Kurre Lab
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Metaphase spread protocol for either murine fetal liver cells or secondary cell lines

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This protocol contains instructions for making metaphase spreads of both fetal liver cells and secondary cell lines. Only the cell preparation steps differ between the two types of cells.

Fetal Liver Cell Preparation:

*NOTE: It is best to start preparing fetal liver cells in the morning because the cells need to incubate for 3-6 hours.

1. Obtain fetal liver and put it into single cell suspension. Aliquot $1.0x10^6$ to $1.0x10^5$ cells to make spreads.

2. For each sample, make 2mL IMDM media with10% FBS, 1% Penicillin-Streptomycin, 50ng/ml mIL3, 50ng/ml mSCF, 0.1µg/ml colcemid.

Notes: If making media for more than one sample, it is good to add an extra mL to the total desired volume as correction for pipette error.

To arrest the cells, colcemid can either be added to each individual sample or to the total volume of media. If adding to the total volume of media, add now and mix well.

3. Spin down the single cell suspension, pour off supernatant, and resuspend in the 2mL of IMDM with cytokines and Colcemid. Pipette to mix.

4. Pipette the 2mL of cells into a well on a twelve-well plate. If the Colcemid was not already added to the media, add it now.

5. Incubate cells at 37˚C (5% CO_2) for 3-6 hours.

6. After incubation, transfer cells to tubes and collect pellet by centrifuging for 5-10 minutes.

7. Pour off supernatant and resuspend in 2-3mL 0.075 M KCl for 20 minutes. Flick tubes to mix.

8. After 20 minutes, add 5 drops of fresh Carnoy's Solution (also called fix) before pelleting again.

Carnoy's Solution: 3:1 methanol to acetic acid. (Needs to be made fresh each day and kept cold.)

9. Resuspend in about 3 mL of Carnoy's Solution, adding dropwise while on level 1 or 2 shake setting of the Vortex Genie.

*Do not pipette to mix. This can break apart the nuclei causing chromosomes to separate from the other chromosomes, leading to incorrect counts.

10. Cover tubes with parafilm and fix cells overnight at 4°C.

11. Just before spreads are made, wash cells twice with fresh fix, and resuspend in 500µl. Transfer cells to 1.5mL microcentrifuge tube to store.

Cells can be stored for several years in a -20°C freezer, but if used often, the storage time is significantly decreased.

Secondary Cell Line Preparation:

1. Expand cell lines. (Plate in a filter top flask and expand until they are in the exponential phase of growth)

2. After expansion, aliquot $1.0x10^5$ to $1.0x10^6$ to cells into a 12 well plate in 2mL cell appropriate media with supplements and 100ng/mL Colcemid.

3. Incubate cells in Colcemid containing media with supplements for 16-18 hours (overnight).

4. After incubation, collect pellet by centrifuging for 10 minutes.

5. Pour off supernatant, and resuspend in 2-3mL 0.075 M KCl for 20 minutes. Flick tubes to mix.

6. After 20 minutes, add 5 drops of fresh Carnoy's Solution (also called fix) before pelleting again.

Carnoy's Solution: 3:1 methanol to acetic acid. (Needs to be made fresh each day and kept cold.)

7.Resuspend in about 3 mL of Carnoy's Solution, adding dropwise while on the level 1 or 2 shake setting of the Vortex Genie.

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Making Metaphase Spreads:

1. Etch coverslips with HCl.

a. Line the bottom of a beaker with cover slips. Pipette 1N or 1M HCl over the top using a 5 mL pipette. CAUTION: HCl is highly volatile. Make sure to do this step in the FUME HOOD.

b. Cover beaker with parafilm or kimwipes underneath aluminum foil.

*If using kimwipes and aluminum foil, be sure not to have any aluminum foil directly exposed to HCl. The HCl will cause the aluminum foil to disintegrate, and pieces will fall into the beaker.

c. Let sit in the hood over night (longer is okay too).

d. Wash 3 times with DI water.

e. Wash in 70% EtOH for 10 minutes.

f. Lean coverslips against something to dry.

2. Prepare a humidity chamber by lining the bottom of a plastic lidded container with damp paper towels. Place container in a water bath at about 55°C. Allow to heat before spreading cells.

3. Label one side of the coverslips with sample names, then flip over so the side with the writing is facing down.

Labeling helps to keep track of the sample and to know which side of the coverslip has the cells on it.

4. Flick or invert cells in tube to mix (the cells settle very quickly). Holding the pipette tip at least 4 inches above the coverslip, pipette 20µl of cells onto it while it is held at a 45° angle.

5. Place cover slip in humidity chamber for 5-10 minutes.

6. Age cover slips 1-3 days in covered box so they don't accumulate dust.

Aging samples is important to get the DNA to adequately adhere to the coverslip.

Hoechst Staining:

1. Prepare Hoechst Staining Solution. Desired concentration is 1µg/mL in 1X PBS.

10µl Hoechst in 100mL PBS

2. Place coverslips cell side up in Hoechst staining solution for 1-3minutes. Using one side of a culture dish works well for staining.

3. While coverslips are in the Hoechst, pipette 10µl fluoromount-G (or other anti-fade mounting medium) onto the middle of a slide. Try to avoid pipetting bubbles onto the slide, as it will cause bubbles when the coverslips are added.

4. After staining, remove coverslips from staining solution. Blot the edges and back of the cover slip to remove excess staining solution, but do not blot the side of the slip with the spreads.

5. Slowly lower coverslip onto the slide. Allow medium to spread out entirely underneath the coverslip.

6. Very carefully dab off excess mounting medium from the edges of the coverslip. Seal with nail polish. Allow to dry thoroughly before imaging.

Imaging:

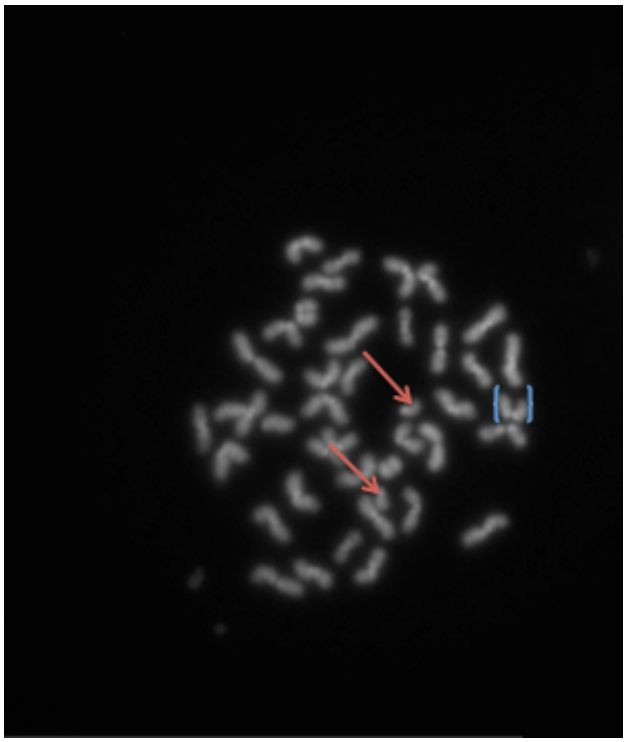
1. Image slides using DAPI under 100x resolution. Make sure to use a microscope with a camera so that pictures can be obtained to count the chromosomes.

2. Only take pictures of spreads where each chromosome can be seen clearly. Make sure there is no cell debris surrounding the spread that could be mistaken for a chromosome.

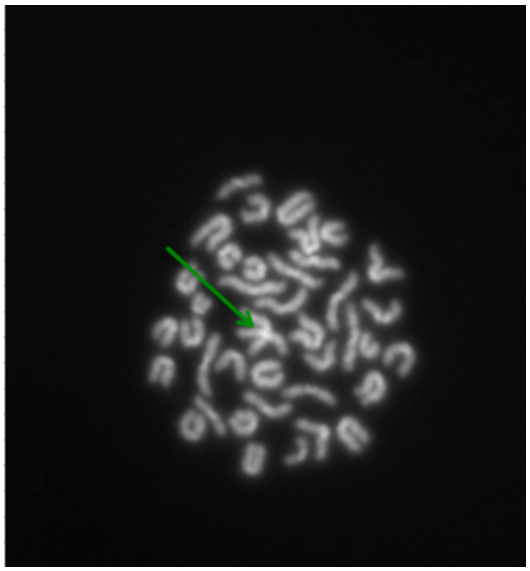
When taking pictures, make sure the exposure is not too high. Images should not be too bright. It will make it hard to differentiate between the chromosomes.

Counting:

1. Count the chromosomes from the pictures. ImageJ cell counter works well.

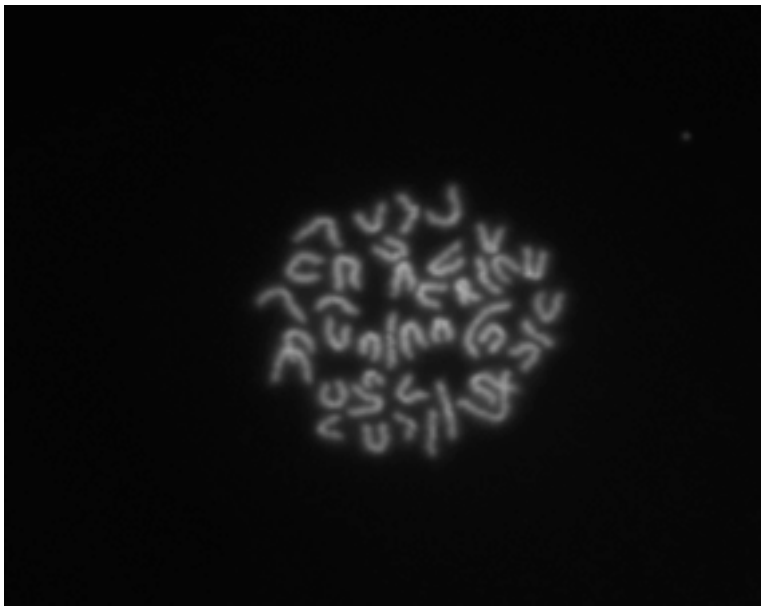


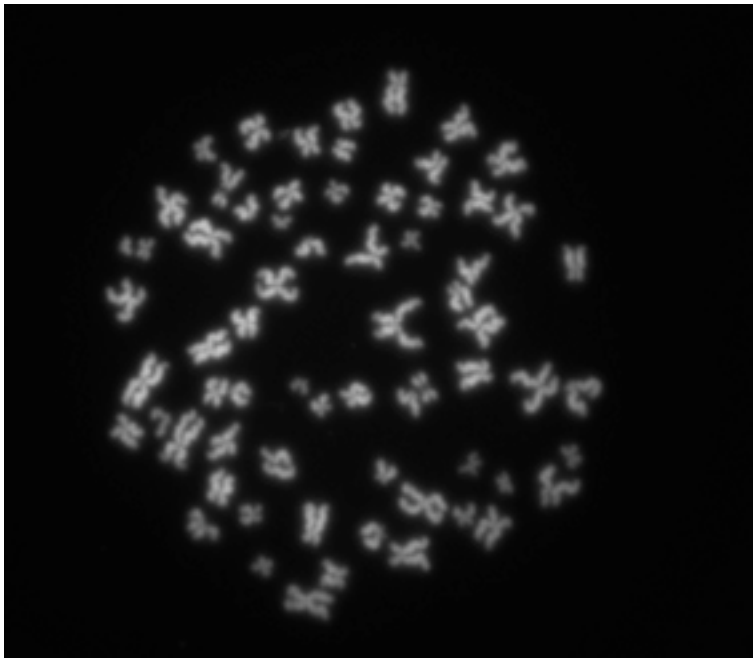
The red arrows point to the two halves of a chromosome that prematurely separated. Watch out for this when counting. The blue brackets surround ONE chromosome whose centromere is spaced more widely apart than some of the other chromosomes in the picture.



In this image, the arrow points to two chromosomes that most likely landed on top of each other when the spread was made. These are counted as TWO separate chromosomes.

Examples of good metaphase spreads:

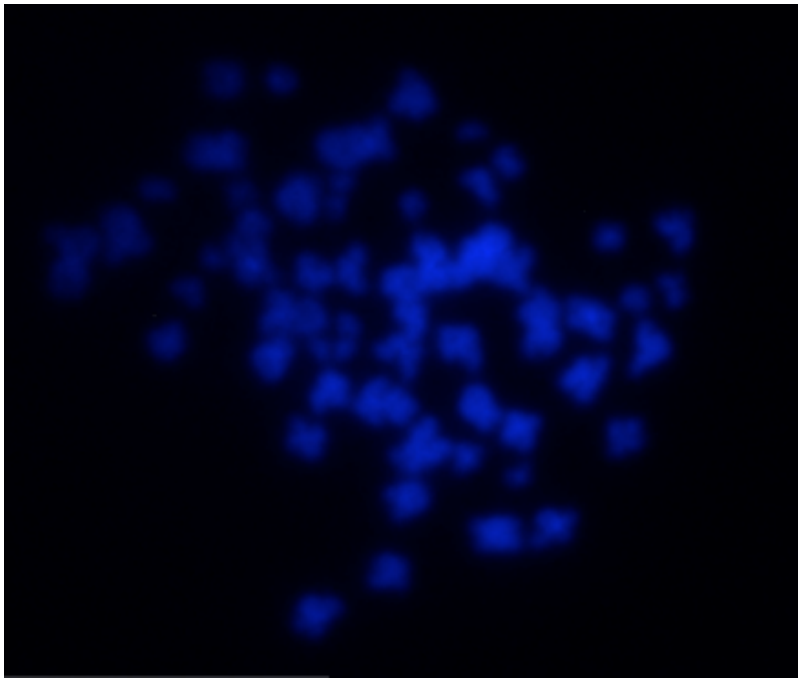




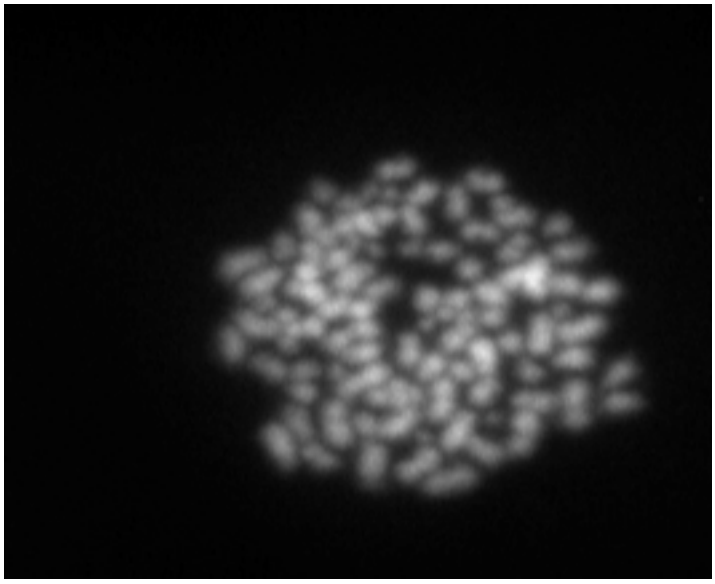
Chromosomes are spread apart from each other to count easily, and there is no surrounding debris.

All chromosomes look different depending on how long the cells spend in Colcemid and what types of cell lines are used.

Examples of bad metaphase spreads:



This image has chromosomes overlapping, making them difficult to count.



In this image the exposure is too high. The shapes of the chromosomes cannot be distinguished.