



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 1 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

CONTENTS

- 1.0 SCOPE AND APPLICATION
- 2.0 METHOD SUMMARY
- 3.0 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE
- 4.0 INTERFERENCES AND POTENTIAL PROBLEMS
- 5.0 EQUIPMENT/APPARATUS
- 6.0 REAGENTS
- 7.0 PROCEDURES
 - 7.1 Site Preparation
 - 7.2 Instrument Set-up
 - 7.3 Leaf Selection
 - 7.4 Dark Adaptation
 - 7.5 Light Level Determination
 - 7.6 Sample Time Determination
 - 7.7 Ambient Conditions Measurement
 - 7.8 Fluorescence Measurement
 - 7.9 Downloading of Stored Measurements
 - 7.10 Data Interpretation
 - 7.11 Battery Operation
 - 7.12 Trouble-Shooting and Problem Prevention
- 8.0 CALCULATIONS
- 9.0 QUALITY ASSURANCE/QUALITY CONTROL
- 10.0 DATA VALIDATION
- 11.0 HEALTH AND SAFETY
- 12.0 REFERENCES
- 13.0 APPENDICES
 - A - Chlorophyll Fluorescence and Key Parameters
 - B - Specialized Field Data Sheet
 - C - Figures



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 2 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

1.0 SCOPE AND APPLICATION

Chlorophyll fluorescence in vivo is a tool that has been used in photosynthesis research, aquatic productivity studies, and stress physiology. When light strikes chlorophyll pigments within the photosynthetic apparatus of a plant, the pigment molecules become excited. Most of this excitation energy is then channeled to the chloroplast's photosystems that drive photosynthesis. Some of this excitation energy (approximately 3%) is re-emitted as fluorescence. A more detailed discussion of chlorophyll fluorescence is presented in Appendix A. Chlorophyll fluorescence is an important physiological indicator as it can reflect the condition of the most basic physiological function, photosynthesis.⁽¹⁾ Environmental stresses such as extremes in temperature, moisture, or nutrition and high intensities of light have been studied for their effects on chlorophyll fluorescence.^(2,3) Anthropogenic stress factors such as pesticides, air pollutants, acid precipitation, increased UV-B radiation, and heavy metals have also been studied.^(4,5,6,7,8,9)

Since plants form the base of the food chain and create habitat conditions for other organisms in an ecosystem, the assessment of the condition of the plants on hazardous waste sites is necessary to fully assess the impact to the ecosystem as a whole.

The instrument used for chlorophyll fluorescence measurement described in this Standard Operating Procedure (SOP) is the CF-1000 (manufactured and supplied by P.K. Morgan Instruments, Andover, MA). This instrument is a portable field fluorometer, which measures chlorophyll fluorescence kinetics in photosynthetic tissue and calculates values of key fluorescence measurement parameters (Refer to Appendix A).

This instrument is intended as a screening tool only, as the source of variation in in vivo chlorophyll fluorescence is not determined by the instrument.

These are standard (i.e. typically applicable) operating procedures which may be varied or changed as required, dependent upon site conditions, equipment limitations or limitations imposed by the procedure. In all instances, the ultimate procedures employed should be documented and associated with the final report.

Mention of trade names or commercial products does not constitute U.S. Environmental Protection Agency (U.S. EPA) endorsement or recommendation for use.

2.0 METHOD SUMMARY

Plants to be evaluated with the chlorophyll fluorescence technique are chosen according to a sampling plan prepared for the site. Plots containing plants to be sampled are selected according to microhabitat similarity and contaminant concentration so that the results are comparable. Plants are chosen randomly within a plot at a rate specified in the sampling plan. Individual leaves are selected according to their level of maturity and position on the plant. According to the requirements of the site, selected leaves and/or plants are identified with flagging tape or survey flags. Leaves are dark adapted for an appropriate length of time, depending upon light conditions. Prior to making actual measurements, the light level to be utilized is determined for each species evaluated. Chlorophyll fluorescence measurements are performed after proper dark adaptation. Data are saved within the memory of the instrument, on computer diskettes, and on specialized field data sheets, Appendix B). Additionally, ambient environmental conditions [i.e., photosynthetically active radiation (PAR) intensity, soil/sediment pH, soil/sediment temperature, air temperature, and weather conditions] are recorded on the field data sheets. Identical procedures are



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 3 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

performed at a reference location (or locations). Within-species comparisons are made using statistical analyses.

Data are prepared for statistical analysis. Data analysis includes within-species comparisons of key fluorescence parameters.

3.0 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

No sample preservation is necessary unless leaves are removed from plants. If leaves must be removed, detached leaves are placed in resealable plastic bags with moist paper towels and must be analyzed within 30 minutes of collection. Detached leaves should be held at approximately the same temperature in which the collection was made.

4.0 INTERFERENCES AND POTENTIAL PROBLEMS

It is imperative that reference and comparison plant plot locations are chosen so that they cover identical microhabitat conditions, to avoid stress factors other than the one(s) being assessed. Drought, chilling temperatures, or other natural environmental stress conditions may mask the effects of contaminants as these conditions will also affect in vivo chlorophyll fluorescence. In practice, it is not possible to locate plots with identical microhabitat/microclimate conditions. However, proper selection of plot locations should keep microhabitat variation to a minimum, and ambient environmental conditions can be monitored during plot evaluations.

This procedure cannot be performed during condensing conditions (wet weather at, or approaching 100 percent relative humidity) with the CF-1000 instrument.

Erroneous readings may result from interference from external transmission sources such as high-voltage transmission lines or powerful radio transmitting equipment.

Data must be collected during the growing season prior to seasonal pigment degradation (senescence) in autumn. Collection of data during senescence, when variations in chlorophyll content exist within and among plant leaves, may lead to highly questionable results.

Plant species that have substantially pubescent or rough textured leaves should be avoided, since these conditions will interfere with fluorescence measurements.

Failure to choose the proper light intensity will result in inaccurate estimates of key fluorescence measurement parameters. Similar inaccuracies will result from improper dark adaptation of the leaf area to be tested.

Intrinsic limitations with fluorescence parameter measurements: Determination of important fluorescence parameters either by the instrument or manually on hard copies of fluorescence traces may be subject to inaccuracies.

Literature on in vivo chlorophyll fluorescence measurements indicate that the fluorescence trace can be affected by stage of development of the leaf, leaf thickness, and dark adaptation conditions. It is therefore imperative that these factors be monitored and matched as closely as possible.



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 4 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

It is unclear from the literature what effects ambient soil and air temperatures have on fluorescence kinetics. Indications exist that, at least for one species, a change in the kinetics of the fluorescence trace occurs when the leaf temperature rises above 20°C, but not necessarily the fast kinetics from which the key fluorescence parameters arise.⁽¹⁰⁾ For these reasons, the soil and air temperatures are recorded during the time the fluorescence measurements are conducted. Additionally, a low temperature limit, (for example, 15°C), should be established below which fluorescence measurements will not be made and a record kept of nighttime temperatures during evaluation.

5.0 EQUIPMENT/APPARATUS

The following equipment and supplies are necessary for chlorophyll fluorescence measurement:

- Portable field fluorometer (Chlorophyll fluorescence measurement system, Model CF-1000, P.K. Morgan Instruments, Andover, MA) in carrying case with accessories including three batteries, two battery chargers, dark adaptation cuvettes, computer communications cable, IBM interface program diskette, and instruction manual
- Spare parts/tools for fluorometer (including 4A slow blow fuses, replacement 35-watt halogen bulb, and replacement cuvette gaskets)
- 10-gallon plastic tote with lid (for equipment transport in the field)
- Field-portable computer
- Light meter (Model LI-190B, LI-COR, Inc., Lincoln, NE) with quantum sensor, case, and instruction manual
- 3 1/4" diskettes
- Soil pH/moisture probe
- Soil thermometer, capable of reading in degrees Celsius
- Ambient air thermometer, capable of reading in degrees Celsius
- Survey flags, flagging tape, stakes and hammer
- Resealable plastic bags (for temporary storage of detached leaves), optional
- Paper towels (to clean/dry off leaves when dusty or wet and for storage of detached leaves when moistened)
- Logbook(s) and field data sheets
- Weatherproof markers

6.0 REAGENTS



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 5 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

This section does not apply to this SOP as no reagents are required for this procedure.

7.0 PROCEDURE

7.1 Site Preparation

Prior to chlorophyll fluorescence measurement, the site will be vegetatively characterized using ERT/SERAS SOP #2038, Vegetation Assessment Field Protocol and species of interest chosen. Select identical or highly similar microhabitats (particularly in terms of light/radiation influx, moisture regime, nutrition, disease incidence, soil/sediment type, and pH) so that comparisons of data between plots can be made. If there are a number of stress factors that will affect chlorophyll fluorescence, data collected from these plots will still be useful provided the test plots only differ from each other by the factor being evaluated. Select individual plants within plots to be sampled randomly. Mark and number each plant with a flag, stake, or flagging tape. A quantum sensor and a soil pH probe are used to aid in the determination of similar microhabitats. Estimates can be made for the amount of shade from trees, other plants and permanent structures during an average day for each plot.

7.2 Instrument Set-up

As a prerequisite to setting up the instrument and using this SOP, the operator must read the CF-1000 instruction manual.⁽¹¹⁾ The instrument is set-up in the following manner:

1. Mount the fiber optic cable in the connector on the front of the instrument.
2. Check calibration with the fiber optic cable, (refer to instruction manual).⁽¹¹⁾ This calibration check should be performed at the beginning of each day and its completion recorded.
3. Set up the file in which you intend to store data (through the memory function), prepare for a test operation ("test" meaning chlorophyll fluorescence measurement). Select appropriate data save mode. It is advisable to select "data save" as this will instruct the instrument to automatically save the fluorescence measurement data. Shut off the power (to save battery power until the tests may be run).

7.3 Leaf Selection

Leaves for measurement are to be representative of the species at the locations examined and must be selected accordingly. Select leaves with the following criteria:

- Leaves are non-senescing and from the same positions within the plant so that chlorophyll fluorescence measurements are comparable and representative. The developmental stage of chosen leaves must be consistent throughout the evaluation. When possible, use the most recent fully developed leaves.
- Select three or more leaves for each plant to be evaluated so that within-plant variation may be discerned. When practical, mark each leaf to be sampled with flagging tape.
- It is imperative that leaves selected have not been exposed to environmental stresses or anthropogenic stress factors (other than the factors being evaluated) that those leaves in other



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 6 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

plots have not been exposed. Chosen leaves should possess no substantial pubescence or visible anthocyanin pigmentation not occurring in all leaves.⁽¹²⁾ The presence of substantial leaf hair will interfere with fluorescence measurements and the presence of the reddish pigment anthocyanin often indicates exposure to excessive light conditions.

In addition, leaves that are adapted to sunny or shaded habitats show distinct differences in their fluorescence traces.⁽¹⁰⁾ Therefore, either sun or shade leaves must be selected, not a combination of both.

7.4 Dark Adaptation

A dark adaptation period, where the portion of the leaf to be measured is kept in darkness, is necessary to insure that the energy in photosystem II is at the baseline level.

The following steps are performed to dark adapt leaves:

1. Affix dark adaptation cuvettes to the leaves. Cuvettes are attached such that no major veins or holes occur in the measurement area covered by the cuvette. It is important that the same side of the leaves be evaluated, as variation of fluorescence measurements have been found between the upper and lower sides of the leaf.⁽¹²⁾
2. Leave the cuvette in place for the required dark adaptation period. A dark adaptation period of 5 - 10 minutes is usually sufficient to dark adapt leaves prior to measurement. However, if incident light reaching the leaves is greater than 500 micromoles of photons per square meter per second ($\mu\text{mol photons/m}^2\text{s}$), a longer dark adaptation time may be necessary to achieve a baseline energy state in the leaves' photosynthetic apparatus. Test for consistency in fluorescence parameter measurement results on a leaf starting with a dark adaptation time of 10 minutes. The required dark adaptation period will often be less than 30 minutes.

It is important that a good light-tight seal is formed between the cuvette and the leaf. This may be a concern, particularly on curved leaves such as Typha (cattail). Leaves should be wiped clean with a damp paper towel if they are wet, dusty, or dirty before affixing a cuvette. When cuvettes are attached to the leaf, a wooden stake may be necessary to provide vertical support so that the weight of the cuvette on the leaf does not damage the leaf or contaminate the cuvette with soil/sediment.

7.5 Light Level Determination

For each plant species to be evaluated, the proper light intensity must be determined. This test is to insure that the excitation from the supplied light is strong enough to reach the maximal fluorescence, F_m , a key fluorescence parameter, (Appendix A).

A photon flux density ($\mu\text{mol/m}^2\text{s}$) of saturating light is necessary for the proper estimation of F_m . Too low a photon flux density and the light is not strong enough to keep all electron acceptors of photosystem II in a reduced state at the P-peak, resulting in an underestimation of the true value for the ratio of variable fluorescence to F_m (F_v/F_m , Appendix A).⁽¹⁾ In this situation, the instrumentation may



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 7 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

Display the message that too low a light level was used to determine a value for F_m . A photon flux density that is too high will overload the instrument detection system.⁽¹¹⁾ In this instance the instrument will display an appropriate error message.

For each species sampled, test varying light intensities on one leaf sample with appropriate dark adaptation times to locate the range of light intensities at which the F_v/F_m ratio is constant. For at least one species (Zea mays), this ratio appears to be constant over a fairly wide range.⁽¹¹⁾

7.6 Sample Time Determination

The length of sample time will dependent upon the species to be evaluated as the kinetics may differ among species. A twenty-five second sample time will normally be used in order to view and calculate key fluorescence parameters. Two hundred seconds or more should be used if an estimate of the terminal fluorescence level value (T) is desired. A sample time of twenty-five to fifty seconds will usually be sufficient to provide a recording in which the shape of the fluorescence curve can be visualized.

7.7 Ambient Conditions Measurement

The following ambient environmental conditions are recorded on specialized field data sheets (Appendix B):

- Photosynthetically active radiation is measured using a quantum sensor with sensor probe at plant height at the beginning and ending of measurements for each plot. If the PAR is highly variable due to cloudy conditions, PAR readings are made intermittently during the fluorescence measurement period. IT IS IMPORTANT TO HAVE THE QUANTUM SENSOR RE-CALIBRATED BY THE FACTORY EVERY TWO YEARS, to insure the accuracy of the readings.
- Soil/sediment pH and moisture condition are determined with a hand-held soil pH/moisture probe.
- Soil/sediment temperature in $^{\circ}\text{C}$ is determined with a soil thermometer.
- Air temperature in $^{\circ}\text{C}$ is determined with a thermometer.
- General weather conditions are noted.
- Characteristics of the soil/sediment type should be visually determined. Ideally, grain size determinations would have been made on soil/sediment samples collected during contaminant level determination prior to plant plot selection.

7.8 Fluorescence Measurement

Notes on the plants being measured must be kept on the specialized field data sheets (Appendix B). Information noted will include the species, plot number, and individual plant-leaf numbers. The associated CF-1000 memory file and sample numbers for fluorometry parameters and the associated file and trace numbers for traces must be recorded on these data sheets as well.



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 8 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

After choosing the appropriate test settings, and when the leaf has been appropriately dark adapted, the free end of the fiber optic cable is inserted into the dark-adaptation cuvette (still attached to the leaf) through the shutter mechanism. Perform the measurement by selecting the "start" in test mode. When the measurement is finished, view the trace as a quality control check and save it if no error messages are displayed. Also, save the measurement data if the automatic data save option is not already on. Measurements for which the instrument gives an error message or show suspect (atypical and non-reproducible) traces for species tested should not be used. The dark adapting cuvette should be left in place and chlorophyll fluorescence re-measured after the appropriate dark adaptation period.

Once a measurement on a leaf is complete, move the cuvette to another leaf that requires dark adaptation. Continue to perform fluorescence measurements, as necessary. Download data onto a computer and replace the battery when appropriate.

The instrument manufacturer indicates that the CF-1000 can hold approximately 2048 sample data and 32 traces before the memory of the instrument becomes full and will no longer accept any data. The instrument display will indicate this condition. When this occurs, data or traces must be deleted or overwritten. Traces can be deleted individually, but whole files of fluorescence parameter measurements (not individual sample data) must be erased from the memory. REMEMBER to save a copy of the desired data onto specialized field data sheets (Appendix B) and into computer files before erasing the data from the instrument.

7.9 Downloading of Stored Measurements

The stored measurements can be downloaded into computer files in the following currently available options:

- Send one file to computer
- Send all files to computer
- Send a trace to the analog port

The manufacturer is presently working on software that will make it possible to send traces to a computer. This software will be available during the second quarter of 1992.

Refer to the manufacturer's instruction manual for procedures on downloading data to a computer.

7.10 Data Interpretation

Key fluorescence parameters measured by in vivo chlorophyll fluorescence describe the fluorescence trace and are briefly explained in Appendix A. Basic interpretation of some of these parameters is described below. In depth explanations and theory behind this technique may be found in the literature.

When a plant is healthy and sufficiently dark adapted (to allow oxidation of all electron acceptors), and when the influx of light is of sufficient intensity, F_v is large in relation to the initial fluorescence (F_o), thus the F_v/F_m ratio will be high (approaching one).⁽¹⁾ If fluorescence is quenched during the rise phase, F_v will be low or absent, and the F_v/F_m ratio will be low (approaching zero). When the peak, or F_m , is reached, fluorescence declines even though the



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 9 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

primary electron acceptor continues to be reduced. This is caused by additional complex combinations of fluorescence quenching⁽¹³⁾ (briefly described in Appendix A).

Each species has its own constant F_v/F_m ratio, (at least in theory). Environmental stress factors that affect Photosystem II (PSII, refer to Appendix A) cause a decrease in this ratio.⁽¹⁴⁾

7.11 Battery Operation

Before performing the first measurement of the day and between within plot measurements, check the charge of the battery. This can be accomplished by selecting "DIAGS" from the "Utilities" menu.

Each fully-charged battery should last for four (4) hours of continuous operation. If the battery voltage falls below 10.8 Volts, a warning message will appear on the display indicating the need for battery replacement. Replace the battery at this time with a spare, charged battery so that proper operation may continue and no loss of data is experienced. It is recommended that data be downloaded to a computer prior to replacement of the battery. The battery that was replaced should be recharged as soon as possible in order to keep it charged for use and to prevent a state of deep discharge.

The manufacturer recommends recharging of discharged batteries for one full night (8 hours) to insure complete charging. Always charge the CF-1000 the night prior to use to assure that the battery is fully charged.

There are several warnings from the manufacturer regarding battery operation:

- The instrument may be connected to a 120 Volt electrical outlet, using a charger connection. This feature provides optimum battery life between applications and maintains a fully charged battery at constant voltage. The warning supplied in this instance is that the charger must be plugged into the wall outlet prior to connection with the instrument.
- Do not leave the battery in a discharged state for several weeks as it may go into a state of deep discharge. If this occurs, the battery will most likely require replacement. The manufacturer suggests that batteries be charged fully at least every 2 to 3 months to prevent this deep discharge condition.
- Do not use any other battery charger than the one(s) provided by P. K. Morgan Instruments, Inc. for this purpose. Some other battery chargers charge at higher rates and will cause damage to the battery and possibly to the instrument.

7.12 Trouble-Shooting and Problem Prevention

If the instrument display does not appear when the power button is pressed, the battery may be faulty and/or drained of all of its power. Replace the battery with a charged battery and retest display. If the battery appears fully charged and all connectors are in place, the fuse may need to be replaced. If it is neither the battery nor the fuse, contact the manufacturer.



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 10 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

To avoid undesirable light penetration through faulty sealing between the dark adaptation cuvettes and leaves one must keep the cuvettes properly aligned, (as cuvettes may require alignment by hand after several uses) and periodically check the cuvette gaskets for need of replacement.

Error messages may occur on the instrument screen. Refer to the instrument instruction manual for explanation and remedies.

Keep the calibration and operation ports free from dust and dirt.

Do not store fiber optic cable tightly wound, this may cause the fibers to break. Store this cable with only one or one and one-half loops in it.

8.0 CALCULATIONS

The following calculations apply to the analysis of fluorescence kinetics. To extract the key parameters of initial fluorescence (F_o) and F_m from the hard copy of the trace, a ruler is placed along the line of the fast rise. F_o is determined as the point of inflection from the ruler. F_m is the total vertical distance from the baseline to the highest point (the P-peak) on the curve before the slow decline. T , or the terminal fluorescence level, is the vertical distance from the baseline to a point on the chart where fluorescence has reached a constant level after the slow decline (Appendix A, Figure 2). Calculations are made as follows:

$$F_m - F_o = F_v$$

F_v/F_m ratio indicates photosynthetic function and is the indicator most often used when assessing effects of hazardous waste.

9.0 QUALITY ASSURANCE/QUALITY CONTROL

The following Quality Assurance/Quality Control (QA/QC) procedures apply:

1. All data must be documented on field data sheets or within site logbooks.
2. Measurements must be made in no contaminated reference sites at a rate of at least 20% of the number made in the contaminated areas.
3. Ambient environmental conditions and any calibrations associated with the instruments must be recorded.
4. Chlorophyll fluorescence traces must be recorded and saved for all samples evaluated.

10.0 DATA VALIDATION

The data will be generated and reviewed according to the QA/QC considerations listed in Section 9.0. Plants will be sampled randomly within plots. Three leaves per plant will be sampled and data will be statistically analyzed. At least 20% of the recorded traces will be analyzed to check for agreement with the instrument's calculation of F_o , F_m , F_v , and $t_{1/2}$ (half rise time from F_o to F_m). If these hand calculations from trace recordings are not within $\pm 10\%$ of the instrument calculations, all calculations must be made from the spectral recordings.



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 11 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

11.0 HEALTH AND SAFETY

When working with potential hazardous materials, follow U.S. EPA, OSHA, and corporate health and safety procedures.

12.0 REFERENCES

- 1) Kapustka, L. A., M. Reporter, with contributors: M. Gordon, J. Fletcher, S. Klaine, and D. Miles, "Evaluating exposure and ecological effects with terrestrial plants: Proceedings of a workshop for the U.S. EPA Exposure Assessment Group," U.S. EPA Region 10, Seattle, WA August 28, 1991.
- 2) Powles, S. B., "Photoinhibition of photosynthesis induced by visible light," Annual Rev. Plant Physiology, Vol. 35, pp.15-44, 1984.
- 3) Smillie, R. M. and S. E. Hetherington, "Stress tolerance and stress-induced injury in crop plants measured by chlorophyll fluorescence in vivo," Plant Physiology, Vol. 72, pp. 1043-1050, 1983.
- 4) Oquist, G. and R. Wass, "A portable, microprocessor operated instrument for measuring chlorophyll fluorescence kinetics in stress physiology," Physiologia Plantarum. Vol. 73, pp.211-217, 1988.
- 5) Miles, D., "The role of chlorophyll fluorescence as a bioassay for assessment of toxicity in plants," American Society for Testing and Materials, Standard Publication 1091 - 1990, pp. 297-307, 1990.
- 6) Li, E. H. and C. D. Miles, "Effects of cadmium on photoreaction II of chloroplasts," Vol. 5, pp. 33-40, 1975.
- 7) Samson, G. and R. Popovic, "Use of algal fluorescence for determination of phototoxicity of heavy metals and pesticides as environmental pollutants," Ecotoxicology and Environmental Safety, Vol. 16, pp. 272-278, 1988.
- 8) Homer, J. R., R. Cotton, and E. H. Evans, "Whole leaf fluorescence as a technique for measurement of tolerance of plants to heavy metals," Oecologia (Berl.), Vol. 45, pp. 88-89, 1980.
- 9) Renger, G. and U. Schreiber, "Practical applications of fluorometric methods to algae and higher plant research," Chapter 19 in Light emission by plants and bacteria, pp. 587-615, 1986.
- 10) Briantais, J. M., C. Verrotte, G. H. Krause, and E. Weis, Chapter 18 In: Light emission by plants and bacteria, 1986. pp. 575-.
- 11) P. K. Morgan Instruments, Inc. "CF-1000, Chlorophyll Fluorescence Measurement System, Instruction Manual," Version 1.02, P.K. Morgan Instruments, Andover, MA, 60 pages, August 1, 1991.
- 12) Bjorkman and Demming, "Photon yield of O₂ evolution and chlorophyll fluorescence characteristics at 77K among vascular plants of diverse origins," Planta, Vol. 170, pp. 489-504, 1987.
- 13) Walker, D. A., "Some aspects of the relationship between chlorophyll a fluorescence and photosynthetic carbon assimilation," In: H. K. Lichtenthaler (ed.) Applications of Chlorophyll Fluorescence, Kluwer Academic Publications, Dordrecht, the Netherlands, pp. 13-20, 1988.



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 12 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

- 14) Krause, H. G. and R. Weis, "The photosynthetic apparatus and chlorophyll fluorescence: An introduction," In: H. K. Lichtenthaler (ed.), Applications of Chlorophyll Fluorescence, Kluwer Academics Publications, Dordrecht, the Netherlands, pp. 3-11, 1988.



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 13 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

APPENDIX A
Chlorophyll Fluorescence and Key Parameters
SOP #2031
November 1994



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 14 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

Chlorophyll Fluorescence and Key Parameters

The theory behind the use chlorophyll fluorescence and the significance of the key chlorophyll fluorescence measurement parameters is offered in the following discussion.

Photosynthesis is the transfer of light energy ideally leading to the production of carbon-based molecules such as glucose. In actuality, not all of the light energy that is absorbed finds its way into carbon-based molecules. When an electron is boosted to a higher energy level, it is unstable and must release its energy and return to the ground state. When light excites chlorophyll molecules in intact plants or chloroplasts, these molecules will release its energy in one of three ways: 1.) the energy is captured in chemical bonds through photosynthesis, 2.) the energy is dissipated as heat, or, 3.) the energy is emitted as fluorescence (light energy). Fluorescence reflects the primary processes of photosynthesis since the fluorescence is inversely related to the energy that is used to fix carbon-based molecules. Thus, the measurement of chlorophyll fluorescence can be used as a tool to evaluate ecologically significant environmental stress.

Figure 1 illustrates the relationships between fluorescence emission and other photosynthetic pathways. To understand the chlorophyll fluorescence process, it is helpful to understand the way in which the photosynthetic system works. Photosynthesis occurs in the thylakoid membranes within the chloroplast of a plant cell and involves light-absorbing pigments such as chlorophyll. These pigments are part of two interacting photochemical systems, photosystem I (PSI) and photosystem II (PSII). The chain of light-initiated reactions begins in PSII. Light energy of wavelengths between 400 and 700 nanometers (nm) is absorbed by PSII antenna pigments and causes a special reactive chlorophyll molecule to become excited. As the electrons continue to be boosted, they are passed to several electron acceptor molecules (the primary one being Q_A) then through a series of electron carriers to the PSI pigment complex. Energy from the electrons is transferred to chemical energy in the formation of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and free inorganic phosphate. When the electrons reach the pigments of PSI, a special reactive chlorophyll molecule in the PSI pigment system becomes excited and passes high energy electrons to the PSI electron acceptor. From the PSI primary electron acceptor the electrons are again passed down in energy level through which nicotinamide adenine dinucleotide phosphate (NADP) is reduced (as the terminal electron acceptor of the light reactions) and along with ATP provides energy to drive the formation of carbon molecules in the dark reactions.

The typical in vivo chlorophyll fluorescence curve is illustrated in Figure 2. This figure shows the typical kinetics and intensity of chlorophyll fluorescence for chloroplasts of leaves after sufficient illumination. For electrons to be accepted, a molecule must be in an oxidized state. After a molecule accepts an electron, the molecule becomes reduced. When light strikes the leaves of a healthy plant with oxidized electron acceptors (a plant that has been in the dark, for instance), there is an initial emission of fluorescence (F_0) from the antenna pigments of PSII. When the electron acceptors become "backed up" with electrons, chlorophyll fluorescence increases to a maximum level (maximal fluorescence, F_m) at the P-peak. The rise from F_0 to F_m is equal to the variable fluorescence (F_v). However, chlorophyll fluorescence is dissipated within several seconds by photochemical (q_Q) and energy-dependant non-photochemical (q_E) quenching.⁽¹⁾ Photochemical quenching exists when the primary electron acceptor passes electrons on to the secondary electron acceptor and can now receive electrons again. Energy-dependent non-photochemical quenching is related to the proton gradient across the thylakoid membrane that exists due to light.

Terminology used in describing fluorescence induction transients is discussed by Lavorel and Etienne.⁽²⁾ The events discussed above corresponding to the fast rise in chlorophyll fluorescence, where F_0 is also called O or "origin" and F_m equals P or "peak". Between O and P, there is an intermediate level (I) followed by a slight dip (D). After P, there is a slow phase characterized by a decline to the quasi-steady state (S), a small rise to a secondary maximum (M) and a final decline to a constant, terminal level (T)(Figure 2). F_v/F_m measurements represent only the early



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 15 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

Photosystem II activities. The second part of the curve represents extremely complex physiological functions of both Photosystems.

References

- (1) Krause, H. G. and R. Weis, "The photosynthetic apparatus and chlorophyll fluorescence: An introduction," In: H. K. Lichtenthaler (ed.), *Applications of Chlorophyll Fluorescence*, Kluwer Academics Publications, Dordrecht, the Netherlands, pp. 3-11, 1988.
- (2) Lavorel, J. and A.-L. Etienne, "In vivo chlorophyll fluorescence," In: J. Barber (ed.), *Primary Processes of Photosynthesis*, Elsevier/North Holland Biomedical Press, Amsterdam, pp. 203-268, 1977.



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 16 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

APPENDIX B
Figures
SOP #2031
November 1994



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 17 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

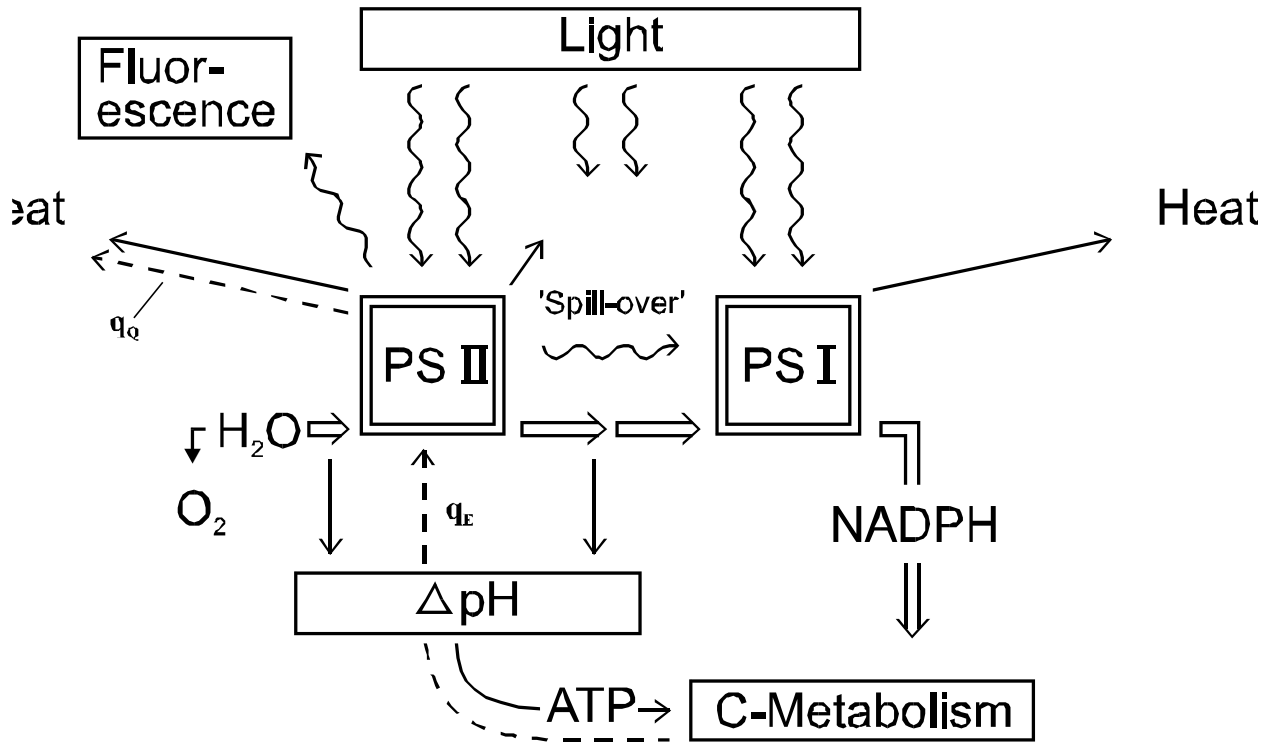


FIGURE 1. Schematic illustrating relationship of fluorescence emission to other photosynthetic pathways. Incoming light energizes the pigment complexes of photosystem II (PSII) and this energy is 1.) used in the production of ATP and NADPH for the production of carbon-based molecules, 2.) emitted as heat, and, 3.) emitted as light (fluorescence). This diagram also shows the relationship of fluorescence quenching: photochemical (q_p) and energy-dependant non-photochemical (q_n) quenching. Adapted from Krause, H. G. and R. Weis, "The photosynthetic apparatus and chlorophyll fluorescence: An introduction," In: H. K. Lichtenthaler (ed.), Applications of Chlorophyll Fluorescence, Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 3-11, 1988.

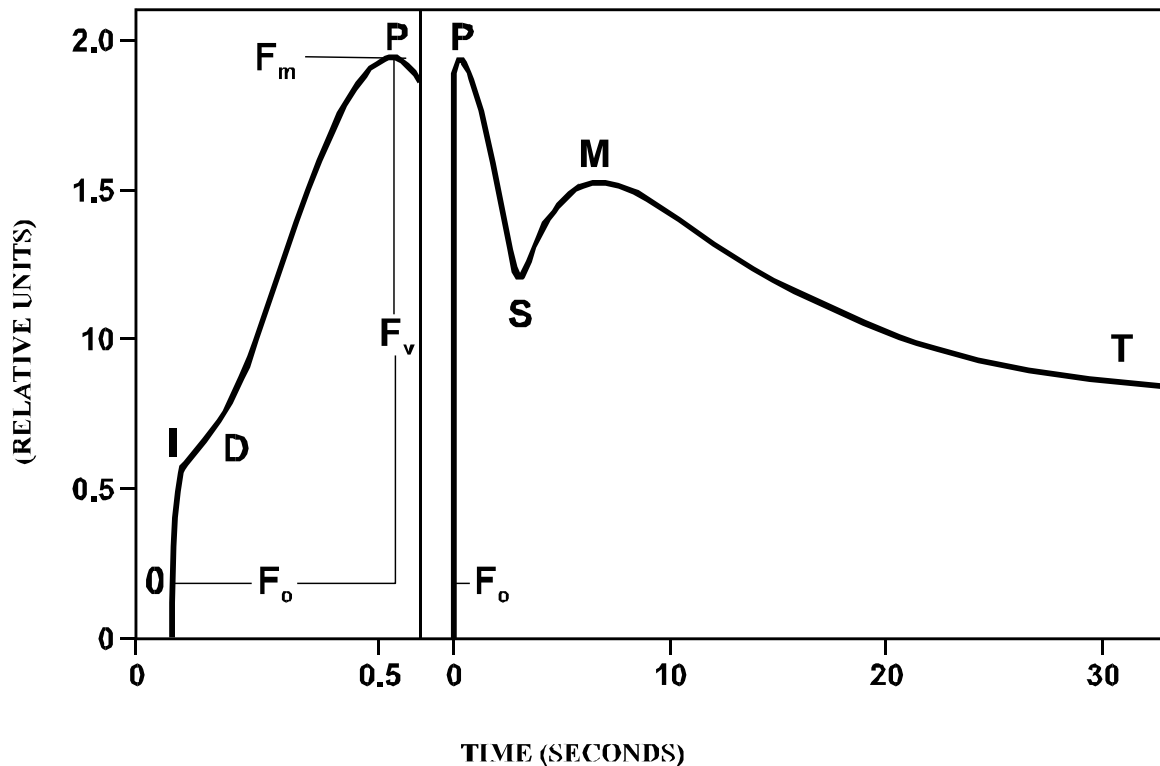


STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 18 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

FIGURE 2. Typical chlorophyll fluorescence curve.



The illustrated fluorescence parameter are defined as follows:

O = Point of initial fluorescence	P = Peak
I = Intermediate fluorescence	S = Quasi-steady state fluorescence
D = Dip	M = Secondary maximal fluorescence
F ₀ = Initial fluorescence	T = Terminal fluorescence
F _v = Variable fluorescence	F _m = Maximal fluorescence

Adapted from Oquist, G. and R. Wass, "A portable, microprocessor operated instrument for measuring chlorophyll fluorescence kinetics in stress physiology," *Physiology Plantarum*. Vol. 73, pp.211-217, 1988 and Kaputska, L. A., M. Reporter, with contributors: M. Gordon, J. Fletcher, S. Klaine, and D. Miles, "Evaluating exposure and ecological effects with terrestrial plants; Proceedings of a workshop for the U.S. EPA Exposure Assessment Group," U.S. EPA Region 10, Seattle, WA, August 28, 1991.

* The timing involved will vary depending upon the species evaluated. The time for fluorescence to reach the terminal level may be longer or shorter than that illustrated.



STANDARD OPERATING PROCEDURES

SOP: 2031
PAGE: 19 of 20
REV: 0.0
DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

APPENDIX C
Specialized Field Data Sheet
SOP #2031
November 1994



STANDARD OPERATING PROCEDURES

SOP: 2031
 PAGE: 20 of 20
 REV: 0.0
 DATE: 11/16/94

In Vivo Chlorophyll Fluorescence Measurement

SITE NAME: CHLOROPHYLL FLUORESCENCE DATA SHEET

REAC, Edison, NJ

SAMPLERS: U.S. EPA Contract: 68-C4-0022 PAGE NO.:

PLOT NO.	SPECIES	PLANT NO.	TIME OF DAY	CF-1000 NO.	TRACE NO.	PAR* INTENSITY (fmol/m²s)	NOTES

* PAR = Photosynthetically active radiation

COMMENTS: _____