inc.), in which the elutriation

fluid was circulated at an initial flow rate of 16.5 ml/min using a MasterFlex peristaltic pump (Cole-Parmer Instrument Inc.) presterilized with 70% ethanol. Elutriation was performed at a rotor speed of 2200 rpm ($683 \times g$) and temperature of 20 °C for 4–5 h. Subpopulations of cells were eluted in F12 medium containing 1% FBS. We have also used the JE-5.0 elutriation system (JE-5.0 Rotor, Elutriator, 5000 rpm, $4704 \times g$, retrievable cells per run 10^{10}), including a Sanderson elutriation chamber (5 ml) and a J-6 M1 centrifuge (Beckman Instruments Inc.) [23]. When the JE-5.0 elutriation chamber is used with the Avanti J-25 and Avanti J-26 XP series and with J6-MI centrifuges equipped with viewport door and strobe assembly, accelerated throughput, shorter run times can be achieved. The JE-6B low-volume elutriator rotor is excluded from the JE-5.0 elutriation system.

The protocol presented here has been developed to enable cell cycle studies in a variety of cell lines [25–27]. The elutriation of $1–2 \times 10^8$ cells in a JE-6B rotor and a J2-21 centrifuge was used most frequently in our experiments [28]. The small JE-6B elutriation system was assembled according to the manufacturer's instructions. Only the flow system (gauge for monitoring back pressure, two three-way valves, T-connector, rubber stoppers, and several feet of Silanistic tubing) was regularly disassembled after elutriation and reassembled before the next elutriation, as the centrifuge was normally used in several other experiments. For shaft, rotor, chamber, pressure ring, stroboscope, and flow system assembly and disassembly, see the instruction manual of the Beckman JE-6 or JE-5.0 elutriator rotor. The rotor speed and flow rate monogram in the manual give the necessary preliminary information on rotor speed (rpm), sedimentation rate, particle diameter (mm), and flow rate (ml/min).

3.6.2 Calibration of Peristaltic Pump

Among the preliminary experiments, the relationship between pump speed and flow rate has to be established. The calibration of the peristaltic pump is done by measuring the flow rates at different pump setting using elutriation buffer. It is recommended to do the calibration before the day of elutriation to lighten the burden of elutriation. The pump can be used within the linear range. In our case, linearity was maintained up to 80 ml/min.

3.6.3 Particle Size Counting

Particle size counters are recommended that produce a particulate size profile and average particle diameter for a given culture. The size limit is set for cells to count them above the limit and cell debris below the limit. The unit is cleaned and first a sample of solution (used for dilution) is analyzed, then the sample of the culture is profiled. The solution profile is subtracted from the culture profile to eliminate solution debris. This yields a debris count and a cell count. More sophisticated units include measurement of the mean particle diameter, which, for cell cultures above the size limit, equates to the average cell diameter.

We have used different counters to determine the sizes of the cells in each fraction. The counter is based on the principle of electronic particle counting, where the amplitude of a voltage pulse, caused by a change in impedance when a particle suspended in an electrolyte is drawn through an electrical field, is proportional to the volumetric size of the particle [29]. From the change in impedance, the diameter and surface area of the particle are calculated, assuming that the particles are spherical.

3.7 Installation of Elutriation System

To operate the elutriation system, install the stroboscope first, as its chamber unit has to be placed at the bottom of the centrifuge. Turn the stroboscope unit in such a way that the flashlight will be under the observation port when the door of the centrifuge is closed. Rotate the wheels on the casting to tighten the chamber of the stroboscope. Run the cables of the stroboscope through the ports of the centrifuge inside the chamber and secure them with split rubber stoppers installed from outside. Connect the cables to the control unit of the stroboscope outside the centrifuge. Turn on the power switch and verify that the lamp of the photocell is lit (*see* **Note 2**).

3.7.1 Installation of Stroboscope

3.7.2 Installation of Elutriator Rotor

Open the door of the centrifuge and install the assembled rotor containing the installed chambers (elutriator and bypass chambers). Place the rotor in the centrifuge (*see* **Note 3**).

3.7.3 Installation of Flow Harness

In addition to the port for the strobe control unit, there are two more ports in the centrifuge chamber, one for the tubing coming from the pump and the other for the outlet tubing. One can use only centrifuges that have these ports. Make sure that the tubing running from the pump to the rotor is placed in the upper (inlet) fitting and the outlet tubing in the lower fitting of the elutriator rotor (*see* **Note 4**). Connect the liquid lines. Secure tubings firmly with split rubber stoppers outside the centrifuge.

3.7.4 Installation of Control Units

The preliminary run serves to test whether the control units are functioning properly and the centrifuge is running smoothly. For the test run, check the control units of the centrifuge and set the elutriation speed of the centrifuge. When the rotor is at speed, reset the counter and push START button (*see* **Notes 5** and **6**). The rotor speed will be displayed in tens of rpm on the counter. Put the timer at "Hold" position to avoid untimely interruption of elutriation, the brake at "Maximum" and set the temperature at 20 °C, and limit its change within ±1–2 °C. On the stroboscope control, turn the FLASH DELAY potentiometer counterclockwise to 0 position and slowly turn it clockwise to bring the image of the elutriation chamber into view, seen through the viewport door of the centrifuge. If you have an old JE-6 rotor, the bypass chamber will come into view first. Keep turning the potentiometer till the

separation chamber is seen. The separation chamber has two screws in it that make it distinguishable from the bypass chamber. Watch the pressure gauge in the flow harness (*see* **Notes** 7 and 8). Rise in pressure indicates the presence of air in the system (*see* **Note** 9). Stop the pump and/or decelerate the rotor. Note that not all the cables and ports are visualized in the scheme (Fig. 4). When the pressure is zero, the rotor speed is set at elutriation speed.

3.8 Synchronization of CHO Cells

1. Prepare 3500 ml of fresh F12 elutriation medium in a 4000-ml flask.
2. Fill up a 3000-ml flask with elutriation medium to the rim. Use this as the permanent medium reservoir. Cover it with aluminum foil until further use.
3. Pour 400 ml of elutriation medium into a 500 ml beaker and use it as a temporary medium reservoir.
4. Set up your centrifugal system (Fig. 4). Pump 100 ml of 70% ethanol through the system. Then pump through PBS solution (200 ml) to remove the 70% ethanol used to provide sterilization. Start the centrifugation to remove bubbles. Remove PBS and the bubbles by pumping 100 ml of elutriation medium through the elutriation system and discard this fraction (*see* **Note** 9).
5. Replace the temporary medium reservoir with the permanent medium reservoir. Fill the cell reservoir (sample mixer) with medium using a large (30 ml) syringe. To flush all the air from the sample-mixing chamber, retract the short needle so that the end is flushed with the inside of the rubber stopper. When the sample chamber is full and no more air is seen leaving the exit flow line, close the bypass valve to make sure that the liquid does not enter the sample chamber. Set the initial flow rate of the peristaltic pump at 16.5 ml/min. Keep the elutriation fluid circulating until the cells are introduced into the sample reservoir. Make sure once more that the bypass valve is closed, the speed of the centrifuge is correct (2200 rpm, $683 \times g$), the refrigeration of the centrifuge is on and set at 20 °C and the timer of the centrifuge is set at HOLD (endless) position. Turn the flash delay potentiometer of the stroboscope clockwise slowly from 0 position to bring the image of the separation chamber into view. Number empty culture bottles (or tubes) for collecting elutriation fractions (100 ml each) and stand in ice. Use the scale on the culture bottle as the volume measure.
6. Load the sample reservoir by passing the concentrated cell suspension ($1–2 \times 10^8$ cells in 10 ml of Ham's medium containing 1% FBS in a 50-ml screw cap tube) through an 18-gauge needle three times just before loading to ensure monodispersion. Use a 10-ml Luer-lock syringe for injecting cells into the loading chamber. This also keeps the needle from jumping off the

hypodermic syringe. The needle attaches to the body of the syringe via a screw-type twist. Remove the needle from the syringe. Close the bypass valve, as elution liquid is not allowed to enter the sample reservoir during loading. Attach the syringe directly to the Luer fitting on the injector valve for sample injection. Open the injector valve and inject resuspended cells into the sample reservoir. After injecting cells slowly into the sample reservoir, close the injection valve. Air is not allowed to enter the system. As cells cannot be completely removed from the 50-ml tube and filled in the sample reservoir, those suspended cells that are left in the tube (~0.5 ml) serve as nonelutriated control.

7. Load cells into the elutriation chamber. To load cells from the sample reservoir to the elutriation chamber, turn the sample chamber upside down and open the bypass valve. Start to collect the first 100 ml fraction flushed from the centrifuge. About 100 ml of liquid is required to remove one subpopulation of cells from the 4.5-ml separation chamber. View the loading of cells through the observation window of the door of the centrifuge. The appearance of a darkening cloud indicates the introduction of cells into the elutriation chamber. After loading, no more cells are allowed to enter the elutriation chamber. Close the bypass valve and turn back the loading chamber to its original position (*see* **Note 10**). The fraction collected during loading is discarded. This fraction contains cell debris and dead cells (*see* **Notes 11** and **12**).
8. To obtain the first elutriation fraction, increase the pump speed from 16.5 ml/min to 19 ml/ml slowly by ~1 ml/min increments and collect the next 100 ml media. Gradually increase the flow rate (or decrease rotor speed) to permit the elutriation of further fractions. Collect consecutively 100 ml effluent volumes from the centrifuge. Immediately after a fraction is collected, take 2×1-ml samples and add 9 ml of saline to each sample for Coulter counting and cell-size measurement. Keep elutriated fractions in ice until further use and shake them gently from time to time to avoid cohesive sedimentation and loss of cells (*see* **Note 13**). We have obtained reproducible experiments by performing centrifugal elutriation at low, increased and high resolution of elutriation and collecting 9, 16, and 30 elutriation fractions, respectively (Fig. 5). Only as many samples should be collected as the small team (2–3 persons) carrying out the elutriation can process on the same day (*see* **Note 14**).
9. To finish elutriation, turn off rotor speed and let the high flow rate drive out residual particles from the elutriation chamber. Let the system run and collect 100 ml more liquid to remove all the remaining particles. Remove the medium at high flow rate and rinse the system with 200 ml of distilled water to lyse

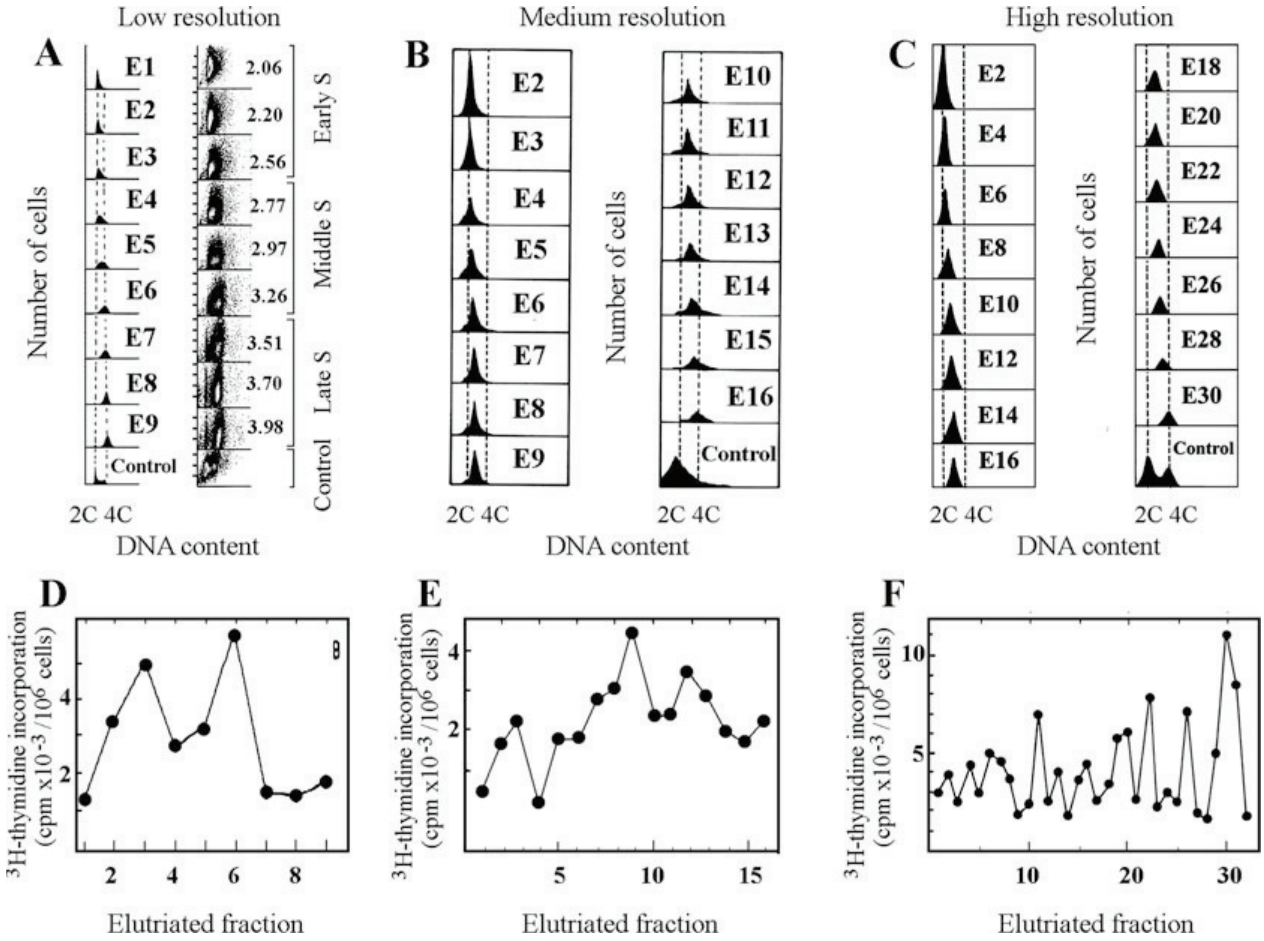

Fig. 5 Centrifugal elutriation at low, medium, and high resolution. Exponentially growing CHO cells were synchronized by counterflow centrifugal elutriation. (**a**) Flow cytometric profiles of DNA content at low (**a**), medium (**b**), and high resolution of elutriation. The cell number is indicated in the ordinate and the DNA content on the abscissa. The average C value (haploid genome content) of elutriated fractions at low resolution (9 fractions, E1–E9) was calculated. For medium resolution 16 fractions (E2–E16) and at high resolution 30 elutriation fractions (E2–E30) were collected. (**b**) DNA synthesis in elutriated fractions at low (**d**), medium (**e**), and high resolution (**f**). ^{3}H-thymidine incorporation is shown in the ordinate and fraction number on the abscissa. 2C- and 4C-values were calculated from the flow cytometric profiles of elutriated fractions [24]. Reproduced with the permission of Banfalvi et al. [30, 31]; Szepessy et al. [32]

any cell left in the system. Cells left in the elutriator chamber may cause pellet formation (*see* **Note 11**).

10. Although almost all parts of the elutriator rotor can be autoclaved, we rinse the system with 70% ethanol to sterilize it. Remove the ethanol by pumping and dry the passages with clean compressed air and leave the rotor assembled. Disconnect the liquid lines and remove the rotor and stroboscope chamber from the chamber. In case of longer storage, remove the elutriation chamber and the bypass (balance) chamber, but store the rest of the rotor assembled.
11. Check the status of your elutriator system by a test run before doing cell separation (*see* **Note 15**).

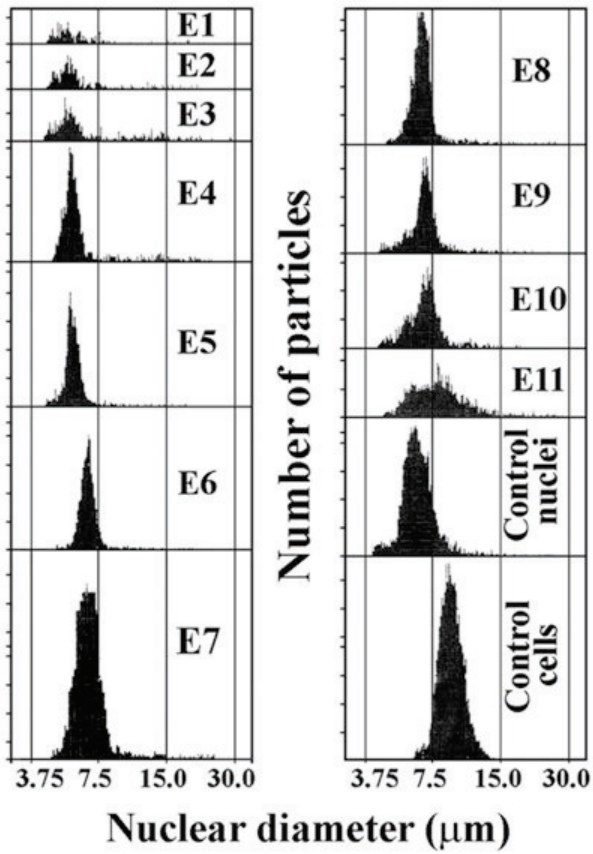

Fig. 6 Centrifugal elutriation of nuclei isolated from murine pre-B cells. Nuclear size is indicated on the abscissa, nuclear number or cell number is given on the ordinate. Cells (1.15×10^8) were subjected to the isolation of nuclei. Elutriation of 6.86×10^7 nuclei was carried out in a JE-5.0 elutriation system equipped with a 5 ml Sandetson chamber and a MasterFlex (Cole-Parmer Instruments) peristaltic pump and a J-6 M1 centrifuge (Beckman Instruments Inc.). Elutriation took place at 20 °C and 3500 rpm ($2305 \times g$). Eleven fractions (E1–E11) were collected, 100 ml each in RPMI medium 1640 supplemented with 1 % FBS. After separation, nuclei were washed with PBS and counted by Coulter multisizer, and nuclear size analysis was carried out. Control nuclei and control cells were not subjected to elutriation. Reproduced with permission of Banfalvi [11]

3.9 Synchronization of Nuclei of Murine Pre-B Cells

1. For the synchronization of isolated murine pre-B nuclei (average diameter 6.8 μm), use higher centrifugal force (3500 rpm, $2305 \times g$).
2. Use a flow rate between 14 and 77 ml/min for murine pre-B cells (average diameter 10.2 μm) and apply a similar flow rate (11–73 ml/min), but a lower centrifugal force (2200 rpm, $683 \times g$) [23, 33]. The synchronization of murine preB nuclei is demonstrated in Fig. 6.

Due to the closer correlation between nuclear size and DNA content than between cell size and DNA content, it could be of potential interest to elutriate subcellular particles to study the fine-tuning of nuclear control. The synchronization of nuclei is expected

to open new vistas for the synchronization of protoplast preparations. The synchronization is based on the nuclear size that is increasing during DNA synthesis and nuclei can be separated by centrifugal elutriation. The introduction of a density gradient during elutriation is not recommended (*see* **Note 16**). To elutriate smaller particles either the centrifugal force has to be increased, or the flow rate decreased. A lower flow rate would limit the resolution, while maximal pump speed (~500 ml/min) should also be avoided as the linearity between the pump speed and flow rate could not be maintained. Turbulence can be minimized by increasing the flow rate slowly from fraction to fraction (*see* **Note 17**).

3.10 Verification of Synchronization

1. Assay the quality of synchronization after centrifugal elutriation in each elutriated fraction. Among the methods used for the isolation and detection of homogeneous populations of cells, several flow cytometric methods are generally accepted. As its introduction, the PI flow cytometric assay has been widely used for the evaluation in different cellular processes in animal models [14] and plant cells [34].
2. Determine the DNA content by fluorometry (*see* Subheading 3.3).
3. Measure simultaneously the cell number and the increasing cell size. This option offers the fastest measurement and is recommended when many fractions are collected and several measurements have to be carried out within a short period of time. A reliable combination is to choose at least two of the three options.

3.11 Synchronized Apoptotic Cells Detected by Forward Scattering

The estimation of cellular DNA degradation during programmed cell death is closely related to the particle size that can be amplified and shows more convincingly the decrease in the cell size of apoptotic cells (Fig. 7a). The quasi 3D representation of forward light scatter analysis in Fig. 7b shows the progression of gamma irradiation-induced apoptosis during 24 h from elutriated fractions 1–9. By means of flow cytometric data, the DNA content of distinct apoptotic cell fractions was estimated and found to be increased from ~0.05 to ~1 C-value [35]. Flow cytometric forward scatter was used to determine the proportion of apoptotic cells relative to unirradiated ones after 24 h incubation. Apoptosis was low in unirradiated cells (~1%) and increased significantly in irradiated cells from 5% to 13–14% (Fig. 7c). To estimate the average value of apoptosis, non-elutriated control populations were also subjected to γ-irradiation and grown in culture medium for 2 and 24 h, respectively. Not only the increase of apoptotic cells from 4.8% to 15.6% were seen in the sub-G_1 marker window, but also the increase in G_1/G_0 and G_2/M phase and the decrease of S phase cells were observed (Fig. 7d). These changes also confirmed the

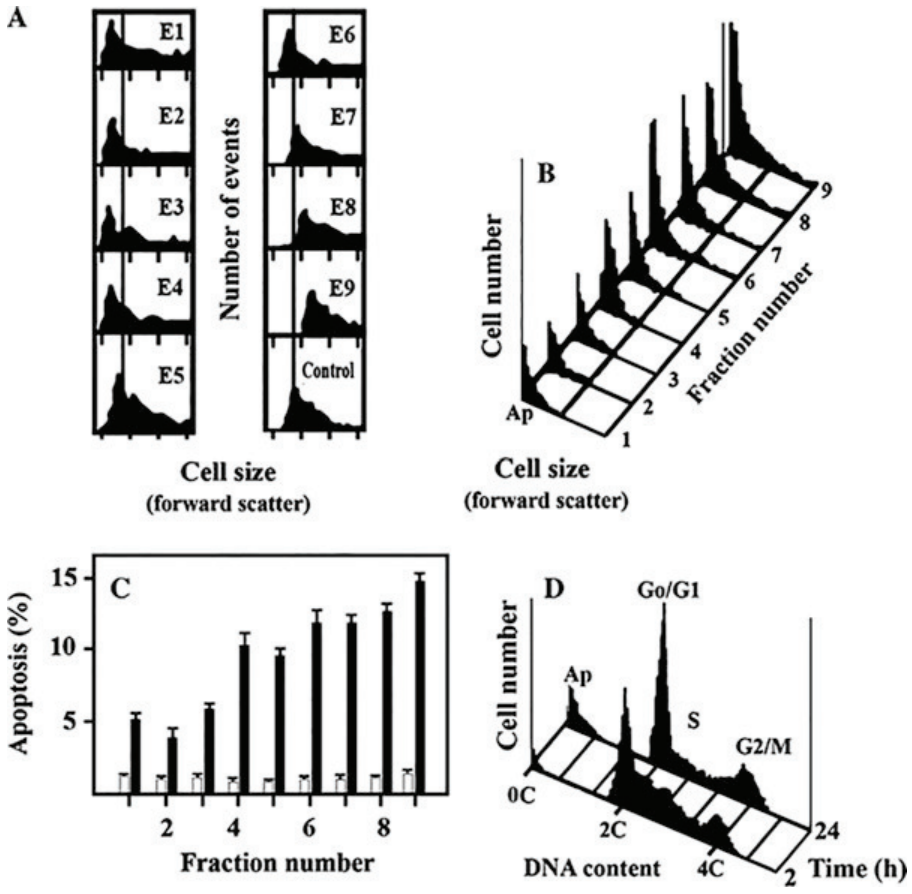

Fig. 7 Apoptotic changes detected by the forward scattering during the cell cycle. Gamma irradiated K562 human erythroleukemia cells subjected to elutriation followed by propidium iodide staining and forward light scatter analysis and found as shrinked cells in the sub-G_1 marker window. (**a**) To follow the apoptotic cell size, a vertical line was placed on the main peak of the irradiated, non-elutriated control. (**b**) After irradiation and incubation for 24 h, apoptotic cells were detected by forward scatter analysis. (**c**) Percentage of apoptotic cells of non-treated (*open square*) and of γ-irradiated (*filled square*) cell fractions. (**d**) Gamma-irradiated cells from non-elutriated populations were cultured at 37 °C. One aliquot of cells was incubated for 2 h, the other for 24 h, then stained with PI and was subjected to forward light scatter analysis. The C-values and major phases of the cell cycle are indicated. *Ap* apoptotic cells. Reproduced with permission of Banfalvi et al. [35]

radiation-induced cell cycle arrest near the G_1/G_0 and at the G_2/M checkpoints [35]. Cells representing different stages of apoptosis can be collected by choosing a somewhat lower centrifugal force for elutriation than for nuclei. By fine-scale elutriation, apoptotic cells can be separated from undamaged healthy cells (*see* **Note 18**). Cold-shock-induced apoptosis has been used without the addition of toxic compounds or antibodies, to synchronize homogeneous apoptotic cell populations [36].

4 Notes

1. *Dead cells*. Avoid the presence of dead and fragmented cells in elutriated fractions. The cell culture has to be kept constantly in logarithmic growth. Best results are obtained when cells are recultivated every day.
2. *Installation of stroboscope*. Make sure that the cables run close to the wall of the centrifuge so that the rotor cannot touch them. In case the photocell of the stroboscope does not work replace the flash lamp.
3. *Placing rotor in the centrifuge*. Be sure that the rotor is properly seated. If the pin of the rotor is improperly resting on one of the castellations, it will lift off when the drive starts. The centrifuge should be perfectly balanced and run smoothly. If you notice any vibration, the balance is imperfect. When the speed of the centrifuge is fluctuating, flow forces are not precisely balanced against centrifugal force to allow proper segregation. Check speed constancy and find the balance between centrifugal force and flow rate in preliminary test runs.
4. *Leaks*. These usually occur when (a) the chamber gasket is put backward; (b) the chamber screws are not tight; (c) tubings and connectors are not properly fitted; (d) there are nicks, scratches, irregularities on O-rings; and (e) permanently lubricated sealed bearings are washed with detergents, which can leach out the lubricant, resulting in a shortened bearing time.
   - *Seal inspection*. Clean the stained stationary seal housing (made of alumina) with soap and water or mild detergent. Abrasive materials should be used neither for cleaning nor for elutriation. Reassemble the bearing assembly (note that the seal mount has a left-handed thread). The seal should be replaced when any damage is visible.
   - *Cross leakage*. The most common problem in centrifugal elutriation is caused by defective or missing O-rings in the shaft assembly. Frequent disassembling and assembling of the elutriator system may result in defective O-rings and leaks. Make sure that all O-rings on the seal screw and transfer tube are in right place, lightly greased. Note, however, that overgreasing may clog the system. Once the subassemblies, as well as the flow system have been assembled and the system proved to be completely sealed without any leakage in the preliminary run, it is not recommended to disassemble it after each elutriation.
   - *High back pressure*. Insufficient spring pressure, which holds the rotating seal against the stationary seal, can be overcome by the back pressure, causing cross leakage. But the most common causes of high back pressure are the

opening of liquid lines in the wrong direction and inadequate de-aeration.

5. *Rotor speed*. As the flow rate relative to rotor speed can be regulated on a much broader and finer scale, it is recommended that during elutriation the rotor speed is held constant rather than using decreasing rotor speed and constant flow rate.
6. *Oscillations in rotor speed*. To avoid temporary changes in rotor speed, constant electric voltage (110 or 220 V) can be secured by plugging the centrifuge to a stabilizer.
7. *Manometer*. Keep the manometer under constant observation at the beginning of elutriation to avoid pressure formation. In the elutriation system, the presence of bubbles is indicated by the sharp increase of the pressure. In such cases, quickly turn off the speed of the centrifuge, which will drive out the bubbles. Repeatedly lower the speed of the centrifuge to remove all the bubbles before elutriation.
8. *Tightness of tubing*. Do not make the connection between the tubing and the rotor too tight; leave some movement for the tubing. As the speed of the elutriation rotors is not high (maximum 6000 rpm, 5080×*g*), vacuum in the centrifuge is not required and could not be maintained due to the liquid lines and electric cables.
9. *Removal of bubbles*. Be sure to fill all parts of the flow harness with liquid until all the bubbles have been removed. Preworm the solutions and the centrifuge to the temperature where elutriation will be performed with the exception of the cells before their loading and the collected fractions which are kept in ice.
10. *Loading cells*. During loading cells into the elutriation chamber, one can line up cells exactly at the boundary inside the elutriation chamber (Fig. 1a): (a) a small increase of the flow rate will push forward the cell population to the boundary; (b) a small decrease of the flow rate will bring the population back to the boundary. Alternatively, the rotor speed could also be changed, but has an opposite effect as changing the flow rate. Such corrections during elutriation are recommended only for advanced users of the system. Basic principle: Do not change conditions during elutriation.
11. *Pellet formation*. Those particles that are not driven out from the separation chamber by the pumping will be pelleted at the entrance of the elutriation chamber as soon as the peristaltic pump stops. The peristaltic pump should not be turned off during elutriation. To remove the sticky pellet, the elutriation chamber has to be disassembled and cleaned.
12. *Damaged cells*. These cells release DNA and tend to stick together. Clumping of cells leads to nonhomogeneous cell populations especially at the final stage of elutriation. Solution:

Avoid rapid pipetting and fast resuspension of pelleted cells, which results in a significant loss of cell recovery, viability, release of chromosomal DNA, and stickiness of damaged cells. We have avoided DNase and EDTA treatment by the gentle treatment and loading of cells.

13. *Cell loss during elutriation*. The possible reasons are as follows: (a) cells are lysed (the system was not properly set up, check solutions for hypotony); (b) cells were left in the mixing chamber; (c) overloading the elutriation chamber results in the loss of smaller (G0/G1 and early S phase) cells; (d) the size gradient was not balanced and cells were not loaded in the elutriation chamber; (e) the centrifuge was stopped or decelerated. The particles concentrate near the increased cross-sectional area of the chamber. Flow velocity is greatest adjacent to the entrance port, the particle concentration is reduced in this area. This effect can be reduced by lowering the centrifugal force and the correspondent flow rates.
14. *High resolution of elutriation*. The danger of collecting too many elutriated fractions is that they cannot be processed. High resolution of elutriation needs the processing of many samples within the same day. Careful planning, the involvement of 2–3 persons in this highly coordinated work, some technical skill and experience are needed.
15. *Unable to judge*. This problem almost always arises when elutriation has not been done before. Consult the JE-6 or JE-5 instruction manual carefully before doing any or further installation. Carry out your first elutriation with an expert and/or discuss the details. Clarify the condition of your rotor by calling your nearest service office and ask for inspection.
16. *Application of density gradient*. Introduction of a density gradient would require a second reservoir. It is not recommended as a second reservoir would make the system too complex to handle. Growth medium with minimum FBS (1%) maintains cell viability and is dense enough to avoid contamination.
17. *Coriolis jetting effect*. Turbulence of particles inside the elutriation chamber can be minimized by increasing the flow rate slowly from fraction to fraction. The collection of many fractions (i.e., small increment in flow rate) also reduces turbulence and contributes to the increased resolution power of elutriation. A small shift (1–2 fractions) in the elutriation profile can be attributed to minor differences in cell growth. This difference can be reduced by strict cell culture conditions and corrected by calculating the C-value (haploid genome content) of each elutriated fraction from the corresponding flow cytometric profile [24].

18. The fine-scale synchronization of apoptotic cells needs specific elutriation conditions to be adapted to cell type and apoptotic treatment.

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References

1. Lindahl PE (1948) Principle of a counterstreaming centrifuge for the separation of particles of different sizes. Nature 161:648–649
2. McEwen CR, Stallard RW, Juhos ET (1968) Separation of biological particles by centrifugal elutriation. Anal Biochem 23:369–377
3. Sanderson RJ, Bird KE, Palmer NF, Brenman J (1976) Design principles for a counterflow centrifugation cell separation chamber. Appendix: a derivation of the equation of motion of a particle under combined centrifugal and hydrodynamic fields. Anal Biochem 71:615–622
4. Pretlow TG 2nd, Pretlow TP (1979) Centrifugal elutriation (counterstreaming centrifugation) of cells. Cell Biophys 1:195–210
5. Meistrich ML (1983) Experimental factors involved in separation by centrifugal elutriation. In: Pretlow TG II, Pretlow TP (eds) Cell separation, vol 2. Academic, New York, pp 33–61
6. Childs GV, Lloyd JM, Unabia G, Rougeau D (1988) Enrichment of corticotropes by counterflow centrifugation. Endocrinology 123: 2885–2895
7. Kauffman MG, Noga SJ, Kelly TJ, Donnenberg AD (1990) Isolation of cell cycle fractions by counterflow centrifugal elutriation. Anal Biochem 191:41–46
8. Bauer J (1999) Advances in cell separation: recent developments in counterflow centrifugal elutriation and continuous flow cell separation. J Chromatogr B Biomed Sci Appl 722:55–69
9. Chianea T, Assidjo NE, Cardot PJP (2000) Sedimentation field-flow-fractionation: emergence of a new cell separation methodology. Talanta 51:835–847
10. Uzbekov RE (2004) Analysis of the cell cycle and a method employing synchronized cells for study of protein expression at various stages of the cell cycle. Biochemistry 69:485–496
11. Banfalvi G (2008) Cell cycle synchronization of animal cells and nuclei by centrifugal elutriation. Nat Protoc 3:663–673
12. Keng PC, Li CK, Wheeler KT (1980) Synchronization of 9L rat brain tumor cells by centrifugal elutriation. Cell Biophys 2:191–206
13. Riccardi C, Nicoletti I (2006) Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 1:1458–1461
14. Doleel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. Nat Protoc 2:2233–2244
15. Terry NHA, White RA (2006) Flow cytometry after bromodeoxyuridine labeling to measure S and G2/M phase durations plus doubling times in vitro and in vivo. Nat Protoc 1:859–869
16. Schmid I, Uittenbogaart C, Jamieson BD (2006) Live-cell assay for detection of apoptosis by dual-laser flow cytometry using Hoechst 33342 and 7-aminoactinomycin D. Nat Protoc 1:187–190
17. Mukhopadhyay P, Rajesh M, Haskó G, Hawkins BJ, Madesh M, Pacher P (2007) Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. Nat Protoc 2:2295–2301
18. Ferlini C, Scambia G (2007) Assay for apoptosis using the mitochondrial probes, Rhodamine123 and 10-N-nonyl acridine orange. Nat Protoc 2:3111–3114
19. van Genderen H, Kenis H, Lux P, Ungeth L, Maassen C, Deckers N et al (2006) In vitro measurement of cell death with the annexin A5 affinity assay. Nat Protoc 1:363–367
20. Quah BJC, Warren HS, Parish CR (2007) Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. Nat Protoc 2:2049–2056
21. Chattopadhyay PK, Yu J, Roederer M (2006) Live-cell assay to detect antigenspecific CD4+ T-cell responses by CD154 expression. Nat Protoc 1:1–6
22. Pittet MJ, Swirski FK, Reynolds F, Josephson L, Weissleder R (2006) Labeling of immune

cells for in vivo imaging using magnetofluorescent nanoparticles. Nat Protoc 1:73–79

23. Offer H, Zurer I, Banfalvi G, Rehak M, Falcovitz A, Milyavsky M et al (2001) p53 modulates base excision repair activity in a cell cycle specific manner following genotoxic stress. Cancer Res 61:88–96
24. Basnakian A, Banfalvi G, Sarkar N (1989) Contribution of DNA polymerase delta to DNA replication in permeable CHO cells synchronized in S phase. Nucleic Acids Res 17:4757–4767
25. Banfalvi G, Nagy G, Gacsi M, Roszer T, Basnakian A (2006) Common pathway of chromatin condensation in mammalian cells. DNA Cell Biol 25:295–301
26. Rehak M, Csuka I, Szepessy E, Banfalvi G (2000) Subphases of DNA replication in Drosophila cells. DNA Cell Biol 19:607–612
27. Banfalvi G (2006) Structure of interphase chromosomes in the nuclei of *Drosophila* cells. DNA Cell Biol 25:547–553
28. Banfalvi G, Littlefield N, Hass B, Mikhailova M, Csuka I, Szepessy E et al (2000) Effect of cadmium on the relationship between replicative and repair DNA synthesis in synchronized CHO cells. Eur J Biochem 267: 6580–6585
29. Coulter WH (1957) High speed automatic blood cell counter and cell size analyzer. Proc Natl Electron Conf 12:1034–1040
30. Banfalvi G, Mikhailova M, Poirier LA, Chou MW (1997) Multiple subphases of DNA replication in CHO cells. DNA Cell Biol 16:1493–1498
31. Banfalvi G, Poirier AL, Mikhailova M, Chou WM (1997) Relationship of repair and replicative DNA synthesis to cell cycle in Chinese hamster Ovary (CHO-K1) cells. DNA Cell Biol 16:1155–1160
32. Szepessy E, Nagy G, Jenei Z, Serfozo Z, Csuka I, James J et al (2003) Multiple subphases of DNA repair and poly(ADP-ribose) synthesis in Chinese hamster ovary (CHO-K1) cells. Eur J Cell Biol 82:201–207
33. Banfalvi G, Ujvarosi K, Trencsenyi G, Somogyi C, Nagy G, Basnakian AG (2007) Cell culture dependent toxicity and chromatin changes upon cadmium treatment in murine pre-B cells. Apoptosis 12:1219–1228
34. Guidozzi F (1997) Enrichment of ovarian cancer cell suspensions by centrifugal elutriation after density gradient purification. Int J Gynecol Cancer 7:100–105
35. Banfalvi G, Klaisz M, Ujvarosi K, Trencsenyi G, Rozsa D, Nagy G (2007) Gamma irradiation induced apoptotic changes in the chromatin structure of human erythroleukemia K562 cells. Apoptosis 12:2271–2283
36. Fransen JH, Dieker JW, Hilbrands LB, Berden JH, van der Vlag J (2011) Synchronized turbo apoptosis induced by cold-shock. Apoptosis 16:86–93