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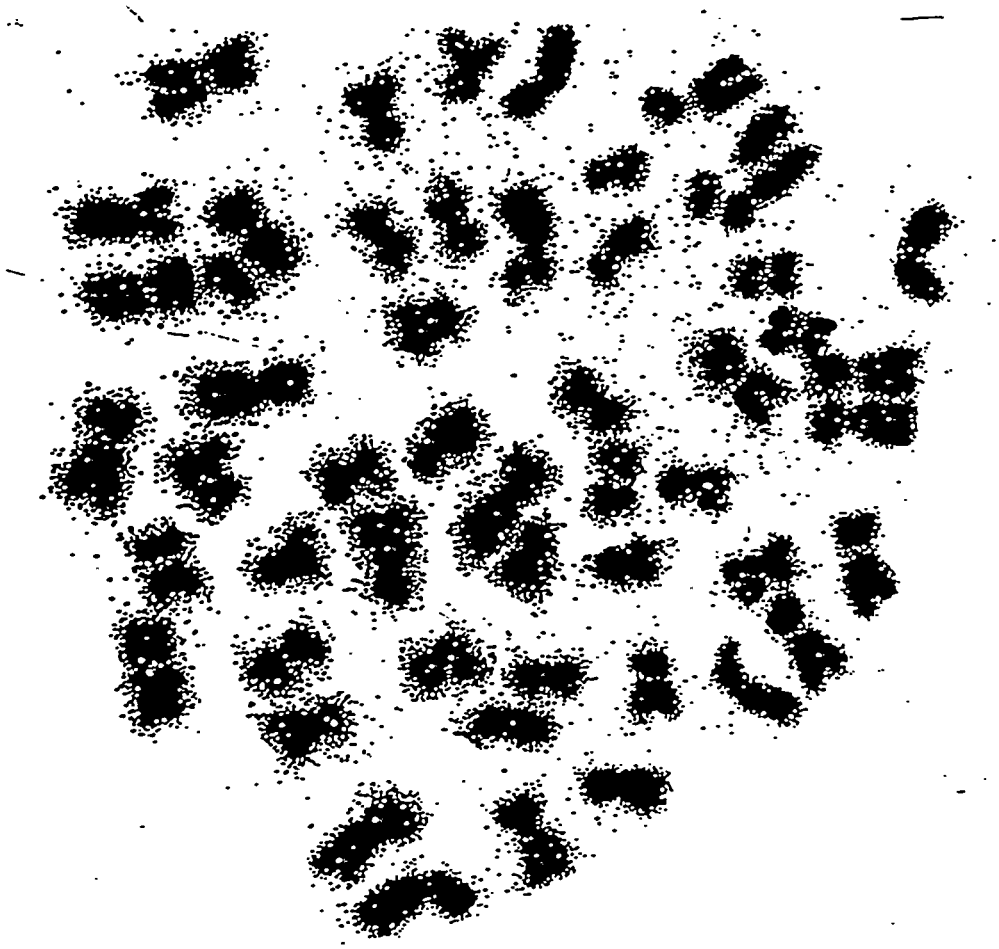
INFORMAL

Cell Synchrony and Chromosomal Protocols for Somatic Cells of Cotton *Gossypium hirsutum*



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Introduction

The intent of this pamphlet is to share information with others who are interested in useful protocols for cotton cytogenetics and cytology. The protocols described are a beginning effort and they are expected to be improved. All methods, results and data given pertain to cultivar MD 51ne (a gift from Hal Lewis, of Scientific Seed, Co.).

We are not satisfied with our results of *in situ* hybridization. So, view Figure 8, page 15 with this in mind. We seek advice on all aspects of this methodology and will send updated protocols as our technique improves.

This pamphlet will be given a Brookhaven National Laboratory Technical Report number. The number will be sent to you in the future, should you need it for a reference.

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I. Seed Germination

A. Day one

1. cultivar MD 51ne; seed, delinted, stored at 4 C
2. rinse seed (about 50 mls worth) in running cold tap water for 6 hours; removal of fungicide & imbibition
3. rinse seed in 100% Clorox for 1 min with mixing - use fork or spatula to mix
4. rinse seed with running distilled water for 5 min.
5. place 3 layers of Whatman no. 1 filter paper (circles, 125 mm) in 15 cm dia. Petri dishes
6. spread 40 to 60 seed on filter paper
7. add 10 ml of deionized water to each dish
8. germinate seed in dark at 26 C for 48 hr

B. Day two

Check seed

II. Cell Synchronization, 5-aminouracil treatment (day three)

A. in morning suspend seedlings having 2.3 to 2.7 cm primary roots on plastic mesh over 1X (full strength) Hoagland's nutrient salt solution, equilibrated to 26 C, pH 6 - make sure the primary roots are in the solution
B. place suspended seedlings in incubator at 26 C and aerate with rapid bubbling
C. grow seedlings in the dark for about 10 hr
D. at the 10th hour lift seedlings, mesh & aerator, and suspend roots in 0.8 mg/ml 5-aminouracil (Sigma, A-4005) dissolved in 1X Hoagland's nutrient solution, 26 C - NOTE: to dissolve 5-aminouracil heat gently with stirring, cool to 26 C
E. treat seedlings for 12 hr with 5-aminouracil, 26 C, aerated

III. Synchronized cell division, treatment recovery (day four)

A. after treatment lift seedlings, mesh and aerator rinse with distilled water, 26 C, then suspend seedlings in fresh 1X Hoagland's nutrient solution, 26 C, return to chamber for recovery in the dark with aeration
B. root meristematic cells will divide synchronously between 3 and 6 hr (Figures 1 & 2); do not expect 100%; see ref. 4.
C. fix root tips with ice-cold 3 parts methanol and 1 part glacial acetic acid; select those that grew 1 cm, are cream-colored, & thin
D. after 10 min, remove fix and add fresh fix
E. store at -20 C

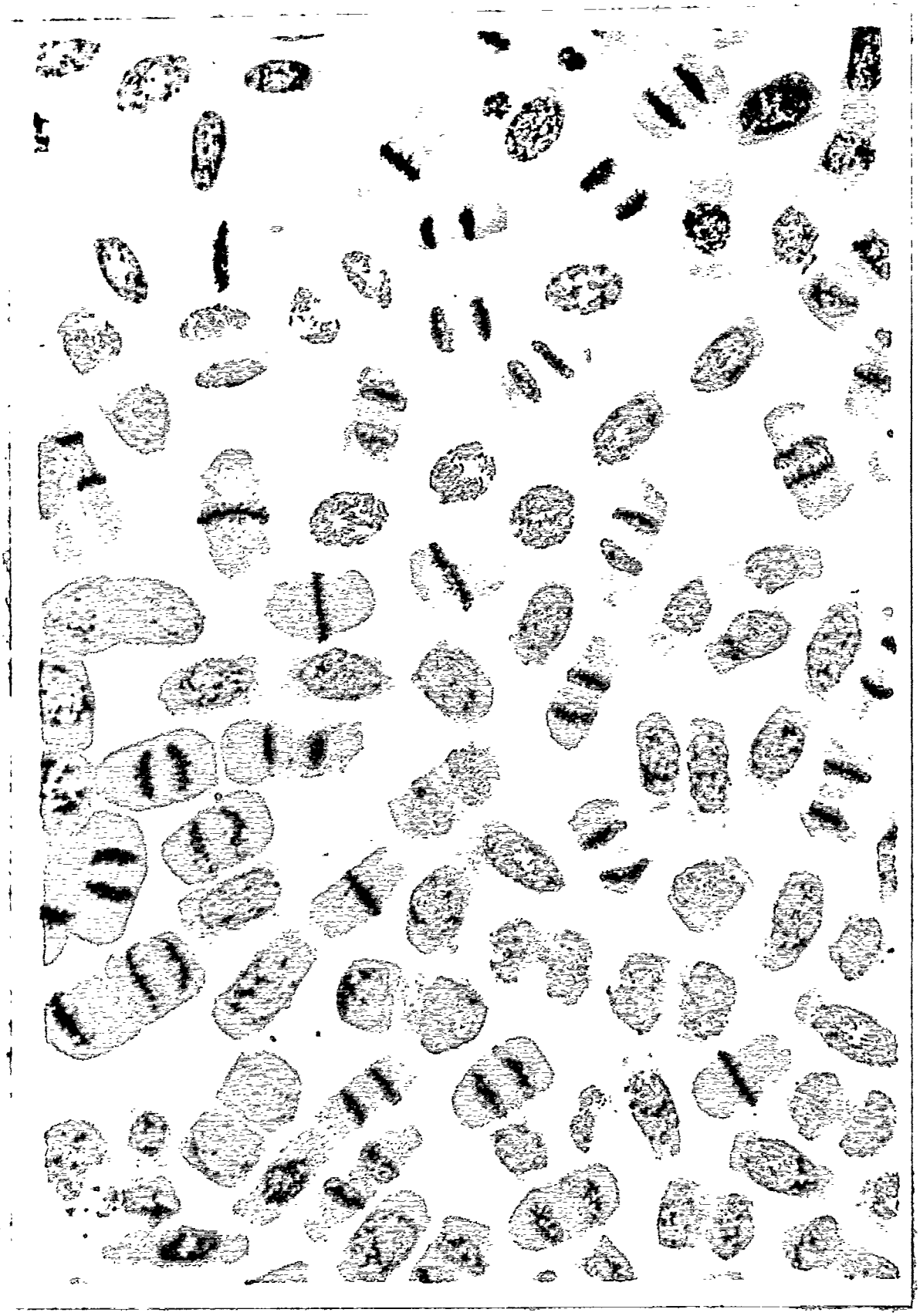

Figure 1. Synchronized cell division 5 hours after 5-aminouracil treatment; squash preparation.

IV. Accumulation of metaphase cells with cycloheximide (Sigma C-6255; CalBiochem, actidione=cycloheximide)

A. at 3.5 to 4 hours after removal of 5-aminouracil transfer seedlings to 1X Hoagland's nutrient solution, 26 C, pH 6, containing 10 ug/ml of cycloheximide; return to incubator and aerate
B. treat for 2 hours
C. fix root tips as described in III above

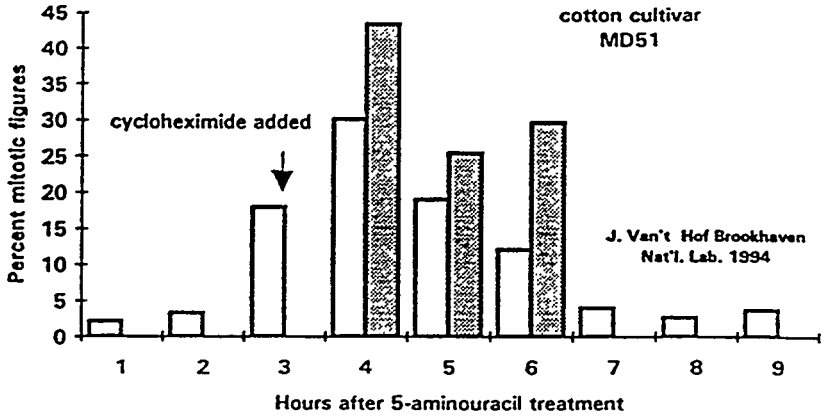

Figure 2. Percent mitotic figures expressed as a function of time after ending 5-aminouracil treatment. Open bars - no cycloheximide added; stippled bars - 10 ug/ml cycloheximide treatment beginning at 3.5 hours. Squash preparations; combined data from three experiments.

V. Feulgen stained chromosomes

A. Feulgen staining:

1. remove root tips from fix
2. suspend in deionized water until they sink
3. cut off 3-4 mm tips
4. suspend tips in deionized water in 1.5 ml Eppendorf tube
5. spin in microfuge for a couple of seconds
6. remove water and add fresh water
7. spin again in microfuge
8. repeat wash-spin twice
9. remove water

10. add 5N HCl, about 200 ul
11. leave in HCl for 30-45 min at room temp.
12. spin in microfuge
13. remove HCl
14. wash with deionized water & spin, repeat three (3) times
15. remove water & add about 200 ul of room-temp Schiff's reagent
16. let stain for about 1 hour; root tips should turn pink in 10 min
17. remove Schiff's reagent
18. wash with deionized water and spin, repeat three times

B. Cell separation after staining

1. remove water
2. add 200 ul of enzyme mix (0.01 gm of 3:1:1 mix in 200 ul water)

   - dry enzyme mix is:

   3 parts cellulysin
   1 part hemicellulase
   1 part pectolyase

   cellulysin [Calbiochem]
   hemicellulase [Sigma]
   pectolyase [Seishin]

3. digest for 30 min at room temp.
4. break-up root tips first by crushing with micropipet tip, then by drawing up into the pipet tip itself
5. continue digestion for another 30 min
6. spin at 1000 rpm (200 x g) in TJ-6 swinging-bucket centrifuge for 1-2 min
   - can use any low speed swinging-bucket head
7. remove enzyme mix
8. add 200 ul deionized water & gently mix by tilting tube
9. spin at 1000 rpm, 1-2 min
10. repeat 8 & 9 above
11. remove water above pellet leaving about 5 ul behind (amount of water left behind depends on the size of the pellet)
12. store on ice
13. clean microscope slide with 100% ETOH until it squeaks
14. gently suspend cells in pellet

15. remove about 0.5 - 1.0 ul of cell suspension with micropipet
16. place in center of cleaned slide equal distance from the edge of the frosted end and the unfrosted end
17. remove about 5 ul of ice-cold fix (3:1)
18. drop the ice-cold 3:1 fix on top of the droplet of suspended cells
    - the action of the fix will spread the cells on the slide's surface
19. air dry
20. add a drop of 100% glycerol to center of spread
21. place coverslip over drop and remove excess glycerol by blotting
22. view chromosomes by either bright field or fluorescence microscopy
    a. Figure 3 Feulgen fluorescence, page 8
    b. Figures 4 and 5, page 9, measurements from cell in Figure 3

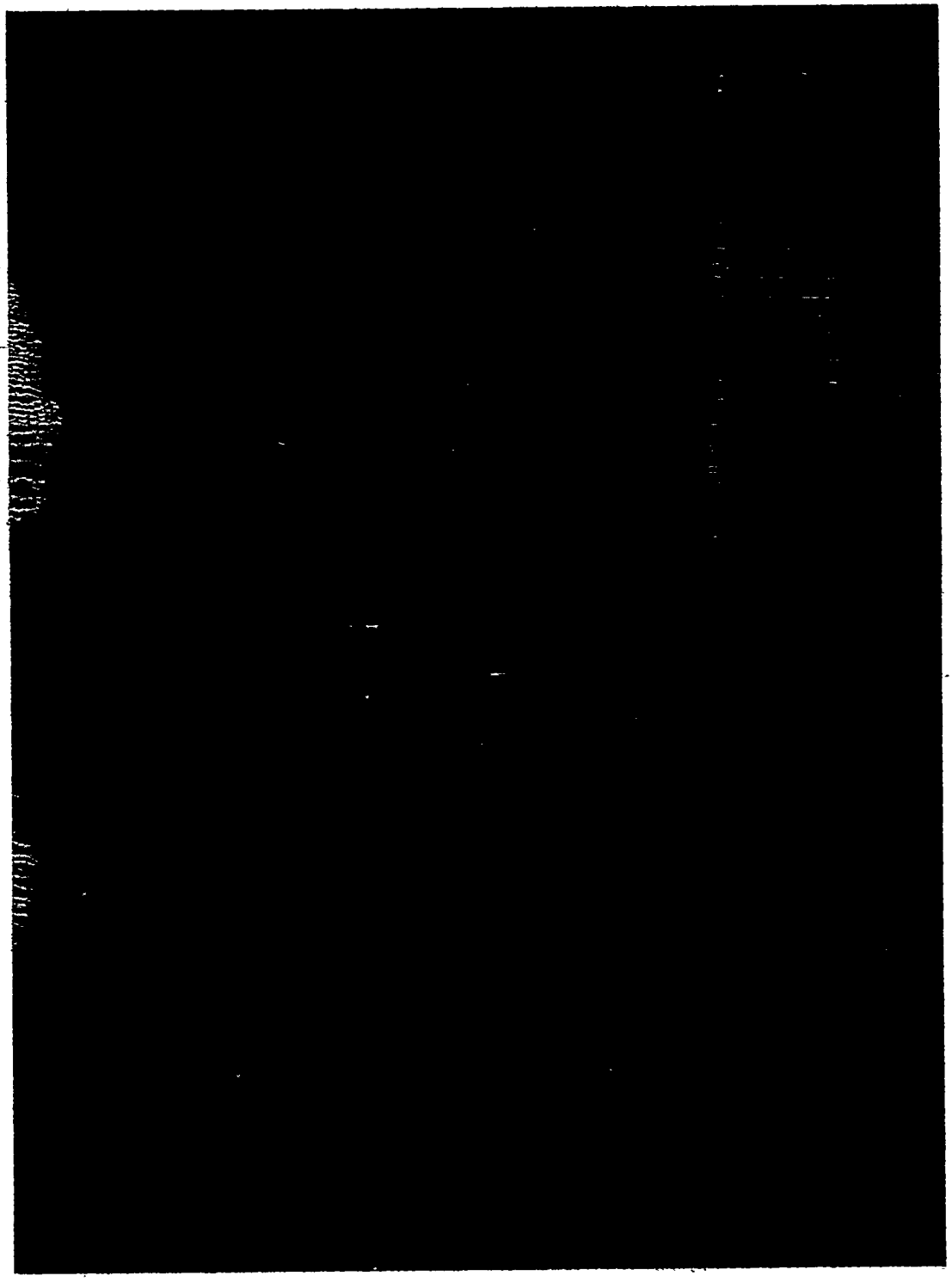

Figure 3. Metaphase chromosomal spread viewed by Feulgen fluorescence. Cycloheximide treated; see Figures 4 & 5 for measurements.

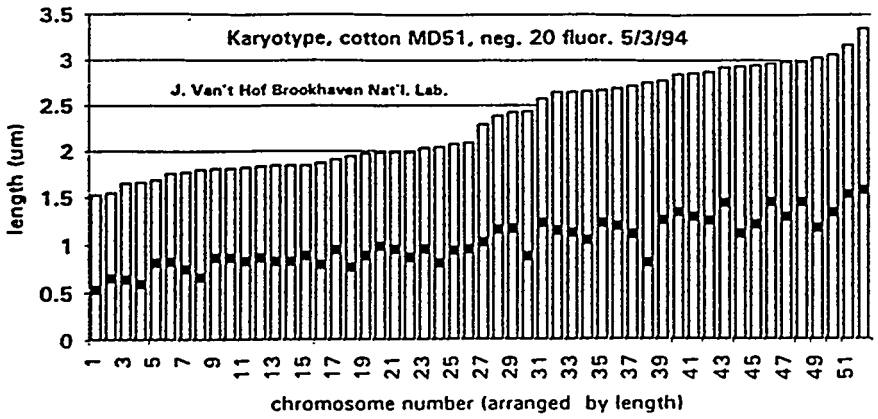

Figure 4. Chromosomes shown in Figure 3 arranged by length. Black squares show centromere position.

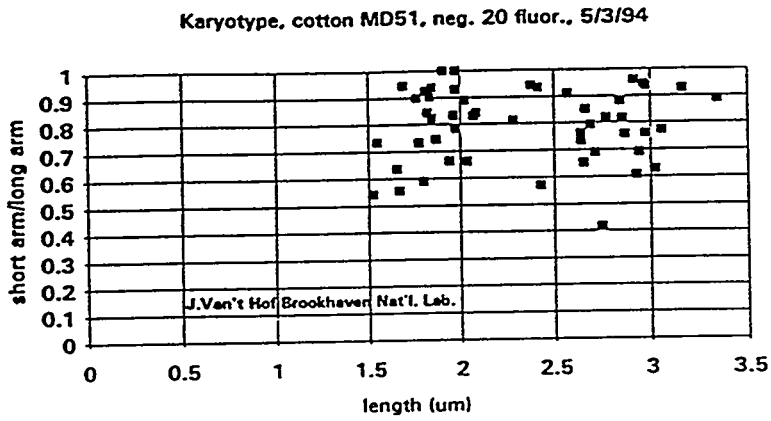

Figure 5. The ratio of short arm to long arm length of each chromosome expressed as a function of its total length. Note the two clusters of datum points.

C. Intensified stain with toluidine blue; can be used for squash and protoplast spreads

1. toluidine blue solution is:

   0.05% toluidine blue
   0.1 M citric acid
   0.2 M Na_2HPO_4, pH 6.8

   Mix and filter before use; store at room temp.

2. streak about 25 ul of stain at 90 degrees to the length of the microscope slide
3. carefully spread stain over cells using the edge of a coverslip; make sure the edge of the coverslip does not touch the surface of the slide
4. add drop of 100 % glycerol to center of stain
5. place coverslip over glycerol drop, cells & stain
6. blot to remove excess stain, glycerol, etc.
7. see Figure 6 for toluidine-stained chromosomal complement and Figure 7 for measurements

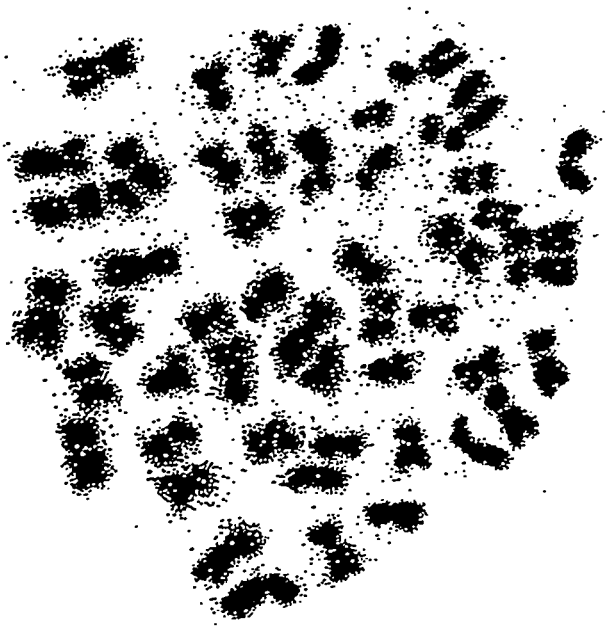

Figure 6. Chromosomal spread from protoplast stained by the Feulgen method and enhanced with toluidine blue. Cycloheximide treated.

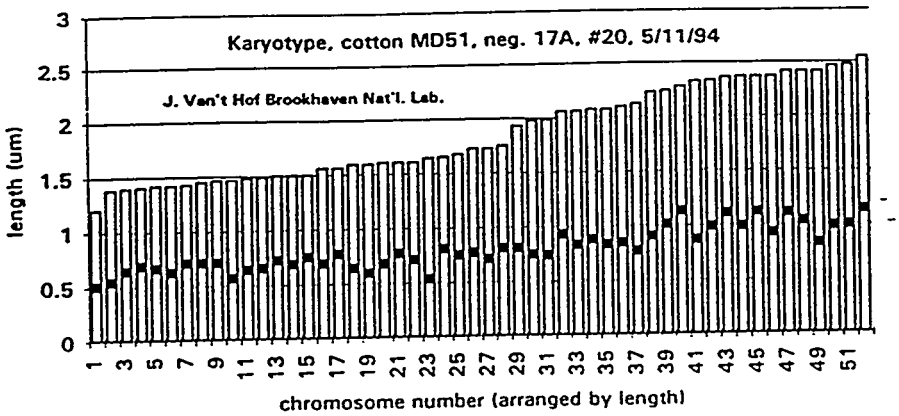

Figure 7. Chromosomes shown in Figure 6 and on cover arranged by length. Black squares show centromere position.

VI. *In situ* hybridization; preliminary results

A. Separation of cells

1. remove 6 root tips from fix & cut off 2 - 3 mm tips
2. suspend tips in ice-cold, sterile-filtered citrate buffer in 1.5 ml Eppendorf tube; spin for 2 sec.in microfuge

citrate buffer is:

0.01 M Na-citrate
0.01 M citric acid titrated to pH 4.6

3. wash with 3 changes of citrate buffer
4. remove buffer
5. add 100 ul of sterile-filtered enzyme
   a. can be the mix of three enzymes mentioned on page (0.01 gm of 3:1:1 mix in 100 ul of citrate buffer pH 4.6) or
   b. 2% pectolyase in citrate buffer, pH 4.6
6. digest for 30 min at 37 C
7. break-up root tips with micropipet tip
8. continue digestion for another 30 min
9. spin at 2300 rpm (800 x g) in TJ-6 centrifuge for 4 min
10. remove ALL BUT 1 MM of supernatant
11. add 200 ul of ice-cold citric acid buffer, vortex
12. spin at 2400 rpm in TJ-6 for 4 min
13. repeat "11" & "12" above
14. again repeat "11" & "12" above
15. remove ALL BUT 1 MM of supernatant
16. add about 200 ul of <u>ice-cold 3:1 fix</u>
17. suspend cells by vortexing, spin as in #12 above
18. repeat #15,16 and 17 above, twice using 100 ul of fix on last spin
19. place on ice

B. Spreading cells on microscopy slides

1. rub 3:1 fix on surface of acid-cleaned slide
2. resuspend cells
3. remove 5 ul with micropipet
4. place tip on slide and slowly dispense 5 ul on its surface
5. air dry
6. check preparation by phase microscopy
7. store slides in box at -20 C until ready to use

C. *In situ* hybridization; squash preparations; VERY PRELIMINARY RESULTS!

1. transfer fixed root tips to 1.5 ml Eppendorf tube
2. add 1 ml deionized water and let stand at room temp. for 5 min
3. spin in microfuge for a second or two and remove water
4. add 1 ml of deionized water and spin again
5. repeat #4 twice
6. add 200 ul of triple enzyme mix (page 6) dissolved in deionized water
7. incubate at room temp. for 30 min; tap side of tube occasionally
8. spin and remove enzyme solution
9. add deionized water and spin; repeat once again
10. add deionized water and place on ice
11. carefully remove root tips (they stick to each other) and place on squeaky clean slide
12. add 4 ul of 45% acetic acid and macerate with glass rod; spread tissue on slide surface by tapping perpendicular to surface of slide
13. place coverslip over macerated tissue
14. blot between absorbent paper; do not move coverslip
15. press with thumb or finger taking care the coverslip will not move
16. place on dry ice; leave for 3-5 min
17. "pop off" coverslip by prying edge with a razor blade
18. place in 95% ETOH; leave overnight
19. air dry
20. place about 100 ul of RNAse (100 ug/ml) on cells, cover with coverslip and incubate 1 hour at 37 C in moist chamber
21. float off coverslip in 2X SSC and rinse with 2X SSC
22. dehydrate through ETOH series (50% -> 70% -> 95% -> air dry)
23. add 20 - 30 ul of probe mix:

formamide ----------	15 ul
50% dextran sulfate ----------	4 ul
4 ug/ul Salmon sperm DNA ------ (sonicated)	2 ul
25X SSC ----------	2 ul
probe (about 30 ng total)------	7 ul
	=======
	30 ul

24. cover with silicon coated coverslip and heat 10 min at 80 C on heated aluminum block
25. seal with rubber cement
26. incubate at 37 C overnight in moist chamber
27. submerge slide in 2X SSC and remove coverslip
28. rinse in 2X SSC 5 min

29. incubate in 50% formamide-50% 2X SSC for 15 min at 37 C
30. repeat # 29 with fresh formamide/2X SSC mix
31. wash in 2X SSC 15 min at room temp.
32. remove slide leaving the surface of cells wetted
33. add a small drop of 100% glycerol, cover with coverslip
34. blot to remove excess glycerol, etc
35. view by fluorescence microscopy; see Figure 8 for result

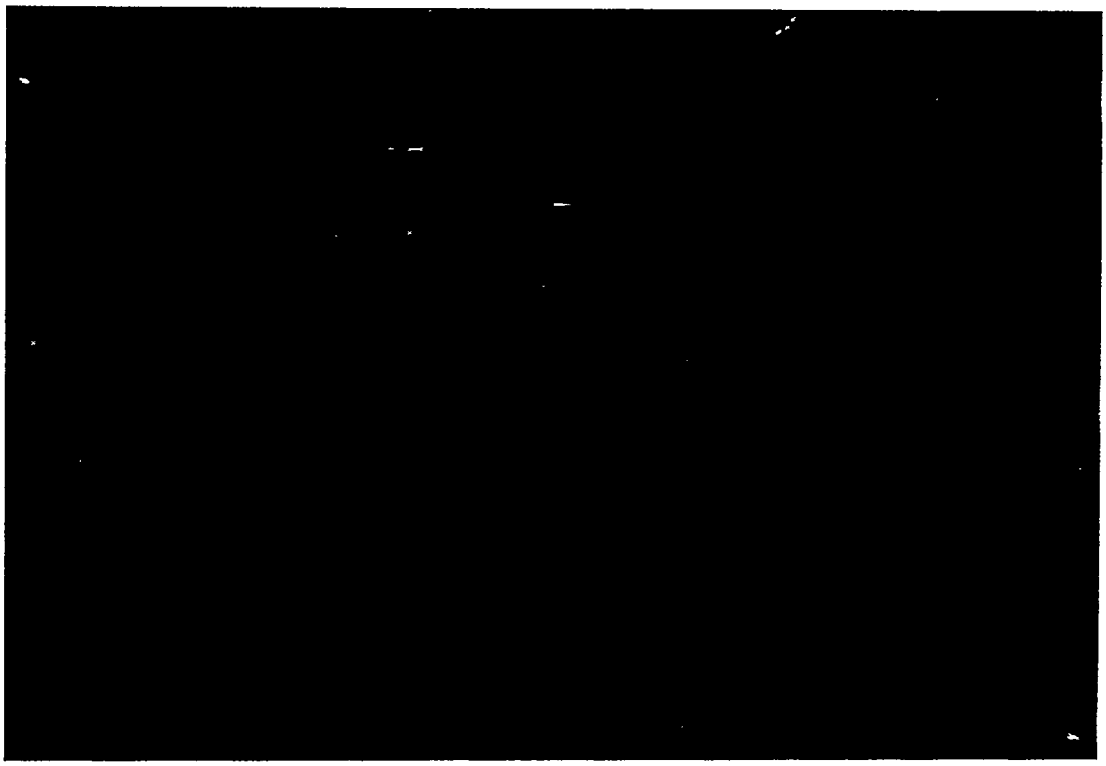

Figure 8. Hybridization of rDNA fluorescent probe to squash preparation. The six spots correspond to the six known loci of the rRNA genes expected in a somatic chromosomal complement. No image enhancement used.

References:

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6. Van't Hof, J. and Lamm, S. S. Site of initiation of replication of the ribosomal genes of pea (*Pisum sativum*) detected by two-dimensional gel electrophoresis. Plant Molecular Biology 20: 377-382, 1992.
   - cell synchrony curve of pea

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