Appendix to

Acute stress in seedlings detected by ultra-weak photon emission

Characterization of Ultra-Weak Photon Emission Measurement Setups and Applications in Environmental Control

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This document presents the setup implementation and its performance evaluation, as well applications in bacterial and seedling's growth. The system is compact and fully automated, with power supply, illumination capabilities and temperature controlled by virtual instrument.

Instrumentation

The measurement system architecture is shown in Fig. 1 as a block diagram consisting of a dark chamber made of stainless steel having a superior chamber for PMT housing and an inferior one fitting a 10-cm diameter Petri's dish to the biological sample, and associated electronics/optoelectronics devices.

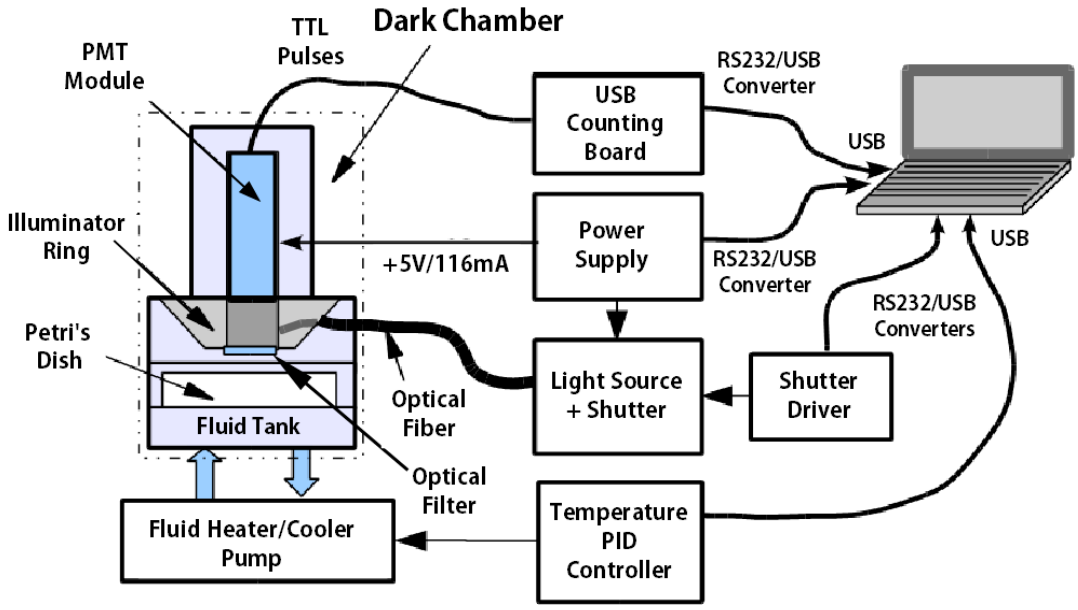

Figure 1. Block diagram of the measurement system.

The whole system is controlled by a LabVIEW® virtual instrument (V.I.) whose front panel is shown in Fig. 2. The V.I. was designed as a state machine controlling the data acquisition and display, auto-saving the data for each desired loop of up to 10 thousand data points.

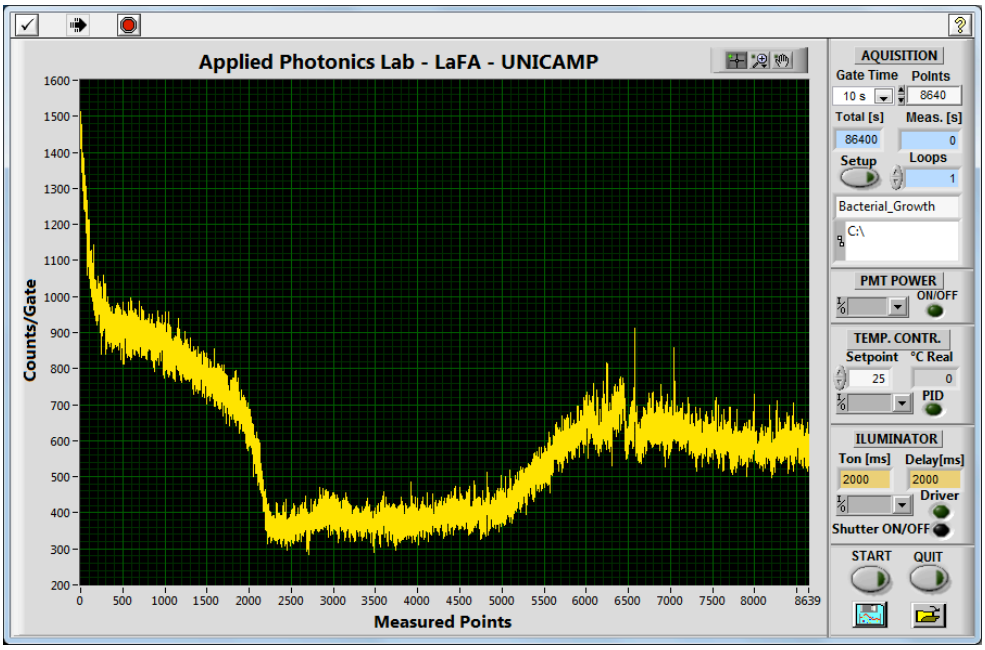

Figure 2. Front panel o data control (example of photon-counts of a bacterial growth test).

The main element of the system is the photomultiplier module (Hamamatsu H7360) being an integrated module comprised by the PMT itself, high voltage power supply and pulse detection and conditioning circuits, having a low dark count noise (17 s^{-1}) and high sensitive photocathode in the visible range [1]. The PMT module is powered by a stable +5V power supply (Agilent E3640A) controlled through RS232 interface by the control program and providing a noiseless and current-limited PMT operation. An USB counting board (Hamamatsu C8855) receives the pulses from the PMT module, integrates them in a time interval selected by the user via control program from 50 microseconds to 10 s, and automatically sends the data to the host PC via USB interface [2].

Sample's bath with temperature control is provided by a circulating fluid loop externally pumped, with a heater/cooler device controlled by a Proportional-Integral-Derivative (PID) controller, operating from -10°C to +50°C (±1°C).

System's illumination capabilities, needed for delayed luminescence experiments, are provided by an external halogen light source guided by fiber optic cable with coupled illumination ring (6.6 cm diameter). Optical filters set can be placed before light coupling into the fiber. A double-blade optical shutter (Uniblitz VS25S2T0) and associated driver module (Uniblitz VMM-D1) are used to control the illumination period and the delay time prior the photon-count to start.

Performance Tests

The background photon emission noise, also known as dark-count noise, generated by environment conditions as temperature, surrounding materials and cosmic-ray, was evaluated for the empty chamber at 5 different temperatures - 10°, 20°, 30°, 40° and 50°C, being measured for 24 hours using integration windows of 10 seconds.

The delayed luminescence generated after stimulation of the dark chamber + sample/dish by an external light source was also evaluated by many series of DL tests using several test conditions as power and period of illumination (white light), and chamber's contents (empty, Petri dish with and w/o solutions - distilled water and, potassium dichromate. For that purpose, three light-exposing periods were tested: 0.5, 2.0 and 10.0 seconds, with inherent electronics delay of 120 ms for the counts. Two different light intensities (54.0 lx and 175.6 lx) could be switched. The integration time was 50 ms, each test taking 90 seconds and repeated 5 times each. Two types of Petri's dish were tested: 10-cm and 6-cm diameter ones, adding always 5 mL of solution when it was the case.

The germination tests used 25 wheat seeds (*Triticum aestivum*) selected randomly from stock preserved in dark, and carefully arranged avoiding light exposure in 6 cm Petri dishes with paper filters and 2 mL of distilled water under stable temperature (20° +/- 1°C). A duplicate test (control) was conducted in a germinating chamber under the same conditions. Germination tests series of 72 hours were used to compare the sensitivity of the new chamber to that of an old one whose sample plate was further away from detector. Parallel tests under identical conditions used a second identical photon-counting chamber for two consecutive 24-hour photon-count tests of samples after spending the first 72 hours in darkness. Another germination test with parallel experiments provided 120 hours of UWPE measurement starting at the germination beginning and at the fourth day one of the two samples were submitted to chemical stress by adding 2 mL of NaCl solution (concentration: 10 g/L). In all germination tests integrating time was 10 s. These tests were checked by germination rates as well as by growth performance, measuring the roots' elongation lengths as recommended by the Organisation for Economic Co-operation and Development (OECD) Guidelines for testing chemicals [3].

UWPE from Escherichia coli and domestic wastewater in three test series are presented and compared to similar tests using the mentioned previous, bigger chamber. Those tests were done according to the standard methods of the American Public Health Association for the examination of water and wastewater [4]. A procedure to determine the growth of coliforms using a commercial chromogenic substrate was used for tests using wastewater samples. The sample was collected from domestic wastewater treatment station and immediately conducted to the laboratorial procedures. Afterwards, the presence/absence of organisms was checked by UV light (365 nm), indicating growth of Escherichia coli in control tests. Additional visualization used the reagent color changes from colourless to yellow - indicating the total coliform growth.

Results and Discussion

Background Noise Evaluations

The results for background (empty chamber) noise are presented in Fig. 3 - histogram plots of noise dependence with temperature inside the dark chamber. It is clear that, as expected, as the temperature increases the dark-count noise increases, but only at 50°C a severe degradation on noise distribution appears. This threshold appears since the photon-count module regularly operates around 30°C. These characterization is worthy when working above 30°C, especially in experiments of bacterial growth, naturally dependent on warm environment.

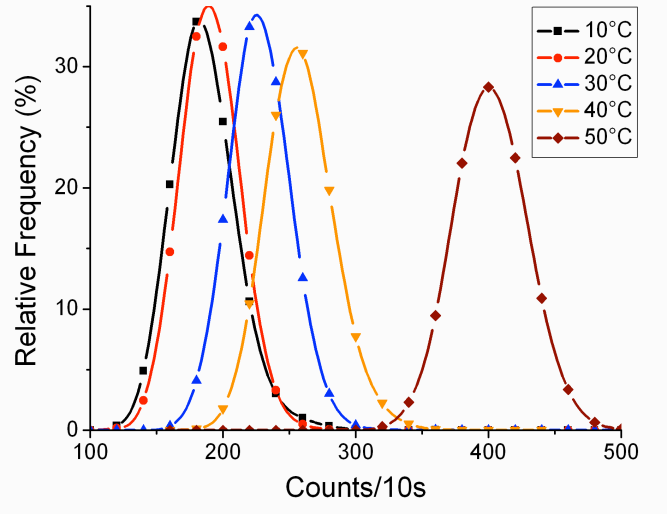

Figure 3. Histograms of the background noise during 24 h for temperatures of 10º, 20º, 30º, 40º and 50º Celsius.

Delayed Luminescence Noise Evaluation

Some of the performed DL tests are shown in Fig. 4 (a-b) for the maximum illumination power (175.6 lx) and minimum illumination power (54.0 lx). Fig. 4 (c) and (d) show the respective final counts after 90 s and peak counts/50ms, in average for 5 successive measurements, for all test conditions. Using data of Fig. 4 it is possible to preview a variety of noise scenarios and so serve as a control table for further tests. The DL peak is affected by the illumination power and duration, but also by adding water or solution to the dish, indicating that some light is absorbed in these new conditions. Water presents higher re-emission (lower absorption) than the $K_2Cr_2O_7$ solution.

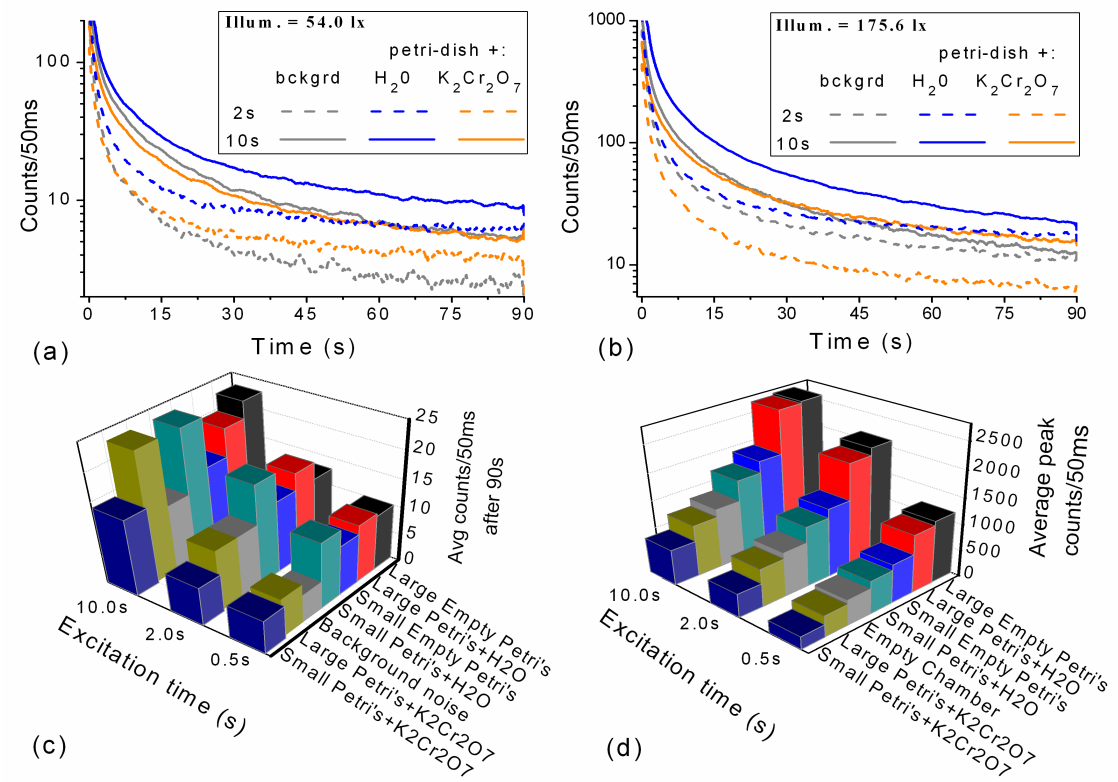

Figure 4: DL curves for noise tests under (a) maximum and (b) minimum illumination power, for excitation time of 2 and 10s; (c) counts after 90s (50ms time window) and (d) DL peak counts (5 tests average).

UWPE in Germination Tests - Wheat Seedlings

Photon-count measurements of wheat seedling's UWPE showed improvement in the signal-to-noise ratio when compared to the previous setup, as shown in Fig. 5 (a) for the 72-hours tests with 25 and 50 wheat seedlings performed in the new chamber compared to similar test with 50 wheat seeds using the old chamber (background noise ~170 counts/10s).

The improvement is related to the smaller sample-to-sensor distance in a three-fold reduction. Fig. 5 (b) shows the data for the 120 hours wheat germination test simultaneously run in two chambers (PMT01 and PMT02), with a stressing solution (NaCl, 10 g/L) added to one of the samples after a period of 48 hours. It is clear the disturbing effect so produced, with a jump in the UWPE counts of the stressed sample when compared to the control one. The tracking of such kind of anomalous UWPE behavior can be applied in environmental monitoring using seedlings as mediator in water/soil quality essays.

Figure 5 (c) shows two rounds of 24 hours germination tests run simultaneously too, but without stress and with photon-count measurements starting at the 3rd day. Each round show different data, as expected in such kind of tests, but in the two curves of the same round the temporal evolution is the same, evolving in perfect synchronism, discounted the inherent PMT gain difference. Fig. 5 (b-c) data were replicated several times and confirm that separated samples have a

common grown rhythm and that parallel UWPE tests can be compared. With so, germination disturbances could be evaluated in real time by photon-count experiments.

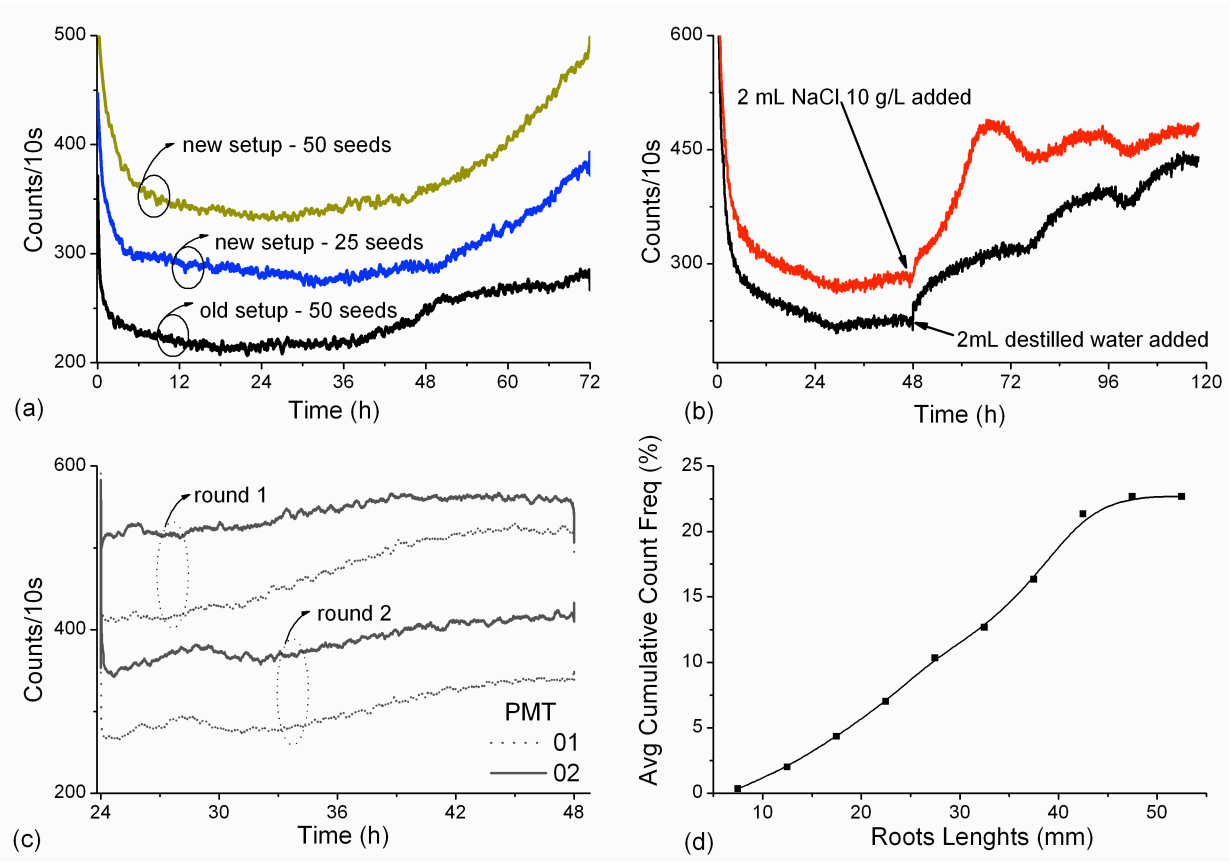

Figure 5. UWPE tests with wheat seedlings: (a) 72-h germination inside dark chamber, comparing old and new setup performance; (b) two simultaneous 120-h tests with NaCl solution added at the 48th hour in one test; (c) two rounds of 24-h (3rd day) tests run simultaneously; (d) germination evaluation for all the tests.

The germination evaluation for all non-stressed tests performed (including not shown here) is shown in Fig. 5 (d) based on root elongation, as recommended by OECD [20] - the average cumulative count occurrence versus root length confirms that germination was successful.

UWPE in Bacteria E. coli Tests

Figure 6 shows a series of tests using coliforms and Escherichia coli growing inside the new dark chamber as well as similar measurements obtained using the old, bigger chamber. The results have even better SNR improvement.

The UWPE patterns reveal the time dynamics related to the microorganisms' growing phases when in culture medium, as described by Carlson and Srienc [23] and also Madigan et al.. [24]. At the initial phase is when the bacterial growth is limited since microorganisms are enzymatically adapting to the substrate (LAG phase). The next phase - the exponential one - microorganisms are in constant growth and use the substrate energy up to 18 hours. Following next is the stationary phase with growth and death rates remaining constant, and the limited nutrient

source starts to decline (after 24h). These classical phases of microorganisms growth briefly described can be tracked by the UWPE measurements plots. Standard tests able to do similar analysis in sanitary control take from 24 to 72 hours - the biophotonic approach can monitor microorganism activity in real-time tests taking about 3 hours. This represents an important result regarding future applications in controlling the efficiency of wastewater treatment facilities.

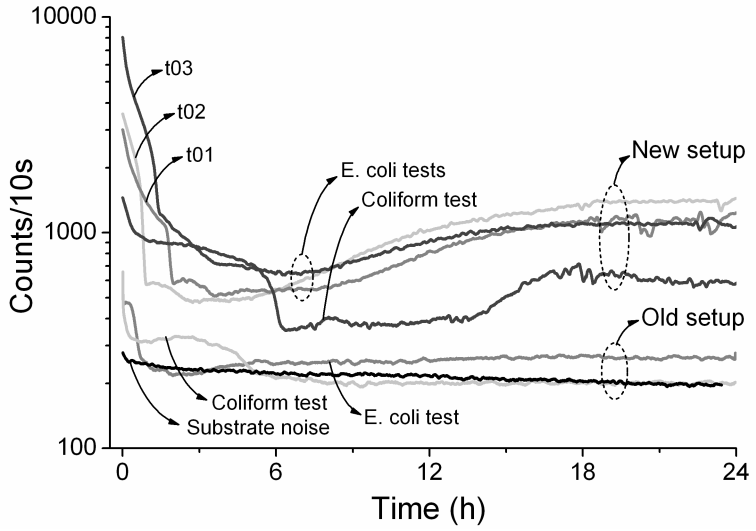

Figure 6: *Escherichia coli* and coliform 24-h growth tests (37°C): results from the new and old chamber.

Acknowledgements

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