C-Fern® Manual: Part B

Culture Instructions for *C-Fern*® Investigations

Leslie Hickok, Thomas Warne, and Stephenie Baxter Duncan

CAROLINA
World-Class Support for Science & Math

Contents

C-Fern® Project
Department of Botany
University of Tennessee
Knoxville, TN 37996-1100
email: cfern@utk.edu
http://cfern.bio.utk.edu

Printed in USA

Published by Carolina Biological Supply Company,
2700 York Road, Burlington, NC 27215

©1998, University of Tennessee Research Foundation

revised, 2004
Development of *C-Fern* has been supported, in part,
by the National Science Foundation and
the University of Tennessee.

Culture Instructions for *C-Fern*® Investigations

Introduction

C-Fern® is an exciting and unique organism that is easy to grow using inexpensive materials and simple growing conditions. To help you get started quickly and easily, follow the Quick Start instructions below. To ensure your success with *C-Fern*, additional detailed information and options are included in the subsequent sections of this document. Please also see the *C-Fern* Web site (http://cfern.bio.utk.edu) and the *C-Fern*® *Manual for Teaching and Research* by Les Hickok and Thomas Warne.

All *C-Fern* materials and supplies are available from Carolina Biological Supply Company. Please refer to the materials lists at the end of these instructions. *C-Fern* products and teaching aids can also be found in the Carolina Science & Math catalog and on the Carolina web pages (www.carolina.com).

Quick Start

C-Fern cultures are started from spores. Surface-sterilized spores of the wild-type and mutant stocks are available through Carolina Biological Supply in 10-mg units. For general use, 4 mL of sterile water should be added to a vial to make a suspension of spores. Spores are then inoculated into 60- × 15-mm petri dishes containing Basic *C-Fern* Medium. Medium is available pre-made or in powder form. Instructions for preparing the medium are detailed in this manual. Use a sterile, disposable transfer pipet to inoculate each petri dish with 3 drops of the spore suspension. This will generate up to 36 cultures with a standard density of >300 spores per dish. Spores should be spread evenly over the agar surface using a sterile bent paper clip or microbial spreader. It is very important to maintain cultures under continuous light and at a temperature of 28°C (82°F). These conditions are easily achieved by using a 15-W screw-in fluorescent bulb to illuminate cultures within a *C-Fern*® Growth Pod™. This setup is described in these instructions and on the Web (http://cfern.bio.utk.edu). Under these conditions, development will proceed as depicted in Figure 1. If the culture temperature is cooler and/or constant illumination is not provided, development will be slower.

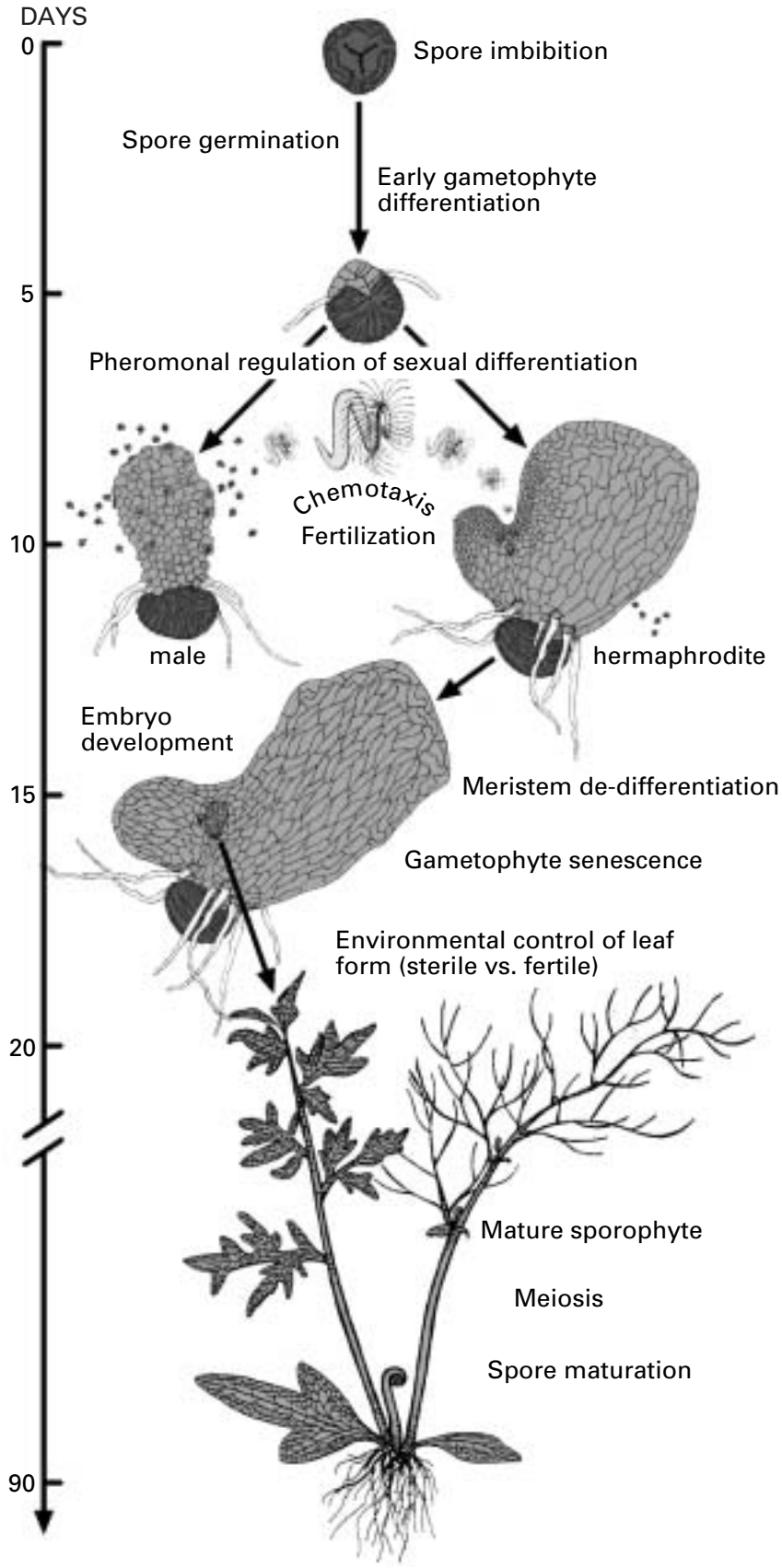


Figure 1. *C-Fern* life cycle stages and timing (sporophyte is not to scale)

Culture Instructions

1. Getting Started

Detailed written exercises are available from Carolina Biological both individually and as a collection of exercises in the *C-Fern*® *Manual*. All *C-Fern* kits contain both Teacher and Student versions of the exercises.

Dried *C-Fern* spores are long lived and are provided by Carolina Biological in small plastic vials, either as pre-sterilized 5- or 10-mg units or as unsterilized 40-mg units. The pre-sterilized spores are very convenient and easy to use—just add the specified amount of sterile water and sow! If using unsterilized spores, the sterilization procedure is simple and efficient (refer to Section 8A).

2. Determining Sowing Density and Number of Cultures

There are approximately 1,250 *C-Fern* spores per mg dry weight. Standard sowing density is approximately 300 spores per 60-mm petri dish. Table 1 provides information for sowing spores at various densities. **Note:** Depending upon technique, there is always some spore loss during sowing procedures. Therefore, the spore numbers given are approximate.

Table 1. Spore-sowing densities

FOR this density	USING 10- or 5-mg vials	ADD this much water (mL) to vial	SOW		MAKE about this many petri dish cultures	WITH this number of spores per petri dish
			This many drops of spore suspension	Plus these additional drops of water[1]		
standard	10	4	3	–	36	300+
standard	5	2	3	–	18	300+
⅔	10	4	2	1	54	200+
⅔	5	2	2	1	27	200+
½	10	2	3	–	18	150+
½	5	4	3	–	36	150+
⅓	10	4	1	2	108	100+
⅓	5	2	1	2	54	100+

[1]Additional water is to allow enough liquid (a total of 3 drops) for spreading spores evenly on the agar surface.

3. Preparing the Culture Medium

Four options for preparing Basic *C-Fern*® Medium are given below: **A)** pre-made bottled medium (melt and pour), **B)** & **C)** powdered medium (mix, autoclave or microwave, and pour), and **D)** preparation and use of stock solutions.

A. *Using Pre-Made Bottled* C-Fern *Medium*

Materials:

Bottled *C-Fern* Medium (Basic *C-Fern* Medium is available in 160- and 400-mL bottles. *C-Fern* Medium is also provided in most kits or kit refills.)

Sterile Petri Dishes, 60 × 15 mm (Petri dishes are also provided in most kits or kit refills.)

Hot Water Bath

Clean Area for Pouring Dishes (or a sterile bench or laminar flow hood)

Melt and pour medium into dishes in advance to allow sufficient time for medium to cool to room temperature and solidify completely. To speed melting, vigorously shake the bottle to break up the medium before placing the bottle in a hot water bath. To melt the medium, loosen the cap and place the bottle into a hot water bath such that the water level is only just above the level of the medium in the bottle. Do not fully submerge the bottle. A cover on the water bath helps the medium to melt faster. In a boiling water bath (100°C or 212°F), the melting time for medium is about 15 minutes for 160 mL and 45 minutes for 400 mL.

Medium should be poured in a clean area free from drafts and traffic. Basic *C-Fern* Medium lacks a carbohydrate source (sugars), so contamination problems, if any, are usually minimal if directions and precautions are followed. Prepare the area by wiping it down with 70% ethanol, 70% isopropanol, or a damp, clean sponge. Open a sleeve of 60- × 15-mm petri dishes by cutting through the end of the sleeve with scissors. Save the sleeve for storage of prepared dishes. Gently swirl the medium in the bottle to be sure that it is thoroughly mixed and completely melted. Remove the bottle cap, then tilt the lid of a petri dish upward just enough to permit pouring of the medium into the dish. Fill dishes about ¾ full, i.e., 15 mL for 60- × 15-mm petri dishes; 160 mL of medium should prepare about 10 petri dishes, and 400 mL about 25 dishes. Do not under-fill dishes. Sufficient medium is needed for proper growth and development of *C-Fern* through the sporophyte stage. Replace the petri dish lid and allow dishes to cool undisturbed. Condensation that may form on petri dish lids during cooling is minimized if dishes are poured and cooled in stacks of 5–10 dishes. Once dishes have cooled and the medium solidified, they may be returned to the plastic sleeves, sealed with tape and stored at room temperature for several weeks prior to use in an investigation. Unused dishes may be stored in sleeves in a refrigerator for several months, but do not freeze them.

B. Using Powdered Basic C-Fern® *Medium*

Materials:

Powdered Basic C-*Fern* Medium
1-L Volumetric Flask or Graduated Cylinder
Distilled or Deionized Water
2-liter Erlenmeyer Flask(s) (per 1 liter medium)
Bacto Agar
Magnetic Stir Plate and Magnetic Stir Bar
pH Meter
1 M NaOH Solution
Autoclave or Microwave
Sterile Petri Dishes, 60 × 15 mm

To prepare 1 L of Basic C-*Fern* Medium, open the packet of powdered medium and add it to 800 mL distilled water in a volumetric flask or graduated cylinder. Rinse out the powder remaining in the packet with distilled water and bring the medium to a final volume of 1 L. For agar-solidified medium, transfer the nutrient solution to a 2-L Erlenmeyer flask and add 10 g (1% w/v) of Bacto Agar (Difco Laboratories). **Note: Some plant tissue culture-grade agars and agar substitutes can result in inhibited or abnormal growth of gametophytes or sporophytes and should, therefore, be avoided.** Adjust the nutrient medium to pH 6.0 using 1 M NaOH. Cover the top of the flask with foil and autoclave the nutrient medium at 120°C/20 psi for 15 minutes, or see the protocol that follows for using a microwave.

Some of the powdered nutrients or agar may not completely dissolve even after autoclaving or microwaving. This is not a problem. Dispense medium into petri dishes, i.e., about 15 mL in a 60- × 15-mm dish and 40 mL in a 100- × 15-mm dish. The dishes should be about ¾ full. This ensures an adequate nutrient and water supply through to the young sporophyte stage. One liter of nutrient medium should pour about 55 60- × 15-mm dishes and 20 100- × 15-mm dishes. Allow the dishes to cool, completely undisturbed. Condensation on petri dish lids is minimized if the dishes are poured and cooled in stacks. Once the dishes have cooled and the medium has solidified, they may be returned to the plastic sleeves, sealed with tape, and stored at room temperature prior to use in an investigation. Unused dishes may be stored in sleeves in a refrigerator for several months. Do not freeze.

C. Using a Microwave to Prepare C-Fern *Powdered Medium*

If an autoclave is not available, a microwave can be used to prepare Basic C-*Fern* Powdered Medium. Although the lower temperatures and shorter times of microwaving cannot guarantee sterility to the degree that autoclaving does, this method has been used repeatedly to successfully prepare contaminant-free medium. **Use caution when heating and handling! Wear safety glasses, use gloves, and do not leave the microwave unattended!**

To prepare 1 L of Basic C-*Fern* Medium, open the packet of powdered medium and add it to 800 mL of distilled water in a volumetric flask or graduated cylinder. Rinse out the powder remaining in the packet with distilled water and bring the medium to a final volume of 1 L. For agar-solidified medium, transfer the nutrient solution to a 2-L Erlenmeyer flask, then add 10 g (1% w/v) Bacto Agar (Difco Laboratories). **Note: Some plant tissue culture grade agars and agar substitutes can result in inhibited or abnormal growth of gametophytes or sporophytes and should therefore be avoided.** Adjust the nutrient medium to pH 6.0 using 1 M NaOH. Cover the top of the flask with plastic wrap and process the solution in a microwave unit as specified in Table 2. You may need to compensate for microwave units of different powers by adjusting the suggested times. Plates should be poured ¾ full.

Table 2. Steps for using a microwave to prepare 1 L of medium[1]

Step	**Microwave Time** (1000-W output unit)	State of Solution[2]	**Post-Microwave Procedure**[3]
1	5 minutes	hot	remove, swirl
2	1 minute	boiling	remove, swirl
3	15 seconds	boiling	remove, swirl
4	15 seconds	boiling	remove, swirl
5	15 seconds	boiling	swirl to mix and pour petri dishes

[1]Be sure to use a vessel at least twice the volume of the medium.
[2]The solution will boil vigorously in Steps 2–5. The medium must be visually monitored constantly so the power can be reduced or turned off briefly if it starts to boil over.
[3]It is important to mix the medium thoroughly by swirling the flask after each step and while pouring the plates.

D. Preparation of Basic C-Fern® *Medium From Stock Solutions*

Materials (Preparation of Stock Solutions):

Macronutrient, Micronutrient, and Fe Salts (refer to Table 3 for list)
Distilled or Deionized Water
1-L Volumetric Flask(s) or Graduated Cylinder
Microbalance
Magnetic Stir Plate and Magnetic Stir Bar(s)
For Fe Stock Solution: Hot plate, 2-L Erlenmeyer Flask(s), Watch Glass
Storage bottle(s)

Table 3. Composition of basic *C-Fern* medium stock solutions and final medium

	Nutrient Components	Stock Solution (g/L)	Final Medium (mL Stock/L)	Final Medium Composition (mg/L)
1	**10× Macronutrients**		**100**	
	NH_4NO_3	1.25		125
	KH_2PO_4	5.00		500
	$MgSO_4 \cdot 7H_2O$	1.20		120
	$CaCl_2 \cdot 2H_2O$	0.26		26
2	**200× Micronutrients**		**5**	
	$MnSO_4 \cdot H_2O$	0.0500		0.25
	$CuSO_4 \cdot 5H_2O$	0.0740		0.37
	$ZnSO_4 \cdot 7H_2O$	0.1040		0.52
	H_3BO_3	0.3720		1.86
	$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	0.0074		0.037
3	**100× Chelated Iron Solution**		**10**	
	$FeSO_4 \cdot 7H_2O$	2.78		27.8
	Disodium EDTA$\cdot 2H_2O$	3.73		37.3

[a]This formulation is based on a medium described in Klekowski, 1969 (Botanical Journal of the Linnean Society 62: 361-377). Higher concentrations of macronutrients in the stock solution are unstable and may form precipitates, as will most combinations of macronutrient, micronutrient, and chelated iron stock solutions.

All components of Basic C-*Fern* Medium should be prepared using high-quality distilled and/or deionized water.

Prepare macronutrient stock solution and micronutrient stock solutions separately by dissolving all listed quantities of components (see Table 3) individually, in sequence, into about 800 mL of distilled water; bring to a 1-liter final volume. Both macronutrient and micronutrient stock solutions can be autoclaved. Autoclaved stock solutions will keep for over 6 months and should be stored in glass at 4°C.

Prepare Chelated Fe-EDTA stock solution by dissolving each component separately in ca. 450 mL of water. On a hot plate, heat the EDTA solution to boiling and then add the hot EDTA solution TO the $FeSO_4$ solution. Cover with a watch glass and boil combined solutions for 1 hour, cool completely, then bring to 1-L volume. Store Chelated Fe-EDTA solution in glass at 4°C.

Materials (Preparation of Final Medium):

Macronutrient, Micronutrient, and Fe Stock Solutions
100-mL Graduated Cylinder
10-mL Pipet
1-L Volumetric Flask or Graduated Cylinder
Distilled or Deionized Water
2-L Erlenmeyer Flask (per 1 liter of medium)
Bacto Agar
pH Meter
Magnetic Stir Plate and Magnetic Stir Bar
1 M NaOH Solution
Foil (to cover flask during autoclaving)
Autoclave
Sterile Petri Dishes, 60 × 15 mm

To prepare Basic *C-Fern* Medium, add the appropriate volume of each of the three stock solutions to about 800 mL of distilled water in a volumetric flask or graduated cylinder and bring to 1-L final volume. For agar-solidified medium, transfer nutrient solution to a 2-L Erlenmeyer flask, add 10 g (1% w/v) agar (Bacto Agar). **NOTE: Some plant tissue culture grade agars and agar substitutes can result in inhibited or abnormal growth of gametophytes or sporophytes and should therefore be avoided.** Adjust nutrient medium to pH 6.0 using 1 M NaOH. Autoclave nutrient medium at 120°C/20 psi for 15 minutes. Dispense medium to petri dishes. Dishes should be about ¾ full—about 15 mL in a 60- × 15-mm dish and 40 mL in a 100-mm dish. This ensures an adequate nutrient and water supply through to the young sporophyte stage. One liter of nutrient medium should pour about 55 60-mm dishes and 20 100-mm dishes. Allow the dishes to cool, completely undisturbed. Condensation on petri dish lids is minimized if dishes are poured and cooled in stacks. Once the dishes have cooled and the medium solidified, they may be returned to the plastic sleeves, sealed with tape, and stored at room temperature prior to use in an investigation. Unused dishes may be stored in sleeves in a refrigerator for several months. Do not freeze.

4. Inoculating (Sowing) Cultures Using Presterilized *C-Fern* Spores

Seven Good Habits for Sowing *C-Fern* Cultures

1. Work very carefully when sowing spores. Secure the vial in a convenient holder.
2. Suspend spores before every sowing.
3. Immediately sow three uniform drops onto the agar surface. Hold pipet at a constant angle.
4. Do not touch the pipet to the agar surface.
5. In most Investigations, use a sterile spore spreader to distribute spores uniformly over the agar surface.
6. Keep the petri dish lid in place as much as possible.
7. Label dishes with name or initials, the date spores are sown, and the treatment code, if any.

Materials:

Surface Sterilized *C-Fern* Spores in a Graduated Spore Vial (appropriate pre-sterilized spores are provided in most kits or refills; spores for wild-type and mutant stocks are also available separately.)

Sterile Distilled Water (provided in most kits or refills)

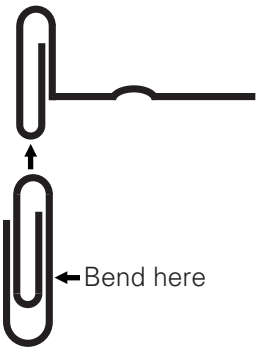
Sterile Transfer Pipet (plastic; provided in most kits or refills)

Sterile Spore Spreader (metal spreader [e.g., a paperclip bent into a T-pin shape] or alternative provided in most kits)

Petri Dishes Containing *C-Fern* Medium (prepared in advance)

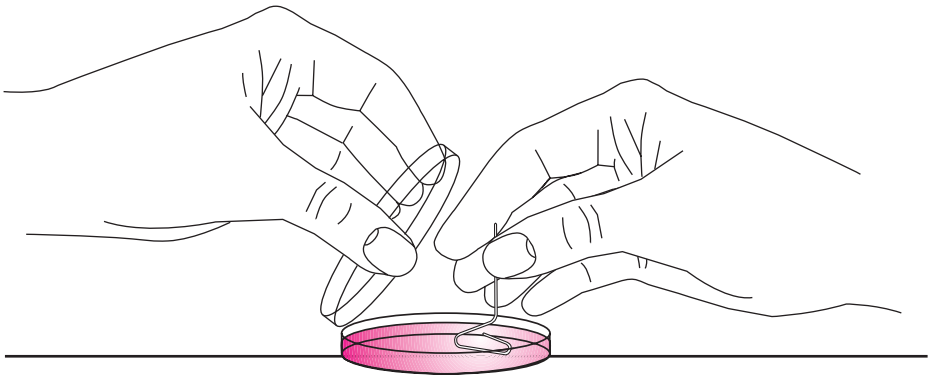
Preparing Spores Before opening any spore vial, be sure that all spores are at the bottom of the vial by tapping the bottom of the vial on a hard surface. Transfer the appropriate amount of sterile distilled water to the spore vial using a sterile transfer pipet. Do not return any water to the sterile water bottle. See Table 1 for the amount of water to add and sowing densities. Wet spores completely by firmly attaching the cap and inverting the vial two or three times. With the cap on, check the bottom of the vial to be sure that all spores have been suspended. Allow the spores to soak for 15 minutes prior to sowing.

Sowing Spores To achieve consistent sowings, spores should be thoroughly suspended between each sowing. Suspend spores gently by drawing the liquid along with the spores in and out of the pipet. To sow, withdraw a small amount of the spore suspension into the pipet and immediately dispense 3 **drops**—not squirts—onto the agar surface. When sowing, tilt the lid of the petri dish upward just enough to permit access of the pipet tip. Do not touch the agar surface with the tip of the pipet. Resuspend the spores in the vial between each sowing by gently squeezing and releasing the pipet bulb.



Spreading Spores To make a sterile spore spreader, bend a paper clip into a "T" shape, as shown in the figure. While holding the straight end, wipe the "T" end with the alcohol prep pad and let it air dry.

Allow the spreader to rest on the agar surface without pressure and move it gently back and forth across the surface of the agar while rotating the dish slowly with the other hand. The goal is to uniformly distribute spores over the entire surface of the medium. This may require some practice but is worth the effort—evenly spread cultures are easy to observe and work with.



5. Maintaining C*-Fern* Cultures

Any number of plant-growing systems can be easily adapted to provide adequate environmental conditions for C*-Fern*. However, in order to attain the growth and timing of development shown in Figure 1, cultures require correct and controlled temperature and adequate lighting. If your temperature and lighting conditions differ substantially from that indicated here, a test run should be carried out to determine when, under your conditions, specific developmental stages will be present for observation and manipulation. The descriptions of two options, Culture Domes and Growth Pods, follow.

A. Culture Domes

Once cultures are inoculated with spores, place them into Culture Domes consisting of clean plastic greenhouse trays covered with transparent humidity domes. For best results, Culture Domes should be thoroughly clean. Both trays and domes are available through Carolina Biological Supply Company and are included in most kits. Culture Domes serve to reduce the possibility of contamination, variations in temperature and humidity, and permit easy handling of a larger number of dishes. Do not tightly seal the petri dishes, for example with Parafilm®, as this can result in poor growth, presumably due to ethylene buildup.

B. Growth Pods

The *C-Fern*® Growth Pod™ can replace or complement the standard Culture Dome that was recommended in early versions of the *C-Fern* Manual. The pod's reduced space requirement, improved temperature control, and increased portability are highly advantageous, especially in situations where the optimum culture temperature of 28°C (82°F) is difficult to attain using the standard Culture Dome. Growth Pods (without light) are available from Carolina Biological, or you can make them yourself, as follows.

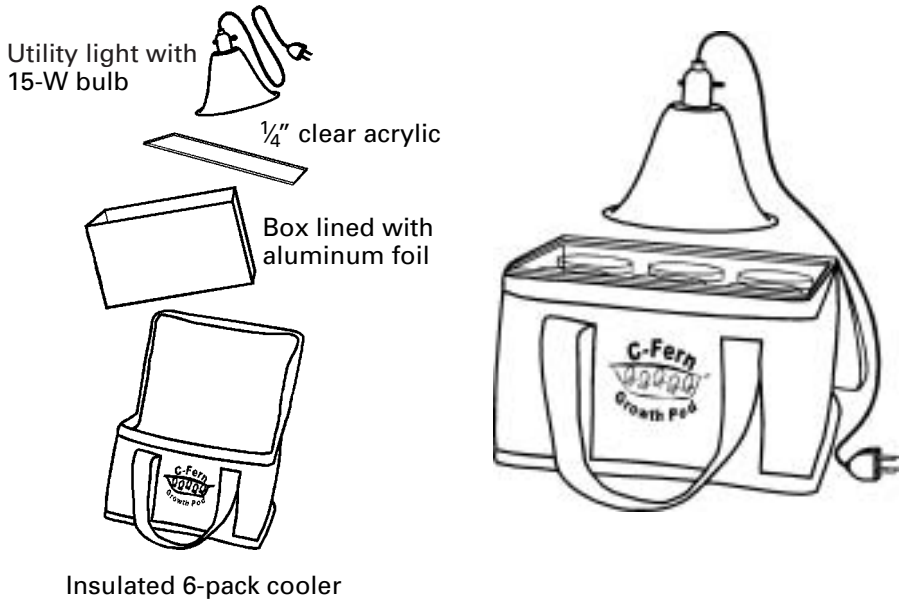


Figure 2. *C-Fern* Growth Pod

The *C-Fern* Growth Pod is made using insulated, vinyl 6-pack coolers or student lunch boxes that are readily available commercially. When fitted with an interior cardboard box, lined with aluminum foil or tape and covered with a fitted lid of ¼″ clear acrylic, the pod can be filled with six stacks of five petri dishes (60 × 15 mm), enough for a class of 30 or more students. A simple lighting fixture consisting of a 6″ reflective dome and a switch (small clamp or utility lights work well) and a 15-W screw-in fluorescent bulb is all that is needed. The newer screw-in fluorescent bulbs are long lasting, highly efficient, and feel only warm to the touch, even when left on continuously. The light can be rested directly on the acrylic lid, upon stacks of petri dishes or small blocks on top of the acrylic lid, or it can be suspended over the top of the lid. The height should be adjusted to achieve a 28–30°C (82–86°F) internal temperature. Small, inexpensive digital thermometers are handy to keep track of temperature. Thermometers that have an "outside" temperature probe on the end of a thin wire are especially useful.

The Growth Pod provides a bright, humid, and warm environment for rapid gametophyte and young sporophyte development. Because of the stacking arrangement of the petri dishes, cultures on the bottom may develop slower than those on the top. Reversing the order of the stacks after 6 or 7 days can

minimize this. However, the slight variation in gametophyte size that results from the stacking can be beneficial for observations and use. For local or long-distance transport, the top of the Growth Pod can be zipped closed to reduce temperature and humidity fluctuations. The light is small, portable, and can be plugged in anywhere.

C. Temperature

The optimum temperature for spore germination and gametophytic development is about 28°C (82°F), as measured *inside* the Culture Dome or Growth Pod. This is somewhat higher than for many other plants. Similar growth and development of gametophytes can be obtained at 26–30°C (79–86°F). However, temperatures lower than this will substantially alter developmental timing; for example, development will take twice as long at 20°C (68°F). It is a good idea to monitor and record the temperature *inside* your Culture Dome or Growth Pod daily. Control of the temperature inside the Culture Dome or Growth Pod can be achieved by adjusting the distance between the light source and Culture Dome or Growth Pod. Once a suitable temperature is achieved, the height of the lights should remain constant during all phases of culture.

A constant temperature within the Culture Dome or Growth Pod reduces the chances of condensation on petri dish lids. If condensation is a problem, cultures may be grown upside down once the sowing water has been absorbed into the agar medium.

D. Light

Note: Continuous illumination is recommended

i. Growth Pod Requirements

A screw-in 15-W fluorescent bulb with a simple fixture, such as a clamp or garage light, is long lasting, safe, and easy to use for light and temperature maintenance. See the above description for constructing Growth Pods.

ii. Culture Dome Requirements

Continuous illumination by two 40-W cool-white fluorescent tubes at a distance of 45 cm from the cultures will accommodate two standard Culture Domes (54 × 27 cm). This will provide about 80 μmoles of photosynthetically active radiation • m^{-2} • sec^{-1}, depending on the age of the bulbs. Two Culture Domes provide enough space for up to 64 individual 60- × 15-mm petri dishes. Smaller or larger setups can be used to serve individual needs. Temperature inside the Culture Dome is more important than light level, so the distance between the Culture Dome and the light source should be adjusted to obtain a temperature near the optimum.

iii. Constructing a *C-Fern* Light Stand

With inexpensive and simple materials, you can easily construct a *C-Fern* light stand.

Materials/tools:

10′ of PVC Pipe (1″ internal diameter, ID)
2 L-Shaped PVC Connectors (1″ ID)
2 T-Shaped PVC Connectors (1″ ID)
4 PVC End Caps (1″ ID)
Tape Measure
Hacksaw
Marker

(The PVC pipe and fixtures in a variety of sizes are readily available in most self-serve home improvement stores.)

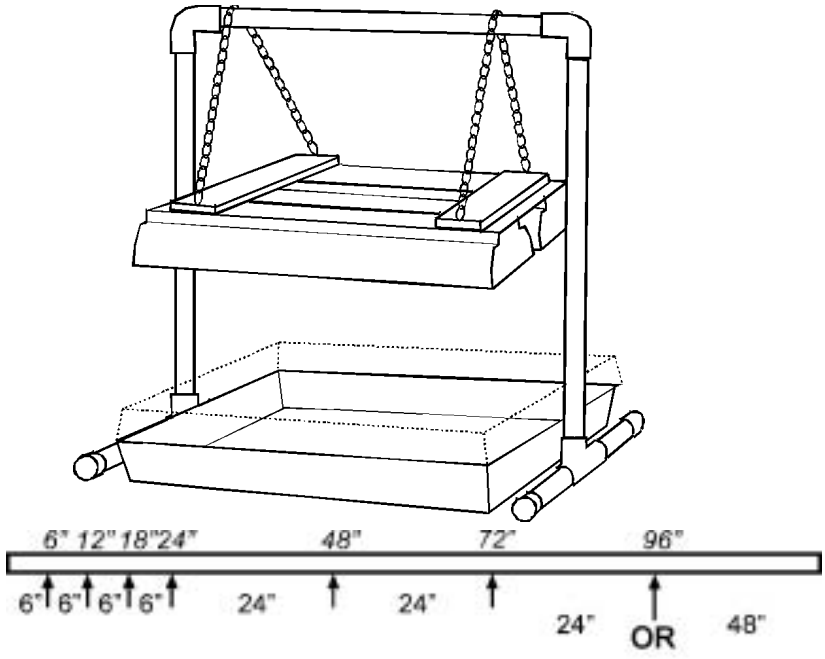


Figure 3. Constructing a *C-Fern* light stand

1. Mark the pipe to the correct lengths:

   4 6″ light bank "feet"
   2 24″ risers
   1 24″ or 48″ crossbeam

2. Cut the pipe with a hacksaw.
3. Construct the light bank by securely pushing the pipes into the appropriate connectors. Adjust the light bank "feet" so they are parallel. Hang the light source from the crossbeam, and you are ready to grow *C-Fern*. Do not glue the PVC pipe if you want the light bank to be portable and easy to store.

When your light stand is finished, you'll need a light source. For *C-Fern*, use a 2-foot fluorescent fixture (4 F20-W cool-white bulbs) OR a 4-foot fluorescent fixture (2 F40-W cool-white bulbs) OR 2 or more dome reflector fixtures with screw-in 15-W fluorescent bulbs. Carolina Biological also carries a light system suitable for *C-Fern*. Grow your *C-Fern* cultures inside a Culture Dome or Growth Pod under continuous light and at a temperature of about 28°C (82°F). Adjust temperature inside the Culture Dome or Growth Pod by varying the distance between it and the lights.

6. Observing Cultures

Observations of germinating *C-Fern* spores, developing gametophytes, and swimming sperm can be conveniently made at low magnification (10×–40+×) using a stereomicroscope. Illumination from below, i.e., with transmitted light, is best. Because the stage of a microscope with transmitted illumination may become quite warm after extended use, students should be encouraged to turn the base light off and to remove the culture plates from the stage when they are not being observed. If cultures are to be observed with the petri dish lids in place, condensation may form on the inside of the lid and obstruct observation. If spare lids from clean unused dishes are available, these can replace the fogged lids. If spare lids are not available, it is possible to remove the condensation by carefully wiping the lids with a clean, preferably sterile, tissue. If the lids are not kept in place during observation, gametophytes may begin to show signs of drying after being open for several minutes. In this case, it is important to remind students to place the lids back on the cultures when the cultures are not being observed. Observations can also be made using a compound microscope (40×–400×) by making wet mounts of some of the gametophytes. Select and remove gametophytes from the culture medium with a sterile probe or toothpick.

7. Sporophyte Culture

Although most student investigations using *C-Fern* concentrate on gametophyte development and sexual reproduction through the early sporophyte stage, 14–21 days following sowing (DFS), extended observations of sporophyte development, including the production of the next generation of haploid spores, are often desirable. Under adequate conditions, sporophytes with mature spores can be grown within 90 DFS. Because of its tropical nature, a large-scale culture of *C-Fern* sporophytes requires a warm and humid greenhouse environment. However, culture of individual sporophytes can be easily accomplished in a terrarium or other suitable vessel. The following instructions are provided for growing individual sporophytes in 2-L clear plastic beverage bottles.

1. One to 3 weeks after water has been added to the gametophyte culture to facilitate fertilization, young sporophytes should be transferred individually to separate 60- × 15-mm petri dishes containing Basic *C-Fern* Medium. Transfer can be made with a clean toothpick or probe so that the root end of the sporophyte is imbedded in the agar and the first leaf is above the agar. Use care to avoid contamination.

2. Place the sporophyte cultures in the Culture Dome or Growth Pod under the same lighting and temperature conditions that were used for gametophyte cultures. Sporophytes should remain under these conditions for 2–4 weeks. Usually, it is not necessary to add water, but if the culture begins to get dry, use a pipet to add sterile distilled water as needed.
3. When sporophytes have produced several roots and leaves, they can be transferred to a bottle "terrarium." If the sporophyte is small enough, both it and the soil mix (see step 4) can be pushed through the mouth of the bottle and adjusted/planted with a long narrow stick, etc. Otherwise, make a circular cut 2 inches below the neck. Cut only ¾ of the distance around the bottle so that a "hinge" is left. The top can now be lifted to provide a larger opening for planting.
4. Place 2 cups of pre-moistened (damp but not wet) ProMix® Potting Soil in the bottom of the bottle. Add 4–6 medium-sized beads of Osmocote® 14-14-14 Fertilizer and mix them into the top ½-inch of potting mix. Make a shallow concave cavity (½″ deep × 1″ diameter) in the center of the bottle.
5. Carefully remove a sporophyte from its petri dish by lifting the agar out of the dish, roots and all. A flat knife or thin spatula works well for this. Remove as much agar as possible without damaging the roots and place the sporophyte (roots down!) in the cavity made in step 4. Gently cover the roots with potting mix and lightly press down on the surface around the sporophyte to give it a firm footing in the potting mix.
6. Add enough distilled water to thoroughly moisten the potting mix and help displace any air spaces between the roots and soil mix. The mix should be moist but not soaked, although excess water will not typically harm *C-Fern*. Subsequent watering can usually be made as needed, usually on a weekly basis. Use distilled or bottled drinking water, if available. **Note:** Constant moist conditions will result in mostly vegetative growth. If fertile leaves with spores are desired, a moderate level of water stress will typically cause a change from vegetative to reproductive leaf production. This can be accomplished by allowing the soil to dry substantially between waterings.

Place the terrarium under the same lighting conditions used for the gametophyte cultures. Warm temperatures and constant 24-hour illumination work best; cold window sills are not recommended. Cooler temperatures and less light will slow development but still allow growth. Other than watering, as described in step 6, little care is needed.

Consult the *C-Fern*® *Manual* or Web site for information on the greenhouse culture of sporophytes.

8. Alternative and Additional Culture Methods

A. *Surface Sterilization of* C-Fern *Spores*

The procedure outlined on the next page describes how to surface sterilize spores that are either collected directly from sporophytes or obtained in bulk, unsterilized lots. Before you begin,

- Make sure the area is clean and free from drafts and traffic.
- Wipe down the area with 70% ethanol, 70% isopropanol, or a damp clean sponge.

Materials:

Bulk Unsterilized C-*Fern* Spores (For information on wild-type and mutant spores, refer to the Resources section or current Carolina catalog.)

Sterile Tube With Conical Bottom

Sterile Transfer Pipets

Sterile Distilled Water

Plain Commercial Laundry Bleach or Equivalent (5.25% sodium hypochlorite solution)

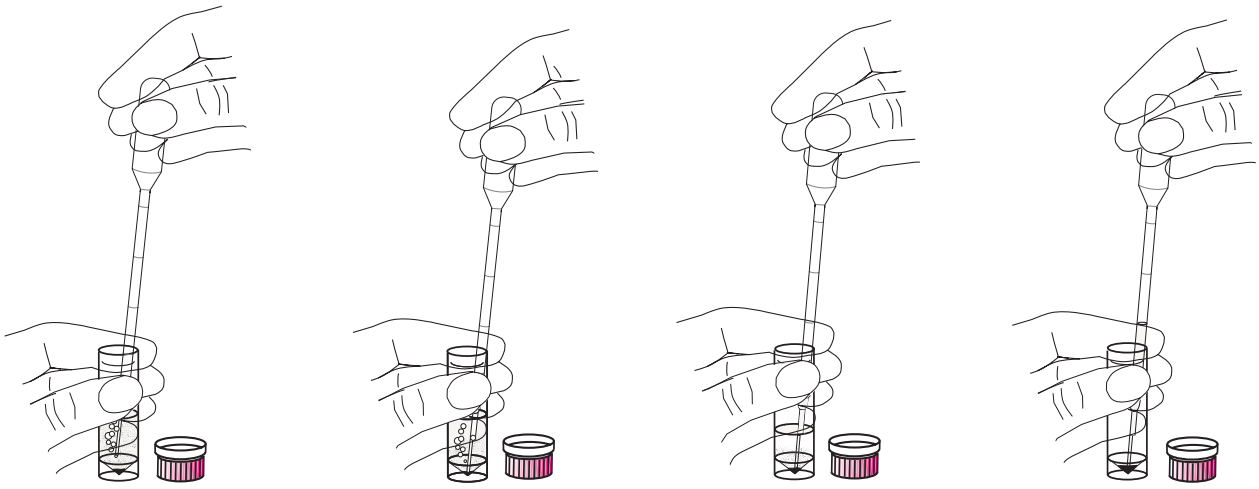
Waste Container

Timer

NOTE: For handling larger amounts of spores (15–250 mg) it may be more convenient to use 12- to 15-mL conical centrifuge tubes. Other materials, such as conical microfuge tubes and automatic pipets, may also be successfully used for spore sterilization, but should be tested first.

Step-by-Step Procedure for Spore Sterilization

1. WEIGH SPORES. Weigh out the spores onto glassine weigh paper and transfer them to a sterile conical tube. Using 4 mL of water, 10-mg spores will sow about 35 petri dishes at a density of 300+ spores per dish.
2. PRESOAK SPORES. Cover the spores with 1–2 mL of distilled water. The spores may be soaked just long enough to become wet, i.e., about 5 minutes, or for up to 24 hours. To hasten the wetting of spores, tightly seal the vial and invert it 2–3 times.
3. REMOVE PRESOAK WATER. Insert the sterile pipet into a conical tube and suspend the spores by bubbling a small amount of air into the water (see Figure 4). While air is slowly bubbling out of the pipet, gently but securely seat the pipet onto the base of the conical tube. Sometimes it helps to gently rotate the pipet tip to seat it properly. Squeeze the bulb to force additional air out of the pipet. When the bulb is released, water should enter the pipet, and the spores should collect around the outside of the base of the pipet tip, provided the pipet is securely seated on the tube bottom (see Figure 4). If you cannot remove the liquid without bringing the spores along, try another pipet. Remember that timing is critical when the sterilizing solution is in the tube! Practice this technique with the presoak water prior to sterilizing the spores. With practice, you should be able to remove the liquid, free of spores, in about 10 seconds or less.
4. SURFACE STERILIZE SPORES. To sterilize, suspend spores in 1–2 mL of 0.875% sodium hypochlorite. Prepare 0.875% sodium hypochlorite by mixing 1 part commercial bleach (5.25%) to 5 parts distilled water. Rinse down the lip and sides of the vial with bleach solution. To ensure that the spore mass becomes evenly suspended, bubble air through a clean, sterile pipet. Surface sterilize the spores for 3 minutes. Remove the bleach solution with a clean, sterile pipet using the technique described in step 3.
5. RINSE SPORES. To rinse the spores, add about 2 mL of sterile distilled water. The pipet used to add the rinse water may be used repeatedly as long as care is taken to prevent contamination with foreign spores. Remove the rinse water with a clean, sterile pipet. Repeat the rinse step 1 or 2 more times. The pipet used to remove the sterile distilled water may be left in the tube for use in sowing spores.
6. SOWING AND SPREADING SPORES. For 10 mg of spores, add 4 mL of sterile distilled water and proceed to the sowing and spreading of spores as outlined in Section 4, Inoculating (Sowing) Cultures Using Presterilized *C-Fern* Spores. Adjust for the density of spores per dish and the number of dishes sown as needed (see Table 1).



Bubbling before "seating" pipet on bottom

Bubbling after pipet seated on bottom

Aspiration of liquid only

Completed aspiration of liquid. Spores remain in bottom of vial.

Figure 4. Spore sterilization procedure

B. DarkStart Method

In some cases, it is desirable to decrease the length of time required to obtain gametophyte material at a particular developmental stage, or give greater control over the timing of particular developmental stages. Because the 10–12 days required to obtain sexually mature gametophytes under standard conditions includes approximately 2 days of imbibition (absorption of water) for dry spores, it is possible to "jump start" cultures by adding water to the vial and immediately placing the sealed vial into a foil bag or covering it with two or more layers of foil. Complete darkness will prevent spore germination in most genotypes (the dark germinator stock, *dkg* is the exception) while still allowing normal imbibition. After 3–7 days in darkness, when spores are sown and placed under lights, germination will occur about 2 days earlier than with the standard light sowing technique.

DarkStart can be used to pre-start a lab so that students sow fully imbibed spores and can more easily observe and/or measure all of the early stages of development. For example, spores "DarkStarted" on a Friday, Monday, or Tuesday could be sown and exposed to light the following Friday to ensure initial germination by Monday and complete germination by Wednesday. DarkStart also increases synchrony of germination in cultures and reduces the frequency of male gametophytes.

C. Hints for Large Classes

The 40-mg vial of unsterilized bulk spores is a convenient size for larger classes. To sterilize a single batch or multiple batches of this quantity of spores, it is advisable to sterilize each 40-mg quantity using the standard techniques described in the Culture Instructions. After sterilization has been completed, transfer the sterile spore suspension to a sterile test tube of larger capacity (e.g., ≥ 20 mL). Then, for each 40-mg quantity, bring the liquid volume up to 16 mL with sterile distilled water. Dispensing 3 drops per dish from a sterile transfer pipet will generate approximately 140 small petri plates. Be sure to keep the spores suspended by repeatedly drawing them in and out of the pipet during the inoculation procedure.

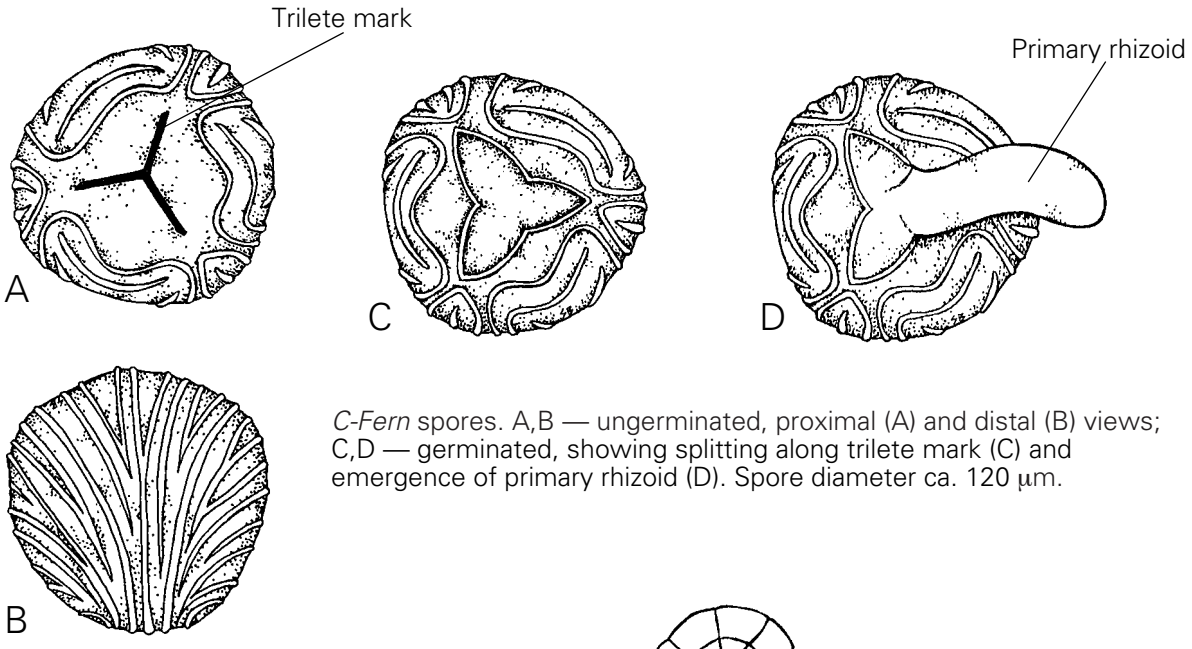
All of the spore suspension can be sown at one time. However, if laboratories are to last for more than 2 days, it may be advisable to sow only a portion (perhaps one-half) initially. This can be conveniently done by separating the sterilized spore suspension into two equal volumes. One volume can be kept in the dark for 1–3 days, as is done in the DarkStart procedure. In this way, cultures of approximately the same age can be scheduled for an entire week of laboratories. This can even be a convenient way of bridging schedule difficulties caused by weekends or vacation days.

D. Dry Sowing of Presterilized C-Fern *Spores*

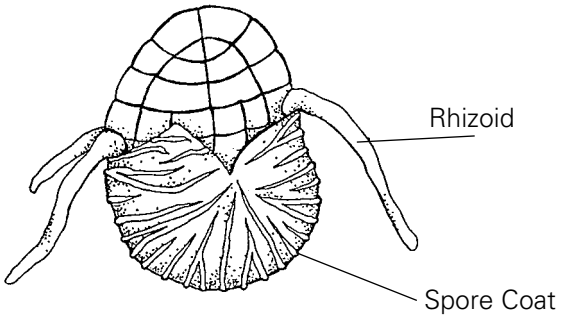
As an alternative to using a liquid suspension of spores for inoculating petri dishes, spores can be sown with a sterile cotton swab. Gently touch the tip of the swab to dry spores in the bottom of the vial. Disperse onto the culture medium by gently tapping the stem of the swab with a finger. Although this method does not produce a uniform distribution or density, it can be useful to quickly establish a few cultures without using the entire vial of spores.

Additional Information

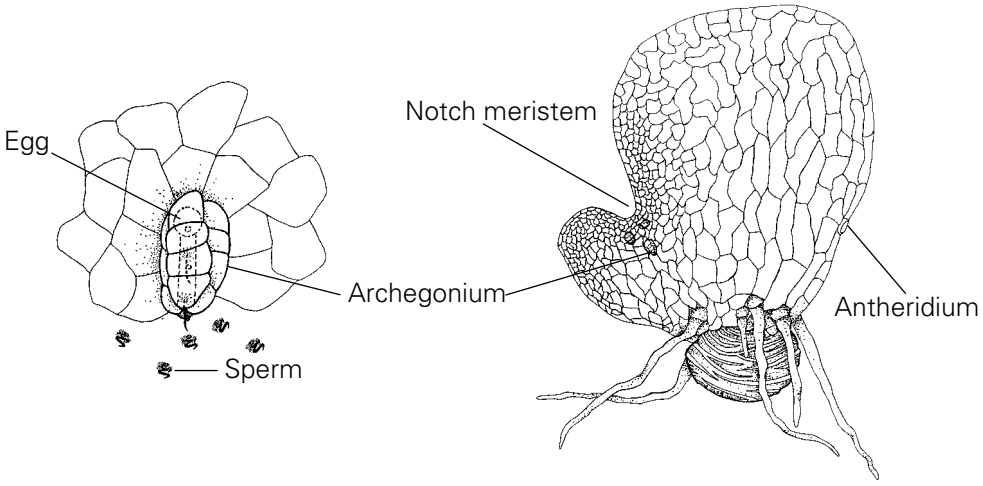
1. Drawings of Gametophyte and Early Embryo Development



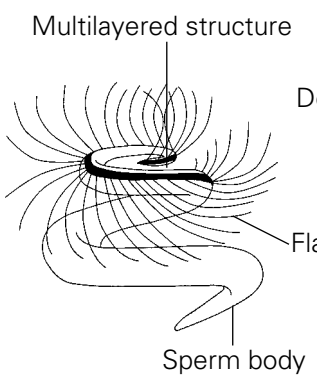
C-Fern spores. A,B — ungerminated, proximal (A) and distal (B) views; C,D — germinated, showing splitting along trilete mark (C) and emergence of primary rhizoid (D). Spore diameter ca. 120 μm.



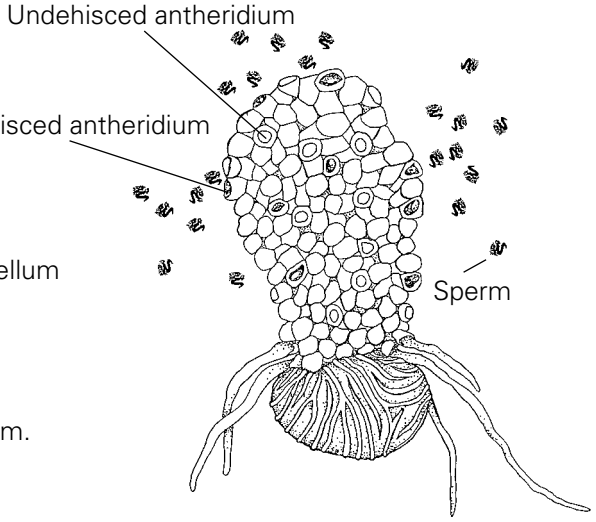
Young *C-Fern* gametophyte, 5 days from start (DFS) of culture. Spore coat diameter ca. 120 μm.



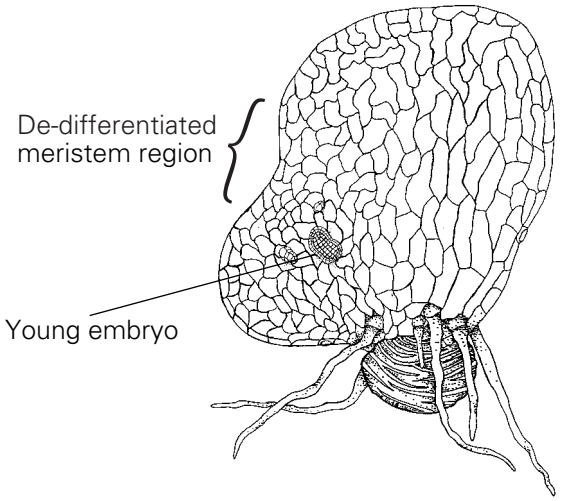
Mature hermaphroditic *C-Fern* gametophyte with archegonia behind the notch meristem and a single antheridium on the margin, ca. 10 DFS. Spore coat diameter ca. 120 μm. Close-up: view of mature archegonium during fertilization. Sperm enter the open neck canal, uncoil, and move toward the egg.



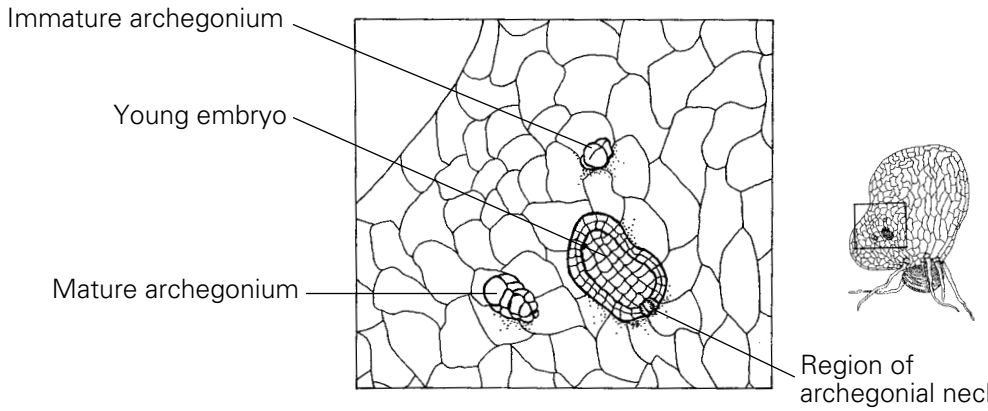
Individual mature sperm, ca. 8.8 × 5.5 µm.



Mature male C-Fern gametophyte, ca. 10 DFS. Sperm are released from the numerous antheridia on the surface of the male gametophyte. Spore coat diameter, ca. 120 µm.



Hermaphroditic *C-Fern* gametophyte, ca. 3 days after fertilization, showing a young sporophyte embryo. The embryo is covered by proliferated archegonial tissue. Following fertilization, cell division ceases in the notch meristem region of the gametophyte and cells enlarge. As the sporophyte continues to develop, the gametophyte eventually dies. Spore coat diameter, ca. 120 µm.



Close-up of hermaphroditic *C-Fern* gametophyte, ca. 3 days after fertilization, showing unfertilized (immature and mature) archegonia and young embryo developing within proliferated archegonial tissue. The remains of the old archegonial neck can be seen at the distal end of the embryo.

2. Independent Student Research Projects

C-Fern is an excellent tool for use in independent student research. Projects can be completely independent of the normal classroom activities or may be integrated with group exercises. The principal goals of independent research are to stimulate students' interest in the subject by encouraging them to formulate questions and subsequently to design appropriate experiments that can provide answers to those questions. This directly involves students in the process of science. Perhaps the most important task of the teacher in this situation is to give helpful but minimal advice and guidance in order to avoid a level of frustration that can dampen students' initial enthusiasm or willingness to undertake a project.

The individual investigations contained within the *C-Fern*® Manual can serve as a starting point to generate questions to pursue. In particular, some of the open-ended questions that are presented at the end of the exercises may be useful. Some of these, along with others, follow. Another source is the bibliography contained in Part C of the *C-Fern*® *Manual*, which gives references for a number of separate investigations that involve a variety of experimental approaches. Use of mutagenesis and selection procedures, determinations of the effects of various growth hormones, comparative tests of the responses of wild type and various mutants to environmental stresses and herbicides, and many other examples are contained within the cited references. Emphasize to the students that many other questions, likely the most interesting ones, haven't even been asked yet!

To cultivate independence in students, it is perhaps best to provide them with only the minimal materials that are needed for *C-Fern* culture. These materials could consist of vials of unsterile spores of the wild type or particular mutants, as well as the basic materials for preparing nutrient medium. In this way, students gain more familiarity with the organism by performing necessary tasks that are often completed by the teacher or others prior to students' involvement with structured exercises. Very good questions often arise from these types of basic manipulations of the organism. Environmental parameters, such as temperature and light, can be conveniently varied by altering the distance between the Culture Dome or Growth Pod and the light source, and/or by utilizing various types of shading material to decrease the amount of incident light on the cultures. Manipulation of other parameters, such as medium composition, pH, sowing density, population composition, light quality, day length, etc., can also be accomplished easily.

3. Questions for Discussion

- How far do sperm swim?
- How do sperm move?
- How do sperm find the archegonium?
- Why are archegonia restricted to the central region of the hermaphrodite?
- Can sperm respond chemotactically to artificial (chemical) signals?
- How do sperm reach the egg after finding the archegonium?
- How do gametophytes limit fertilization to 1 and rarely 2 events?
- What happens to gametophytes after a fertilization event?
- What happens to meristem activity after a fertilization event?
- What type of signal elicited by a fertilization event is responsible for stopping meristem activity?

- What are the patterns of cell division and expansion that yield the heart-shape of the hermaphrodite?
- What are the effects of gravity on developing gametophytes?
- How long will unfertilized gametophytes live?
- Other than using a bioassay, what approach could you use to show that a chemical substance was responsible for controlling development of sexual types in C-*Fern*?
- Do all gametophytes secrete the pheromone?
- When are gametophytes sensitive to the pheromone?
- How could you chemically characterize the active agent?
- How could you design a bioassay to detect other agents that control development?
- What might be the advantage of controlling the sex ratio in the cultures?
- What other factors might be important in controlling the sex ratio in cultures?
- How could one determine if there was a genetic difference that controlled sexual type in gametophytes?
- How could you test the relative fitness of different genotypes, e.g., wild type versus polka dot?
- Can you think of an advantage to having more males at higher densities? How could you test your hypothesis?
- How do higher densities result in more males within a population?
- What might be the advantage of having fewer males at lower densities?
- How do changing proportions of males influence the kinds of mating that can occur?
- What factors limit sporophyte growth at high densities?
- Can sporophytes form without fertilization?
- Can gametophytes develop directly from sporophyte tissue?
- What role does the meristem play in maintaining the hermaphroditic gametophyte?
- Can microsurgery on gametophytes demonstrate alternate paths of development?
- Can sporophytes develop normally if the young embryo within the archegonium is cut away from the gametophyte?
- What effect on development is caused by adding sucrose to the medium?
- What factors are important in sporophyte development?
- Can sporophyte development be speeded up?
- What is the difference between vegetative and fertile leaves on sporophytes?
- Do rhizoids respond to the direction of gravity?
- Do gametophytes show a phototropic response?
- Do sporophytes show a phototropic response?
- What wavelengths of light are necessary to induce germination?
- Does gametophyte growth and development respond to different wavelengths of light?
- Which phase, gametophyte or sporophyte, is more important in limiting the distribution of C-*Fern*?
- Under what conditions is vegetative development more important than sexual development?

- Do rhizoids have a specific function?
- If a meristem is surgically removed, will a new one form? What factors could influence this?
- Is distance between gametophytes important during their sexual development?
- Do sperm swim best in distilled water or in some other medium?
- Following meiosis, how long does it take a spore to mature?
- How long can sperm swim? What environmental factors are important in determining this?
- What happens when *C-Fern* cultures are grown upside down or on their side? Why?

4. Resources

Sources for Materials and Additional Information

A. Selected References

Copeland, E. B. 1942. Edible ferns. *American Fern Journal* 32: 121–126.

Hickok, L. G., S. Baxter Duncan, and T. Warne. 2002. *C-Fern* and the living Punnett square. *Carolina Tips* 65: 1–3.

Hickok, L. G., T. R. Warne, and R. S. Fribourg. 1995. The biology of the fern *Ceratopteris* and its use as a model system. *International Journal of Plant Science* 156: 332–345.

Hickok, L. G., T. R. Warne, and M. K. Slocum. 1987. *Ceratopteris richardii:* Applications for experimental plant biology. *American Journal of Botany* 74: 1304–1316.

Hickok, L. G., and T. R. Warne. 1998. *C-Fern* Manual. Carolina Biological Supply Company. Burlington, NC.

Hickok, L. G., T. R. Warne, S. L. Baxter, and C. T. Melear. 1998. Sex and the *C-Fern*: Not just another life cycle. BioScience 48: 1031–1037.

Lloyd, R. M. 1973. Systematics of the genus *Ceratopteris* (Parkeriaceae), I. Sexual and vegetative reproduction in Hawaiian *Ceratopteris thalictroides. American Fern Journal* 63: 12–18.

Lloyd, R. M. 1974. Systematics of the genus *Ceratopteris* Brongn. (Parkeriaceae) II. Taxonomy. *Brittonia* 26: 139–160.

Lloyd, R. M.1993. Parkeriaceae—the water fern family. Pages 119–121 in Editorial Committee, ed. *Flora of North America North of Mexico*. Oxford University Press, New York.

Renzaglia, K. S., and T. R. Warne. 1995. Ceratopteris: An ideal model system for teaching plant biology. *International Journal of Plant Science* 156: 385–392.

Renzaglia, K. S., T. R. Warne, and L. G. Hickok. 1995. Plant development and the fern life cycle using *Ceratopteris richardii. American Biology Teacher* 57: 438–442.

Tryon, R. 1990. Pteridaceae in K. Kramer and P. Green, Eds. *The families and genera of vascular plants. Vol. 2. Pteridophytes and Gymnosperms.* Springer Verlag, New York.

B. Additional Information

For more details on availability of *C-Fern* educational materials and culture conditions or to offer comments and suggestions, please contact:

Carolina Biological Supply Company
2700 York Road
Burlington, NC 27215
1-800-227-1150
www.carolina.com

C-Fern® Project
Department of Botany
The University of Tennessee
Knoxville, Tennessee 37996 USA
cfern@utk.edu

Web: **http://cfern.bio.utk.edu** A source for additional information on using *C-Fern* in teaching and research. Research ideas, protocols, an image gallery, and an extensive bibliography are included.

C. C-Fern *Educational Products Offered by Carolina Biological Supply*

***C-Fern* Supplies and Support Materials**	
Catalog #	**Description**
RN-15-6715	*C-Fern*® Growth Pod™
RN-97-1944	Lighting System
RN-15-9705	Pro-Mix™ Potting Soil
RN-15-9765	Osmocote® 14-14-14 Fertilizer
RN-15-6780, 81	Pre-made Basic *C-Fern*® Medium (160 mL and 400 mL)
RN-15-6782	Powdered Basic *C-Fern*® Medium (for 1 L)
RN-21-5840	Sterile Disposable Transfer Pipet
RN-74-1346	Sterile Plastic Petri Dishes, 60 × 15 mm
RN-79-6200, 01	Bacto Agar (100 g and 500 g)
RN-19-8697	Sterile Distilled Water
RN-66-5666	Culture Dome Bottom (plastic greenhouse tray)
RN-66-5874	Culture Dome Top (clear plastic dome)
RN-88-9573	1 Molar Sodium Hydroxide (1M NaOH)
RN-15-6775	*C-Fern*® Manual for Teaching and Research Contains background information on *Ceratopteris*, detailed culture instructions and a current set of laboratory investigations, as well as other support and technical information to use in the classroom and laboratory.
RN-15-6787	*C-Fern*® Image CD-ROM
RN-15-6793	*C-Fern*® Culture Instructions and Background Information
C-Fern® Investigations (Instructor's Manuals):	
RN-15-6794	Sex in a Dish
RN-15-6796	More Pressures of Life
RN-15-6797	Genetics in Action
RN-15-6795	The Pressures of Life
RN-15-6798	Battle of the Sexes
RN-15-6799	Chemical Attraction

C-Fern® Kits

C-Fern kits demonstrate a variety of biological principles. With the exception of the Meet the *C-Fern* kit, all kits are designed for classroom use by 30 students. All kits (except the Chemotaxis kit) come complete with pre-sterilized spores, petri dishes, *C-Fern* medium, other needed supplies, and detailed directions for students and teachers. In addition to the kits, all necessary supplies and support materials for *C-Fern* culture can be purchased individually.

Catalog #	Description
RN-15-6702	**Sex in a Dish: The *C-Fern*® Life Cycle Kit.** A dynamic introduction to the life cycle of this unique plant, a good prelude to other investigations. This exercise provides a clear illustration of organ and tissue differentiation in the developmentally simple gametophytes. Gametophyte sexual development, swimming sperm, fertilization and embryo development are directly visible using a dissecting microscope and simple culture equipment. Materials for 30 students. Experiment time is 21 days with optional extended observations.
RN-15-6704	**The Pressures of Life—Population Density and *C-Fern* Development Kit.** Two distinct investigations carried out sequentially. Quantitatively explore the effects of population density. First, the influence density has on gametophyte sex ratio, and second, the effects of sporophyte competition. Materials for 30 students. Experiment time is 14–35 days.
RN-15-6706	**More Pressures of Life Kit.** Couples the Pressures of Life exercise with an exercise in serial dilutions.
RN-15-6708	**Genetics in Action—Mendelian Genetics Kit.** Students visualize basic principles of Mendelian Inheritance in *C-Fern* by following the segregation of a visible marker, polka dot, in both the F_1 gametophyte and F_2 sporophyte generations. Students sow spores of an F_1 hybrid (wild type × polka dot) to produce F_1 gametophytes. By adding water to mature F_1 gametophytes, students visualize random fertilization events that produce the F_2 sporophyte generation. Hypotheses generated from observations of F_1 gametophytes are then tested by analysis of phenotypes and ratios in the F_2 sporophyte generation. The large sample numbers provided from class and individual data sets allow meaningful use of the Chi-square test. This investigation demonstrates the Mendelian principles of segregation, random fertilization, and dominance/recessiveness and provides hands-on experience in data acquisition, hypothesis formation, and testing. Materials for 30 students. Experiment time is 14–28 days.
RN-15-6712	**Battle of the Sexes—*C-Fern*® Sexual Differentiation Kit.** Students actively explore the nature of sexual differentiation in *C-Fern*. Although all *C-Fern* gametophytes develop from genetically identical spores, their development into males or hermaphrodites is environmentally controlled by a pheromone-like system. Students investigate this phenomenon experimentally by repeatedly inoculating culture dishes over a period of 7 days and analyze the resulting sex ratios. Students formulate testable hypotheses to account for the presence and frequency of each sexual type in different aged inoculations. In addition to the wild type, this exercise uses a maleless strain (*her1*) which is insensitive to the pheromonal system. Materials for 30 students. Experiment time is 14 days.
RN-15-6714	**Chemical Attraction—*C-Fern*® Sperm Chemotaxis Kit.** An amazing experiment that tests the ability of sperm to detect and swim to a chemical signal (positive chemotaxis). Using sperm released from 12- to 18-day-old gametophyte cultures, students document the responses to a series of test solutions, including a pair of stereoisomers that elicit distinctly different responses. All manipulations and observations can be made using a stereomicroscope (dissecting scope) at 20× or higher and using transmitted (bottom) illumination. Materials for 30 students. Experiment time is ½ to 1 hour, not including culture time. **Note:** This kit is supplemental to other kits and does not include spores or culture materials.

Representative *C-Fern* Spore Stocks (see the current Carolina Biological Catalog for a complete listing)			
Catalog # (10 mg pre-sterilized, 40 mg unsterilized)	**Stock**	**Symbol**	**Description**
RN-15-6728, 29	Wild Type	RNWT1	Basic *C-Fern* genotype
	Single Gene Nuclear Mutants		
RN-15-6738 (10 mg only)	Dark Germinator	*dkg*	Does not require light to initiate spore germination.
RN-15-6743 (40 mg only)	ABA Tolerant	*abr48*	Gametophytes tolerant to the plant growth regulator, abscisic acid.
RN-15-6744, 45	Maleless	*her1*	Does not form male gametophytes; insensitive to the male-inducing pheromone.
RN-15-6746 (10 mg only)	Non-Etiolated	*det30*	Gametophytes do not show elongation response when grown in the dark.
RN-15-6764, 65	Highly Male	*him1*	Gametophytes exhibit "supermale" morphology. Forms high numbers of males, even in isolate cultures, with increased numbers of antheridia on both males and hermaphrodites. Homozygous sporophytes exhibit narrow-pointed leaves in young sporophytes and a curling of the leaf tips in older sporophytes.
RN-15-6767 (40 mg only)	Sleepy Sperm	*zzz1*	Sperm are released normally, but swimming is delayed. After a few minutes, some sperm "wake up" and swim slowly to moderately, while the rest show only slight movement and remain close to the gametophyte. Excellent for observing at high magnifications (>50×). Self fertile.
RN-15-6768 (10 mg only)	Slow-Mo Sperm	*slo1*	Sperm are released normally and swim immediately. All but a few show very slow movement. Excellent for observation at high magnification (>50×). Self-fertile.
RN-15-6722 (40 mg only)	Bubbles	*bub1*	This curious mutant appears normal until Day 10, but then begins to show massive swelling of a few to many cells on the gametophyte. Some cells become so large that they burst. Expression is variable but easy to observe. Self-fertile. Some irregularity can also be seen in sporophytes.

(continued)

Representative C-*Fern* Spore Stocks *(continued)* (see the current Carolina Biological Catalog for a complete listing)			
Catalog # (10 mg pre-sterilized, 40 mg unsterilized)	**Stock**	**Symbol**	**Description**
F_1 Hybrids (Monohybrid)			
RN-15-6760, 61	F_1 Polka Dot	*CP/cp*	Spores from an F_1 sporophyte produce gametophytes in a 1:1 ratio of polka dot mutants to wild type. The recessive mutation results in a 3:1 ratio in the F_2 sporophyte generation.
RN-15-6772 (10 mg only)	F_1 Pale	*PAL1/pal1*	Spores from an F_1 sporophyte produce gametophytes in a 1:1 ratio of pale mutant to green wild type. Pale gametophyte mutants grow nearly as well as wild type and are easily distinguished. The recessive mutation results in a 3:1 ratio in the F_2 sporophyte generation.
F_1 Hybrid (Dihybrid)			
RN-15-6756 (40 mg only)	Pale 1–Pale 2	*PAL1/pal1*	PAL2/pal2 A dihybrid with 2 non-allelic pale mutations showing a 1:3 (green:pale) segregation in the gametophyte stage and 9:7 (green:pale) in the F_2 sporophyte stage. Useful as a challenging unknown for students, especially when compared with the F_1 monohybrid pale.