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Construction of Advanced Fluorescence Systems for Automatic Measurements of Phytoplankton Biomass, Physiology, and Photosynthetic Rates aboard R/V Araon

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본 보고서를 “서남극 아문젠해역 생태계와 해양순환 변동 연구”과제의 위탁연구“아라온 탑재 식물플랑크톤 광합성생리특성 측정장비 개발”과제의 최종보고서로 제출합니다.

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Summary

The purpose of this project was to construct two miniaturized bench-top instruments, called **F**luorescence **I**nduction and **Re**laxation (mini-FIRe) Systems, for measurements of the abundance and physiological status of phytoplankton in the ocean. One FIRe instrument is design for discrete sample analysis; the second one – for continuous underway measurements in a flow-through cuvette. These instruments have been installed aboard R/V ARAON and will be used for basic research at KOPRI laboratories. In contrast to previous FRRF and FIRe fluorometers invented and developed by the Rutgers team, the new instruments exhibit enhanced (ca. 10x) sensitivity and provide a greater quantity of physiological parameters in real-time. The extreme sensitivity of the new instruments makes them invaluable for field work in the open ocean.

1. Research contents

1.1. Assessment of the photosynthetic activity of phytoplankton and other photosynthetic organisms using Variable Fluorescence Technique

Rapid and non-destructive assessment of the physiological state of photosynthetic organisms relies on the use of Fast Repetition Rate Fluorometry (FRRF) and its technological successor Fluorescence Induction and Relaxation (FIRe) technique. These technologies have been invented and developed by the Rutgers team. The basic approach for assessment of the viability of the photosynthetic organisms relies on the measurement and analysis of chlorophyll "variable fluorescence" profiles, a property unique to the photosynthetic machinery (reviewed by Falkowski et al, 2005). The "variable fluorescence" technique relies on the relationship between chlorophyll fluorescence and the efficiency of photosynthetic processes and provides a comprehensive suite of fluorescent and photosynthetic parameters of the organism. The optical measurements are sensitive, fast, non-destructive, and can be done in real time and *in situ*.

The principles of this patented methodology and realized instrumentation are established in peer-reviewed literature (Falkowski and Kolber 1995; Kolber at al., 1998; Gorbunov et al., 2000, 2001; Gorbunov and Falkowski 2004). Originally developed for studying phytoplankton in the water column, the FRR technique provided unprecedented information about the functioning of phytoplankton communities and the impact of environmental factors controlling primary productivity in the ocean (e.g., Falkowski and Kolber 1995; Falkowski and Raven 2007; Behrenfeld et al., 1996; Coale et al, 2004; Falkowski et al, 2004). The use of bench-top and submersible FRR and FIRe fluorometers became an integral part of most biological oceanographic programs in the U.S. and in the world.

1.2. Fluorescence Induction and Relaxation technique

Fluorescence **I**nduction and **Re**laxation (FIRe) technique has been developed to measure a comprehensive suite of photosynthetic and physiological characteristics of photosynthetic organisms (Gorbunov and Falkowski 2005). The FIRe technique is based on recording and analysis, at high temporal resolution, of fluorescent transients induced by a sequence of excitation flashes of light with precisely controlled intensity, duration and intervals between flashes (Fig. 2 and Gorbunov and Falkowski 2005). The measured parameters characterize photosynthetic light-harvesting processes, photochemistry in Photosystem II (PSII), and the photosynthetic electron transport down to carbon

fixation. Because these processes are particularly sensitive to environmental factors, the FIRe technology provides the basis for identification and diagnostics of natural (nutrient limitation, photoacclimation and photoinhibition, thermal stress, etc.) and anthropogenic stressors (such as pollutions).

The FIRe instrument (Fig.1) incorporates a set of ultra-bright Light Emitting Diodes (LEDs) to excite fluorescence from a target organism. To selectively excite different functional groups of phytoplankton, three sets of LEDs are used: blue (450 nm with 30 nm half-bandwidth), green (530 nm with 40 nm half-bandwidth), and orange (590 nm with 30 nm half-bandwidth). The induced fluorescence is recorded using an ultra-sensitive APD detector in the red spectral region (680 nm with 20 nm bandwidth) at the sampling rate of 1 MHz. The LEDs generate microsecond flashes with peak optical power density up to 1W/cm^2 in the sounding volume to ensure fast reduction of PSII reaction centers within a single photosynthetic turnover (< 50 μs).

The FIRe instrument is equipped with an Actinic Light Source (ALS) for measurements of photosynthetic rates as a function of irradiance (i.e., photosynthesis-versus -irradiance curves). The ALS is computer-controlled via the FIRe data acquisition software. Analysis of these photosynthesis-versus-irradiance curves provides the maximum rate of photosynthetic electron transport (Pmax) and the light-saturating parameter (Ek) that are used for modeling the rates of primary production from FIRe fluorescence measurements (Gorbunov and Falkowski 2005).

The optical chamber of the FIRe instrument can accommodate two types of cuvettes or test tubes (12mm quartz cuvettes or 25 mm test tubes, respectively). The smaller cuvettes can be used for analysis of small-volume samples (e.g., in the laboratory experiments). The larger cuvettes are proven to be more convenient for analysis of field sample in the open ocean.

The FIRe instrument includes data acquisition and data processing software packages.

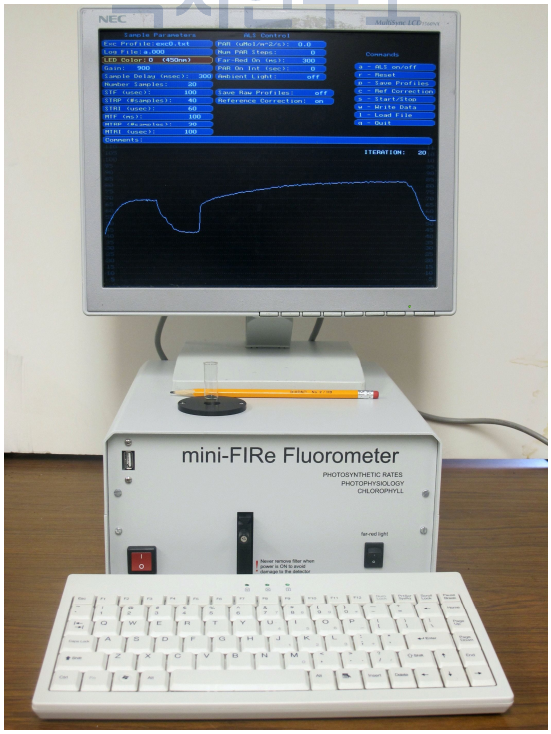

Figure 1. Miniaturized Kinetic FIRe Fluorometer.

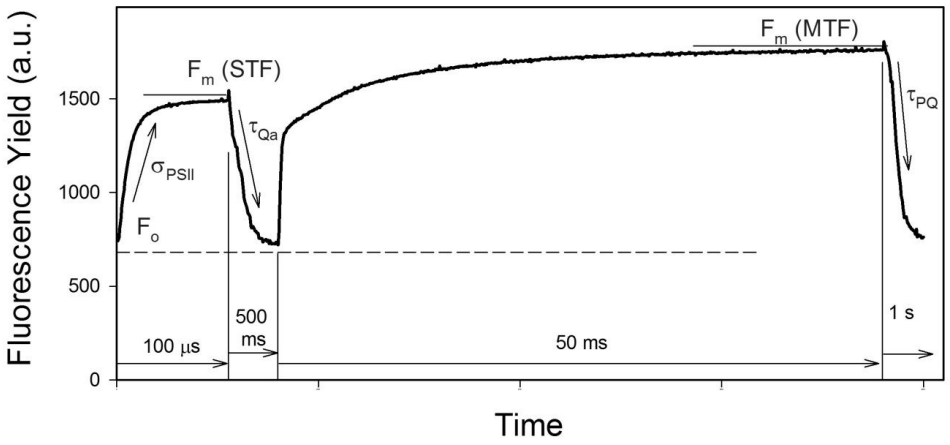

Figure 2. An example of the fluorescence kinetic profile recorded with a FIRe instrument. The kinetics of fluorescence yields is recorded with microsecond time resolution and includes four phases: (1st phase, 100 µs) a strong short pulse of 100 µs duration (called Single Turnover Flash, STF) is applied to cumulatively saturate PSII and measure the fluorescence induction from Fo to Fm(STF); (2nd phase, 500 ms) weak modulated light is applied to record the relaxation kinetics of fluorescence yield on the time scale of 500 ms; (3rd phase, 50 ms) a strong long pulse of 50 ms duration (called Multiple Turnover Flash, MTF) is applied to saturate PSII and the PQ pool; (4th phase, 1 s) weak modulated light is applied to record the kinetics of the PQ pool re-oxidation over a time scale of 1s. Analysis of the Phase 1 provides: the minimum and maximum fluorescence yields (Fo, Fm); the quantum efficiency of photochemical charge separation in PSII, Fv/Fm(STF); the functional cross-section of PSII, σ_{PSII}; and the connectivity factor (p). Phase 2 provides time constants for the electron transport on the acceptor side of PSII (i.e., re-oxidation of the Qa acceptor). Phase 3 provides Fm(MTF) and Fv/Fm(MTF). Phase 4 reveals the time constant for the electron transport between PSII and PSI (re-oxidation of the PQ pool).

1.3. List of parameters recorded with FIRe instrument

- Minimum and maximum fluorescence yields in dark adapted state (F_o, F_m);
- Effective, minimum, and maximum fluorescence yields in light adapted state (F', F_o', F_m');
- Maximum and effective (*) quantum yield of photochemistry in Photosystem II, PSII, (F_v/F_m and $\Delta F'/F_m'$);
- Functional absorption cross sections of PSII (σ_{PSII}) at three wavelengths;
- Efficiency of energy transfer between photosynthetic units ("connectivity factor");
- Time Constants for electron transport on the acceptor side of PSII (Q_a to Q_b, Q_a to Q_b^-);
- Time Constant for photosynthetic electron transport between PSII and PSI;
- Electron Transport Rate, ETR, as a function of irradiance;
- Coefficients of photochemical quenching (q_p) and non-photochemical quenching (NPQ);
- Maximal photosynthetic rate, initial slope, and photosynthetic turnover time (from F versus E curves).

1.4. Specifications for Mini-FIRe Fluorometer System:

- Extreme Sensitivity: 0.005 - 100 mg/m3 of chlorophyll-a (higher concentrations can be sampled by adding a Neutral Density attenuating filter)
- Excitation light sources: blue (maximum emission 450 nm, 30 nm bandwidth), green (maximum emission 530 nm, 40 nm bandwidth), orange (maximum emission 590 nm, 30 nm bandwidth) for selective excitation of different functional groups of phytoplankton.

- Emission detection: 680 nm (chlorophyll-a) and 880 nm (bacteriochlorophyll-a), other wavelengths can be selected using a replaceable emission filter.
- Dimensions: 10 x 5 x 12 inches

The design of the FIRe instrument for continuous underway measurements is similar to that for discrete sample analysis, but this instrument is equipped with a dedicated flow-through cuvette system. In contrast to previously used flow-through cuvettes, the new system is much less susceptible to biofouling. The prototype of such cuvette design has been tested by Rutgers/KOPRI team during the 2013 Araon cruise in the Amundsen Sea.

The FIRe instrument and data collection software package are coupled with a GPS receiver to collect and store GPS data in real-time, together with fluorescence measurements. The FIRe instrument can be connected to an external GPS receiver (e.g., Garmin 18x) or plugged into the ship GPS line.

1.5. Installation of FIRe instruments aboard RV Araon and field tests.

The first FIRe instrument has been delivered to KOPRI in June 2015 and has been used during a 2015 summer Arctic cruise of R/V Araon. The second instrument has been delivered to KOPRI in October 2015. Both instruments will be used during the 2016 cruise of RV Araon in the Amundsen Sea in January –February 2016 (Chief Scientist Dr. S.H. Lee). KOPRI personnel have been trained to conduct measurements, analyze data, and maintain the instruments during prolonged oceanographic expeditions.

1.6. Biophysical Backgrounds of Variable Fluorescence Technique

At room temperature, chlorophyll *a* fluorescence mainly arises from PSII. When the PSII reaction centers are in the open state (with Q_a oxidized), the fluorescence yield is minimal, F_o. When the Q_a is reduced (e.g, by exposure to strong light), the reaction centers are closed and the fluorescence yield increases to its maximum level, F_m. To retrieve F_o and F_m, the FIRe technique records, with microsecond resolution, the induction of fluorescence yields induced by a strong saturating flash of light (~ 100 μs long, called a Single Turnover Flash, STF) (Phase 1 in Fig. 1). The rate of fluorescence induction is proportional to the functional absorption cross section of PSII, σ_{PSII}, whereas the relative magnitude of fluorescence rise, F_v/F_m, is defined by the quantum efficiency of photochemistry in PSII. The shape of fluorescence induction is controlled by the excitonic energy transfer between individual photosynthetic units and is defined by a "connectivity factor", p (Kolber et al. 1998). Thereby, the fluorescence induction is exponential in the absence of energy transfer ($p = 0$) and becomes sigmoidal when p increases to the maximum values of ~ 0.5 to 0.7.

The kinetics of electron transport on the acceptor side of PSII (i.e., Q_a re-oxidation) is assessed from analysis of the fluorescence relaxation kinetics after the STF (Phase 2 in Fig. 1). The fluorescence kinetics consists of several components, because the rate of Q_a re-oxidation depends on the state of the second quinone acceptor, Q_b, which works as a mobile two-electron acceptor:

$$Q_a^- Q_b \rightarrow Q_a Q_b^- \text{ (150 - 200 μs)} \quad (1)$$

$$Q_a^- Q_b^- \rightarrow Q_a Q_b^{=} \text{ (600 - 800 μs)} \quad (2)$$

$$Q_a^- \_ \rightarrow Q_a^- Q_b \rightarrow Q_a Q_b^- \text{ (~ 2000 ms)} \quad (3)$$

The reaction (3) corresponds to the conditions when the Q_b is initially out of its binding site on the D1 protein. Also, a fraction of inactive reaction centers with damaged electron transport may contribute to the slowest component in the relaxation kinetics. FIRe software processes the relaxation kinetics using a 3-component analysis to retrieve time constants for electron transport (i.e., Q_a reoxidation, τ_{Qa}).

The time constant $\tau_{PSII-PSI}$ for the electron transport between PSII and PSI is retrieved from analysis of the fluorescence relaxation kinetics following the Multiple Turnover Flash (MTF, Phase 3 and 4 in Fig. 1). Under most physiological conditions, this time constant is determined by the rate of plastoquinone (PQ) pool re-oxidation and is an order of magnitude slower compared to τ_{Qa}.

Measurement of FIRe fluorescence parameters over a range of ambient irradiances permits one to reconstruct the rates of photosynthetic electron transport, P_f, as a function of irradiance (photosynthesis-versus-irradiance curves) (Kolber and Falkowski, 1993). P_f is proportional to the product of irradiance and the quantum yield of photochemistry measured under ambient light ($\Delta F'/F_m'$). Analysis of these photosynthesis-versus-irradiance curves provides the maximum rate of photosynthetic electron transport (P_{max}) and the light-saturating parameter (E_k). Photosynthesis-versus-irradiance measurements are conducted using a FIRe Actinic Light Source (ALS) which is computer-controlled via FIRe data acquisition software.

2. Expected Impact

2.1. Academic impact

Oceanic ecosystems contribute ~ 50% to the global primary production. Current observations of primary producers on large spatial scales are based on remotely-sensed estimates of Chl a biomass or routine measurements of chlorophyll fluorescence. Incorporation of new FIRe instruments into longer-term monitoring programs provides information about phytoplankton physiology and is important for our better understanding of how environmental factors affect oceanic ecosystems. In addition to field research, the new FIRe instrumentation can be used for basic and applied research in KOPRI laboratories.

2.2. Economic impact

The proposed FIRe systems can be used in a wide range of applications, including:

- Basic and applied photosynthesis research
- Environmental monitoring of phytoplankton and other aquatic plants, as well as terrestrial plants
- Water quality monitoring
- Assessment of primary productivity in aquatic ecosystems
- Assessment of environmental stressors, including pollutions
- Identification and prediction of toxic algal blooms in coastal waters
- Eco-physiology.

3. References

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4. Publications resulted from this project

Lin H., Kuzminov F.I., Park J., Lee S.H., Falkowski P.G., and Gorbunov M.Y. (2016) The fate of photons absorbed by phytoplankton in the global ocean – *Science*, in press.

Park J., M Y. Gorbunov, B. Bailleul, H. Lin, F. I. Kuzminov, P. G. Falkowski, E. J. Yang, SangHoon Lee (2016) - Distinct differences of phytoplankton physiology in two polynyas of the Amundsen Sea, Southern Ocean – in preparation.

Appendix A.

Appendix A and B include brief manual for the FIRe instrument and operational software. More detailed information is provided in the Full Manual provided with the FIRe instruments.

5. Quick Start Manual for FIRe Fluorometer

5.1. Power Requirements

The FIRe System can be powered with any AC voltage ranging from 85 to 250V and can be plugged in directly (without voltage converter) to an electrical outlet anywhere in the world or at any research vessel. Depending on the country, an outlet adapter may be needed.

5.2. Initial Connections and Installations

Connect a power cord, monitor, and keyboard to the connectors on the back panel of the instrument (Fig. 2). Make sure that the emission filter is installed (the removable filter holder slot is on the front panel). To avoid damage to the detector by strong light, never remove filter when the instrument is ON.

Turn ON switches on the front panel of the FIRe and on the monitor.

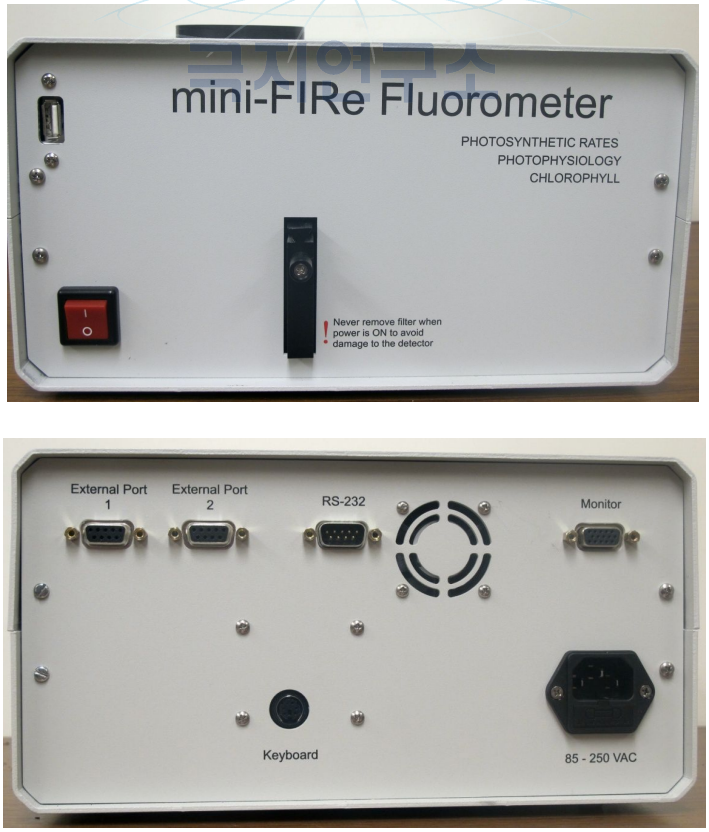

Figure 3. Front and rear panels of the FIRe instrument. (**A**) The front panel includes the main

ON/OFF switch (in the left corner), USB connector, a slot for emission filter holder, and switches for BLUE, GREEN, or ORANGE excitation. The filter holder comes with two interference filters installed – red one (678 nm) for chlorophyll fluorescence detection and IR (880 nm) for bacteriochlorophyll. Other filters can be installed if needed - e.g. for phycoerythrin or phycocyanin fluorescence.

(**B**) The rear panel includes the main power connector (85-250 VAC), VGA monitor connector, serial port (RS-232) connector, PS2 keyboard connector, two External Port connectors, and a cooling fan. The two External Ports are identical – any of these can be used for Actinic Light Source or Fiber Probe. RS-232 can be used to connect a GPS receiver.

Figure 4. Mini-FIRe instrument can accommodate small (12 mm diameter) or large (25mm diameter) cuvettes. While using large cuvettes, the 12mm cuvette Delrin holder must be removed from the FIRe (as shown on the right panel – just rotate the 12mm adapter with your fingers and move it up). Corning 25 mm test tubes with flat bottom can be used and are recommended for field work. Standard test tubes (12mm x 75mm) serve well as small cuvettes. Small cuvettes must be used for measurements of fluorescence versus irradiance curves using an Actinic Light Source (ALS). For most accurate measurements, the cuvette must be filled with at least 40 mm of sample (~4 mL in a small cuvette and ~ 15 mL in a large cuvette).

Appendix B.

6. Software for Running Discrete Samples (program FVIEW)

FVIEW is the data acquisition program used to collect fluorescence profiles on discrete samples. It enables the user to manipulate adjustable parameters and control the data acquisition process.

6.1. Starting a measurement

To run FVIEW, type FVIEW from DOS prompt:

C:\FIRE\FVIEW and press [ENTER]

6.1.1. Choose the name of Log File to store your data

First, you need to select a name of the data file to save your data (Fig. 4).
Use the arrow keys (←,→,↑,↓) to go to the 2nd line of the left menu '**Log File:'** and enter the name of the destination file (i.e., the file where the fluorescence profile is to be saved, e.g., ALGAE1.000).

To enter the file name, press [Space Bar], type the name with extension (e.g., ALGAE1.000), and press [Enter]. The file name must not exceed 8 letters/numbers (DOS requirement) and should have the extension .000. When a fluorescence profile is saved, the extension will increase automatically by one (.001, .002, and so on).

Tip: It is strongly recommended to save the whole series of measurements (e.g., samples collected from different depths at a given field station or a time series) under the same name and extension from 000 to NNN. In this case, the FIRe processing program (FPRO – see below) will be able to run all these files at once that will reduce your time for data processing.

6.1.2. Start/Stop data acquisition

To start a measurement, press the **s** key on the keyboard. The program will start the data acquisition until the preset number of acquisition 'Number Samples:' is reached.
The data acquisition may be stopped by pressing **s** at any time.

Commands: **s** – start or stop;
w – write (save) data file;
q – quit the program.

Note: The command letters are *case sensitive*, so "**S**" and "**Q**" will not work.

If necessary, adjust the GAIN, and restart data acquisition (press **s** to start a new data acquisition).

6.1.3. Adjust Gain

To accommodate a wide range of Chl-a concentrations in natural phytoplankton and laboratory cultures, the electronic gain of the detector unit is adjustable over the range of two orders of magnitude. The **GAIN** (in relative units) can be varied in the range from 0 to 2400 and is selected from the main program menu. We suggest the **GAIN** to be changed with an increment of 200 (i.e., 0, 200, 400,…, 2000, 2400). In this case, the measurements conducted at different GAIN values can easily be compared by using the conversion table in Appendix A. The actual electronic **Gain Value** is a nonlinear function of **GAIN.**

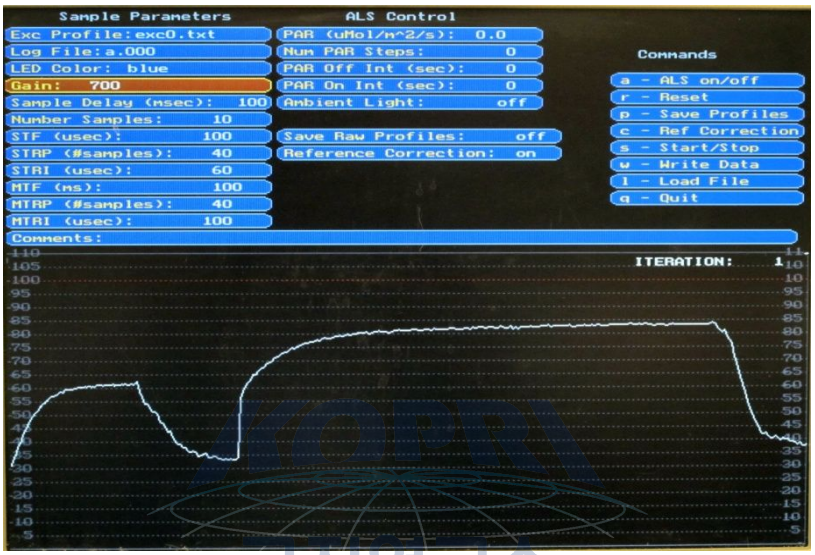

Figure 4. Typical FIRe Fluorescence Profile Acquired with FVIEW Program. Both STF and MTF protocols were used. If MTF is not set (MTF=0 and MTRP=0), only STF Induction and Relaxation will appear in the left part of the screen. GAIN was chosen correctly and the maximum fluorescence level is within the upper 50% or the window and below the red dotted line.

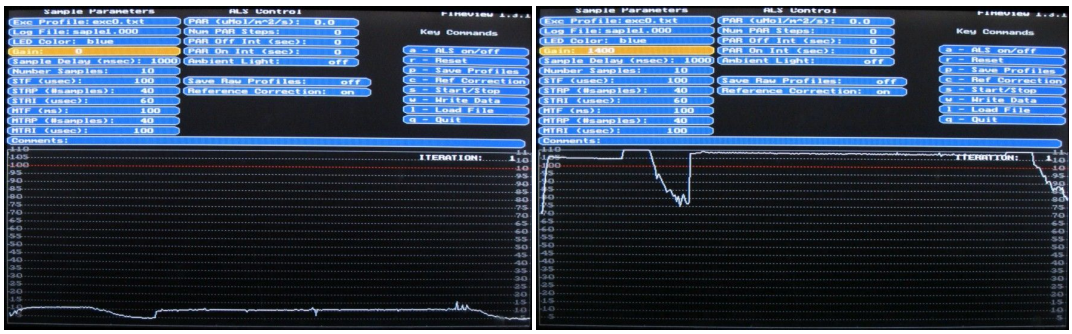

Figure 5. (left) FIRe fluorescence profile with GAIN set too low. (right) FIRe fluorescence profile with GAIN set too high. In the first case the GAIN needs to be increased, in the second – to be decreased.

6.1.4. Save a data file

To save the acquired fluorescence profile, press '**w**' (i.e., **w**rite). The data files (SAMPLE1.000, etc.) will automatically be saved in the same directory from which **FVIEW**

is run (i.e. C\:LAB). When the profile is saved, the extension of the destination file will automatically increase by one (i.e. the next file will be SAMPLE1.001). The program is now ready to conduct the next measurement.

Note: If you do not press '**w**' after completing a measurement it will be lost when a new measurement is performed.

6.1.5. Number of Acquisitions (Number of Samples:)

The number of acquisitions (or '**Number Samples**' in the main menu) allows the user to vary the number of repeated profiles taken for a single fluorescence profile. If the fluorescence signal is low (e.g. oligotrophic waters) relative to the noise, a larger number of acquisitions (e.g., 100) will increase the signal-to-noise ratio and provide better results.

Note that you do not need to wait until the preset number of acquisitions is finished. You may stop acquisition at any time by pressing '**s**'.

6.1.6. Single Turnover Flash

The single turnover flash (**STF**) duration is set to a default value of 100 μs. This duration can be adjusted up or down depending on the shape of the STF induction curve. If the fluorescence profile does not reach saturation plateau by the end of the STF (small σ_{PSII}, e.g. in cyanobacteria), the STF should be lengthened (up to 200-300). If the saturation peak is reached very early in the STF (large σPSII), the STF should be shortened to ensure that multiple turnovers are not induced.

Operational range for STF is between 10 and 300.

The default FIRe protocol utilizes only STF (MTF and MTRP are both set to 0 in the SETUP.txt configuration file). In order to add MTF protocol, you need to change MTF and MTRP (MTF=100 and MTRP=40 will be optimal values for most applications).

6.1.7. Selecting (and changing) the color of excitation light.

The Multi-Color version of the mini-FIRE instrument includes several different color LED excitation sources (e.g., 450 nm, 470 nm, 500 nm, 530 nm, 590 nm). Each color is assigned a number (0,1,2,3,4) in the FVIEW program. To change the color, you should go to "Color:" line in the main menu and select/change an appropriate color by entering the number from 0 to 4.

6.1.8. Adjusting other parameters and choosing an optimal protocol.

The default set of FIRe parameters is chosen to provide optimal sampling protocol for most samples. The next chapter describes in detail all FIRe parameters and when and how they may be adjusted.

6.1.9. Finishing measurements

When you finish, quit the sampling program by pressing '**q'**. Turn the FIRe instrument OFF.

Note: It is strongly recommended to keep the FIRe instrument OFF when it is not used to prolong the lifetime of the detector.

6.2. Processing of Discrete Sample Data (program FPRO)

FPRO is the data analysis program used to process fluorescence profiles acquired with FVIEW program. This program fits the fluorescence profiles with the biophysical model and calculates photosynthetic parameters and fluorescence yields (Fo, Fm, Fv/Fm, Sigma-PSII, and others).

Note: To run the program on a separate PC, copy the program (FPRO.exe), together with the file **EGAVGA.bgi** (a graphic driver). The program does NOT run without this graphic driver. The program FPRO must be stored in the same directory as the data files. Depending on the Windows version, you will need to install a DOS emulator (e.g., DOSBOX) or Windows XP emulator on your computer.

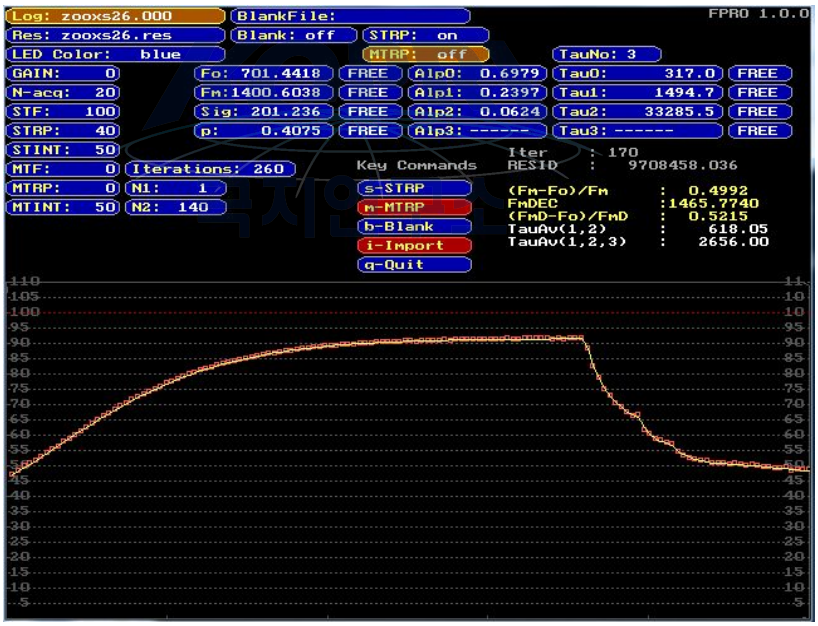

Figure 6. Operation of the FIRe data processing program (FPRO.exe).

From the FPRO main window, enter the name of the first data file to be processed (e.g. SAMPEL1.000) next to the **Log**: field. To enter the file name, press [Space Bar] and when finished typing the name, press [Enter].

To begin processing, hit '**i**' to **i**mport the data file. The program will start processing all the files in the series, that is files that have the same root name with an incrementing suffix such as .000, .001, .002 etc.

The calculated photosynthetic parameters will be automatically saved in an ASCII file with the extension *.RES (e.g., SAMPLE1.RES).

STF Relaxation and/or MTF Relaxation processing may be disabled by using the **s** and **m** commands, respectively. Note that disabling of the STF Relaxation processing also disables the MTF Relaxation processing. Disabling STF Relaxation processing is important during analysis of F_versus_E measurements with an ALS (see "Using Actinic Light Source" below).

Note: If there is a break in the extension number sequence (e.g. A.003 is missing), the program breaks after the last consecutive file (i.e., A.002). Then you have to restart the program and enter the name of the next available file (i.e. A.004).

GAIN correction: FPRO automatically corrects fluorescence yields (Fo, Fm, Fv) for gain. In other words, if you run two samples (e.g., a concentrated one at Gain=0 and a diluted one at Gain=2400) and the fluorescence levels were similar in the FVIEW window, the Fo, Fm values in the results file (*.res) will differ by a factor ~ 100 (proportionally to the difference in chlorophyll concentration).

Gain-corrected Fm values can be used as **a proxy of chlorophyll-a biomass**, as well as for calculations of algal **growth rates** (from changes in Fm over time) is lab experiments.

주 의

1. 이 보고서는 극지연구소 위탁과제 연구결과 보고서입니다.

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