Advanced Cell Biology Lab 1.
Organelles

January 14, 2013

Background There 14 transmission electronic microphotographs of cells from bacteria, protists, plants and animals. Choose 10 and proceed to the next step.

Determine organelles On each photo chosen, find at least three organelles and label them. Use arrows to connect labels and places.

Guess the origin of cells For each photo chosen, answer two questions:

1. Is that prokaryotic cell? Why?
2. Is this cell photosynthetic? Why?

Put your answers nearby to photo.

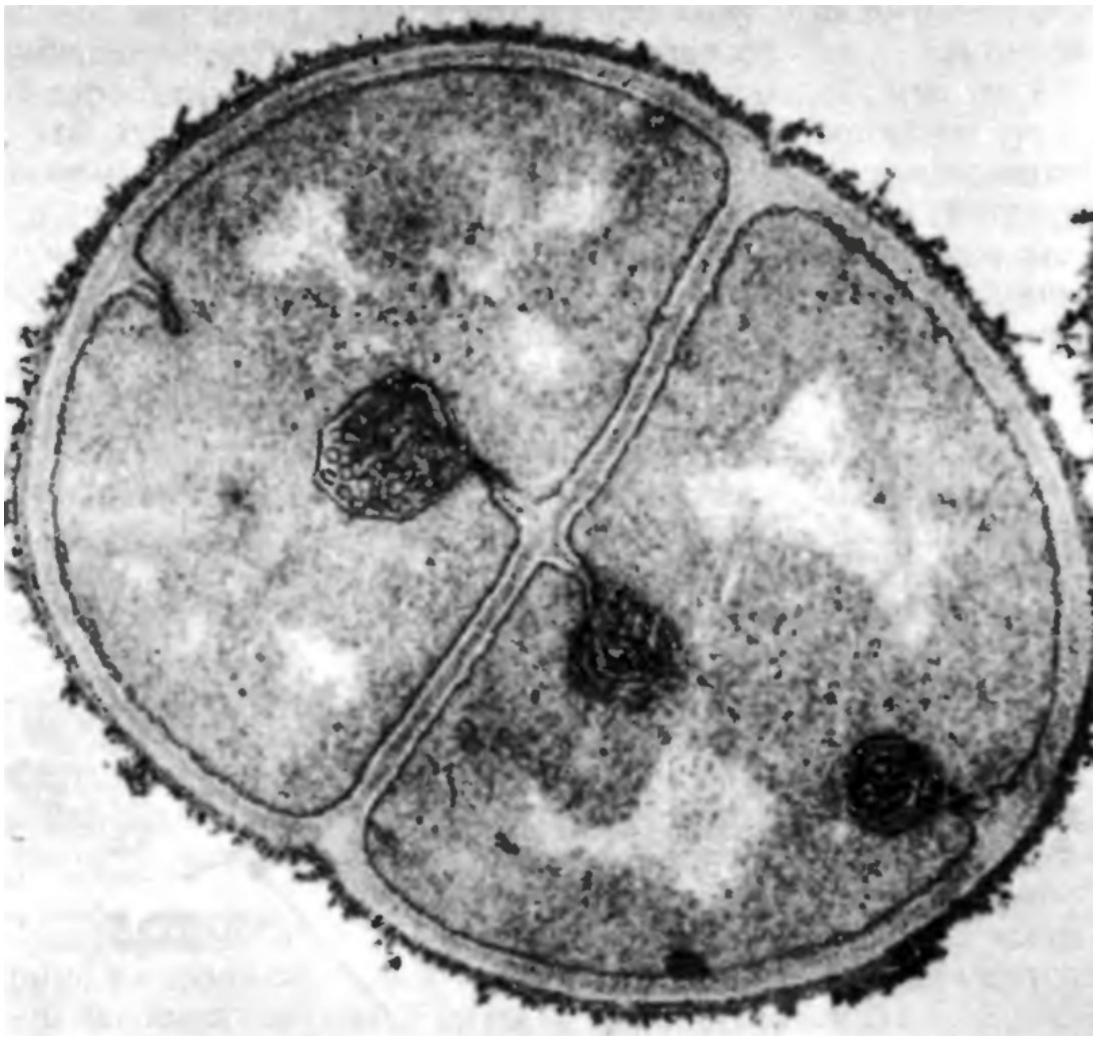


Fig. 1.

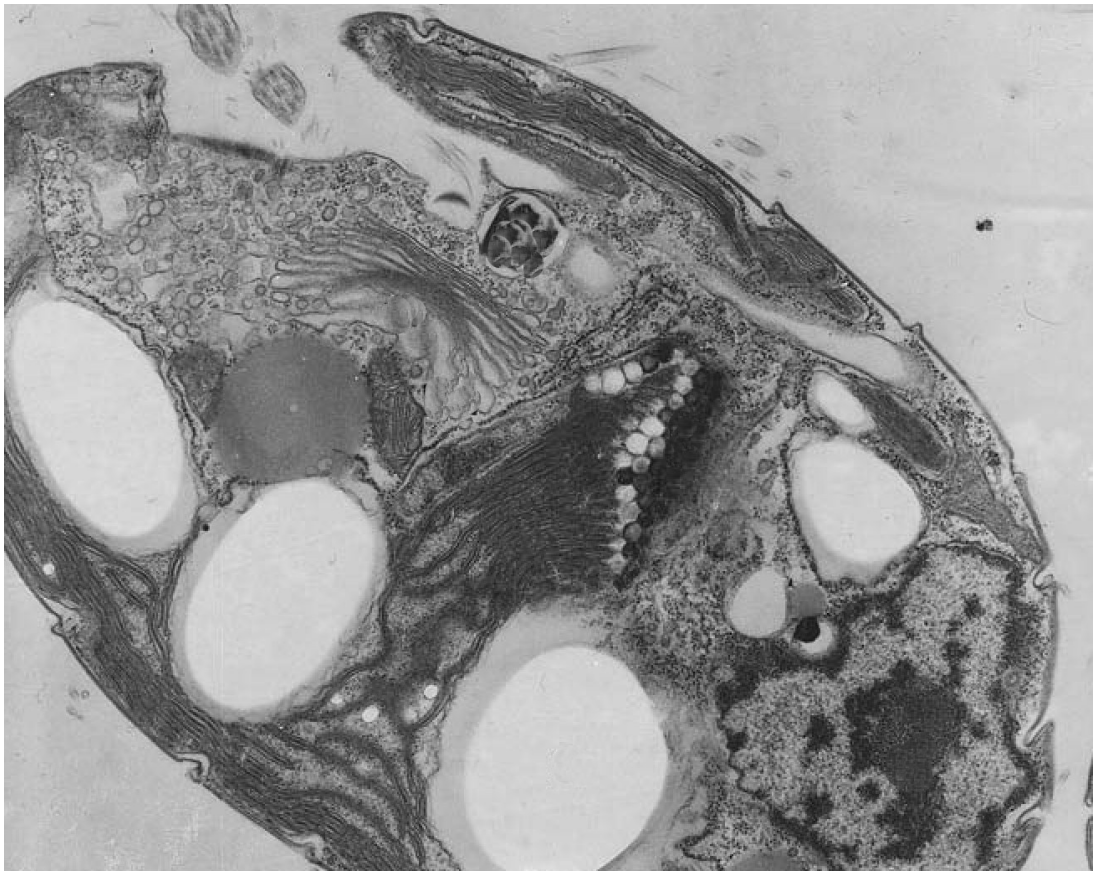


Fig. 2.

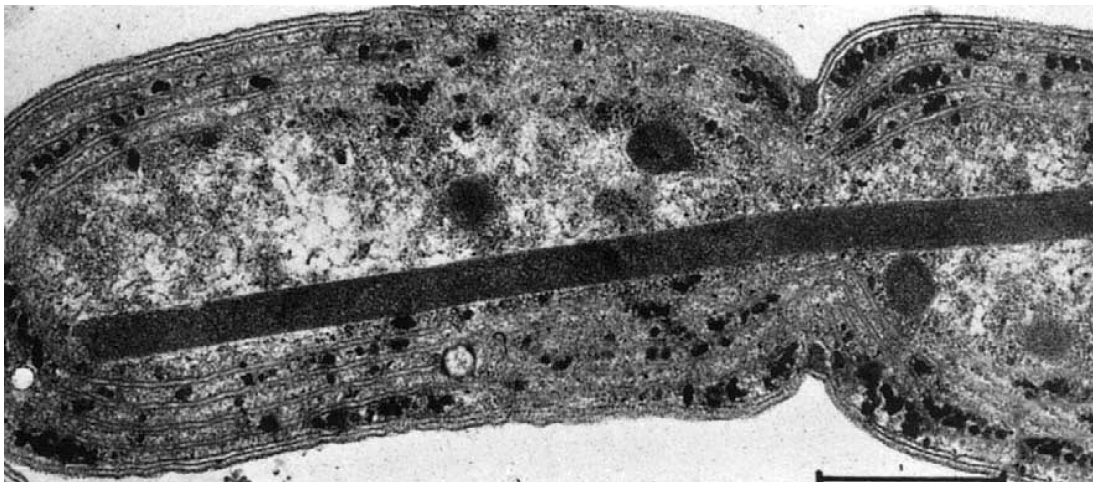


Fig. 3.

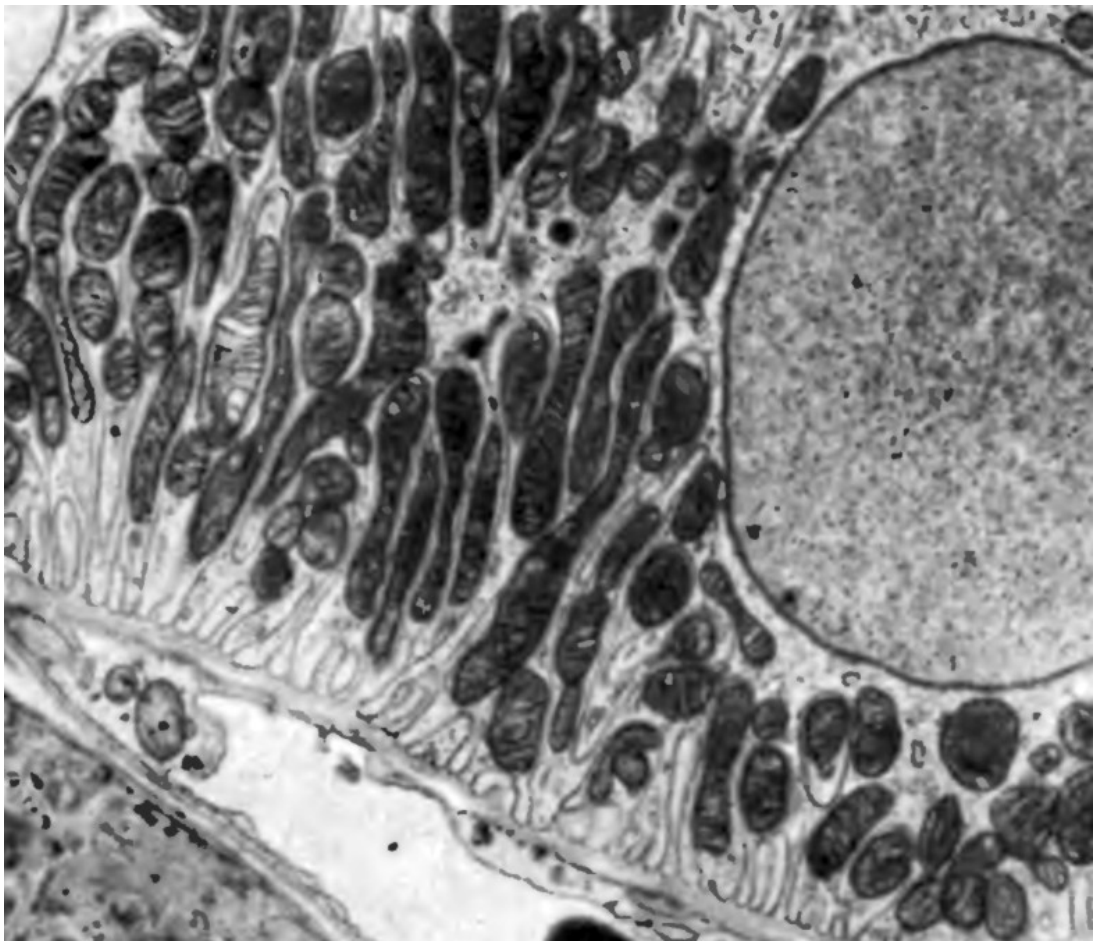
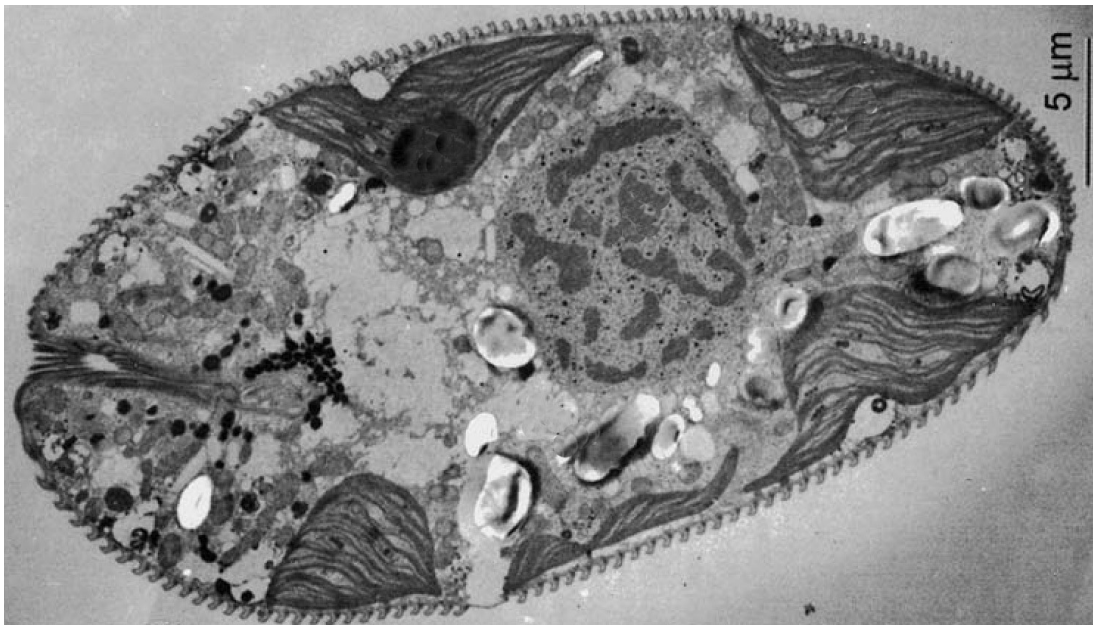


Fig. 4.



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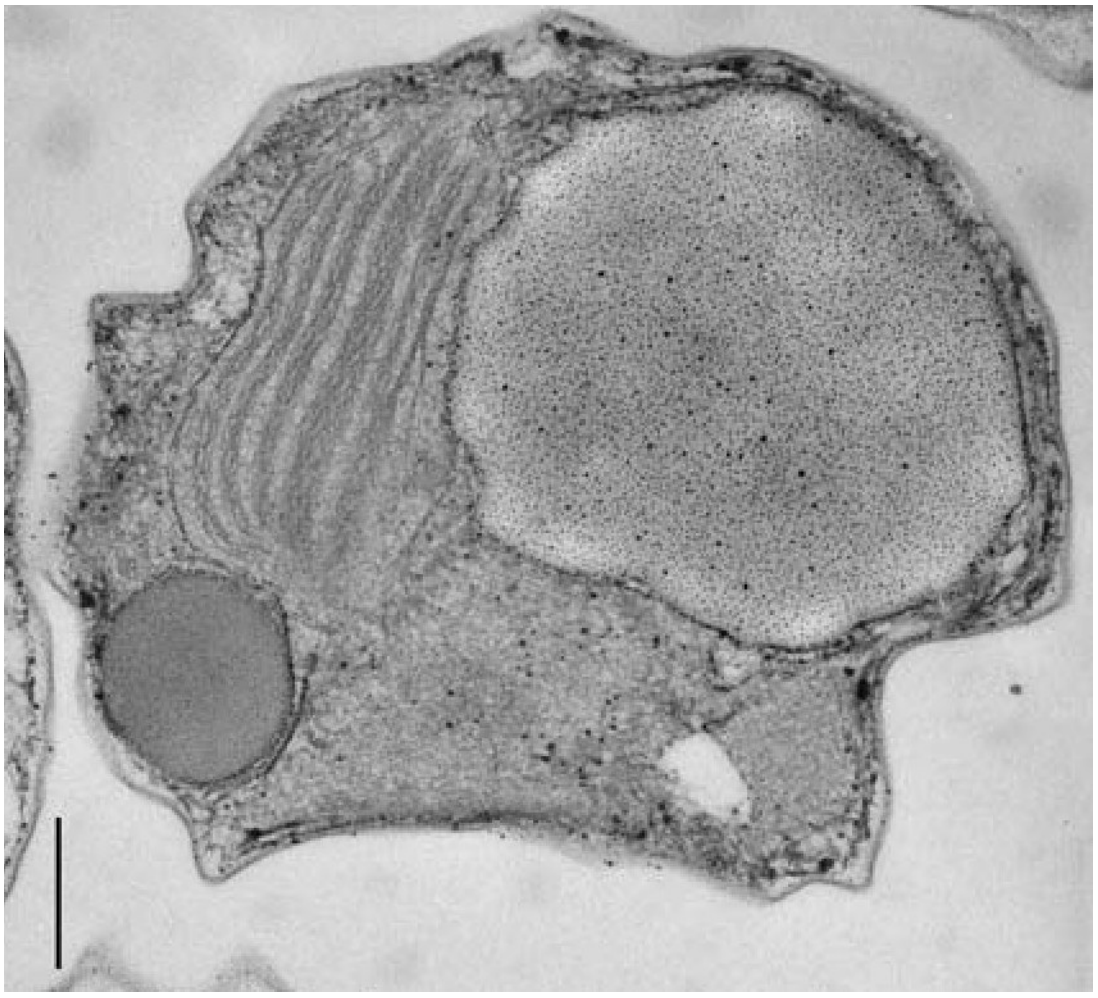

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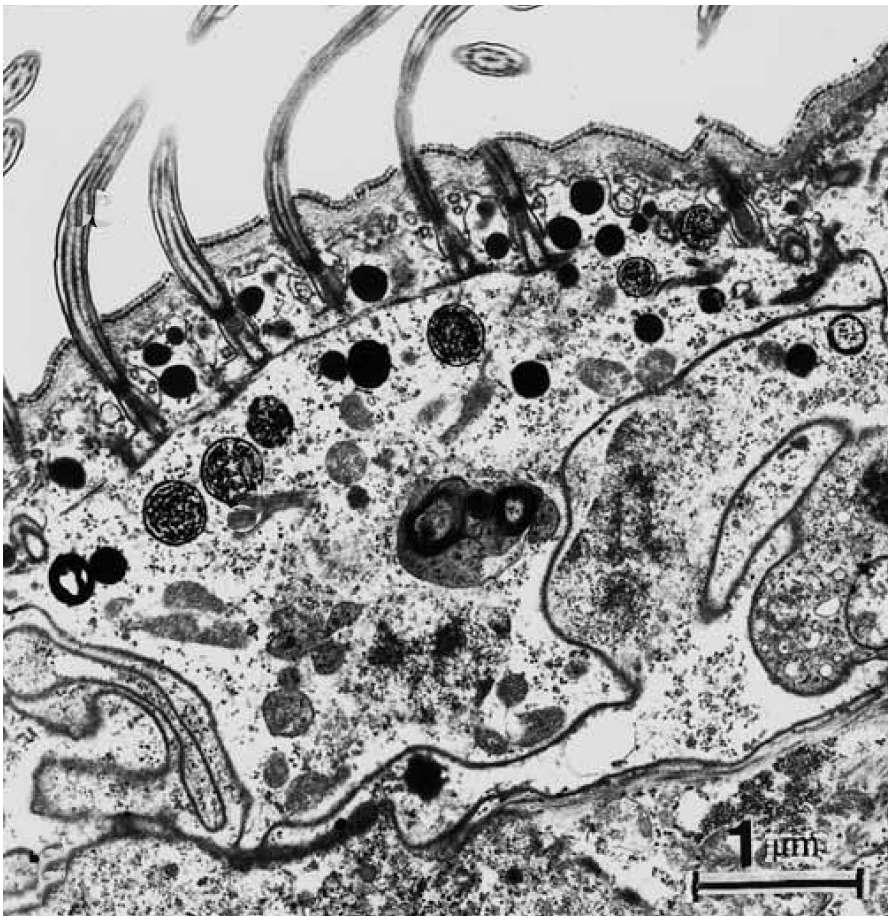

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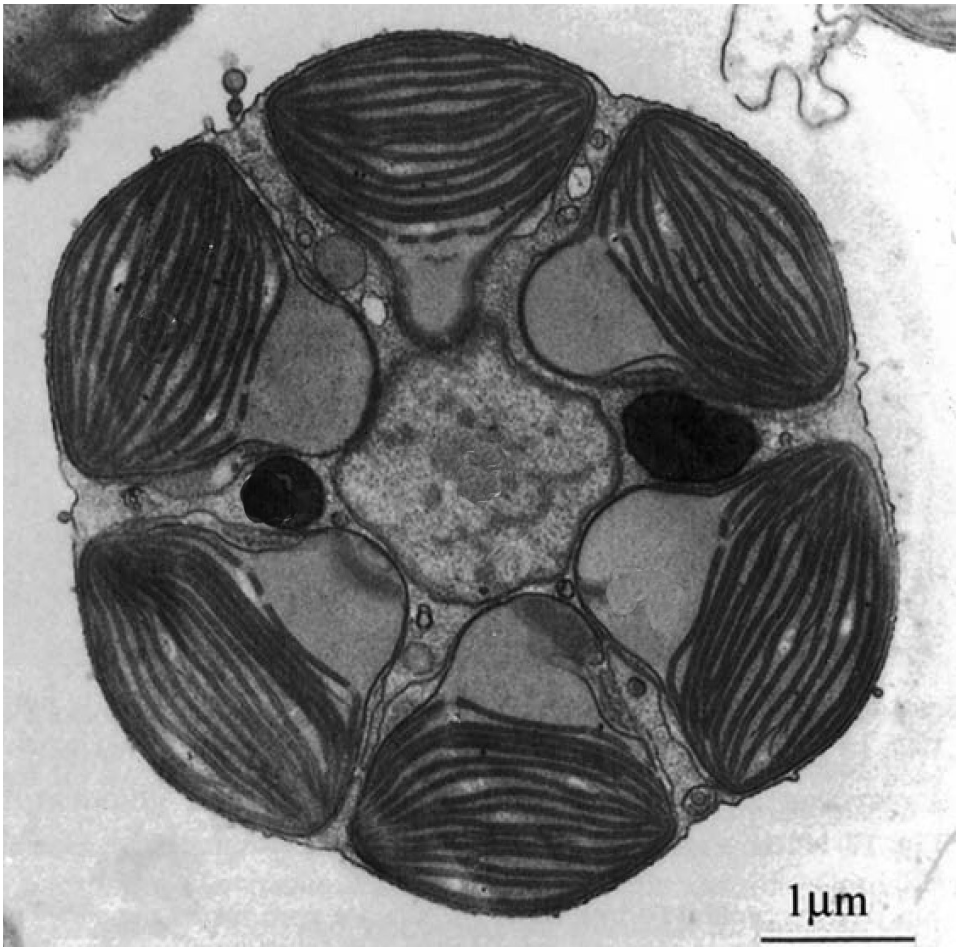

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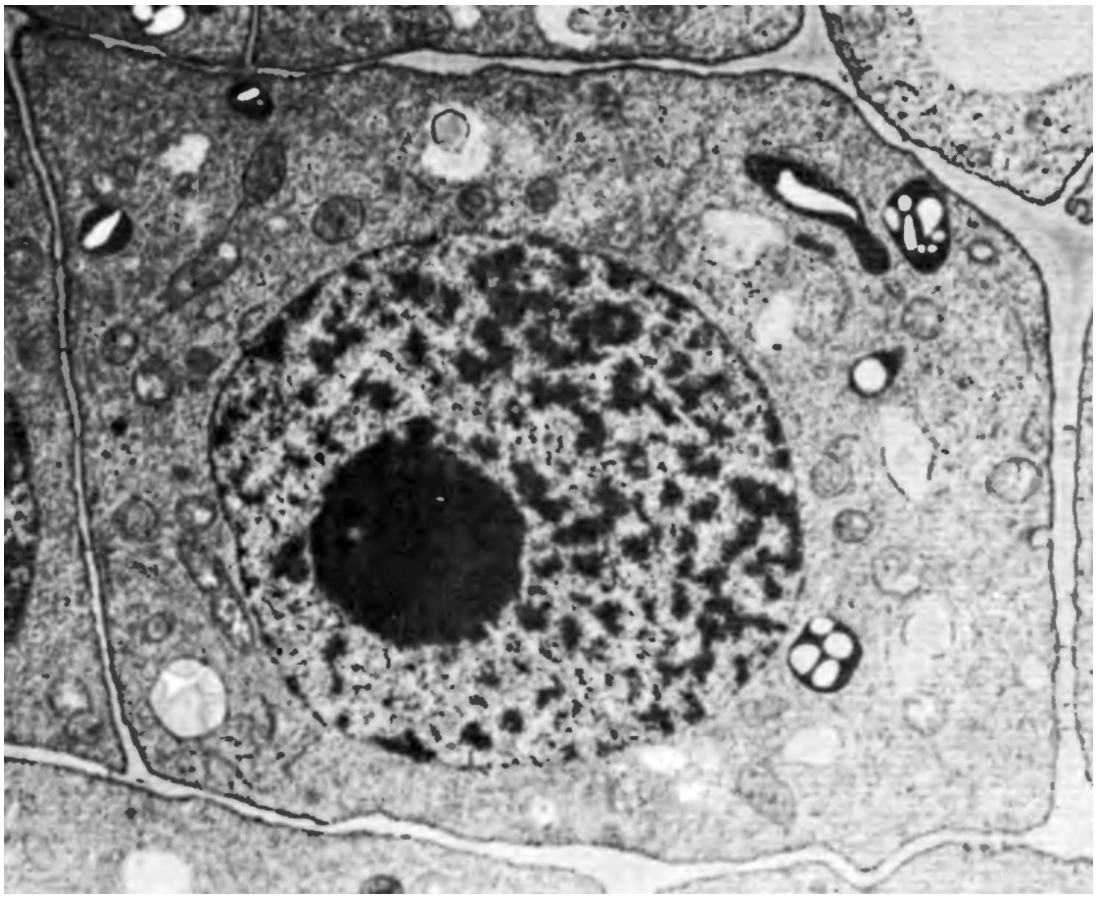


Fig. 9.

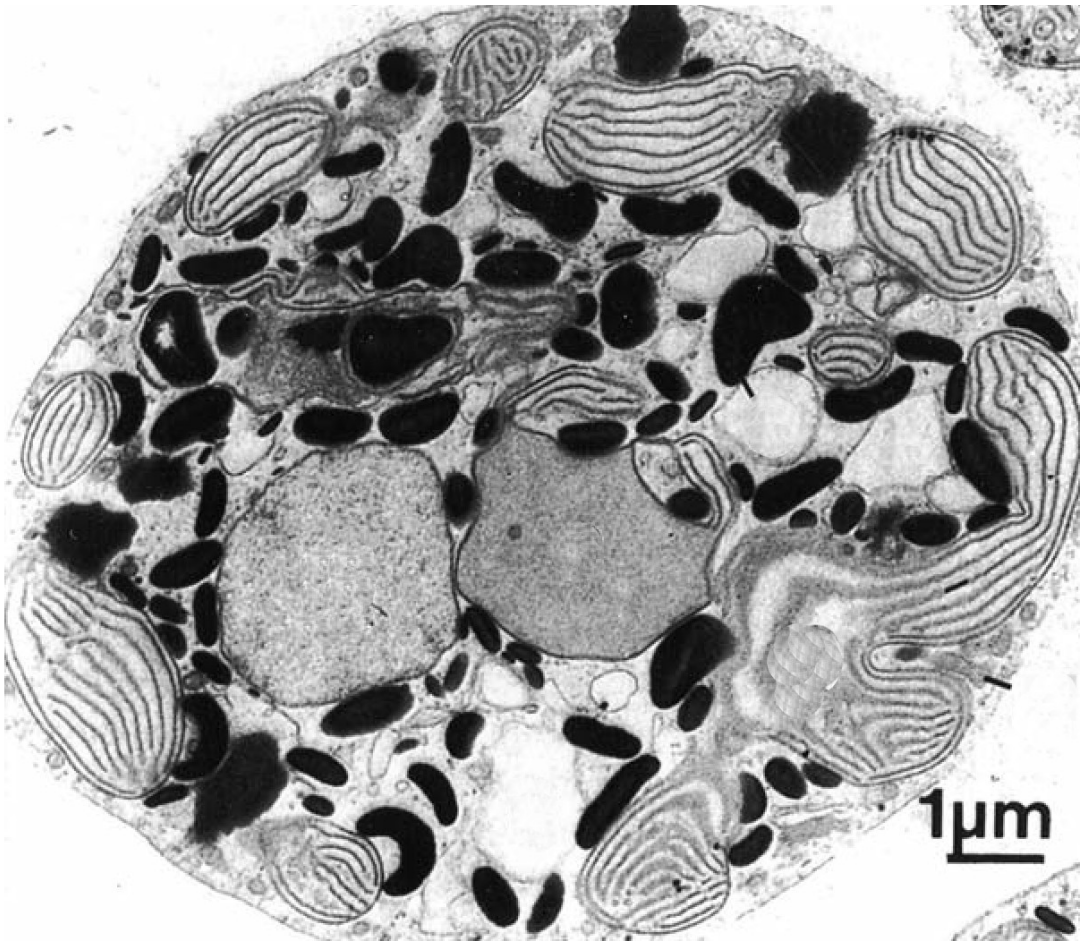


Fig. 10.

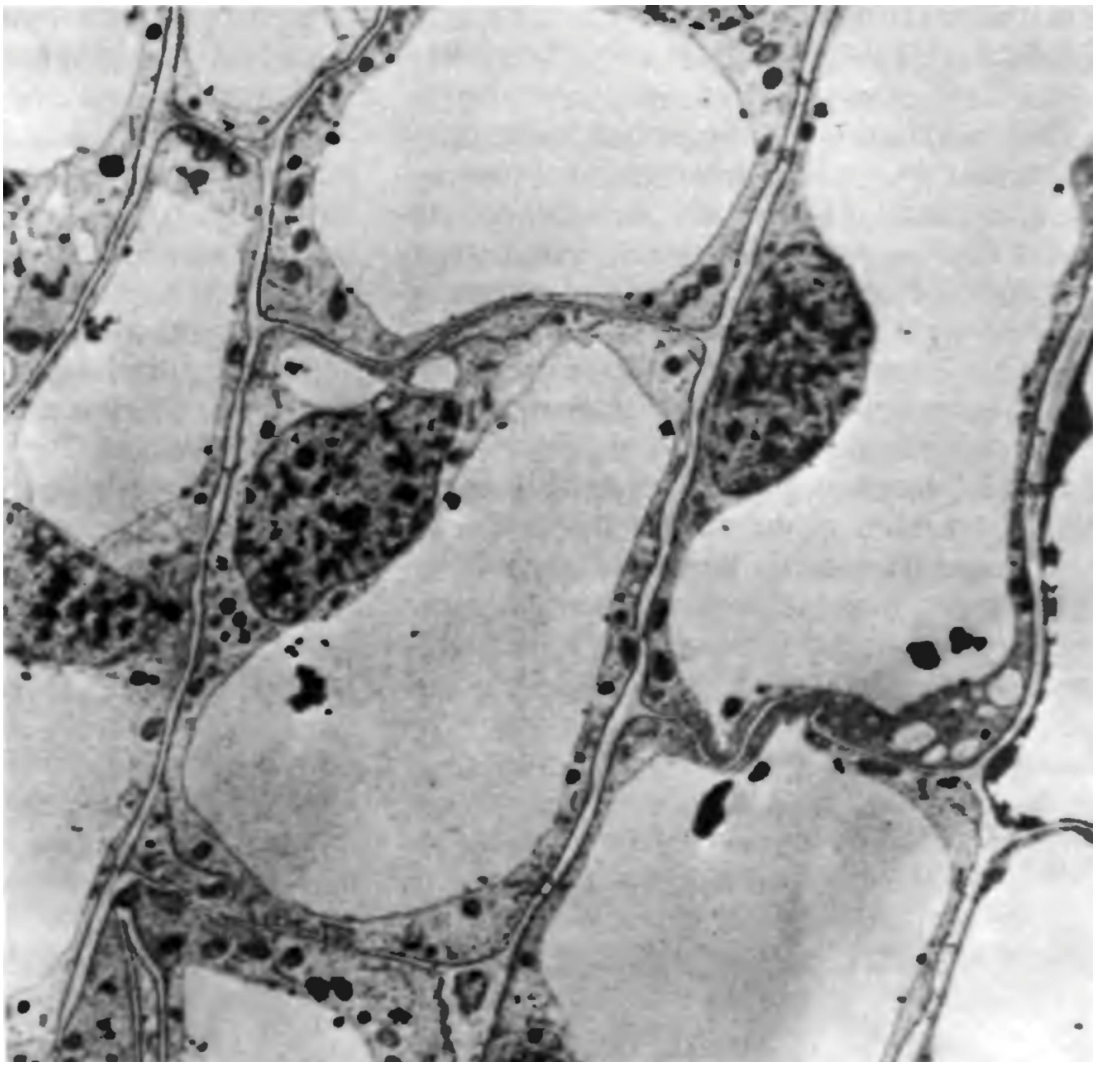


Fig. 11.

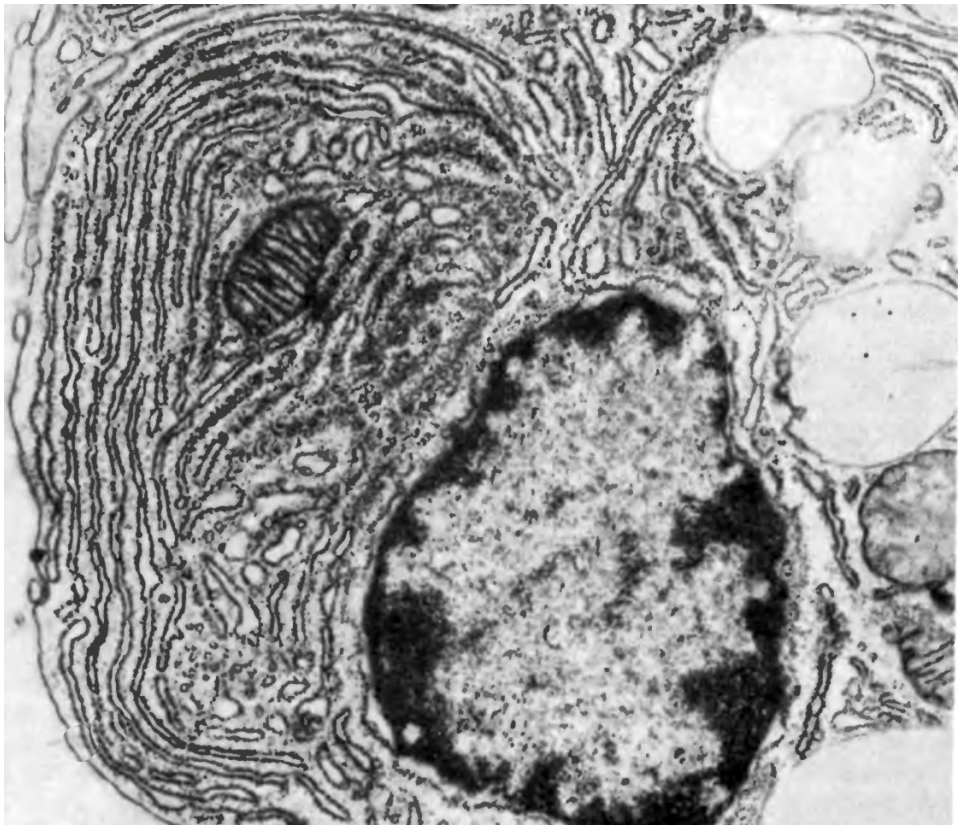


Fig. 12.

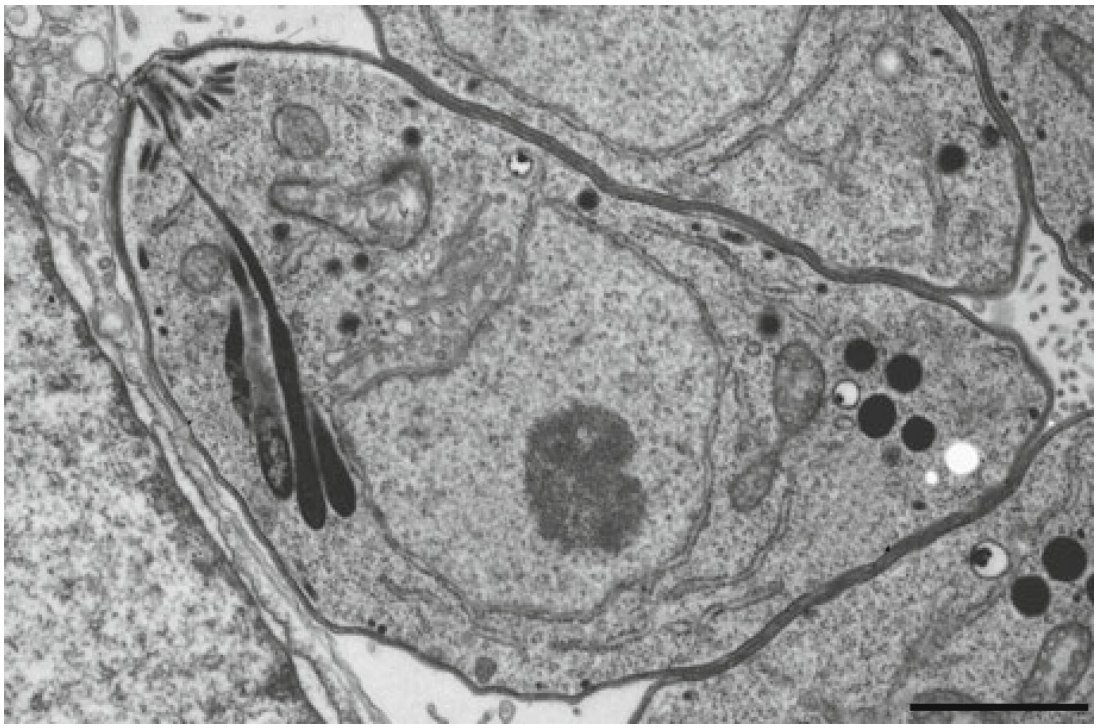


Fig. 13.

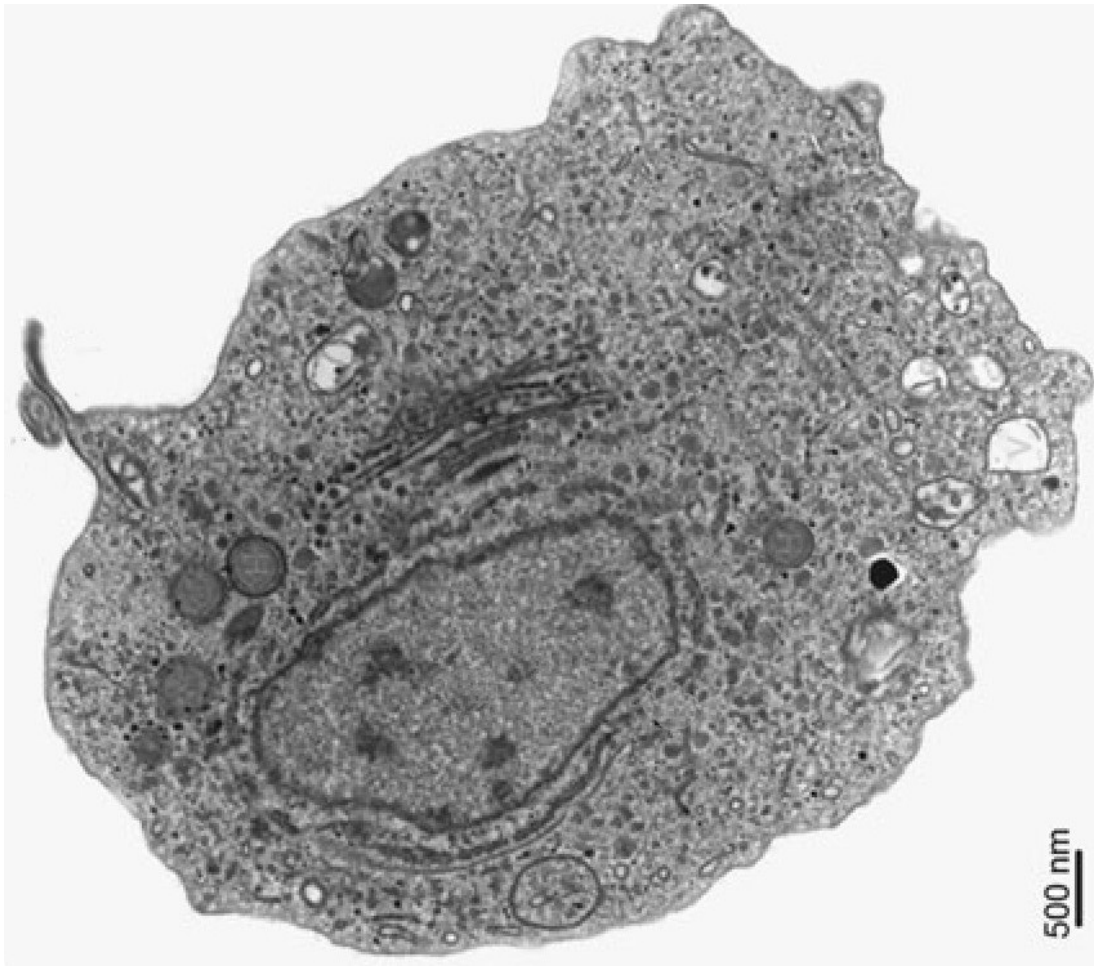


Fig. 14.

Advanced Cell Biology Lab 2.
Protein folding

January 28, 2013

Background FoldIt is a free computer game intended to help in science research of protein structure. Solving FoldIt puzzles, player will contribute to medical biology, because every protein has an astronomical number of possible conformations, and finding low energy conformation is an extensive task even for contemporary computer systems. Instead of computers, FoldIt employs much more powerful thing—human brain.

Assignment

1. **Read** the introductory page from a FoldIt Web site (`http://fold.it/portal/info/science`), make sure you followed additional links to RCSB Protein Data Bank (like "Amylase" etc.)
2. **Skip this if FoldIt is already installed!** [**Download** Windows version of FoldIt from `http://fold.it/portal/download/windows` and **install** it for current user]
3. **Run** FoldIt, **skip the update**, **choose "Play offline"**. **Do not** register! (If you like the game, you can do it later on your computer).
4. Your goal is to **solve** all "Tutorial Puzzles" (for proper grade, it is important to solve at least 50% of them)
5. Please work in pairs. If the puzzle seems to be hard, do not hesitate to **consult** with other pairs, with FoldIt Wiki (`http://foldit.wikia.com/wiki/FoldIt_Wiki`) and especially Tutorial Puzzles Wiki (`http://foldit.wikia.com/wiki/Tutorial_Puzzles`).
6. Prepare the short **report**: your name, today's date, how many puzzles have been solved, how much time has been spent.

Advanced Cell Biology Lab 3.
DNA extraction

February 4, 2013

Background This lab starts a series of labs wher we will do all steps of DNA analysis, from DNA extraction to the creation phylogeny tree. The goal is to understand elucidate relationships between species of *Plantago* (plantains). Many species of this large (> 200 species) genus are not yet studied genetically. I have 28 herbarium (dry plant collection) samples of these species, and we use them as a basis of our collective research.

Warning! Since these labs are research, be ready for negative results.

Assignment We will extract DNA with MO Bio PowerPlant DNA Isolation Kit. The detailed protocol is given below.

Notes:

1. We will work in pairs, every pair will extract DNA from four samples. Since there are 8 pairs and 27 samples, some samples will be shared between pairs. If you have a shared sample (P2, P3, P4, P6, P9), do not use more than a half of it.
2. Every tube in the process (you will need 4 samples $\times$ 5 tubes = 20 tubes) should be labeled with extraction ID twice: on lid and on side. Always use black markers with stable ink.
3. Before every step, consider which pipetter and which tip to use, basing on the amount of liquid you have.
4. Use gloves at all times.
5. Use forceps to operate with herbarium samples.
6. Every DNA sample should be weighted before step 1, and extraction ID should be assigned for every extraction item. Table of samples is below. Please fill cells which you are responsible for. **This is your report** (see the next page).

Report

Extraction *Plantago* # 5

Conditions: MO Bio Power Plant DNA isolation kit

Date:

ID	Collection ID	Species	Weight, *g*	Your names
5-1	P1	*Plantago media*		
5-2	P2	*Plantago krasheninnikovii*		
5-3	P3	*Plantago krasheninnikovii*		
5-4	P4	*Plantago krasheninnikovii*		
5-5	P6	*Plantago urvillei*		
5-6	P7	*Littorella uniflora*		
5-7	P8	*Plantago arachnoidea*		
5-8	P9	*Plantago komarovii*		
5-9	P10	*Plantago minuta*		
5-10	P11	*Plantago canescens*		
5-11	P12	*Plantago griffithii*		
5-12	P13	*Plantago lagocephala*		
5-13	P14	*Plantago loeflingii*		
5-14	P15	*Plantago lessingii*		
5-15	P16	*Plantago minuta*		
5-16	P17	*Plantago polysperma*		
5-17	P18	*Plantago evacina*		
5-18	P19	*Plantago griffithii*		
5-19	P20	*Plantago squalida*		
5-20	P21	*Plantago altissima*		
5-21	P22	*Plantago argentea*		
5-22	P23	*Plantago carinata*		
5-23	P24	*Plantago monosperma*		
5-24	P25	*Plantago schwartsenbergii*		
5-25	P26	*Plantago pusilla*		
5-26	P27	*Plantago subulata*		
5-27	P28	*Plantago lagopus*		
5-28	P2	*Plantago krasheninnikovii*		
5-29	P3	*Plantago krasheninnikovii*		
5-30	P4	*Plantago krasheninnikovii*		
5-31	P9	*Plantago komarovii*		
5-32	P6	*Plantago urvillei*		

Protocol (see the next page)

Please wear gloves at all times

This protocol is written for the first time user. It is designed to be informative and describes each step in detail.

1. To the PowerPlant® Bead Tubes provided, add up to 100 mg (0.1 g) of plant tissue sample, followed by the addition of 550 µl of PowerPlant® Bead Solution.
   NOTE: THE DNA EXTRACTION EFFICIENCY WILL IMPROVE BASED ON THE CONDITION OF HOMOGENIZATION
   What's happening: After your sample has been loaded into the PowerPlant® Bead Tube, the next step is a homogenization and lysis procedure. The PowerPlant® Bead Tube contains a buffer that will (a) help wet the tissue surfaces, and (b) protect nucleic acids from degradation.

2. Gently vortex to mix.

   What's happening: Gentle vortexing mixes the components in the PowerPlant® Bead Tube.

3. **Check Solution PB1**. If Solution PB1 has precipitated, heat solution to 60°C until the precipitate has dissolved before use.

   What's happening: Solution PB1 contains SDS and other disruption agents required for complete cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down proteins, fatty acids and lipids associated with the cell membranes. If it gets cold, it will form a white precipitate in the bottle. Heating to 60°C will dissolve the SDS and will not harm the SDS or the other disruption agents. Solution PB1 can be used while it is still warm.

4. Add 60 µl of Solution PB1 and invert several times or vortex briefly.

5. Place the PowerPlant® Bead Tubes in a water bath at 65°C

   What's happening: Heating the plant tissues help in homogenizing them in the following step.

6. Vortex at maximum speed for 20 minutes.

7. Make sure the PowerPlant® Bead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature.
   CAUTION: Be sure not to exceed 13,000 x *g* or tubes may break.

8. Transfer the supernatant to a clean 2 ml Collection Tube (provided).

   Note: *Expect between 400 to 500 µl of supernatant at this step. The exact recovered volume depends on the absorbency of your starting material and is not critical for the procedure to be effective. The supernatant may be dark green in appearance and still contain some tissue debris. The presence of carry over tissue debris or a dark color in the mixture is expected in many plant types at this step. Subsequent steps in the protocol will remove both carry over tissue debris and coloration of the mixture.*

9. Add 250 µl of Solution PB2 and invert the tubes to mix the contents. Incubate at 4°C for 5 minutes.

   What's happening: Solution PB2 contains a reagent to precipitate non-DNA organic and inorganic material including plant polysaccharides, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

10. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

11. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2.2 ml Collection Tube (provided).

What's happening: The pellet at this point contains non-DNA organic and inorganic material including plant tissue debris, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

12. Add 1 ml of Solution PB3 and invert the tubes at least 5 times to mix the contents. Incubate at room temperature for 10 minutes.

What's happening: Solution PB3 is 99% isopropanol and will precipitate DNA along with some organic contaminants. Most of the co-extracted impurities will be removed at this step.

13. Centrifuge the tubes at room temperature for 15 minutes at 13,000 x *g.*

14. Discard the supernatant and resuspend the pellet in 100 µl of Solution PB6. Note: The tubes do NOT have to be air dried as residual isopropanol will not affect the process.

What's happening: Isopropanol will precipitate and pellet the DNA. The pellet at this point contains relatively pure DNA along with some organic contaminants; mostly polysaccharides and phenolics depending on the plant tissues processed, leaving a majority of the contaminants in solution.

15. Shake to mix Solution PB4. Add 500 µl of Solution PB4 and vortex briefly to mix.

What's happening: Solution PB4 is a high concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solution salt concentrations to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the Spin Filters.

16. Load the entire volume (600 µl) onto a Spin Filter and centrifuge at 10,000 x *g* for 1 minute.

What's happening: DNA is selectively bound to the silica membrane in the Spin Filter device in the high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.

17. Remove the Spin Filter basket, discard the flow through and replace the Spin Filter basket back in the tube.

18. Add 500 µl of Solution PB5 and centrifuge at room temperature for 30 seconds at 10,000 x *g*.

What's happening: Solution PB5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residual salts and other contaminants while allowing the DNA to stay bound to the silica membrane.

19. Discard the flow through from the 2 ml Collection Tube.

What's happening: This flow through fraction is non-DNA organic and inorganic waste removed from the silica Spin Filter membrane by the ethanol wash solution.

20. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.

What's happening: This second spin removes residual Solution PB5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in Solution PB5 can interfere with many downstream DNA applications such as PCR, restriction digests, and gel electrophoresis.

21. Carefully place Spin Filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution PB5 onto the Spin Filter.

Note: *It is important to avoid any traces of the ethanol based wash solution.*

22. Add 50 µl of Solution PB6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).

    Note: *Placing the Solution PB6 in the center of the small white membrane will make sure the entire membrane is wetted. This will result in a more efficient and complete release of the DNA from the silica Spin Filter membrane. As Solution PB6 (elution buffer) passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution PB6 (10 mM Tris) which is a low salt solution.*

23. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.

24. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). Solution PB6 does not contain any EDTA. To concentrate DNA see the Hints and Troubleshooting Guide.

NOTE: Occasionally, plants such as grapes, cotton, sunflower, strawberry, pine needles, etc. will yield DNA with inhibitors, which may prevent target sequences from amplifying in PCR. Under such circumstances, it is suggested to use the clean-up protocol provided. Alternatively you can dilute the template DNA one to several fold, for successful PCR.

Thank you for choosing the PowerPlant® DNA Isolation Kit.

Advanced Cell Biology Lab 4.
DNA discovery: discussion

February 11, 2013

Background Discovering of DNA was the result of long and complicated research, from Koltsov (1927) "template" hypothesis to series of papers in Nature (1953). Our goal will be to elucidate this chain of research through the review and discussions of **three main papers**: Avery et al. (1944), Wilkins et al. (1953) and Watson & Crick (1953a and 1953b). All papers are openly accessible on the Nature "Double Helix" Web page: `http://www.nature.com/nature/dna50/archive.html`

Assignment

1. Download and read three mentioned papers.
2. Join one if three presenting teams and participate in making Power Point presentation about particular paper. There are multiple resources in Internet with a title like "How to read a scientific/research paper" (like `http://www.google.com/search?q=how+to+read+scientific+research+paper`) which could be of some help.
3. Prepare questions for two other presenting teams. Since we all know that these papers are really great, the most important will be to understand *weaknesses* of each paper—in other word, *directions of further research*. Please also try to emphasize how features of DNA help to extract it (Lab 3).
4. Present a presentation, answer all questions. It is important that all members of team participate!
5. Evaluate all teams (including yours) on 1–10 scale.

Advanced Cell Biology Lab 5 and 6.
Polymerase Chain Reaction (PCR)

February 25 and March 4, 2013

Part 1. PCR preparation and running

Background

Shortly following the discovery of DNA structure, important experiments showed DNA is replicated in a semiconservative manner, in which each strand serves as a template for synthesis of a new strand, (key experiments by Messelson and Stahl). The sequence of the template strand determines the sequence (complementary) of the new strand. Arthur Komberg added to the understanding of DNA replication with his identification of the enzymes that catalyze the DNA polymerization reaction. These enzymes, DNA polymerases, first isolated from *Esherichia coli* ("*E. coli*"), perform 5' to 3' DNA synthesis.

Many details of DNA replication were worked out in the last 50 years. We know that a host of proteins work together at a replication fork to unwind DNA and maintain a single stranded template. We know that DNA polymerases must have a short existing stretch of nucleic acid with a free 3' OH group on which to add new nucleotides (nucleoside triphosphates, dNTPs). In cells, this primer is a short RNA molecule.

Much of the work in DNA replication was based on in vitro experiments, using a purified DNA template and showing DNA replication could occur in *E. coli* or in vitro, in the presence of the essential elements used in the cell. (DNA polymerase, primers, dNTPs). In 1988, Kary Mullis published his invention of a DNA replication method that is arguably one of the most important advances in molecular biology. The technique, called Polymerase Chain Reaction (PCR) is a super sensitive method for replicating any region of DNA using a fast, simple method of consecutive rounds of DNA synthesis.

Mullis realized DNA synthesis can occur with a minimum of components if one can mimic the action of some proteins in other ways (like manipulating temperature). PCR can be performed with as few as 4 ingredients:

1. Template DNA (to copy or amplify)
2. dNTPs (dATP, dTTP, dCTP, dGTP)
3. Short DNA/RNA sequences to prime synthesis of the DNA template (**primers**)
4. DNA polymerase enzyme

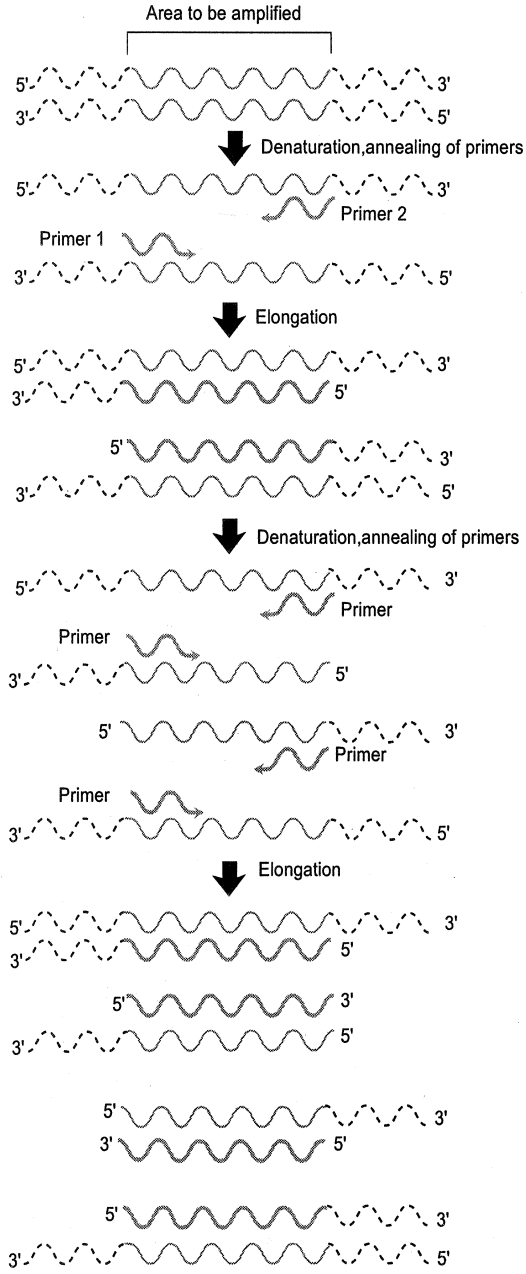
PCR requires that you know at least some of the linear sequence of the DNA of interest (**template**). The sequence information is used to design short DNA primers (10–20 nucleotides in length) that are complementary to the ends of the DNA segment of interest. These primers serve the same purpose as the RNA primer used in DNA replication inside cells and allows replication of the DNA in between the primers.

Like all replication, PCR also requires DNA molecules to copy. This template DNA can be purified from cells (genomic DNA) or be cloned DNA with much more limited sequence (small plasmid).

Finally, PCR requires the DNA replication enzyme (DNA polymerase) and ingredients (nucleotides, dNTPs: A, T, G and C) and a way to mimic the events of cellular replication. One of the key events of DNA replication is strand separation. In PCR strands are separated by high heat. Primers are

not made in the test tube, but are added and allowed to anneal to the ends of the DNA template (by adjusting the temperature). Once primers are annealed the DNA polymerase can synthesize DNA as it does in cells, by linking together dNTPs according to the single stranded template. We use a special, heat-stable, DNA polymerase so that multiple rounds of PCR can be done without adding new enzyme. The enzyme, *taq* polymerase comes from *Thermus aquaticus*, a thermophilic bacterium.

The essential components for DNA synthesis are combined in one tube and placed in a thermal cycler. The tubes are subjected to repeated cycles of heating (to denature the template to its single strands) and cooling (to allow complementary binding of primers to the region to be amplified). The specific region amplified in PCR depends entirely upon the primers used. A third temperature may be used according to the active temp for the polymerase (see PCR figure below).



PCR

Typically 30–40 rounds of repeated cycles are used to create enough copies of the DNA to be visualized (e.g. by DNA electrophoresis). The number of copies of the DNA of interest is dependent on the number of cycles of PCR done. For each starting copy there will be 2n copies produced where n is the number of cycles.

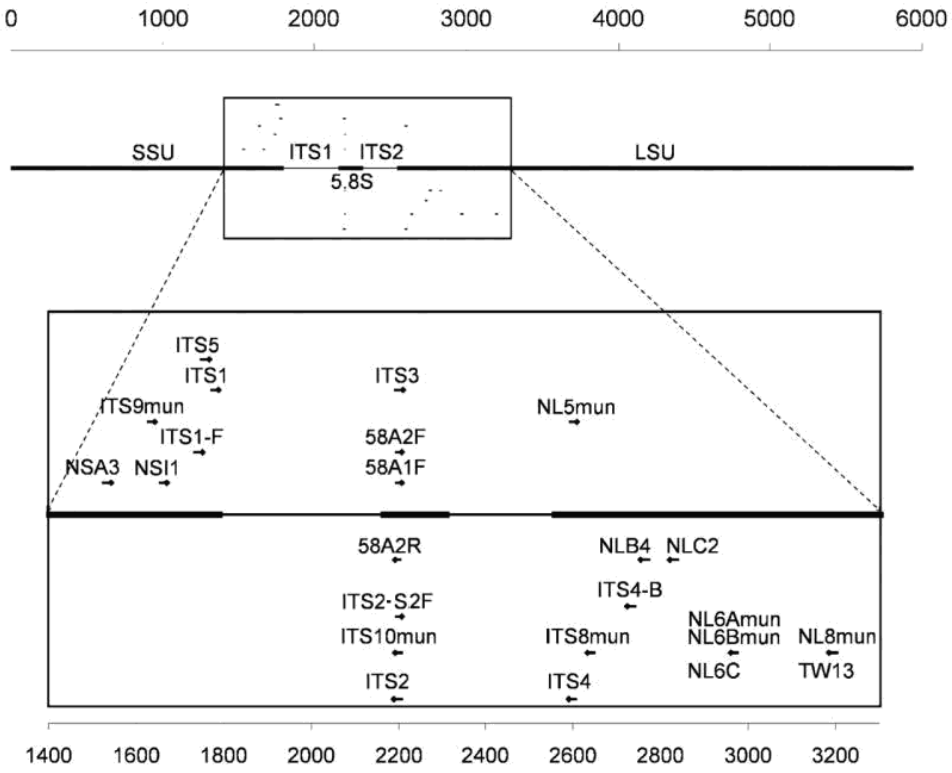
The exponential amplification of DNA allows many applications, including detection of rare DNA sequences. PCR is used to detect viral or bacterial DNA in humans and animals. It is used to produce probes for genetic analysis. Most procedures that match a suspect's DNA profile to the crime scene utilize PCR.

Assignment

The template are 23 samples of a genomic DNA of plantains (*Plantago* spp.) obtained on the previous lab. I will also add here 11 DNA samples of rowans (*Sorbus* spp. and *Aronia* spp.). Everybody will work with 2 samples. *It is very important to remember extraction ID from a tube!*

We will need to amplify a variable fragment located inside nuclear DNA, between genes encoding two ribosomal RNAs (see the figure below)—so-called "ITS2", internal transcribed spacer 2. The region size should be about 400 base pairs. This is well-known **barcoding** region for plants and fungi.

Today we will set up PCR on a single template, ans use one primer set: primers ITS4 and ITS2-S2F.



Different primers amplifying internal transcribed spacers: ITS1 and ITS2.

Materials

1. 2 thin walled PCR tubes
2. 2 tubes of template DNA
3. Forward primer ITS2-S2F, 10 µM
4. Reverse primer ITS4, 10 µM
5. 10× PCR buffer
6. 25 mM $MgCl_2$
7. 10 mM dNTPs mixture (2.5 mM each)
8. *Taq* polymerase (5 units per 1 µl)
9. ddH_20 (double distilled water)

Procedure

1. Wear gloves!
2. Label your PCR tubes *on cap and on side* according to your DNA extraction ID. **Keep tubes on ice** for the duration of the procedure steps.
3. Pipette 11.8 µL ddH_20 into each PCR tube.
4. Pipette 2 µL 25 mM $MgCl_2$ into each PCR tube.
5. Pipette 2 µL 10× PCR buffer into each PCR tube.
6. Pipette 1 µL dNTPs into each PCR tube.
7. Pipette 1 µL forward primer into each PCR tube.
8. Pipette 1 µL of reverse primer into each PCR tube.
9. Pipette 0.2 µL of *taq* polymerase into each tube.
10. Pipette 1 µL of DNA template into each PCR tube.
11. Set a pipette to 20 µL and gently mix by pipetting the entire mixture. Try not to introduce bubbles and drops on the tube walls. If drops or bubbles appear, short centrifugation (4–6 sec) may remove them.
12. Cap the tubes tightly and place in thermal cycler (wait for instructor's assistance!)

Thermal cycler settings:

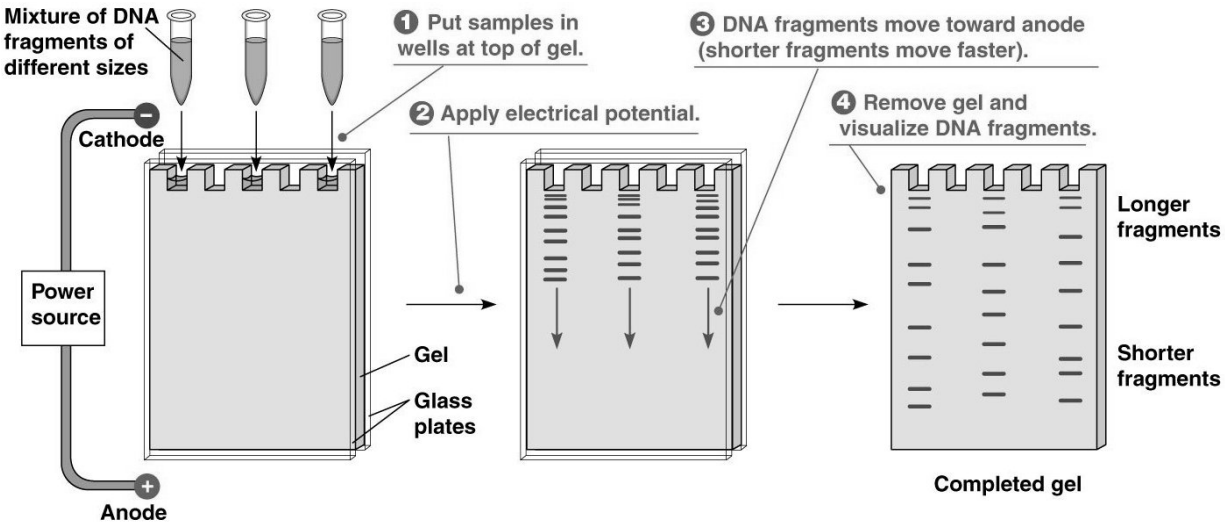
1. 94°C 5 min (premelt: complete denaturing)
2. Then 35 cycles of:
   (a) 94°C 1 min (denature)
   (b) 51°C 1 min (annealing)
   (c) 72°C 2 min (extension)
3. 72°C 10 min (final extension)
4. 4–10°C for unlimited time (stable storage of DNA)

Part 2. Analysis of PCR: agarose gel electrophoresis

Background

Today we will determine our success in amplifying the ITS2 region by PCR. You should have 2 PCR samples in which you used 2 primer pairs to analyze.

A standard method for analyzing the size and amount of DNA produced in a PCR reaction is **agarose gel electrophoresis**. Agarose is a product of seaweed that can be melted and then solidified, forming a gel/matrix for separation of DNA. The gel is submerged in a buffer which allows an electrical potential to be applied. DNA is loaded into wells made in the gel and an electrical current is applied. DNA is highly negatively charged and will migrate through the pores of the gel toward the positive pole. DNA fragments of different sizes will migrate different distances in a given period of time (see below). DNA is typically visualized by staining the whole gel with a dye that is taken up by DNA. One common dye is called SYBR Safe. This fluorescent dye binds between the paired bases of the nucleotides and is detectable in special kind of lightening.



DNA electrophoresis.

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the $\log_{10}$ of either their molecular weights or number of base pairs, a roughly straight line will appear.

Materials

1. **PCR samples**
2. **An electrophoresis chamber and power supply**
3. **Gel casting tray**
4. **Sample combs**, around which **molten agarose** is poured to form sample wells in the gel.
5. **Electrophoresis buffer**, usually Tris-borate-EDTA (TBE) or Tris-acetate-EDTA (TAE).
6. **Loading buffer**, which contains the dye ("Blue juice") and something dense (e.g. glycerol) to allow the sample to sink into the sample wells, and one or more tracking dyes, which migrate in the gel and allow visual monitoring of how far the electrophoresis has proceeded.
7. **SYBR Safe**, a fluorescent dye used for staining nucleic acids.
8. **Transilluminator** which is used to visualize SYBR-stained DNA in gels (best visible in a dark room).
9. **Camera** to photograph a gel.

Procedure

A. Prepare 1% agarose gel following steps below. USE EYE PROTECTION and OVEN MITTS!

1. Prepare casting tray for gel. Use the masking tape to dam the ends.
2. Place a comb in the casting tray.
3. Weigh 1.5 g agarose for 100 ml gel (1.5% w/v agarose)
4. Measure 100 ml electrophoresis buffer (1× TBE—mixture of Tris base, boric acid and EDTA) in flask (125 ml or larger)
5. Add powdered agarose to electrophoresis buffer, swirl gently and plug with paper towel.
6. Heat in microwave till boiling starts—watch carefully.
7. Remove from microwave and GENTLY swirl
8. Return to microwave and bring just to boil
9. Remove from microwave and GENTLY swirl
10. Check for undissolved crystals—if none stop here. If not dissolved repeat heating.
11. Let cool 5 minutes
12. Put on gloves (latex)
13. Add 10 μL SYBR Safe 10,000× to the warm agarose.
14. Pour the gel slowly into the casting tray and allow it to solidify.
15. Place your gel into a plastic box, cover it with electrophoresis buffer

B. Prepare DNA samples and load the gel

1. Wear gloves!
2. Instructor will load the DNA TrackIt "ladder" (standard sample) into margin wells
3. Put two drops of 1 μL of cold Blue juice on the hydrophobic parafilm
4. Mix each drop with 4 μL of one of your PCR samples
5. Set pipette to 5 μL
6. Carefully load 5 μL of each drop into wells of the gel (wait for demonstration!)

C. Loading and running the gel

1. Place the lid on the gel, noting the orientation of the electrodes. Run the gel at 100 Volts for 30 minutes.
2. View on transilluminator, noting presence or absence of DNA in your lanes, and differences in migration of DNA.
3. Document and discuss your results. Using the DNA ladder, estimate the sizes of the DNA fragments you amplified. Plot the migration of the DNA ladder fragments and compare migration of your PCR-amplified fragments.

Advanced Cell Biology Lab 7.
Learning multiple sequence alignment through Phylo game

March 25, 2013

Background Phylo game is a multiple sequence alignment competition. Like protein folding, multiple sequence alignment is very computer-intensive; common algorithms sometimes do not provide with the most optimal solution. In that case, human brain is needed.

Assignment

1. **Look** to the "About" and "Tutorial" on Phylo Web site: `http://phylo.cs.mcgill.ca`
2. **Go** to "Play" menu, choose "Random" game and 3 sequences.
3. After you finish with 3 sequences, play with 5 and finally with 8 sequences. You may change a game if you believe it is too complicated or unclear.
4. Your goal is to **complete** three games.
5. Please work in pairs.
6. Prepare the short **report**: your name, today's date and how much time has been spent for each of three games and the score which was reached in every game.

Advanced Cell Biology Lab 8.
AB1 and BLAST

April 8, 2013

Background Sequinator machine normally returns results as "AB1" **trace file**. In such file, every type of nucleotide is represented as a line of different color. Peaks of lines correspond with the position of nucleotide in DNA. Unfortunately, trace files usually contains noise, especially on ends. Sometimes, DNA quality or primer efficiency is low, and peaks are not well expressed. Also, most of sequinators do not sequence well fragments exceeding 1,200 base pairs. In these cases, we try to sequence fragments from both ends (with forward and reverse primers) and then **assemble** them (manually or with specific software).

As you see, human input is needed to finalize sequencing. The main goal is to take out noise, assemble forward and reverse sequences (if needed), make the resulted textual sequence and check it with DNA database.

The checking process is normally a GenBank BLAST search. This search returns both **names of organisms** and **names of genes**. This information will help to confirm if you sequenced the right piece of DNA (i.e., primers worked well) from right organism (i.e., not a symbiont or contaminant).

Assignment

1. Listen carefully for explanations
2. Download zipped folder with the program and sample files from `http://ashipunov.info/shipunov/school/biol_250/finchtv_lab_250_08.zip`
3. **Unzip** folder to the desktop, delete downloaded archive. Run "FinchTV.exe" program. Open samples from "SampleData" subfolder.
4. Edit and assemble (if needed) forward and reverse sequences. Save results to the text file (for example, use Notepad).
5. Run the BLAST search to determine which gene and which organism was sequenced.
6. Make a report with answers on the following questions:
   (a) Sample 1. The organism is probably _____ ,
   the sequenced DNA is probably _____
   (b) Sample 2. The organism is probably _____ ,
   the sequenced DNA is probably _____
   (c) Sample 3. The organism is probably _____ ,
   the sequenced DNA is probably _____

Advanced Cell Biology Lab 9.
Quantitative PCR (qPCR): the theory and interpretation of results

April 15, 2013

Background

Conventional PCR does well to detect the presence of the DNA that the primer pair targets. Conventional PCR detects the amplified product (amplicon) by an end-point analysis—running the DNA on an agarose gel after the reactions are completed.

If the target DNA sequence is not there, no amplicon will appear on the agarose gel. If as little as a single DNA molecule that contains the target DNA sequence is in the sample. The amplification by 25–30 cycles is sufficient to generate detectable amplicons via electrophoresis. Thus, conventional PCR makes a highly sensitive assay for specific DNA sequence, which is useful for the diagnosis of diseases, especially viral types. It is also a rapid, highly sensitive and specific assay for microbes in environmental samples. It is, of course the assay of choice for genotyping human samples as you have seen in the PV92, Alu dimorphism analysis. Results of such assays are often used in paternity testing and in crime scene analysis.

Real-time PCR is based on the same principles as conventional PCR. The reaction requires both forward and reverse primers bracketing the target region (amplicon), nucleotides, and a DNA polymerase such as *Taq*. However, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses—in "real time". The difference is the addition of a fluorescence chemistry, which enables product amplification to be monitored throughout the entire real-time reaction using specialized thermal cyclers equipped with fluorescence detection modules. The measured fluorescence reflects the amount of amplified product in each cycle. Real-time PCR results can either be qualitative (presence or absence of a sequence) or quantitative (number of copies of DNA). Real-time PCR that is quantitative is also known as qPCR. The main advantage of using real-time PCR over conventional PCR is that real-time PCR allows you to determine the starting template copy number with accuracy and high sensitivity over a wide dynamic range. Conventional PCR can at best be semi-quantitative and the methods to obtain quantitative data can be quite complicated. One advantage of conventional PCR is better determination of the sizes of the amplified PCR products using conventional gel electrophoresis. Therefore, separating the real-time PCR products on a gel following amplification allows the visualization and determination of the size of the amplified products.

How Real-Time PCR Works

To best understand how real-time PCR works, think of what is happening in a PCR reaction. During the first cycles of a PCR reaction, the amount of amplicon doubles. The amount of amplicon after each cycle then multiplies exponentially in proportion to the starting amount of template in the sample. At some point, this doubling slows as the amount of substrate, nucleotides, and primers become used up. The DNA polymerase also becomes less active after the prolonged heating within the thermal cycler. The loss of doubling efficiency results in a plateau effect and the amount of amplicon produced with the later thermal cycles is no longer proportional to the amount of template DNA in the sample (Fig. 1). After enough cycles, all amplicons reach a certain maximum concentration, regardless of the initial concentration of template DNA.

The key to determining the quantity of original template DNA present in a sample during amplification is to examine the initial thermal cycles before reaching the plateau region of amplification. To

do this, the level of amplification is monitored continuously during the thermal cycling. Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (cycles 1–18 in Fig. 1) even though PCR product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is called the threshold cycle, or CT. Since the CT value is measured in the exponential phase when reagents are not limited, real-time qPCR can be used to reliably and accurately calculate the initial amount of template present in the reaction.

The CT of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background. Thus, the reaction will have a low, or early, CT. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. The most commonly used chemistries for real-time PCR are the DNA-binding dye SYBR Green I and TaqMan hydrolysis probe. We provide an overview of SYBR Green I fluorescence chemistry below.

SYBR Green I is a DNA dye that binds non-discriminately to double-stranded DNA (dsDNA). SYBR Green I exhibits minimal fluorescence when it is free in solution, but its fluorescence increases dramatically (up to 1000-fold) upon binding to dsDNA (Fig. 2). As the PCR reaction progresses, the amplified product accumulates exponentially, more SYBR Green I binds, and fluorescence increases. The advantage of using SYBR Green I is its simplicity. This is similar to the action of ethidium bromide, but unlike ethidium bromide, SYBR Green I does not interfere with DNA polymerases, so it can be added directly to a PCR reaction mixture. SYBR Green I also has less background fluorescence than does ethidium bromide, is able to detect lower concentrations of double-stranded DNA, and is not hazardous.

Optimizing qPCR

Since real-time quantitation is based on the relationship between initial template amount and the CT value obtained during amplification, an optimal qPCR assay is absolutely essential for accurate and reproducible quantitation of your particular sample. The hallmarks of an optimized qPCR assay are:

- Linear standard curve
- Consistency across replicate reactions

A powerful way to determine whether your qPCR assay is optimized is to run a set of serial dilutions of template DNA and use the results to generate a standard curve. The template used for this purpose can be a target with known concentration (for example, nanograms of genomic DNA or copies of plasmid DNA) or a sample of unknown quantity (for example, total cell DNA—cDNA). A standard curve is constructed by plotting the log of the starting quantity of template (or the dilution factor, for unknown quantities) against the CT value obtained during amplification of each dilution. The equation of the linear regression line, along with Pearson's correlation coefficient (r) or the coefficient of determination (R^2), can then be used to evaluate whether your qPCR assay is optimized. Ideally, the dilution series will produce amplification curves that are evenly spaced, as shown in Fig. 3. If perfect doubling occurs with each amplification cycle, the spacing of the fluorescence curves will be determined by the equation 2^n = dilution factor, where n is the number of cycles between curves at the fluorescence threshold (in other words, the difference between the CT values of the curves). For example, with a 10-fold serial dilution of DNA, $2^n = 10$. Therefore, $n = 3.32$, and the CT values should be separated by 3.32 cycles. Evenly spaced amplification curves will produce linear standard curves, as shown in Fig. 4. The equation and r values of the linear regression lines are shown above the plot.

Assignment

In today's lab we will analyze results of qPCR optimization experiment. This experiment utilized serial dilutions of the same template to illustrate the quantitative application of real-time. Each sample amplified the available template exponentially. The more starting template, the quicker the amplified product has been accumulated. Samples with more template reach the threshold for fluorescence detection sooner (at an earlier cycle) than samples with less template. We call this threshold cycle the CT value for the experiment.

We have three graphs (Figures 3, 4, and 5) and the table which reflects the results of qPCR. Please review them, discuss within a group and answer these questions:

Fig. 3.

- What is the average difference between CT for every group of dilution?

- Why CTs for same dilutions are not exactly the same?

Fig. 4.

- This graph summarizes our dilution scheme and the corresponding CT. Why is the log value used?

Fig. 5.

A very important final check on your qPCR run is analysis of the endpoint product. Since SYBR green will show amplification of any double-stranded product, it is essential that you check to see that a single, expected product is produced. To check this, at the end of the run, the thermal cycler goes through continuous series of temperature changes (from the annealing temp up to about 95°C). The melt curve plots the change in fluorescence (negative first derivative of fluorescence vs. temperature). A sharp peak will form at the temperature at which fluorescence changes rapidly. This corresponds to the melting temperature (Tm) for an amplification product in the tube.

- As the temperature increases, what will happen to the PCR products?

- What is the melting temperature (Tm) in the experiment?

Table 1.

- Please estimate the unknown dilution. Explain how you did it.

Figures and tables

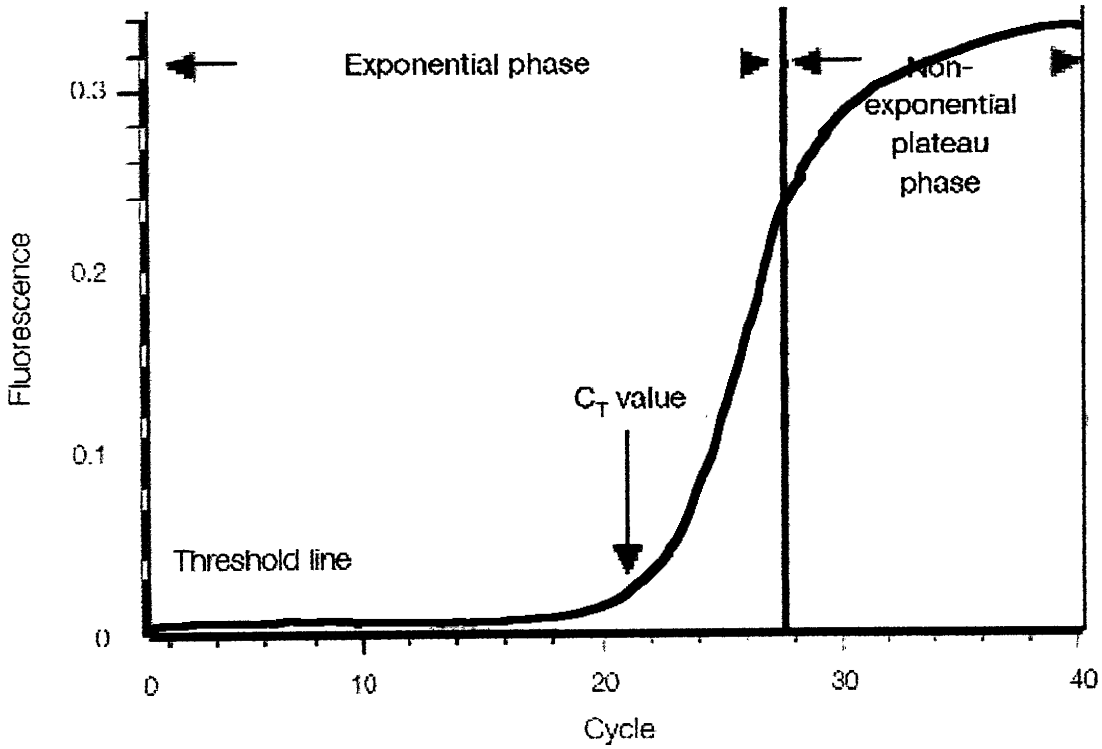


Fig. 1. Amplification plot. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds and reaction components are consumed, the reaction slows and enters the plateau phase.

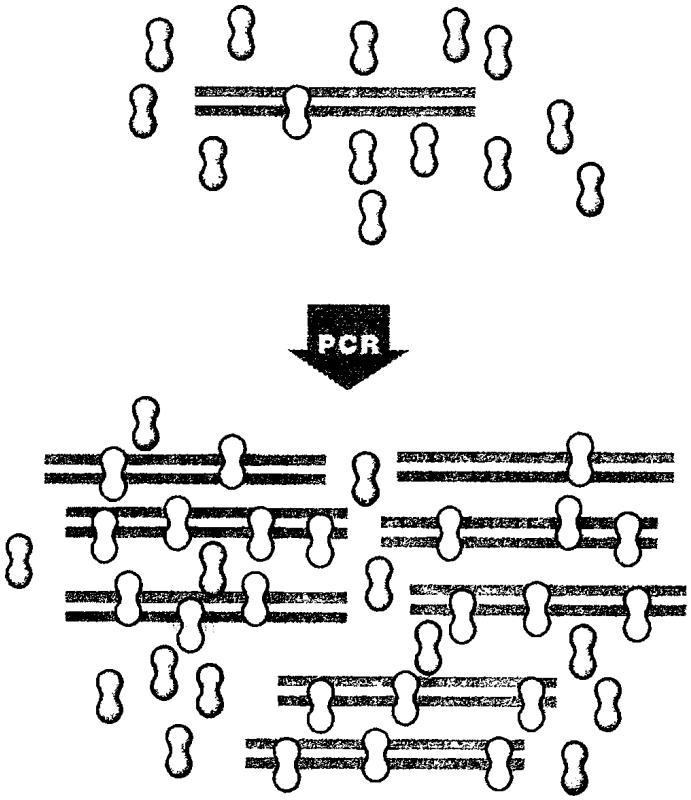


Fig. 2. SYBR Green I.

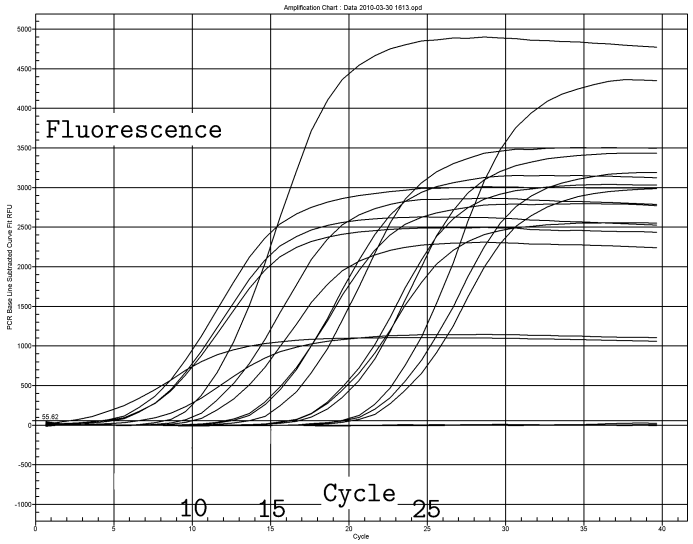


Fig. 3. Generating a standard curve to assess reaction optimization. A standard curve was generated using a 10-fold dilution of a template amplified on the real-time system. Each dilution was assayed in triplicate. Graphs are amplification curves of the dilution series.

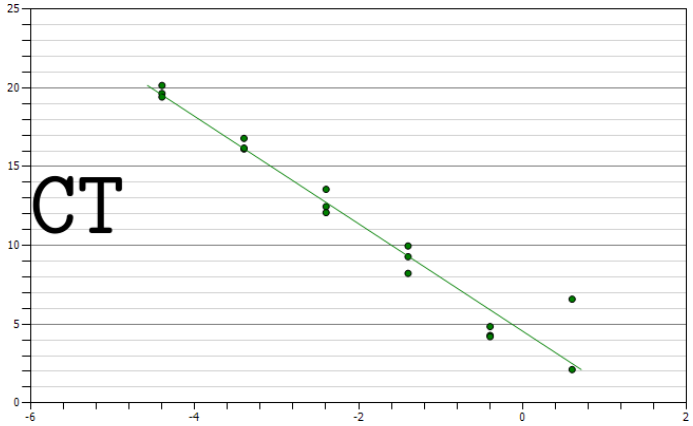


Fig. 4. Standard curve with the CT plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the r value are shown above the graph.

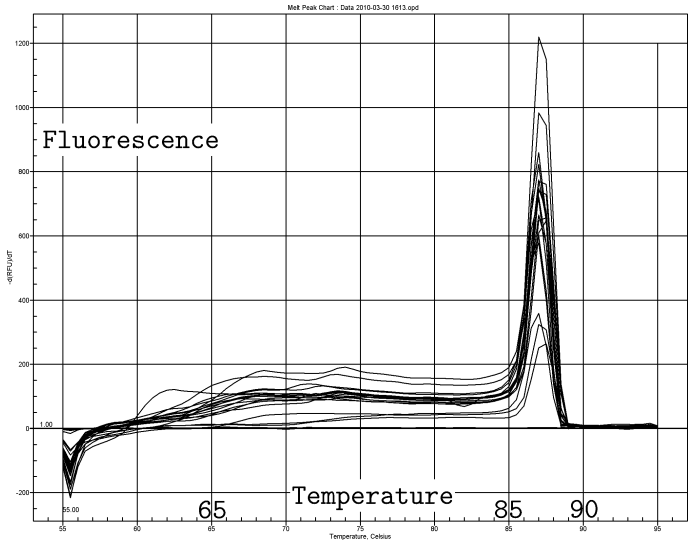


Fig. 5. Melt curve for the products made during the qPCR amplification.

Table 1. Numerical results of dilution experiment. One of dilutions is unknown.

Well	Fluor	Identifier	Threshold Cycle (CT)	CT Mean	CT Std. Dev
A01	SYBR1	undiluted	6.59	04.35	3.159
A02	SYBR1	undiluted	2.12	04.35	3.159
A03	SYBR1	undiluted	NA	00.00	NA
B01	SYBR1	1:10	4.29	04.45	0.349
B02	SYBR1	1:10	4.85	04.45	0.349
B03	SYBR1	1:10	4.21	04.45	0.349
C01	SYBR1	1:100	9.97	09.16	0.877
C02	SYBR1	1:100	9.29	09.16	0.877
C03	SYBR1	1:100	8.23	09.16	0.877
D01	SYBR1	1:1000	13.56	12.71	0.767
D02	SYBR1	1:1000	12.47	12.71	0.767
D03	SYBR1	1:1000	12.09	12.71	0.767
E01	SYBR1	1:10000	16.80	16.37	0.375
E02	SYBR1	1:10000	16.12	16.37	0.375
E03	SYBR1	1:10000	16.18	16.37	0.375
F01	SYBR1	?	20.16	19.74	0.374
F02	SYBR1	?	19.65	19.74	0.374
F03	SYBR1	?	19.43	19.74	0.374

Advanced Cell Biology Lab 10.
Making phylogenetic tree from sequences

April 19, 2013

Background In February and March, we extracted DNA and amplified barcoding ITS2 DNA fragment from several species of Eurasian plantains, species which never been genetically researched before. Now we need to answer question: where do these species belong?

Plantains (*Plantago*) is a big genus which has several subgroups. Our species may belong to any subgroups. Of course, morphology already told us what this place could be but DNA should provide the better answer simply because DNA has more characters than morphology. In addition, DNA sequence is almost free from subjective factors.

For subgroups centers, we will use four the most characteristic ("typical") species, worldwide-distributed (and often invasive) plantains: *Plantago major* (big plantain), *P. media* (hoary plantain), *P. maritima* (sea plantain) and *P. lanceolata* (narrowleaf plantain). We did not sequence these species, but GenBank contains information about their ITS2. We will also need outgroup. It is known that *Aragoa* (from American Andes) is a closest outside relative of plantains.

Assignment

1. Download our 11 sequences (from 7 species of question) from course Web site, save the file to the desktop. This file is in FASTA format (text file where each sequence is started with string like ">sequencename")
2. Find and download five ITS2 sequences (four "typical" and outgroup *Aragoa*) from GenBank. To find ITS2 sequences, use "internal transcribed" as search keywords.
3. Merge all sequences in one FASTA format text file (do not forget ">" sign!)
4. Paste them to on-line ClustalW alignment program (`http://www.genome.jp/tools/clustalw/`), save the alignment file
5. Upload your alignment to the PhyML (`http://phylogeny.fr`)
6. Manually re-arrange (with outgroup) and redraw the tree, find most "primitive" and "advanced" ingroups
7. Answer questions:
   (a) Where do each of our questionable species belong to?
   (b) What is a most "primitive" and "advanced" plantain on the phylogenetic tree?

Advanced Cell Biology Lab 11.
Cell cultures

April 29, 2013

Background

I. Introduction

In today's lab, we will practice the handling of human cells growing in culture. Much of what we know about the function of specific cells comes from studying cells/cellular activities *in vitro* (outside the organism). All the techniques used to maintain and manipulate cells *in vitro* are referred to as tissue culture or cell culture. Two major goals of growing cells in culture are to:

1. Provide the necessary nutrients and environment for cells to divide regularly, but NOT to change in major characteristics (i.e., inhibit differentiation)
2. Maintain sterility (prevent bacterial and fungal contamination)

Cell lines are established from tissues by dissociating the cells from the tissue and providing them the proper conditions for their growth. Dissociated cells can divide for some period of time, but then cease to divide or senescence. True cell lines require some genetic change to become immortal (divide forever). This can occur spontaneously (cancer cells), or be induced with chemicals or cancer-causing animal viruses. Hematopoietic cell lines are established from blood cells, often from individuals with leukemia or lymphoma. In this case the cells have already undergone the genetic changes that make them grow indefinitely while in the person having leukemia or lymphoma.

Cell lines usually require specific nutrients formulated in a specific media. Other factors called *growth factors* are provided in serum. Fetal calf/bovine serum (FBS) is often used as a supplement to the specific medium providing growth factors, some of which are still undefined. Cell culture media often has an additive to monitor the pH. As cells become more crowded, waste accumulates, lowering the pH. pH changes can be seen as a change in color of the media with these additives.

Cell lines grow in one of two ways: either attached to a substrate (such as a plastic flask), or in suspension (floating around). Hematopoietic cells such as lymphocytes grow in suspension, whereas macrophages tend to attach to a substrate. Fibroblast cells (e.g. from skin) tend to grow as attached cells. Today we will practice with suspension cell culture techniques using a cell line derived from a leukemia patient. The cell line is called **THP-1**. Some cells are viable, but some are dead, and these will be stained with Trypan Blue because it will go inside dead cells (e.g., cells with damaged membrane) only.

II. Using a Counting Chamber

For microbiology, cell culture, and many applications that require use of suspensions of cells it is necessary to determine cell concentration. A device used for determining the number of cells per unit volume of a suspension is called a counting chamber. The most widely used type of chamber is called a *hemacytometer*, since it was originally designed for performing blood cell counts.

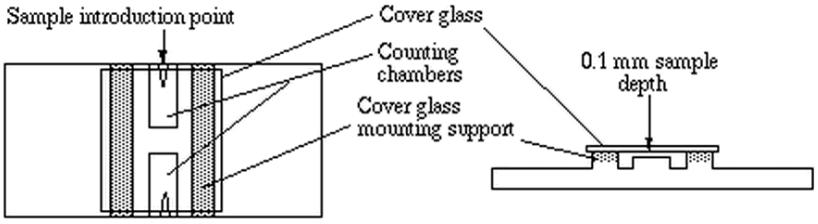


Fig. 1. Hemacytometer.

To prepare the counting chamber the mirror-like polished surface is carefully cleaned with lens paper. The coverslip (coverglass) is also cleaned. Coverslips for counting chambers are specially made and are thicker than those for conventional microscopy, since they must be heavy enough to overcome the surface tension of a drop of liquid. The coverslip is placed over the counting surface prior to putting on the cell suspension. The suspension is introduced into one of the V-shaped wells with apipette. The area under the coverslip fills by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered. The charged counting chamber is then placed on the microscope stage and the counting grid is brought into focus at low power.

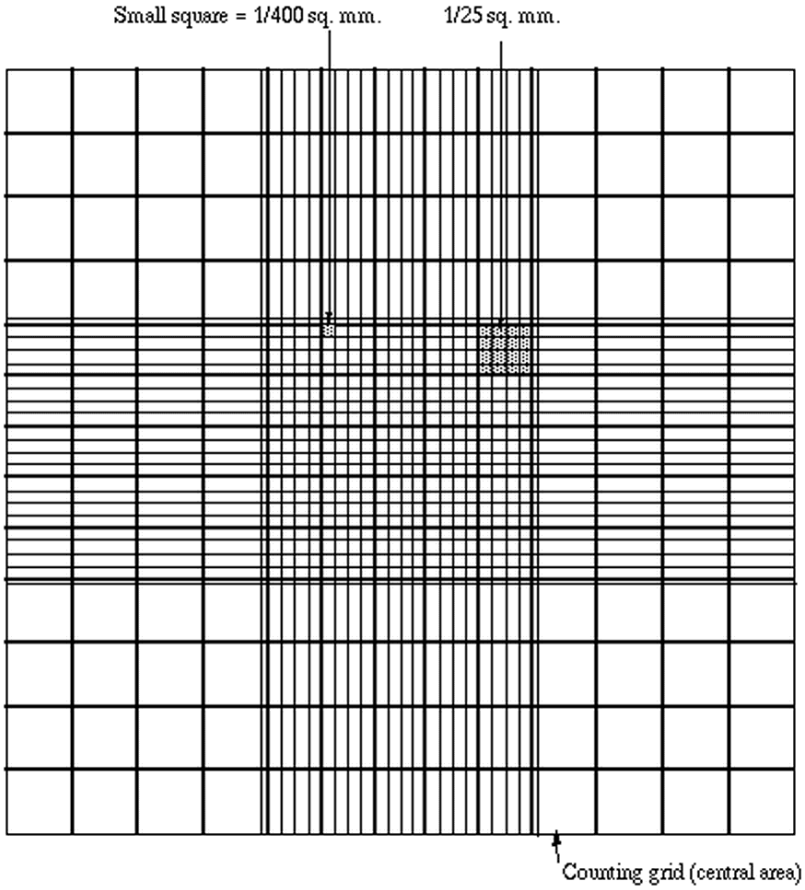


Fig. 2. Counting grid.

It is essential to be extremely careful with higher power objectives, since the counting chamber is much thicker than a conventional slide. The chamber or an objective lens may be damaged if the user is not not careful. One entire grid on standard hemacytometers with Neubauer rulings can be seen at 40× (4× objective). The main divisions separate the grid into 9 large squares (like a tic-tac-toe grid). Each square has a surface area of one square mm, and the depth of the chamber is 0.1 mm. Thus the entire counting grid lies under a volume of 0.9 mm-cubed.

Suspensions should be dilute enough so that the cells or other particles do not overlap each other on the grid, and should be uniformly distributed. To perform the count, determine the magnification needed to recognize the desired cell type. Now systematically count the cells in selected squares so that the

total count is 100 cells or so (number of cells needed for a statistically significant count). For large cells this may mean counting the four large corner squares and the middle one. For a dense suspension of small cells you may wish to count the cells in the four 1/25 sq. mm corners plus the middle square in the central square. Always decide on a specific counting pattern to avoid bias. For cells that overlap a ruling, count a cell as "in" if it overlaps the top or right ruling, and "out" if it overlaps the bottom or left ruling.

Suppose that you conduct a count as described above, and count 187 particles in the five small squares described. Each square has an area of 1/25 mm-squared (that is, 0.04 mm-squared) and depth of 0.1 mm. The total volume in each square is $(0.04)\times(0.1) = 0.004$ mm-cubed. You have five squares with combined volume of $5\times$ $(0.004) = 0.02$ mm-cubed. Thus you counted 187 particles in a volume of 0.02 mm-cubed, giving you $187/(0.02) = 9350$ particles per mm-cubed. There are 1000 cubic millimeters in one cubic centimeter (same as a milliliter), so your particle count is 9,350,000 per ml.

Cells are often large enough to require counting over a larger surface area. For example, you might count the total number of cells in the four large corner squares plus the middle combined. Each square has surface area of 1 mm-squared and a depth of 0.1 mm, giving it a volume of 0.1 mm-cubed. Suppose that you counted 125 cells (total) in the five squares. You then have 125 cells per 0.5 mm-cubed, which is 250 cells/mm-cubed. Again, multiply by 1000 to determine cell count per ml (250,000).

Sometimes you will need to dilute a cell suspension to get the cell density low enough for counting. In that case you will need to multiply your final count by the dilution factor. For example, suppose that for counting you had to dilute a suspension 10 fold. Suppose you obtained a final count of 250,000 cells/ml as described above. Then the count in the original (undiluted) suspension is $10 \times 250{,}000$ which is 2,500,000 cells/ml.

Assignment

1. Become familiar with THP-1 cell morphology and in vitro growth characteristics
2. Become familiar with hemacytometer technique
3. Determine cell concentration (# cells/ml culture media) and viability (% of non-stained with Trypan Blue cells)
4. ***Provide a resulted cell concentration, viability and a short description of how you calculated it***

Materials

1. Flask of THP-1 cells
2. Trypan Blue
3. Microfuge tube
4. Bleach, ethanol, lab tissue
5. Sterile pipette 2–20 μL
6. Hemacytometer

Procedure

[*Instructor's part is in italics*]

1. Put on gloves
2. Be sure to carefully clean the hemacytometer. Lift off the coverslip and squirt off the coverslip and slide with 10% bleach, into the plastic waste cup. Then squirt off both with 70% ethanol and shine with a lab tissue. When dry, return to the box.
3. *Obtain your flask from the incubator*
4. *Look at it using the inverted microscope*
5. *Loosen the cap on one of your flasks. Gently loosen the cells from the flask bottom. Try to get as many off as possible. Do not remove the cells yet.*

6. *Mix 2 mL of cell suspension with 2 mL of Trypan Blue in a separate tube*
7. *Make 200 μL aliquotes of mixture in microfuge tubes*
8. Using a hemacytometer, charge both sides with cells from microfuge tube (use 10 µL pipetting)
9. Count cells and determine cell concentration (# cells/ml) and viability using both sides
10. Clean the hemacytometer again; be sure to put all waste in a plastic container (**not** in a sink!)