Lab Manual
for
Introductory Cytogenetics
PLNT3140

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Photo credits: Kenny So

General Lab Rules for Cytogenetics

- Keep the lab tidy. Failure to do so will result in the loss of open lab privileges.

- Great care should be taken with the microscopes. Once the light source has been properly centred (see Appendix1) there should be very little adjustment needed. Take the time to ask your demonstrator for assistance if you are having difficulties maintaining a clear field of view. Immersion oil should only be used on the drawing and photo microscopes to keep the general use microscopes clean. If you find an eye piece or objective has debris on it, it can be gently cleaned with a Kimwipe or cotton swab. Make sure you turn off your microscope and cover before leaving the lab.

- Never use alcohol or acetone to clean the microscopes as it can destroy the lens cement. Immersion oil can be cleaned off of objectives or slides using a cotton swab moistened with petroleum ether.

- Focus in an upward direction when using the coarse adjustment and use the fine focus when focussing downward. Preventing contact between the objective and your slide will avoid costly damage to your microscope.

- The petroleum ether should be kept at the sink and away from any open sources of flame.

- **Extinguish alcohol burners immediately after use.**

- MSDS sheets for all chemicals can be found in the lab.

- **Late penalties** for lab assignments will be assessed at the same rate as course assignments.

- Any slides that a student prepares are for their personal use. Slides are considered individual work, slide sharing will not be tolerated.

- If for some reason you cannot attend the lab contact the demonstrator by email or by phone.

Lab 1
Mitosis of *Vicia faba*

The first lab is to introduce you to techniques in slide preparation that at first may seem to be frustrating but are necessary to develop your own methods that you will serve you for a long time. My experience has been that the students with the most difficulty in the first two-labs find the final project a piece of cake. A little bit of forethought into what type of material you are dealing with and what you are trying to accomplish goes along way in the cytogenetics lab.

Dividing cells of young seedling plants are located in the shoot and root apical meristem. These areas are very easy to dissect out of freshly germinated healthy material .Collecting root tips is one of the easiest methods of checking the chromosome number.

The general procedure of the squash or smear technique for root tips involves six steps:

- Germination of seeds to obtain young root tips
- Pretreatment of the tips to arrest cells at metaphase and enhance chromosome observation
- Fixation to preserve the material
- Hydrolysis to soften cell walls and make the root tip malleable
- Maceration, staining and slide preparation
- Observation and documentation

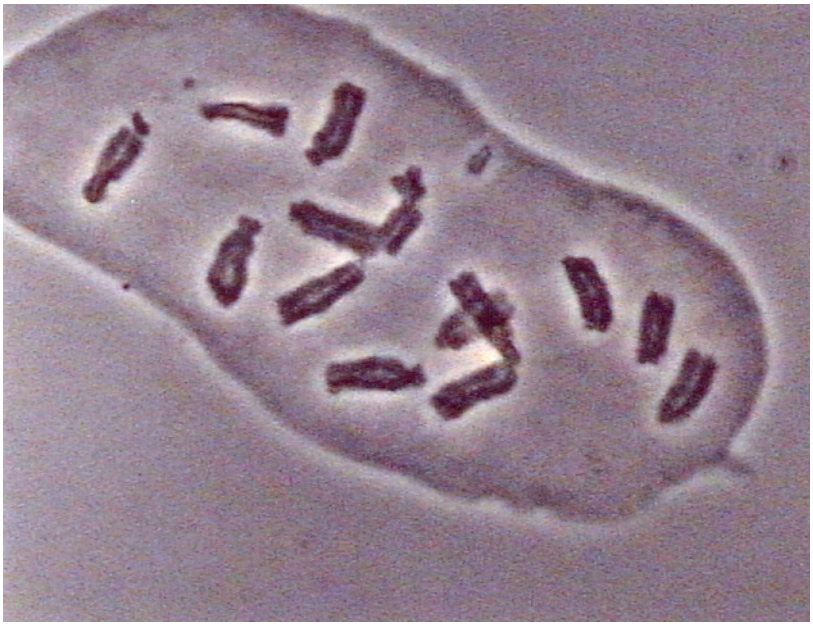

Figure 1 Metaphase in Vicia faba 2n=11, Magnification 260X

Procedure

Figure 2 Materials required for preparing root tip squashes. From top left clockwise: microscope slides; prepared and stained root tips; acetocarmine stain; alcohol lamp; matches; forceps; razor blade; cover slips.

Germination and pre-treatment: *Vicia faba* seeds are soaked in warm water for 3-4 hours before planting in moistened vermiculite. When roots are 2-3 cm long, the last 1 cm of root is taken off and placed in ice water over night at 4 ºC (pre-treatment).

Fixation: Roots are transferred to Farmers Fixative (see appendix 3) at 4º C overnight.

Storage: Roots are stored in 70% ethanol 4 ºC.

Hydrolysis: Three or four root tips should be placed in a clean vial with a 1 N HCl solution at 60 ºC for 8-10 minutes. Length of hydrolysis and concentration of HCl will vary for different species.

Staining and Slide Prep: Select a root tip and blot it on a paper towel.

Place the root tip on a clean microscope slide and check for the root tip, it should be slightly pointed and appear opaque. A dissecting microscope is handy if you're not sure. Place a drop of the prepared aceto-carmine on the tip and dissect the smallest bit of the root tip that you can (1-2 mm).

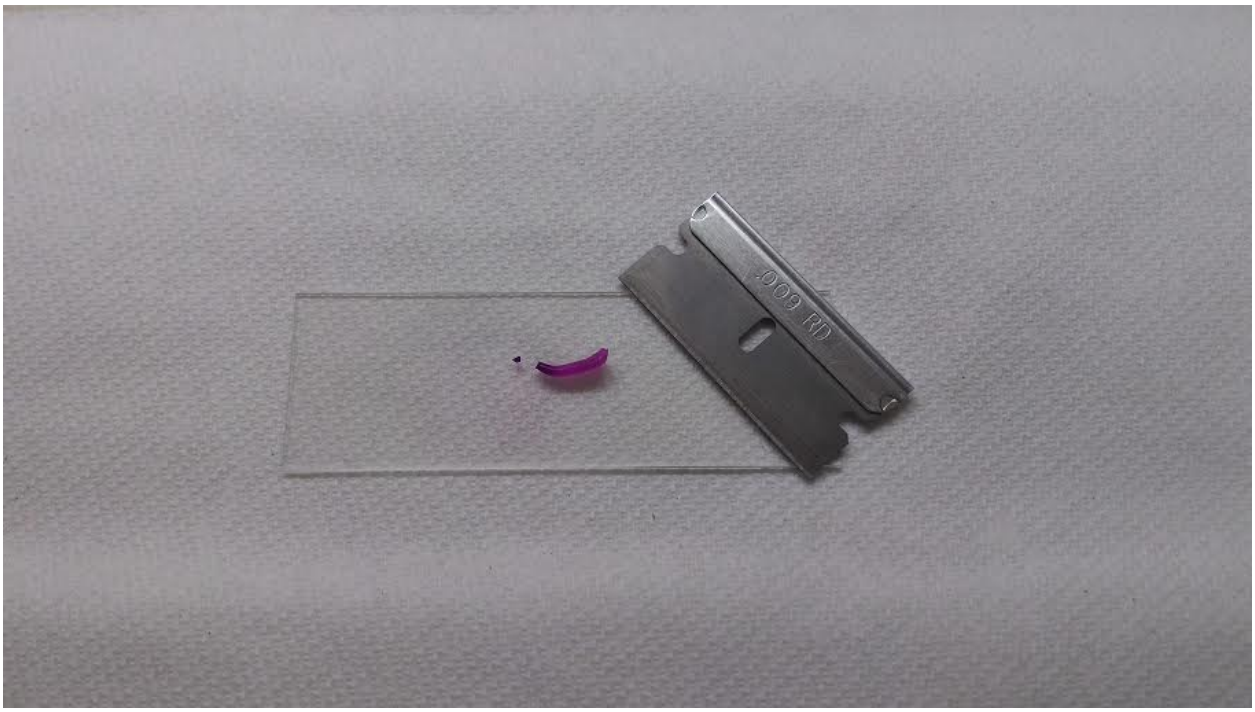

Figure 3 Using a razor blade, cut off a 2-3 mm section from the root tip.

Chop it in half if possible, the smaller the section you prepare here the better the chance of getting a single layer of cells to observe.

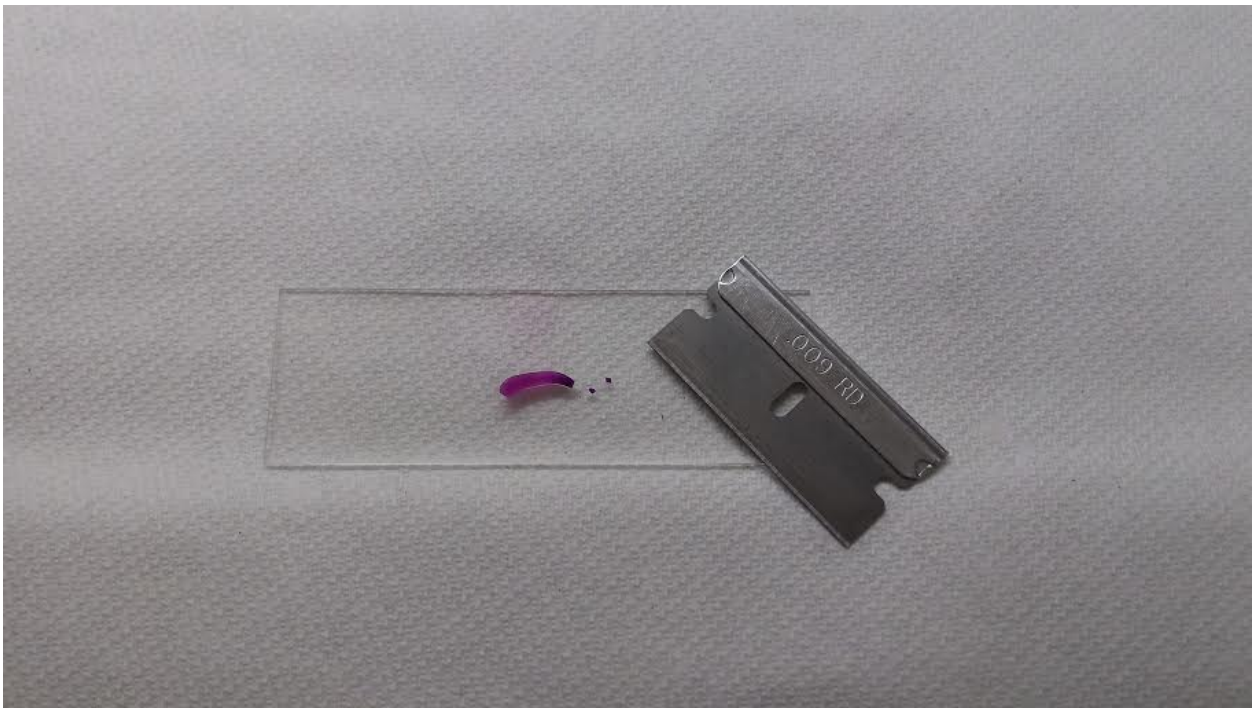

Figure 4: Cut the root tip in half longitudinally.

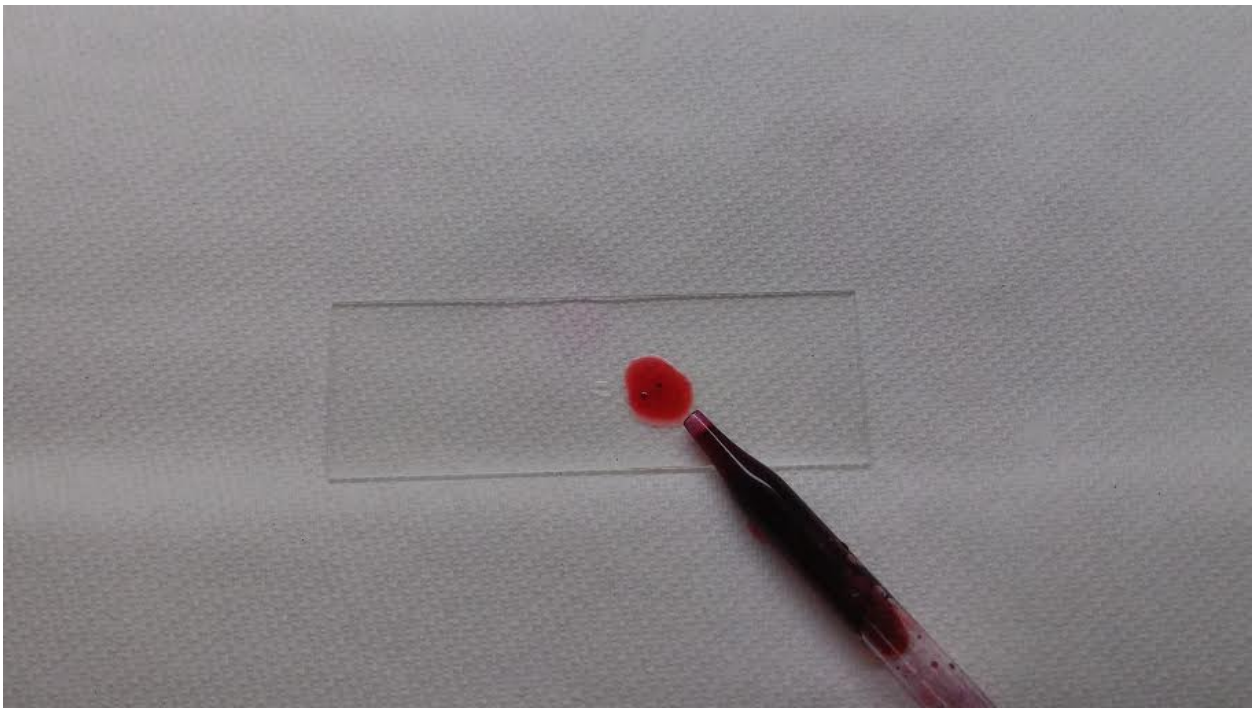

Figure 5: Place a single drop of acetocarmine stain on the root fragments.

Place a cover slip on the slide at an angle, slowly lowering it onto the stain will prevent bubbles from forming.

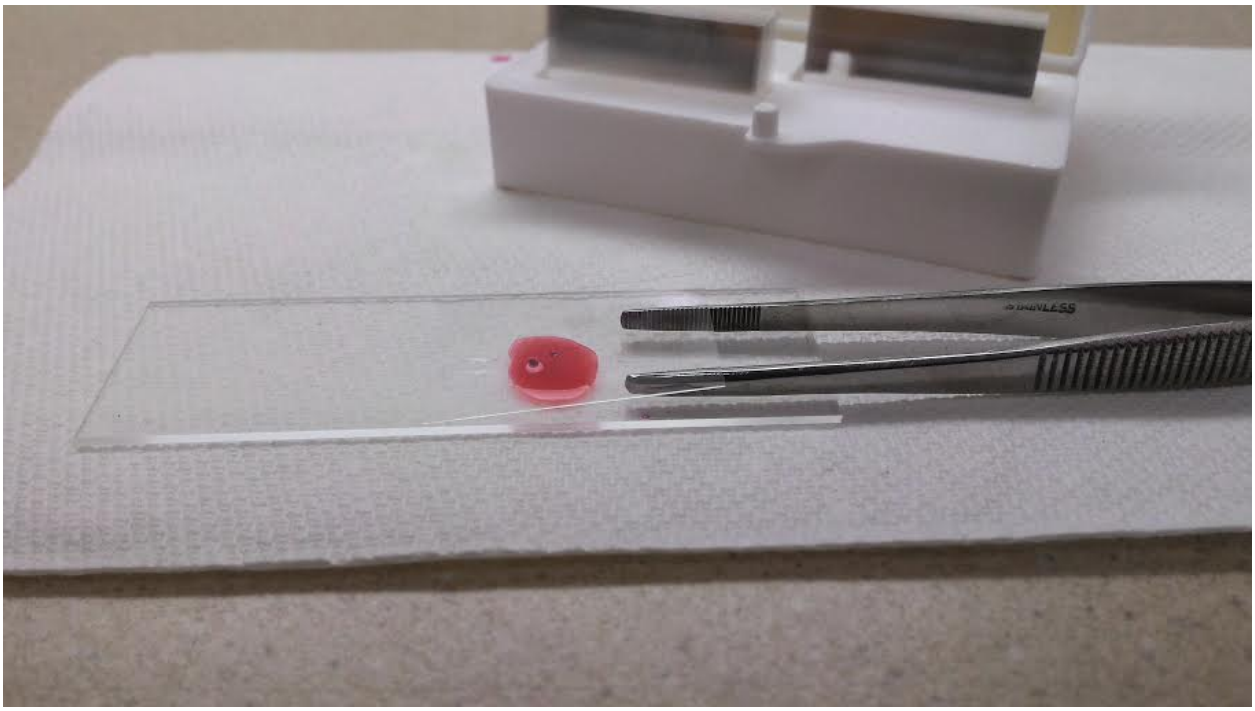

Figure 6: Carefully lower the coverslip on the stain and root.

Hold one edge of the cover slip down with a paper towel and tap on the area of the root tip with the blunt end of a dissecting needle or the eraser end of a pencil. Apply enough pressure to squash the root tip out under the cover slip, but if you break the cover slip it will allow oil to seep in when you attempt to use the oil immersion objective of the drawing microscope.

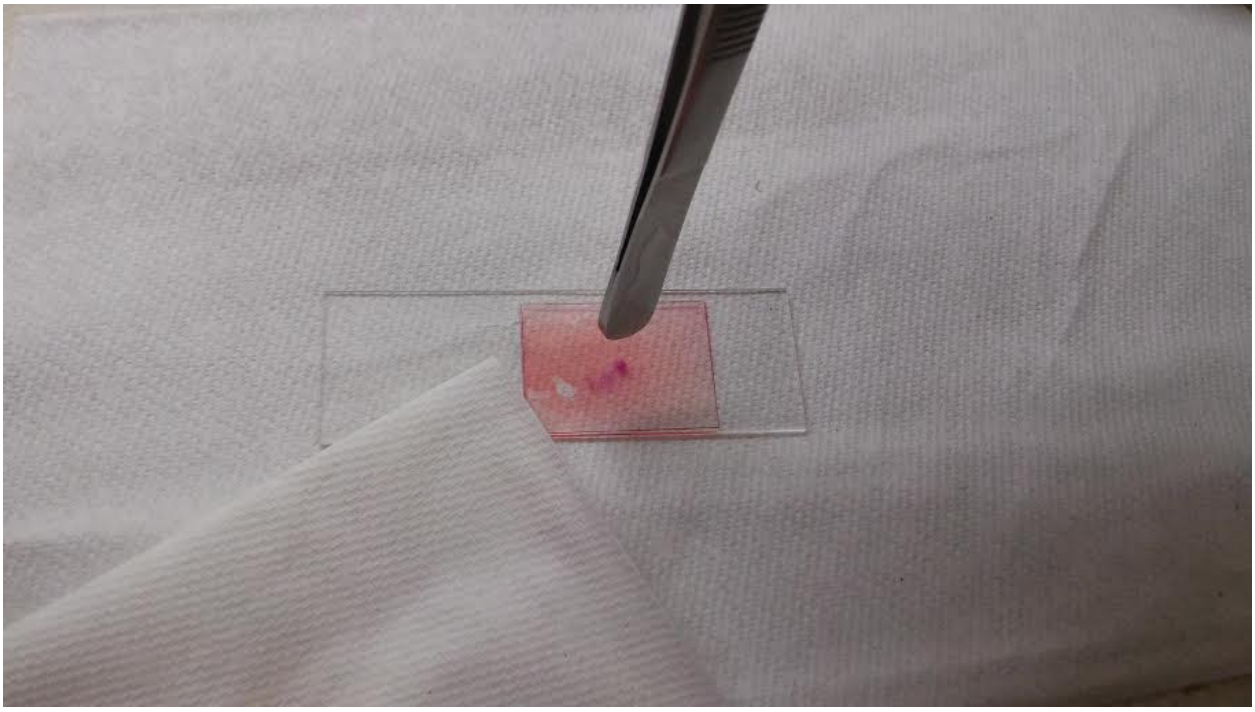

Figure 7: Hold the corner of the coverslip with a paper towel to soak up extra stain. Tap on the root tissue with the back of your forceps to disperse the tissue.

Use the alcohol burner to heat the slide slightly and then tap the cover slip down again to get an even flatter preparation. By the time you are finished your root tip should be barely visible on the slide.

Figure 8: Pass the microscope slide 8-10 times over an alcohol lamp. DO NOT hold the slide over the flame too long or the stain will boil off.

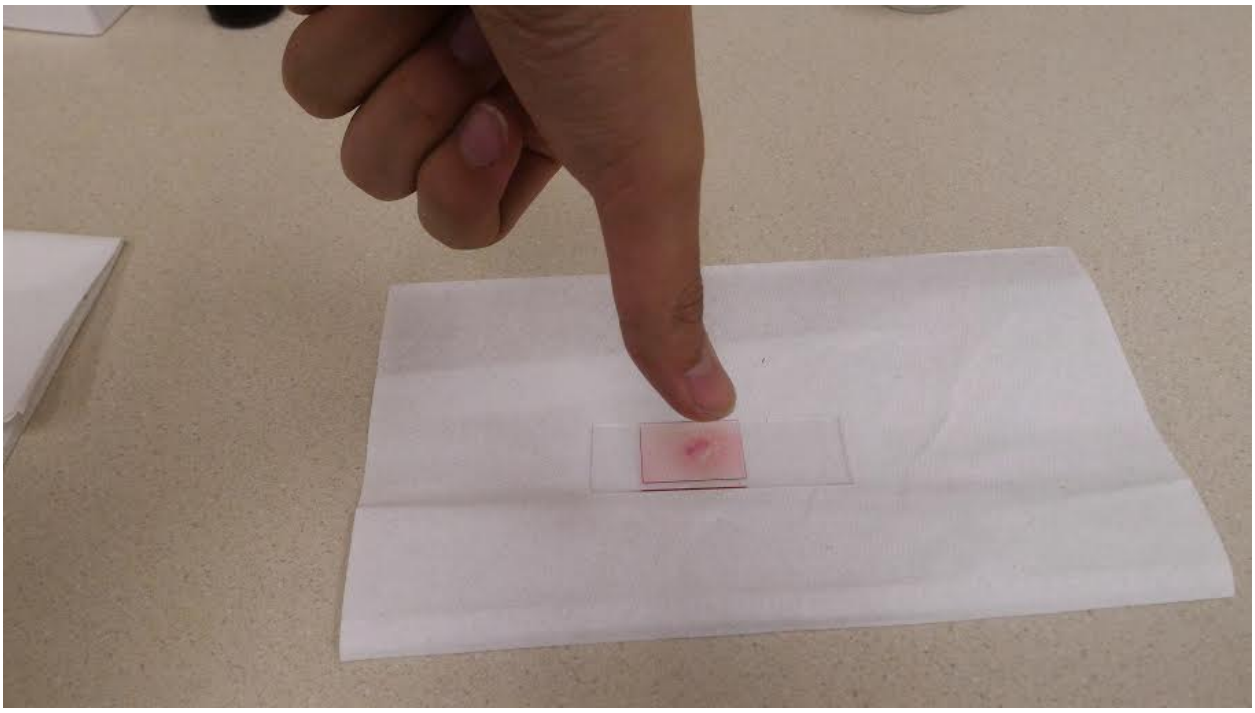

Figure 9: Once cooled, use your thumb to press the coverslip to flatten the remaining root tissue.

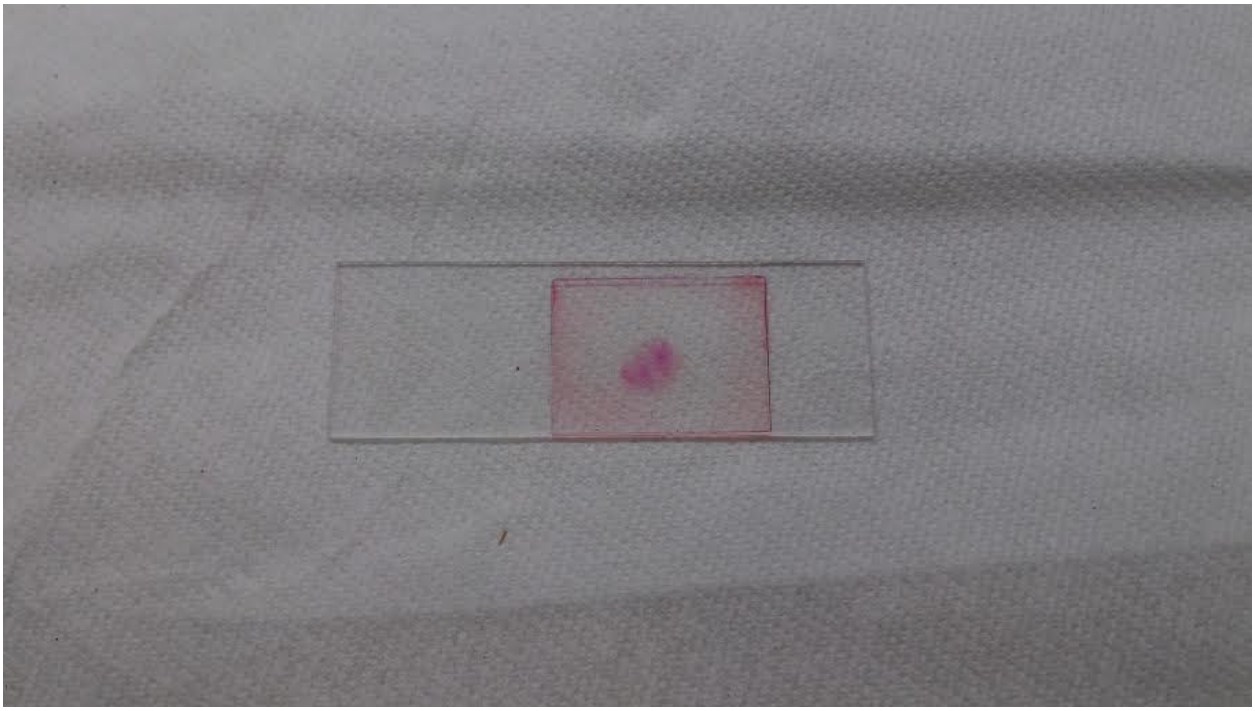

Figure 10: If properly prepared, you will not see any intact root tissue left. The root tissue should appear as a smear. You will likely observe chromosomes along the periphery of the smear.

Observe: Place your slide on the microscope at 10X power and locate the dividing cells. Remember the root apical meristem is located slightly behind the root tip, protected by a layer of cells that slough off as the root pushes itself through the soil. The cells should be small and cube shaped with a well-defined nucleus. Scan at 10X for any cells in mitosis, move to the 40X objective (phase 2) to check the exact stage. By the end of the period you should be able to identify prophase, metaphase, anaphase and telophase. Once you have found a metaphase cell to draw, prepare your slide for transfer to the drawing microscope. Use a slide transfer method described in appendix 2 and seal the slide with Cytoseal™ 60 (Fisher Scientific 23244257).

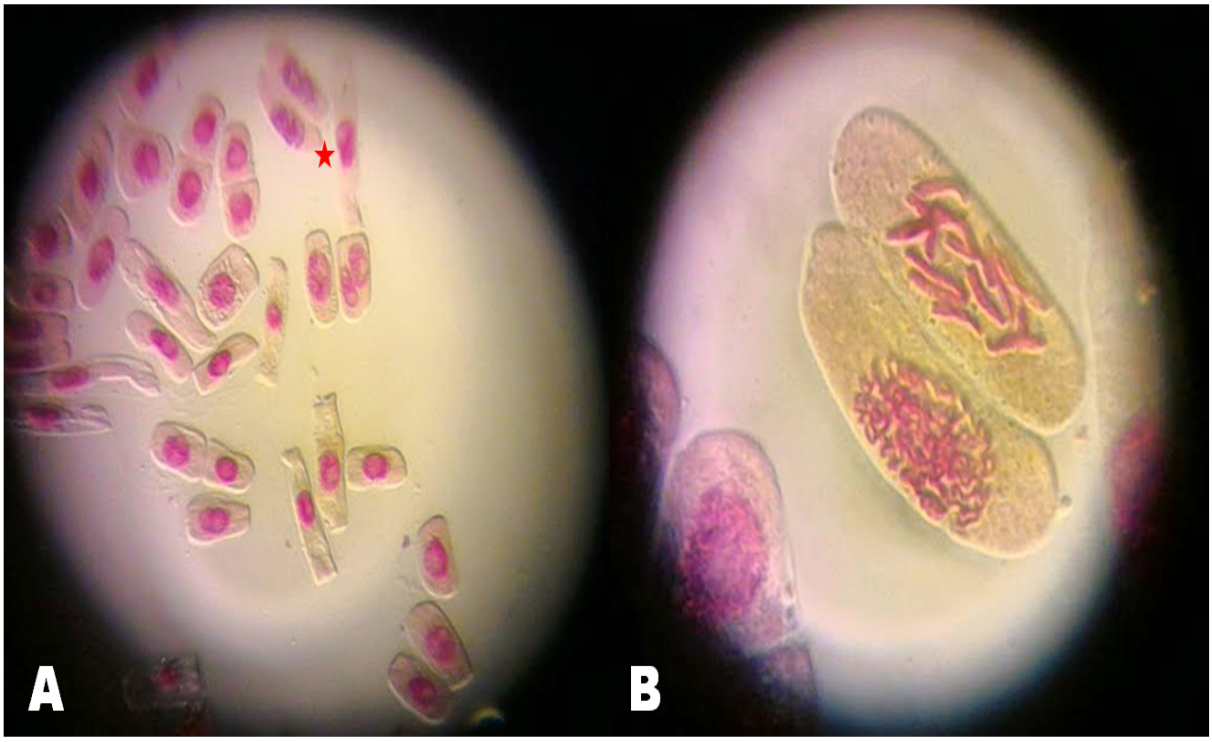

Figure 11 Vicia faba chromosomes observed in root tips at (A) 400x and (B) 1000x magnification.

Drawing: The presentation for grade includes one drawing of a metaphase cell using the camera lucida. Only the outline of the chromosome structure is necessary. The drawing should be done on an unlined eight x 10 sheet of white paper. Include the magnification, species, and chromosome number. Label the two types of chromosomes.

Camera Lucida

Several methods are available for registration and reproduction of microscopic images: freehand drawings, microprojection, photography, digitization etc. In the past, drawing an image was the only way for recording a microscope image. Few users of the microscope possess the requisite talent to make accurate freehand drawings of specimens observed under their instrument and those who possess this ability realize the difficulty of judging the size and proportion of the individual parts.

For this reason a drawing aide, the camera lucida, was devised to bring the microscope image and the drawing paper and pencil in the same plane. This is done by the combination of a mirror and prism with an adjustable set of screens for reducing the strength of the beams of light from the microscope and paper surface to produce equality. The drawing procedure involves sketching the outline, and for this lab the location of the centromere. Advantage of the camera lucida over other methods of recording is that all the chromosomes need not be on the same plane as you should be able to focus up and down a small amount to obtain all of the details. You will find it helpful to keep both eyes open while you draw the chromosomes. If you find it difficult to see both the image and the end of your pencil, cover the drawing with your hand, check the location of the ends of the arms and resume drawing.

Lab 2

Karyotype of *Hordeum vulgare* (2n=14)

In this week's lab, we will expand on last weeks procedure by characterizing the chromosomes or karyotype of barley. Essential to the lab report is a good drawing with a scale added for measuring the length of the chromosomes (karyogram), including the exact location of the centromere and any secondary constrictions. These measurements and ratios will be used to pair the chromosomes and prepare an idiogram (a diagramatic sketch or interpretive drawing) of barley.

The protocols are essentially the same as in lab one, but we will switch to Feulgen stain, which is specific for the chromosomes only. Feulgen stain is a bit more tricky to work with than aceto-carmine. Feulgen stains everything it comes into contact with purple, has a limited shelf life and if it contaminates the ethanol storage solution or the HCl it will make future staining of the chromosomes impossible.

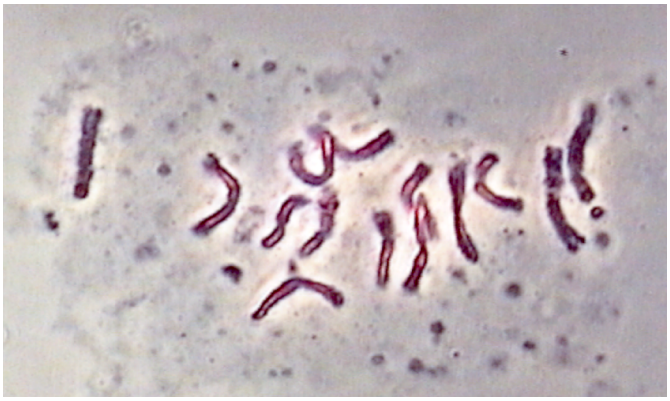

Figure 12 ***Hordeum vulgare stained with Feulgen stain. 2n=14, Magnification 1260X***

Procedure

Pre-treatment: Clean barley seed is placed on moistened filter paper in the dark until roots emerge and are 2 cm long. Root tips are harvested and placed in ice water overnight at 4ºC.

Fixation: Farmers solution at 4ºC overnight.

Storage: 70% ethanol at 4ºC.

Hydrolysis: Transfer the root tips to 1N HCL at 60ºC for 8-10 minutes. Rinse the root tips with distilled water and transfer to a clean vial..

Staining: Add the Feulgen stain and place the root tips in the dark. Staining will take between 10-20 minutes. Be sure to keep any tweezers that have come in contact with the stain separate or clean with Comet and rinse thoroughly. The chlorine in the cleaner will deactivate the stain.
Once the root tips have stained dark purple excise the tip and place in a drop of 45% acetic acid on a slide. Macerate carefully and apply a cover slip. Feulgen stain will fade over time so be sure to place your remaining root tips back in the dark. Check your slide carefully for a good metaphase cell. Remember the more detail at 40X the easier it will be to draw and therefore measure at 100X.

Photo: After finding the appropriate metaphase cell, take the chromosomes photos as carefully as you can under the Leica microscope. Take care to note the exact location of the centromere, the satellite length and the location of the end of each arm. Find the lines in the centre of the circle and focus under at 100X as you did with your photo. Carefully add the scale below your photo. Draw vertical lines below your photo spaced as they are on the slide, the space between two lines represents 10 microns. Use this scale by converting it to mm and measure your chromosomes and then convert back to microns for the final report. Include in your report this photo with the scale, a table showing the measurements and pairing and the karyotype represented as an idiogram. The idiogram is drawn longest to shortest chromosome. The labeling on your original drawing should also represent your pairing ie. change the original labels to represent the pairing you have made through the measurements and ratios.

Lab 3
Mitotic Aberrations

The purpose of this lab will be to explore the effect of a known mitotic inhibitor on the cell division of onion roots. *Vicia faba* 2n=12 (or Onion *Allium cepa* 2n=16) roots will be treated with the chemical and then divided among the students for observation. A report will be handed in by each student in the standard lab report form.

Procedure: *Vicia faba* seeds or onion bulbs are rooted in moistened vermiculite at room temperature until 3-4 cm long. Solutions are prepared of maleic hydrazide (0.1, 0.2, 0.4, 0.8, and 1.6 mM) and distilled water control. The rooted bulbs are placed in beakers so only the roots are immersed in the treatment solutions. The roots are allowed to sit in the solutions for 2 hours at room temperature. The bulbs are then rinsed well with tap water and placed back in the vermiculite for a 24 hour recovery period. Roots are then removed and placed in Farmers Fixative for 16 hours at 4^{o}C. Roots are then transferred to 70% ethanol for storage.
Hydrolysis: You will be assigned one treatment to score along with one other person, take these roots and transfer them to 1N HCl for 10-12 minutes at 60^{o}C.
Staining: Root tips will be stained as before with aceto-Carmine.
Scoring: Carefully scan your root tip at 10X and locate the dividing cells. These are the cells of interest and care should be taken to count a representative sample of them. Count 300 cells per root tip and record the number and type of each mitotic cell. To do this without recounting material over again, start on one side of the dividing group, lets say upper left, and count the cells in your field of view and make note of the dividing cells on the form provided. Keep moving to the right one field of view at a time until you come to the end of the area then move down one field of view and continue counting to the left. Aberrations will be scored in anaphase cells. The type of aberrations may give you a clue to the mode of action of this chemical. Add up the columns on the sheet before handing it in to the demonstrator who will photocopy it and return it to you. All the data will be tabulated and put on the course web site so you can make your final calculations for the report.

Report: Include in the report a short introduction, a materials and methods section should refer to the lab manual with any changes in the procedure, a data section with the mitotic indices and inhibition for all the treatments, include a sample calculation for each using you raw data, and a discussion section. Be sure to include the sources of error in your discussions.

$$\textbf{Mitotic Index} = \frac{\textbf{\# of dividing cells}}{\textbf{Total \# of cells}}$$

$$\textbf{\% Mitotic Inhibition} = \frac{\textbf{Mitotic Index in Control-Mitotic Index in Treatment}}{\textbf{Mitotic Index in Control}} \times 100$$

Lab 4
Meiosis of Normal Diploids

We will leave mitosis behind and finish the lab with a study of meiosis. Work on your project will continue until the last week of classes when the project is due. Giving the project as much attention as you can in the early weeks will get you a good start on your photos before the end of term where most people find it difficult to find any extra to spend in the cytogenetics lab.

Meiosis is the production of a haploid nuclei from a diploid cell. The joining of two separate haploid nuclei is the basis for sexual reproduction in eukaryotes. Meiosis consists of two separate divisions with only one DNA replication phase. The first division separates homologous chromosomes, and the second division separates sister chromatids.

Plant material for this type of study can be either the microspore phase in this case pollen mother cells from the anther or megaspore cells from the ovary. *Secale cereale* spikes have been collected and dissected from the sheaths of field grown plants. In contrast to the mitotic stages which are continuous, meiosis is synchronised and takes place over a very short period of time. The spikes are harvested in the early summer over a one week time period to get a wide range of material to capture the meiosis event. The lengthiness of this lab is due in part to the fact that the correct anther stage change with the growing conditions in the field which vary from year to year depending on soil moisture and temperature conditions. Once the correct anthers are found, several different stages can usually be seen on one slide.

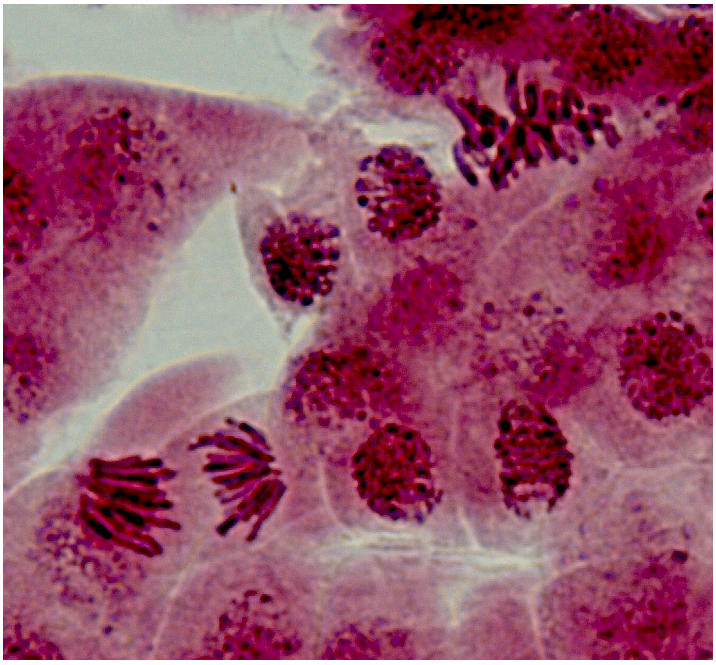

Figure 13: Meiosis of Secale cereale Pollen Grain showing several stages of meiosis

Procedure: Staging of the *Secale cereale* (Rye) from previous years indicates that harvesting the spikes or inflorescence when they are in the boot stage, about half to three-quarters of the way up the sheath will give anthers of the right size. These are collected in bulk and placed in Farmers fixative overnight at 4ºC. The spikes are then placed in storage solution of 70% ethanol at 4ºC.

Figure 14: Prepared rye inflorescences in Petri plate with 70% ethanol. Red triangle indicates a single floret separated from the inflorescence. The opaque anthers can be distinguished from the transparent glume, palea, and lemma.

Anther selection: As was noted in the introduction sampling the right material for observation will take some trial and error. First select a spike that has anthers that are white or pale yellow. You should be able to locate these visually through the papery glumes of the spikelets. Any anthers that appear grey will likely have mature pollen grains. There will also be variation in maturity along the length of the spikes.

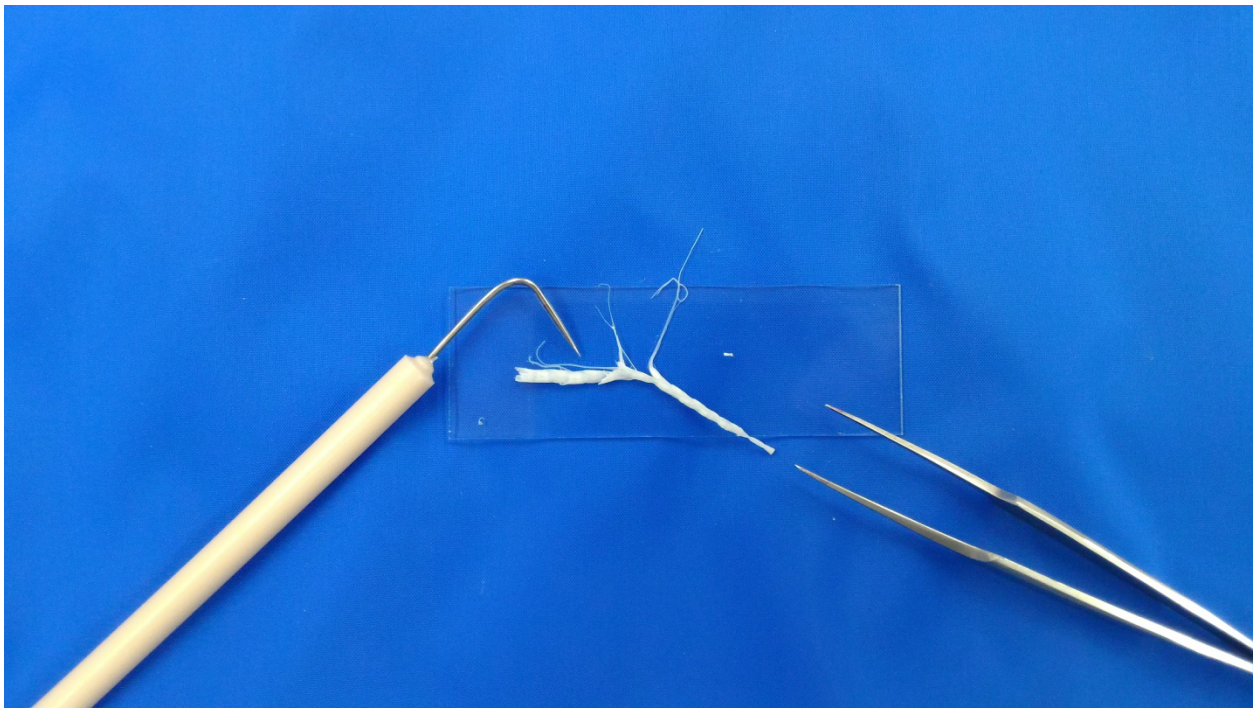

Figure 15: Extraction of the anther from inflorescent. A floret approximately half way up the inflorescence is selected and the anther (red triangle) is removed from the spiklet using a dissecting needle and forceps. The inflorescence can be returned to the Petri plate.

The most mature pollen can be found in the centre and least mature at both the top and the stem end of the spike. Sampling anthers from three or four places along a spike will give you the best range of maturity of the pollen. If you note the length of these anthers, you can use this as a guide when re sampling.

Slide Preparation: To make a good slide for photography, make sure to remove all the large debris from the slide. Any small fragments of a glume or anther wall will affect the focus and therefore the quality of your pictures. Carefully pull the anthers out of the spikelets and collect them on a slide. Add a drop of aceto-Carmine and cut the anthers across the middle. Line several anthers up side by side to save time. The pollen will be released from the anthers with a gentle squeeze from the tweezers. Remove the anthers and any other debris. Apply the cover slip careful so not to add any bubbles, and examine the pollen under low power. Heat the slide gently if you are seeing to movement of the stain under the cover slip. Once you have found good examples of the following stages the lab demonstrator will take the pictures for you. Seal the edges of the cover slip with nail polish or with bees wax if your cell is close to the edge. Prepare a marker slide for transfer to the photomicroscope.

Report: One picture of each of the following labeled appropriately:

1. Diakinesis
2. Metaphase I
3. Anaphase I
4. Metaphase II or Anaphase II

Appendix 1
Aligning the Light Source of your Microscope

A properly aligned light source is essential for both your comfort and proper illumination using phase contrast objectives. Each microscope should be aligned by first focussing then centring the light coming from the illuminator at the bottom of the microscope.

1. Take a test slide and place it in the microscope slide holder, make sure the cover slip is on the top side.

2. Turn on the light source and focus on the specimen at 10X power. The phase contrast condenser should be in the **J position**. Turn the **lamp field stop** until you see a small circle or hexagon of light.

3. For optimum illumination this circle should have a crisp outline and be in the centre of your field of view. Check for the knob on the side of the microscope that raises and lowers the **condenser only** (do not use the fine or coarse focussing knobs for the objectives). Look through the eye pieces while moving the condenser up or down to obtain a sharp outline of the circle of light from the previous step. The condenser is now set at the correct depth for the thickness of slide you are using. To centre the light, use the **two screws** set in the side of the housing that holds the condenser. These move the condenser in the holder, left or right, up or down, to centre the circle of light in your field of view. Note: If you have to turn screws more than half a turn either way the top lense of the condenser may be slightly out of position. Place it in the upright position and start from the beginning.

Once you have aligned the light, open up the lamp field stop at the bottom of the microscope before moving on to the phase contrast portion.

For Phase Contrast

1. Move the phase condenser from the **J position** to the number indicated on the side of the objective you are using, for the 10X objective go to the **1 position**.

2. Remove one eyepiece and replace it with the **telescoping eyepiece**. When phase contrast is out of alignment, two rings, one grey and one white will be visible against a dark back ground.

3. Carefully check the front and side of the phase contrast condenser. There are two places for adjustment, one a **knurled knob** at the front and one a **set screw** at the side of the condenser that should be loosened to slide back and forth. Look through the telescoping eyepiece and move the rings until they are one on top of the other. The phase contrast portion of the microscope is now in alignment and the set screw should be retightened.

4. Be sure to move your condenser to the correct position each time you move between objectives. The correct position number is written on the side of each objective, 40X requires the number 2 setting.

Under phase contrast specimens should be much easier to visualize. Stained chromosomes will appear darker or even black, sometimes with a fluorescence around the edges. Over time you will be able to tell when your microscope will need to be realigned. Once your cells lose the contrast or appear bubble

like its time to realign. Depending on the age and wear on your microscope you may have to go through the whole procedure more than once during your lab session.

Appendix 2

Cell location

One of the most difficult things to do is to relocate a cell that you are interested in from memory. Once you have the cell of the correct stage it is very important to record the coordinates of the cell and to make some effort to find a landmark on the slide that you can use to find the cell again. The landmark could be a piece of debris or a unique looking group of cells.

Each microscope in the lab has both a vertical and horizontal scale on the stage which you can use to record coordinates that represent the location of the cell you are interested in. Look at the scale on the side or top of the stage. There are actually two scales for each reference point, one that is stationary and one that moves as you move the stage (vernier). The vernier has ten places, with the first line dotted. Simply use the dotted line as a reference, recording the numbers on the stationary stage (eg 36). To record the decimal place use the line on the vernier scale that lines up best with the stationary side as the decimal point. For example if the dotted line is slightly greater than 36, and the second line of the vernier scale matches up with a line on the stationary scale this would be recorded as 36.2. If the dotted line is less than the 36 and the seventh line of the vernier scale matches up with a line on the stationary scale the coordinate would be 35.7.

Unfortunately the scales are different for each microscope. There are several ways to move from one to another. Field finders can be purchased that have a grid marked with coordinates that can be seen under low power. Simply centre your cell, replace your slide with the field finder slide without moving the stage and read the coordinates through your eyepieces. Reverse this procedure on the microscope you want to transfer to.

If you don't have a field finder available to you, simply replace your slide with a clean slide as above, turn down the lamp field stop in the base of your microscope to make a small circle of light and mark this with a fine tipped marker. Mark your slide and the finder with an arrow so you know in which orientation to put the slides in the microscope slide holder. Place your finder slide in the stage of the microscope that your transferring to and locate the dot again . Replace this slide with your own and you should see your cell.

Appendix 3
Solution Index

The following list of solution is for your information only. All solutions are prepared for you by your demonstrator. Most cytogenetic texts have a list of the most popular solutions, a good reference is R.J. Singh, Plant Cytogenetics, 2003, CRC Press.

Fixing Solutions

The purpose of the fixing solution is stop the life process with minimal cell disruption or distortion of tissues. Essentially the perfect solution will replace aqueous solution with solutions that will protect the structures of interest from damage during the physical handling and storage of the specimens.

Farmers Fixative

- Ethyl Alcohol (95%) 30 ml
- Glacial Acetic Acid 10 ml

All tissue used in this lab has been treated at some time with Farmers Fixative. It is widely used in plant and animal materials. Acetic acid has a swelling action and the alcohol has a shrinking action on the protoplasm.

FAA (formalin-acetic-alcohol)

- Formaldehyde (37-40%) 10ml
- Glacial Acetic Acid 5 ml
- Ethyl Alcohol (95%) 50 ml
- Water 35ml

Carnoy's Fixative

- Absolute Ethyl Alcohol 60 ml
- Glacial Acetic Acid 10 ml
- Chloroform 30 ml

Preparation Of Stains

- Aceto-Carmine Add 1 gram of certified carmine dye to 100 ml of boiling 45% glacial acetic acid. After rapid cooling the stain is filtered. A drop or two of 45% acetic acid saturated with ferric acid can be added. Too much will precipitate the carmine dye. Ready made preparations of this stain can be purchased relatively inexpensively, results will vary, and its useful to test several preparations if possible.
- Aceto-Orcein A stock solution is prepared at 2.2% American synthetic orcein in glacial acetic acid. A 50% dilution is then made to prepare working stock solution.
- Feulgen stain. Note: stain preparation takes several days and it is difficult to store for more than one month. Pour 200 ml of distilled boiling water over 1 gram of basic fuchsin, cool to 50° C and filter into a brown bottle. Add 30 ml of 1 N HCL followed by 3 grams of $K_2S_2O_5$ (potassium metabisulfite). Allow the solution to decolorize for 24-48 hours in a dark place at room temperature. Add 0.5 gram of decolorizing vegetable charcoal. Shake for one minute and filter into a clean dark bottle. Store in the dark.

Appendix 4
Printing a Photo by Leica Microscope

1. Turn on Leica microscope (green switch, bottom right).
2. Load a slide.
3. Adjust lens, focus, filter, amount of light, etc.
   *if use 10X lens, match the white line on filter to the yellow stripes; if use 40X lens, match the white line on filter to the blue stripes; if use 100X lens, match the white line on filter to the white stripes.
4. After adjustment and find a good view under the microscope, double click “INFINITY ANALYZE” icon on computer desktop.
5. Under “Capture Control”, click “White Balance” or “Area WB” if needed.
6. Under “Camera Control”, click “Continuous Auto Exposure” to view the image on computer.
7. Change “Average Intensity” to adjust amount of light. For the best resolution of a photo, turn the light intensity to the maximum on the microscope.
8. Do not change “Gain”, leave it as “1”.
9. Keep “Gamma” as “1”.
10. If you need to make a measurement on the image, click “Measure” on tool bar, then click “Point to Point”. After a cross was shown on the screen, move it to where you want to make the measurement, click it then drag the cross to the end point, then click it again, and then the measurement will be shown on the image.
11. Always remember to click “Adjust” then “Magnification & Units” to change magnification (ex. 10X, 40X or 100X) for your measurement.
12. After making all the adjustments, click “File” and “Save” to save your image, then click “Print” to print.