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REPORT

Optimizing chromosome yield: a comparative analysis of harvesting, preparation and waste recovery methods

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ABSTRACT

In mitotic chromosome preparation, it is crucial to maximize chromosome yield for downstream cytogenetic analysis. Using HeLa cells as a model adherent cell, we assessed and compared the recovery of chromosomes from the entire process as well as the fraction of chromosomes that would generally become discarded in the standardly used trypsinization and mitotic-shake-off chromosome preparation methods. A higher chromosome yield for polyamine (PA) and methanol acetic acid (MAA) chromosomes was achieved using the mitotic-shake-off method compared to trypsinization. Moreover, mitotic arrest using colcemid or nocodazole gave similar PA and MAA chromosome yields in the commonly collected fractions. Interestingly, when comparing the fractions that would usually be discarded in the mitotic-shake-off, for colcemid-treated cells compared to nocodazole-treated cells, a greater number of PA chromosomes was recovered from the former. Our results show that chromosomes can be retrieved from the waste media. These recovered chromosomes display a suitable morphology in all chromosome preparations, suggesting that in conditions where high chromosome yields are required, utilizing the mitotic-shake-off method and recovering the generally discarded chromosome fraction together with the commonly used fraction would aid in maximizing chromosome yield.

METHOD SUMMARY

In this study, we performed a thorough evaluation of chromosome yield obtained from the standard chromosome preparation methods. We varied four parameters involved in the process as well as comparing the yield obtained from both generally collected and discarded fractions.

MULTIDISCIPLINARY ABSTRACT

Maximizing chromosome yield is crucial for chromosome preparation for downstream cytogenetic analysis. In this study, we assessed and compared the recovery of chromosomes from the entire process including the fraction of chromosomes that would generally become discarded in the standardly used preparation methods. A high chromosome yield was achieved when mitotic cells were selectively collected using mitotic-shake-off method compared to trypsinization, regardless of mitotic inhibiting drugs (colcemid or nocodazole) used. Interestingly, a decent amount of chromosome yield was recovered from the fractions that would usually be discarded in the mitotic shake-off in colcemid-treated cells. Our results show that chromosomes can be retrieved from the waste media. These recovered chromosomes display a suitable morphology in all chromosome preparations. This suggests that in conditions where high chromosome yields are required, utilizing the mitotic shake-off method and recovering the generally discarded chromosome fraction together with the commonly used fraction would aid in maximizing chromosome yield.

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1. Background

Chromosome preparation for chromosome analysis is used in a number of biological areas such as in genetics, medicine, molecular biology, and many other fields [1]. For any chromosome preparation, the quantity and quality of the chromosomes is essential for any downstream applications, such as their use in conventional and molecular cytogenetics [2–4]. Protocols to prepare mitotic chromosomes can often result in a diminished chromosome yield [5]. As the sample preparation involves multiple steps ranging from tissue culture, mitotic arrest, fixation/preservation and slide preparation, each step must be carefully considered [6]. Determining the correct methodology is a crucial step, as various technical variations can be used to prepare chromosomes. There are two main types of chromosome preparations that are widely used. The first includes methanol acetic acid (MAA) fixed chromosomes [7], used for conventional karyotyping and Fluorescent in Situ Hybridization (FISH) [8]. These chromosomes are ideal for the detection of chromosome rearrangements, including those found in genomic disorders, and are also used in many laboratory settings for research purposes [9,10]. The other type is the "in solution" polyamine (PA) chromosomes [11] mostly used in flow cytometry to generate whole chromosome probes for FISH, genome sequencing, structural studies in microfluidic devices, and identifying chromosomal proteins [10,12–22].

Methods for preparing chromosomes have been well-established [2,6]. The quality of the chromosome preparation is assessed via microscopic visualization using a standard light microscope [9,23]. Chromosomes are arrested at the pro/metaphase stage of the cell cycle following the addition of microtubule depolymerizing agents such as colcemid or nocodazole [24]. The mitotic shake-off method, originally reported by Terasima and Tolmach [25], exploits the fact that cells attached to plastic culture dishes round up during mitosis and can be dislodged by physical agitation. This method is especially effective for adherent cells synchronized in metaphase [26–28]. The cells that remain on the surface of the flask are generally discarded as they are not in the mitotic stage. An alternative method is to add trypsin solution to cells treated with the mitotic inhibitor. In the case of trypsinization, the supernatant prior to the addition of trypsin is usually disposed of. For both the mitotic shake-off and the trypsin method, the cell membrane is then ruptured using a hypotonic buffer [29–31], and chromosomes are fixed either by MAA [7] or kept "native" by suspending them in a PA buffer [11].

As in both the trypsin and shake-off methods, a fraction of chromosomes are lost in the process. Herein this work, the chromosomes of such discarded cell fractions are recovered and analyzed based on their yield and morphology both in PA and MAA suspensions. Additionally, two mitotic inhibitors, colcemid and nocodazole, were compared to determine their effect on the chromosome yield, morphology and recovery rate in the discarded cell fraction of trypsinization and mitotic shake-off methods.

2. Materials and methods

2.1. Cell culture and synchronization

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-Glucose supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Life Technologies, UK), 1% GlutaMAX (Gibco, Life Technologies, UK) and 1% Penicillin/Streptomycin (Gibco, Life Technologies, UK) (together as complete media) at 37 °C under humidified 5% CO_2. For these experiments, HeLa cells were seeded at 2.5×10^5 cells in 15 ml of media in a T75 flask (~15% confluency) overnight. On the following day, cells (~20–30% confluency) were synchronized with a concentration 2.5 mM thymidine (Sigma, UK) in a complete medium (187.5 µl of 200 mM Thymidine stock solution into 15 ml cell culture medium) for 22 h. The thymidine was removed by pipetting and the cells washed twice using 1× Phosphate-buffered Saline (PBS) (Gibco, UK). The cells were incubated in a fresh completed medium for 8 h (with conditions as above), then synchronized by adding the same concentration of thymidine (~50-60% confluency) for an additional 15 h. Following the second thymidine block, the cells were washed with PBS and re-cultured in the fresh complete medium in the incubator. To obtain the mitotic cells, 5 h after being released from the second thymidine block, either colcemid (Thermo Fisher Scientific, UK) or nocodazole (Sigma, UK) were added to the cells in the growth medium, both at 0.1 µg/ml in 15 ml cell culture medium (150 µl of 10 µg/ml colcemid stock solution, 0.3 µl of 5 mg/ml nocodazole stock solution). The cells were then incubated with the mitotic inhibitors for 6 h before harvesting.

2.2. Chromosome preparation

A flow diagram showing the complete chromosome sample preparation procedure with conditions used is shown in Figure 1. Chromosomes were harvested for both the mitotic shake-off (MS) method [25] and the general trypsinization method (non-MS) [23]. In the MS, mitotic cells round up and easily detach from the culture surface and accumulate in the medium after knocking the bottom of the culture flask on a table. The medium containing mitotic cells was collected and referred to as mitotic shake-off supernatant (MSS). The remaining cells in the flask were collected after adding 1X trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, Life Technologies, UK) and collected as a trypsinized fraction in the mitotic shake-off (MST). On the other hand, for non-MS, which is the trypsin method for harvesting chromosomes [23], the cell culture media was first collected as a supernatant fraction (non-MSS). After that, the cells in the flask were collected by adding 1X trypsin-EDTA, thus generating the trypsinized fraction (non-MST) or the second fraction from the same flask. A comprehensive summary of the above chromosome preparations together with the Sample IDs they were allocated with, is shown in Table 1.

Chromosomes were isolated in a PA buffer according to [5,10]. In brief, collected cells were spun down at 150 ×g for 5 min. The cell pellet was resuspended in 5 ml of 75 mM potassium chloride (KCl) (VWR, UK) (hypotonic solution) and incubated at 37 °C for 15 min. Following the incubation, the cells were spun down at 200 ×g for 5 min, and the pellet was resuspended in 1 ml of the PA buffer (15 mM Tris-hydrochloric acid (Tris-HCl) (Sigma, UK), pH 7.2, 80 mM KCl, 20 mM sodium chloride (NaCl; Sigma, UK), 2 mM EDTA (Sigma, UK), 0.2 mM spermine (Sigma, UK) and 0.5 mM spermidine (Sigma, UK) containing 0.12% digitonin (Sigma, UK). The cells were left on ice for 10 min and the suspension was vortexed vigorously for 2 min, then centrifuged at 190 ×*g* for 3 min at 4 °C. The supernatant was collected and centrifuged at 1,750 ×*g* for 10 min at 4 °C. After centrifugation, the chromosome pellet was resuspended in a 500 µl PA buffer and stored at 4 °C. MAA chromosomes were prepared as previously described [16,23]. Simply, the collected cells were spun down at 150 ×g for 5 min, and the cell pellet was resuspended in 5 ml of prewarmed 75 mM KCl and incubated at 37 °C for 10 min. Following the incubation, the cells were spun down at 200 ×g for 5 min. The pellet was resuspended in 5 ml of an ice-cold 3 methanol: 1 acetic acid (MAA) (Sigma, UK) solution and mixed thoroughly by flicking the tube. The tube was further incubated for 5 min at 4 °C. Following incubation, the cells were spun down at 200 ×g for 5 min and washed twice with 500 µl of the MAA solution. The samples were stored at 4 °C.

2.3. Assessment of chromosome yields

PA chromosomes obtained from the different preparations were initially diluted by a factor of 2 in the PA buffer and then stained with 5 µg/ml Hoechst 33342 (minor groove-binding blue fluorescent DNA probe) (Thermo Fisher Scientific, UK) as a general stain to validate chromosome morphology. 10 µl of the diluted chromosome solution was added to a hemocytometer (Supplementary Figure 1). A fluorescence microscope with a 10× objective lens was used to acquire images. Twenty gridded squares (0.04 mm^2 each) at the center of the hemocytometer were counted and averaged and multiplied by a dilution factor of 2 and 2.5×10^5 (an inverse of a volume of the 0.04 mm^2 gridded square) [32] to obtain the average number of chromosomes per ml (see Supplementary Figure 1). Chromosomes were counted twice using a hemocytometer to generate an average and standard deviation (SD) between the two counts (Supplementary Table 1).

While PA chromosomes are dispersed in the solution and are not part of distinct spreads, the MAA chromosomes display as clear chromosome spreads and nuclei. Thus, instead of using the hemocytometer as previously described for the PA samples, the number of spreads and nuclei per field of view per condition could be recorded instead. Hence, for MAA-prepared chromosomes, 12 µl of the MAA solution was dropped onto a glass slide, air-dried at room temperature (RT), and then stained with 5 µg/ml of Hoechst 33342. The number of chromosome spreads and nuclei were averaged from 5 fields of view per chromosome preparation, and the SD was calculated between the different fields of view (Supplementary Table 2). All images were taken using a 10× objective lens on a Nikon microscope (Ni-E) using NIS element software (version 5.42.01).

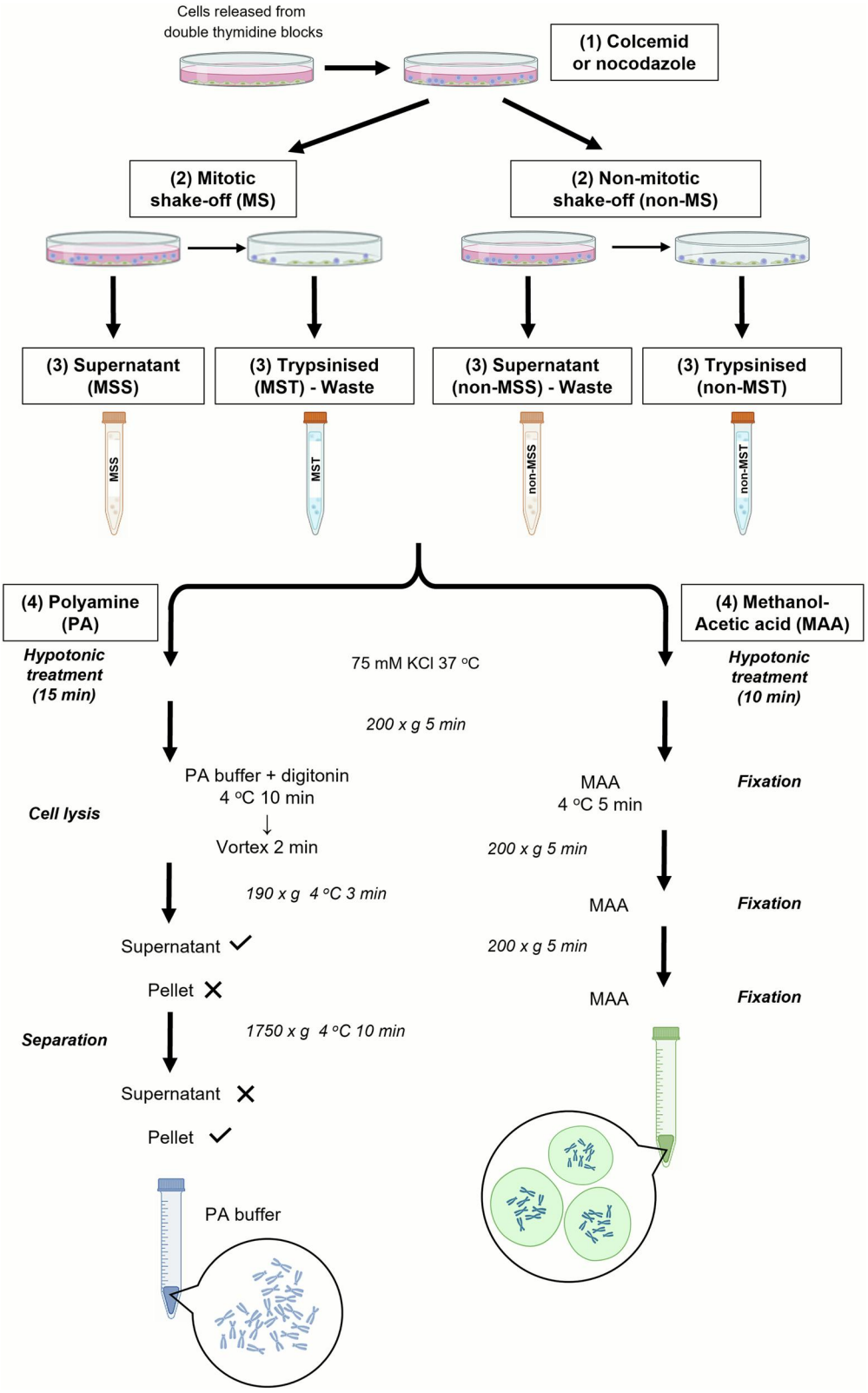

Figure 1. Flow diagram showing chromosome preparation steps. Four parameters in the preparation were compared: 1) Mitotic inhibition using colcemid or nocodazole; 2) Cell harvesting with or without mitotic shake-off (MS or non-MS); 3) Parts of culture collected supernatant (S) or trypsinized (T); and 4) Chromosome extraction using polyamine buffer (PA) or methanol-acetic acid (MAA).

Table 1. Conditions used for the different chromosome preparations.

Sample ID	Mitotic inhibitor	Cell harvesting	Part collected	Chromosome preparation
A1	Nocodazole	MSS	Supernatant	PA
A2	Nocodazole	MST	Trypsinized	PA
A3	Nocodazole	MSS	Supernatant	MAA
A4	Nocodazole	MST	Trypsinized	MAA
A5	Colcemid	MSS	Supernatant	PA
A6	Colcemid	MST	Trypsinized	PA
A7	Colcemid	MSS	Supernatant	MAA
A8	Colcemid	MST	Trypsinized	MAA
A9	Nocodazole	non-MSS	Supernatant	PA
A10	Nocodazole	non-MST	Trypsinized	PA
A11	Nocodazole	non-MSS	Supernatant	MAA
A12	Nocodazole	non-MTS	Trypsinized	MAA
A13	Colcemid	non-MSS	Supernatant	PA
A14	Colcemid	non-MST	Trypsinized	PA
A15	Colcemid	non-MSS	Supernatant	MAA
A16	Colcemid	non-MST	Trypsinized	MAA

MS: Mitotic shake-off; non-MS: nonmitotic shake-off (trypsinization); MSS: the supernatant fraction MS; MST: the trypsinized fraction of MS; non-MSS and non-MST are the supernatant and trypsinized fractions of the non-MS method; PA: Polyamine extraction; MAA: Methanol acetic acid extraction. For each method, the supernatant in the flask (collected by pipetting) and the cells at the bottom of the flask (collected by trypsinization) are indicated as supernatant and trypsinized fraction in the table, respectively.

2.4. Assessment of chromosome quality

For PA preparations, a chromosome solution containing 5 µg/ml Hoechst 33342 was dropped onto a glass slide and covered using a coverslip. For MAA prepared chromosomes, a hanging-drop cast (from ~10 cm height) onto a glass slide was performed and left to dry at RT. Once dry, a drop (~10 µl) of 5 µg/ml of Hoechst 33342 was added to the slide for 10 min. Following staining, the slide was rinsed twice with 1×PBS and covered using a coverslip. PA chromosome images were taken using a 63× oil objective lens on a Nikon microscope (Ni-E) using NIS element software (version 5.42.01). MAA chromosome images were taken using a 63× oil objective lens using a Zeiss Axio Z2 microscope with Isis software.

High-quality chromosomes should exhibit a typical structure, including a compact structure, a clear centromeric constriction, individualized chromatids and distinguishable p and q arms. In addition, for MAA preparation, samples with a higher ratio of chromosome spreads to nuclei were considered favorable for this study.

3. Results and discussion

In this study, we prepared chromosomes from the two most commonly used chromosome preparation methods (MS and non-MS) and also from their normally discarded "waste" media. Both the yield and quality of the chromosomes were evaluated from native (PA) and fixed (MAA) preparations. Again, these are standard chromosome preparation methods. To our knowledge, this is the first study to investigate further recovery of chromosomes from the waste cell culture media that is usually discarded. This idea is not new for mammalian cell culture as waste media is generally recycled and used in secondary microbial fermentation to produce new recombinant protein products [34]. However, we have applied this mammalian cell culture process to chromosome preparations.

Our data shows that chromosomes can be further prepared not only from non-waste media but also from waste media, with both PA (Figure 2) and MAA (Figure 3) preparations. Among non-waste media, the supernatant fraction of the mitotic shake-off method (MSS) gave the highest number of chromosomes from all the preparations. A1 (nocodazole-treated) and A5 (colcemid-treated) gave the highest chromosome count at 5.38±0.18 million and 4.69±0.44 million chromosomes per ml, respectively (Figure 2, Supplementary Table 1). Similarly, for the MAA preparations, A3 (nocodazole-treated) and A7 (colcemid-treated) gave the highest number of chromosome spreads, 16.6±3 and 10.8±2.6 spreads per field of view, respectively (Figure 3, Supplementary Table 2). Our results showed that a higher number of metaphase chromosome spreads were found from preparations with nocodazole compared to colcemid. In agreement with this, previous studies conducted in HUES-2 cells [23] and H9 hESCs [35] showed that with the same concentration and incubation time, nocodazole treatment provides a higher number of mitotic arrested cells in the culture than the colcemid treatment. Furthermore, in combination with

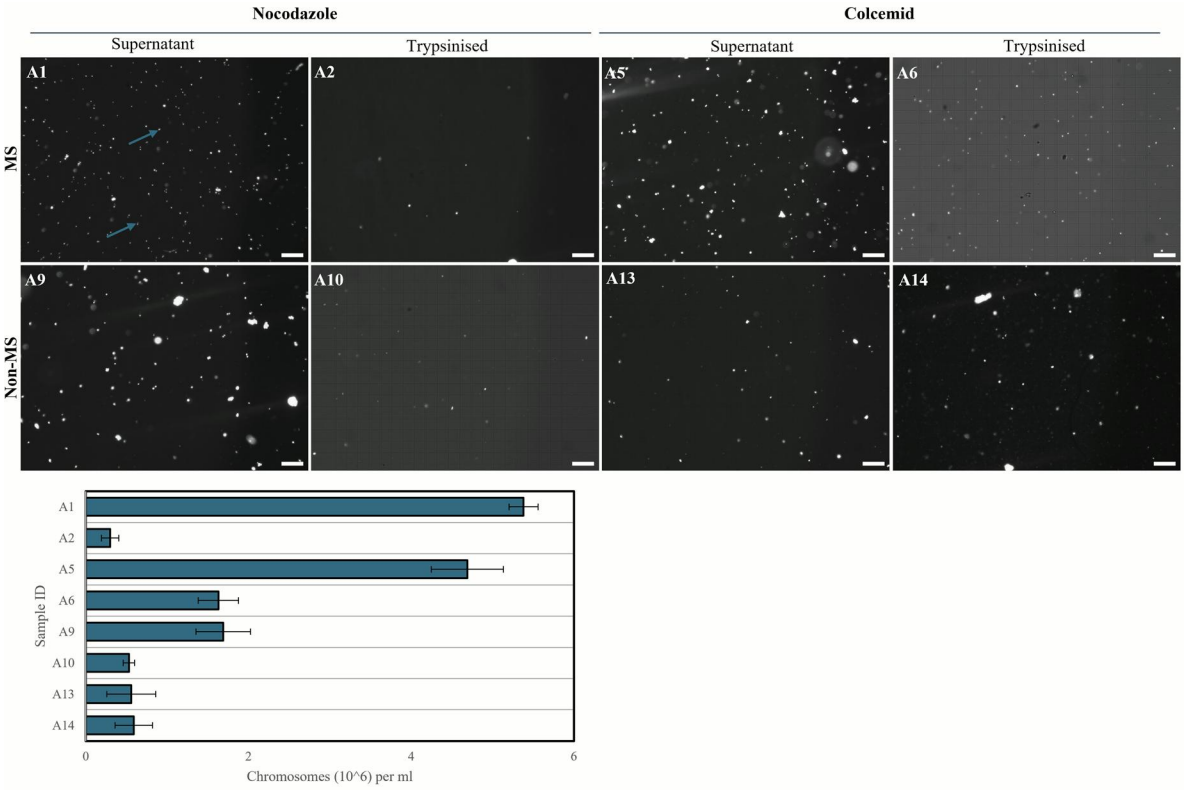

Figure 2. Polyamine (PA) chromosome count for different preparation conditions. Single chromosomes are indicated using blue arrows in A1 as an example. All samples were stained with 5 µg/ml Hoechst 33342 and imaged with a 10× objective lens. The bar graph shows the average number of chromosomes per ml for each preparation condition. The error bars represent the standard deviation from two different counts. Scale bars = 500 µm.

double thymidine block, Yao et al. [36] found that with nocodazole treatment, more than 99% of sheep skin fibroblast cells collected by mitotic shake-off (dish patting) were G2/M arrested cells, while colchicine treatment gave about 88% of G2/M cell fraction from total cells collected by MS. These findings suggest that MSS fractions from nocodazole treatment (A1 and A3) in our study should have higher numbers of mitotic arrested cells, resulting in higher chromosome yields than those from colcemid treatment (A5 and A7). It is possible that nocodazole-arrested mitotic cells easily detached from the culture surface, and with MS, fewer mitotic cells remained attached to the flask surface in nocodazole treatment. Hence, fewer chromosomes were found in MST of nocodazole treatment (A2) compared with colcemid (A6).

For cells harvested without a mitotic shake-off method (non-MS), chromosomes are usually prepared from the trypsinized fraction (non-MST). As shown in Figure 2 and Supplementary Table 1, we obtained 0.53 ± 0.07 and 0.59 ± 0.23 million chromosomes per ml from nocodazole- (A10) and colcemid-(A14) treated non-MST samples, respectively, which is nearly 10× fewer PA extracted chromosome yields compared to the supernatants collected from MS samples (A1 and A5). Similar to PA, MAA preparation from non-MST samples (A12 and A16) had 8 ± 3.3 and 7.8 ± 1.3 chromosome spreads per field of view, which is lower compared to the MS supernatant samples (A3 and A7, Figure 3 and Supplementary Table 2). Unlike MSS samples (A3 and A7), there were a large number of interphase nuclei in both non-MST samples (A12 and A16, Figure 3).

Chromosomes were recovered from all waste cell culture media (MST and non-MSS) with either PA or MAA preparations. In PA preparation (Figure 2 and Supplementary Table 1), a high number of chromosomes of 1.63 ± 0.25 million chromosomes per ml was obtained from A6 (colcemid-treated, MST) and 1.69 ± 0.34 million chromosomes per ml from A9 (nocodazole-treated, non-MSS). In contrast, other waste media (A2, MST; A13, non-MSS) gave much lower number of chromosomes (0.3 ± 0.11 and 0.56 ± 0.3 million chromosomes per ml). This is consistent with our hypothesis above that with nocodazole treatment, cells are easily detached from the flask surface, even without MS, leaving a few mitotic cells attached to the culture surface. On the other hand, some colcemid-treated cells remain attached to the

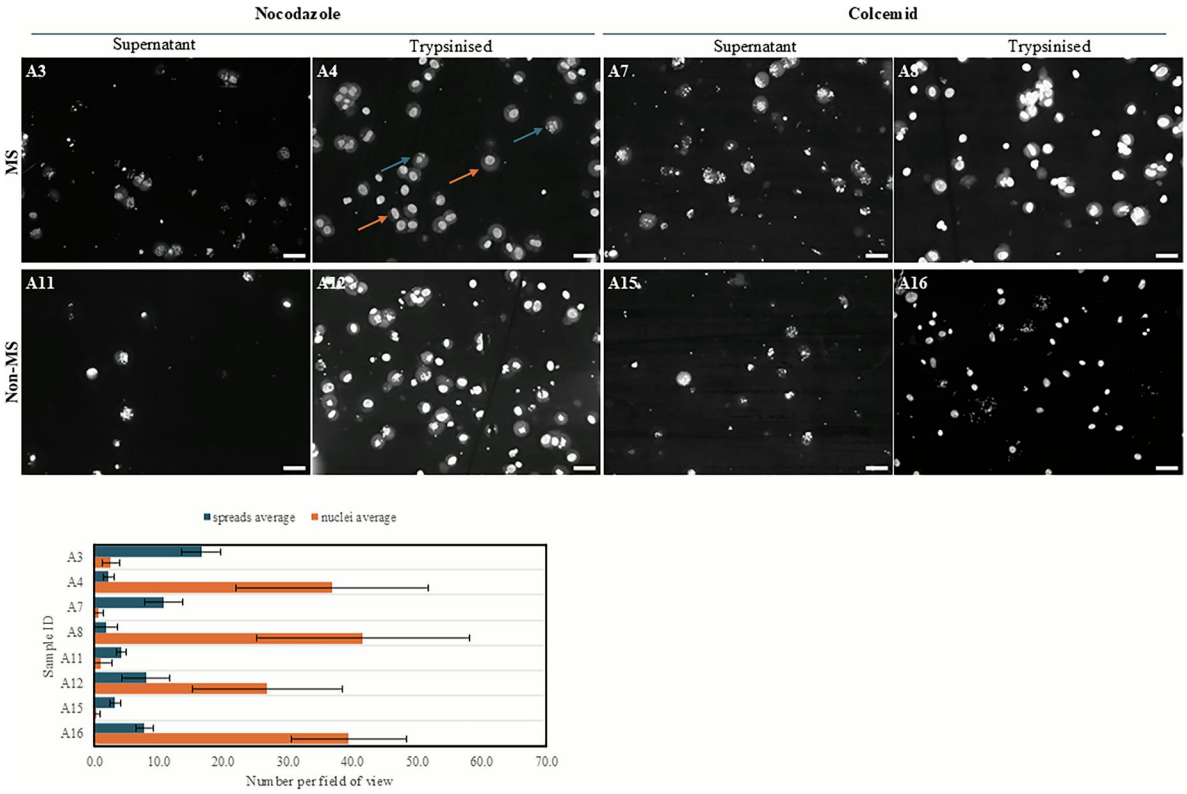

Figure 3. Methanol acetic acid (MAA) chromosome count for different preparation conditions. Chromosome spreads and nuclei are indicated using blue and orange arrows, respectively, in A4 as an example. All samples were stained with 5 µg/ml Hoechst 33342 and imaged with a 10× objective lens. A bar graph shows the average number chromosome spreads (blue) and nuclei (orange) for each preparation condition. The error bars represent the standard deviation determined from 5 fields of view. Scale bars = 500 µm.

flask after MS, and therefore, must be collected by trypsinization. In MAA preparation (Figure 3 and Supplementary Table 2), there was a subtle difference in the number of chromosome spreads, having 2-4 spreads observed in each field of view among all samples (A4 and A8 for MST, A11 and A15 for non-MSS). However, in A4 and A8, there were more than 30 interphase nuclei present in the same field of view.

Despite the difference in chromosome yield among each preparation, morphology of chromosomes extracted from cells from the routine collected fraction and waste media were similar. PA and MAA chromosomes extracted from all samples demonstrated a typical chromosome morphology (Figures 4 and 5), with a centromeric constriction, individual chromatids, and distinguishable chromosome p/q arms. For samples A2 and A6, the morphology is not so clear in the images as the chromosomes are in different orientations in liquid, hence making it difficult to resolve the images. Similarly, to PA chromosomes, MAA chromosomes displayed a classical chromosome morphology. Chromatids in all chromosomes are separated and remain attached only at the centromere. These chromosome spreads are suitable for downstream cytogenetic analysis as they are well spread on the glass slide.

Our study confirms the preference for using the mitotic shake-off method to prepare either PA chromosomes or MAA chromosome spreads from HeLa cells compared to the simple trypsinization (non-mitotic shake-off). However, the yield can be improved further by trypsinizing cells left attached. In addition, when mitotic shake-off is implemented with nocodazole, the largest chromosome yields are obtained.

Taking these results together, the number of PA chromosomes can be maximized by using mitotic shake-off supernatant and then further recovering more chromosomes from the bottom of the flask by trypsinization, especially when using colcemid, as the chromosomes recovered from a nocodazole shake-off sample is minimal. On the other hand, for MAA preparation, although chromosomes could be

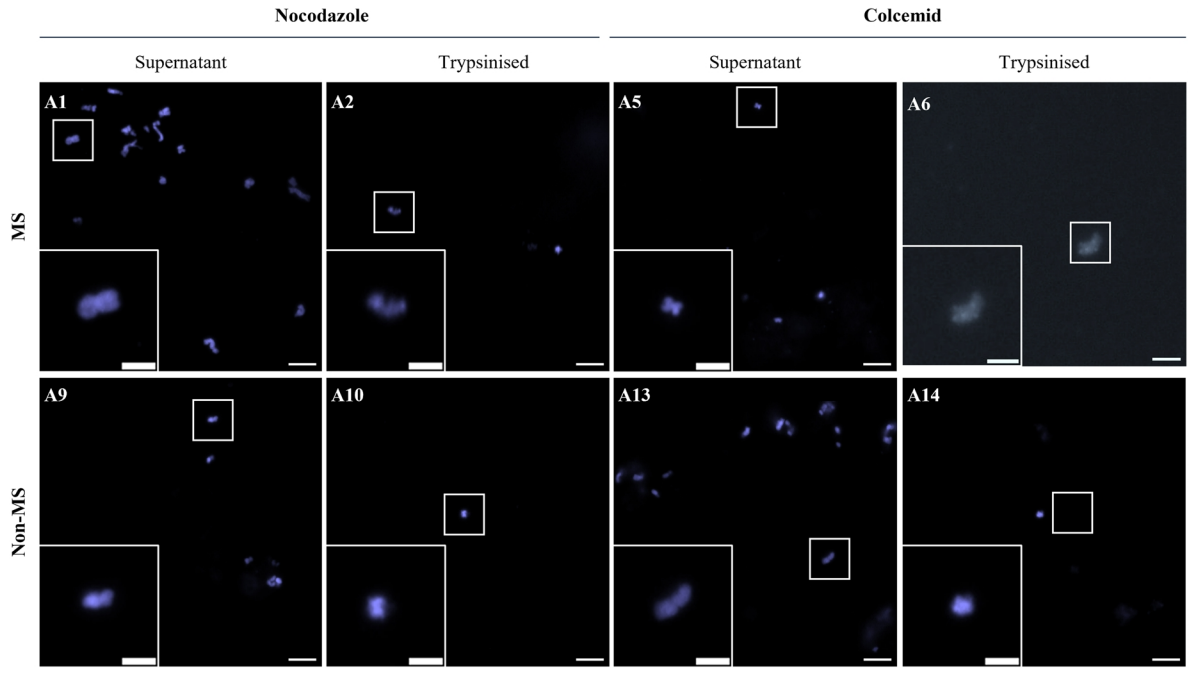

Figure 4. PA chromosome morphology from different chromosomes preparation. All samples were stained with 5 µg/ml Hoechst 33342 and imaged with a 63× objective lens. All images are unprocessed. Scale bars = 5 µm and 2 µm for magnified chromosome images.

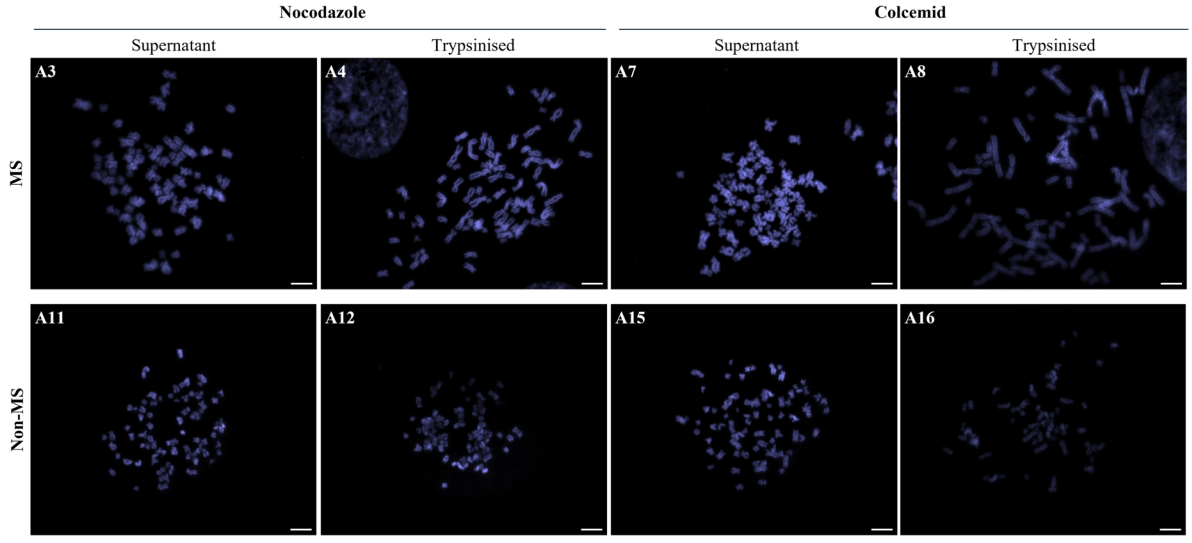

Figure 5. MAA chromosome spreads morphology from the different chromosome preparation conditions. All samples were stained with 5 µg/ml Hoechst 33342 and imaged with a 63× objective lens. All images are unprocessed. Scale bars = 5 µm.

recovered from the trypsinized fraction after the mitotic shake-off, a trade-off would be the presence of a higher fraction of nuclei that could obstruct the finding of chromosome spreads.

This methodology of doubly recovering chromosomes will be useful for any adherent cell lines that rounds up during cell division as demonstrated using HeLa cell line as an example in this study. However, this methodology described here may need further improvements to be applicable to certain specialized adherent cell lines, such as PtK cells (derived from the kidney of a rat kangaroo), that do not round up during mitosis [37], or cell lines derived from blood that grow in suspension, such as lymphocytes. It is clear that harvesting both supernatant and trypsinized fractions maximizes the number of chromosome spreads and chromosome yield, which will be useful for downstream analysis such as stem cells/induced

pluripotent stem cells (iPSCs) [23] where the starting sample amount can be limited. We emphasize that the recovery may vary among different cell lines, which remains to be tested.

4. Conclusion

In this study, we evaluated factors that may influence the yield of PA and MAA chromosomes and their cell waste recovery. Mitotic shake-off plus trypsinization in combination with either nocodazole or colcemid treatment gave the highest yield for both PA (~5 million chromosomes per ml) and MAA chromosomes (10-15 chromosome spreads with less than 5 nuclei per field of view). The highest yield from the waste material alone was obtained from the mitotic shake-off method that had been treated with nocodazole. Hence from this study, the yield of chromosomes obtained was maximized by combining both the standard chromosome preparation method together with the discarded chromosome waste material.

5. Future perspective

Our study demonstrates the potential for increasing chromosome yield by recovering chromosomes from both routinely collected and waste media fractions, providing a more efficient and resourceful approach to chromosome preparation. This methodology, particularly when implementing mitotic shake-off and nocodazole treatment, has shown promising results in HeLa cells and can be adapted to other adherent cell lines. One promising direction is the refinement of recovery techniques for waste media-derived chromosomes to enhance both yield and quality. This may involve modifications in drug treatment, media composition, and incubation times to maximize mitotic arrest and chromosome release. In addition, by integrating this method into cytogenetic workflows, researchers working with rare or precious cell lines, such as iPSCs and primary cultures, can increase the efficiency of karyotyping and other chromosome-based analyses, ultimately advancing studies in genetics, cancer research, and regenerative medicine.

Article highlights

- Obtaining high quality and quantity of chromosomes is essential for cytogenetic analysis.
- In standard chromosome preparation procedure, chromosomes are extracted only from a fraction of mitotic cells harvested from the culture using mitotic shake-off.
- To our knowledge, there is no report assessing if chromosomes can be recovered from discarded fractions.
- Yield of chromosome was compared by varying four parameters in the chromosome preparation method: 1) mitotic inhibitors, 2) cell harvesting techniques, 3) parts of culture collected, and 4) chromosome extraction using polyamine buffer (PA) or methanol-acetic acid (MAA).
- Morphology of chromosomes obtained from all fractions was examined using fluorescence microscopy.
- Standardly collected fractions with the MS method of nocodazole-treated cells produced the highest chromosome yields (both PA and MAA).
- In discarded fractions, maximum PA and MAA chromosomes yield was obtained from cells harvested in supernatant of the non-MS after nocodazole treatment.
- Morphology of chromosomes collected from all fractions is comparable.
- Maximum chromosome yield can be achieved when chromosomes are extracted from cells collected with MS and waste fraction of MS.

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Project administration: Stanley W. Botchway and Mohammed Yusuf

Disclosure statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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