A MODIFICATION OF THE "MOORHEAD TECHNIC" FOR CHROMOSOMAL ANALYSIS

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THE purpose of this paper is to present a simple technic for the preparation of cells for chromosomal analysis. The origin of this technic dates from 1952 when Makino and Nishimura[1] employed a technic involving water pretreatment fixation, and crushing of animal tissue. Sachs,[2] in 1952, also described a hypotonic pretreatment squash technic that was used and was modified by subsequent workers.[3]

The type of tissue that was originally used for the determination of the chromosome number in man was neoplastic tissue, testicular tissue, or normal cells grown in vitro. Using these tissues and the aforementioned technics, the best estimate that workers could achieve of the correct diploid number of chromosomes in man was 48.[4] However, in 1956, according to Ford and Hamerton,[5] Tjio and Levan published observations on four therapeutically aborted Swedish embryos in which they could find only 46 chromosomes. Ford and Hamerton[5] in the same year reported similar results in their study of the freshly removed testes of three men. Their preparations were made with the pretreatment with hypotonic solution, fixation with acetic acid and methanol, and the squash technic.

The arresting effect of colchicine upon growing cells has been known for many years.[6] Thus it was natural that this drug should be used in the early technics for the examination of chromosomes in cells grown in vitro. Ford and Hamerton[7] in 1956 published a method employing colchicine and hypotonic citrate before squashing the cells.

In 1958, Rothfels and Siminovitch[8] suggested an air-drying technic to supplant the traditional squash technic for flattening chromosomes. They grew monkey renal cells in suspension and added colchicine for from 12 to 18 hours before harvesting. They suspended the cells in quarter-strength Tyrode's solution for 10 minutes, and fixed them in a 1:3 acetic acid—methanol solution. The suspension was then dropped onto a slide that was allowed to dry in a horizontal position. They found that chilling of the fixative was unnecessary. They also found that a longer period of incubation with colchicine caused less clumping of the chromosomes than did a lesser period of from two to five hours, as advocated by Ford and Hamerton.[7] This paper was a significant contribution in technic, and led the way to refinements by many other investigators.

Nowell,[9] in 1960, reported a meticulous study on the variable environmental conditions affecting tissue culture. He ruled out any appreciable effect on mitosis by changes of temperature, pH, oxygen tension, carbon dioxide tension, plasma,

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or of cell concentration, and the amount of agitation over at least as wide a range as might be found in vivo. He found that Phytohemagglutinin* was the only factor that caused a significant stimulation in the rate of mitosis.

In 1960 the technic of Moorhead, Nowell, Mellman, Battips, and Hungerford[10] was published. It used the pretreatment of earlier technics on short-term cultures of peripheral blood stimulated with Phytohemagglutinin, and the air-drying technic of Rothfels and Siminovitch.[8] The "Moorhead technic" with its many modifications[11-16] has become the standard method in use by most laboratories today.

We also have modified this technic for use in a standard hospital laboratory.† The technic, as outlined below, is simple, reliable, and inexpensive. Furthermore, only 1.0 ml. of blood is used per culture, which reduces the total amount of blood drawn from the patient. The Phytohemagglutinin is added to the culture medium rather than to the blood directly, thus using it only for its mitotic-stimulating effect, and not for its cell-agglutinating properties. The addition of Colcemide‡ to the culture, for from 18 to 24 hours before harvesting, produces a larger number of adequate metaphase spreads than the six-hour colchicine incubation time as advocated by Moorhead and associates.[10] We have found that the temperature of the hypotonic saline and of the fixative is of no importance in the production of good mitotic spreads. The length of time the cells stand in each of these solutions is important, the optimum time being 20 minutes.

Photographs of the cells are made with a Carl Zeiss Ultraphot II. The entire light arrangement is set for phase microscopy, but the 100X oil immersion lens is used instead of the phase lens. Pictures are taken on Kodak Contrast Process Ortho film, and are developed for five minutes at 70 F. in DK-60 developer. *Figure 1* shows a low-power view of a good preparation with an adequate number of cells arrested in metaphase. *Figure 2* shows a typical cell from which the chromosomal analysis *Figure 3* was made. This technic has been used on 130 cultures, with only 5.4 percent failures.

Steps in the Modified "Moorhead Technic" for Chromosomal Analysis§

I. Drawing of blood

1. Heparinize a sterile syringe.
2. Draw at least 3 ml. of blood.

Phytohemagglutin (Difco Laboratories) is a partially purified mucoprotein extract of* ***Phaseolus vulgaris* *(red kidney bean).*

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‡Desacetylmethyl colchicine, supplied through the courtesy of the Ciba Pharmaceutical Company, Summit, New Jersey.

§Superscripts A-G refer to "Notes on Technic," which follows this section.

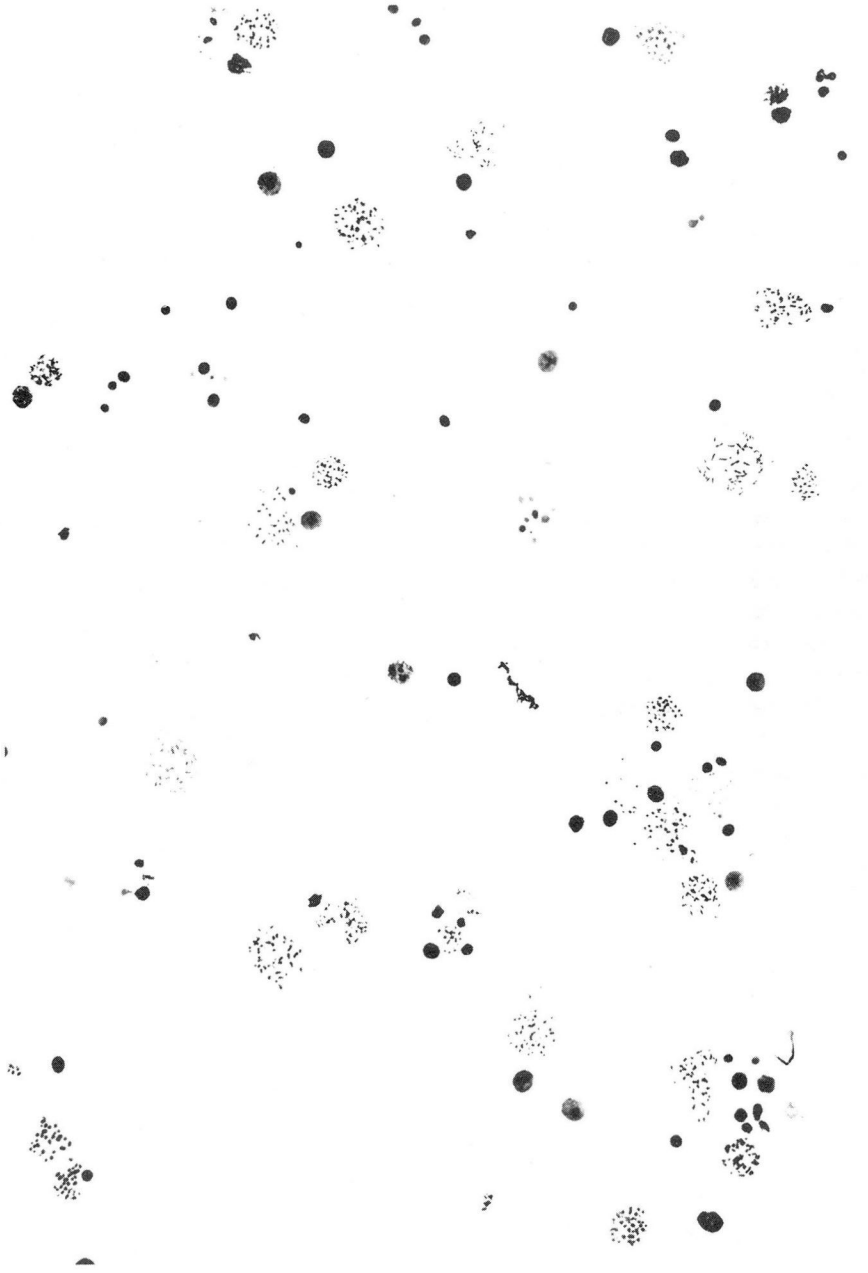

Fig. 1. View of spread showing large number of cells in mitosis, Wright's stain; magnification X150.

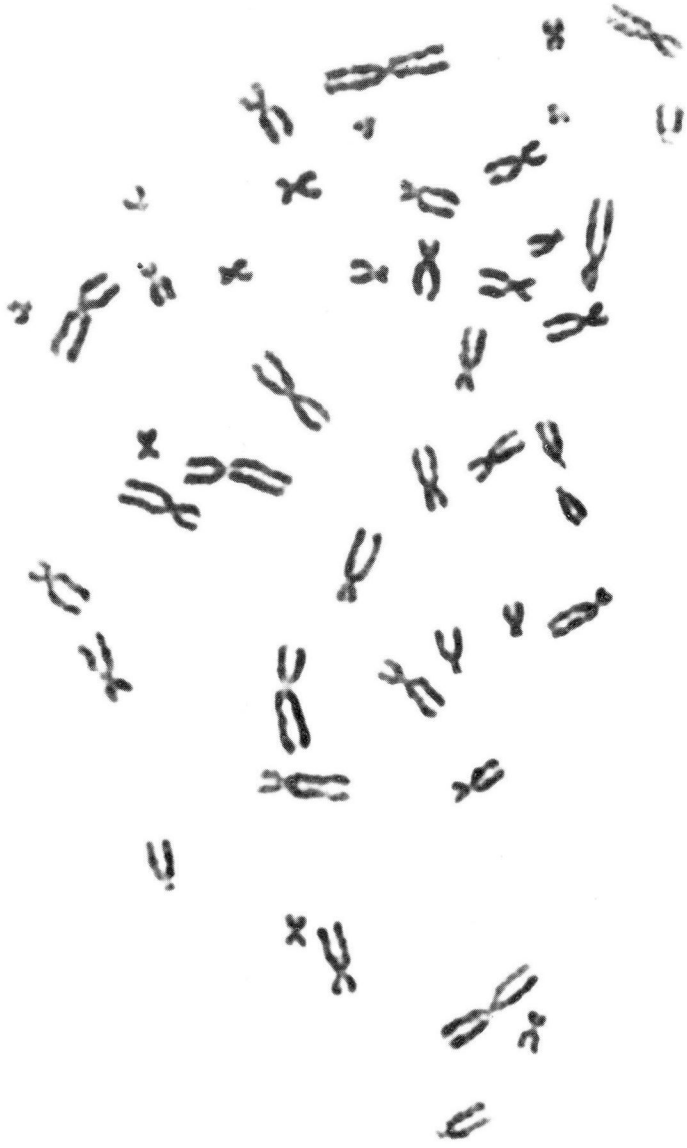

Fig. 2. View demonstrating detail of chromosomes; magnification X2100.

II. Culturing of blood

1. *All glassware* that comes in contact with the blood, from this step on, *must* be cleaned with acid.[A]

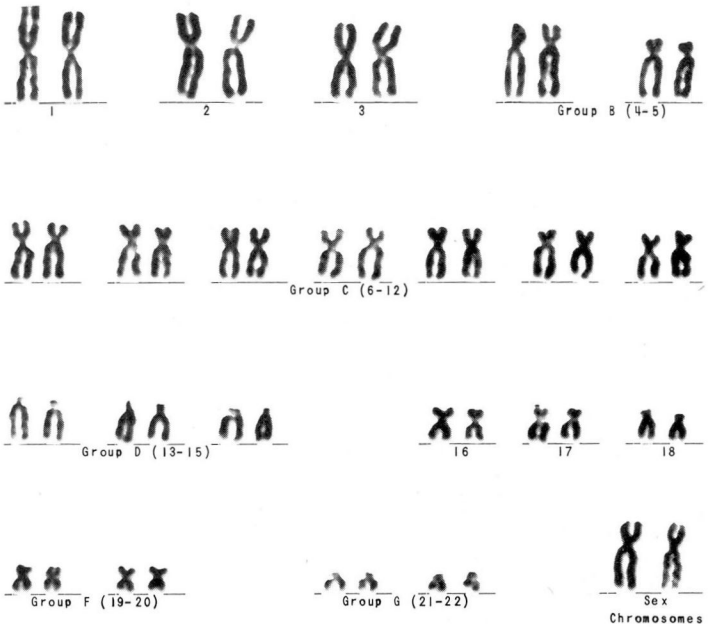

Fig. 3. Karyotype constructed from photomicrograph in *Figure 2.*

2. Place 1 ml. of whole blood into each of two sterile Rourke-Ernstene sedimentation rate tubes.
3. Spin at 1500 rpm for ten minutes.
4. With a sterile Pasteur pipet, draw off all the plasma, making sure to include the buffy coat.
5. Place this in a sterile culture bottle containing 5 ml. of medium.[B]
6. Follow the same procedure for the other tube of blood, to give duplicate cultures.
7. Place in a 37 C. incubator and allow to stand undisturbed for two or three days.
8. At the end of two or three days, add 3 drops (0.05 ml.) of *Colcemide* (*Ciba*)[C] to each bottle, swirl thoroughly, and replace in incubator for from 18 to 24 hours.[D]

Through this point, all technic must be kept as sterile as possible. However, sterility is not necessary in the following steps.

III. Harvesting of culture

1. Pour each culture into a 12-ml. centrifuge tube.
2. Spin at 1100 rpm for five minutes.

3. Remove as much supernatant fluid as possible with a Pasteur pipet.
4. Add 3 ml. of 0.25% buffered saline.[E]
5. Mix gently with a Pasteur pipet.
6. Spin at 1100 rpm for five minutes.
7. Remove all excess supernatant fluid and again add 3 ml. of buffered saline; mix with pipet.
8. Let stand for 15 to 20 minutes.
9. Spin at 1100 rpm for five minutes.
10. Remove all excess supernatant fluid.

IV. Fixing of cells

1. Add 3 ml. of freshly prepared fixative,[F] and mix with pipet.
2. Spin at 1100 rpm for five minutes.
3. Remove all excess supernatant fluid with pipet.
4. Add 3 ml. of fixative, and mix with pipet.
5. Let stand for 15 to 20 minutes.
6. Spin at 1100 rpm for five minutes.
7. Remove all but about 0.5 ml. of the supernatant fluid; mix with pipet.
8. With pipet, drop on *cold*, *wet*, slanted slides.[G]
9. Air-dry quickly.

V. Staining of cells—(Wright's stain)

1. Wright's stain for two minutes.
2. Add phosphate (PO_4) buffer (pH 6.4) and water for 12 minutes.
3. Mount with Canada balsam.

VI. Culture bone marrow with the same technic, using 0.5 ml. of bone marrow. Leave in culture only 24 hours before harvesting.

Notes on Technic

A. Acid cleaning

1. Thorough rinsing in acid cleaning solution—concentrated sulfuric acid (H_2SO_4) and potassium dichromate ($K_2Cr_2O_7$).
2. Ten (10) rinsings in tap water.
3. Ten (10) rinsings in distilled water.
4. Two (2) rinsings in double-distilled water.

B. Culture medium

1. Difco TC 199 . . . . . 100-ml. bottle
2. Phytohemagglutinin P (Difco) . . . 0.5 ml.
   (good for one month)

3. Heparin, sodium . . . . . 1,000 units
4. Penicillin G (freshly prepared) . . . 20,000 units
5. Streptomycin . . . . . 0.04 gm.

C. **Colcemide Injectable Solution (Ciba),** 1-ml. ampules each contain 1 mg. of *Colcemide* per milliliter. This is diluted in 99 ml. of sterile isotonic lactated Ringer's solution, and is handled with sterile syringe technic.

D. **If this technic is inconvenient** (for instance, if a culture was started on a Thursday), the *Colcemide* can be added 1½ hours before harvesting, although this will give a low yield of mitotic figures. The timing is important, for, if the *Colcemide* is left in for 2 to 4 hours, it may cause clumping of the chromosomes. If left in for 18 to 24 hours, this clumping is lost. However, longer than 24 hours causes the *Colcemide* to lose its arresting effect and the cells will proceed on through mitosis.

E. **10% stock solution of buffered saline**

Chemical formula	Grams
NaCl (sodium chloride)	90
Na_2HPO_4 (sodium phosphate, dibasic, anhydrous)	13.65
$NaH_2PO_4 \cdot H_2O$ (sodium phosphate, monobasic, hydrate)	2.15 or
[$NaH_2PO_4 \cdot 2H_2O$ (sodium phosphate, monobasic, dihydrate)	2.43]

This is made up to 1 liter with distilled water, and the pH is adjusted to 7.4.

F. **Fixative** is made by adding 1 part glacial acetic acid to 3 parts absolute methyl alcohol.

G. **Preparation of slides**

1. Rinse slides in acid alcohol (absolute methyl alcohol and acetic acid).
2. Before using, put the slides into ice water and do not remove until immediately before use.

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