

NEW PLATFORM OF BIOSENSORS BASED ON FLUORESCENCE DETECTION FOR ENVIRONMENTAL APPLICATIONS

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ABSTRACT

Multilights is an instrument for fluorescence detection on biosensor applications, It permits working simultaneously with different types of biomediators (that can be fluorophores or photosynthetic). This article describes the different systems that form the instrument, like measurement cells, signal conditioning, digital electronics, data management, etc. Also instrument testing results are shown. A real application for Multilights will be water contamination detection (especially pesticides and herbicides) using photosynthetic materials.

Keywords: *Biosensors, Biomediator, Fluorescence, Kautsky Curves, Pesticides, Herbicides, Water Pollution*

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NUEVA PLATAFORMA DE BIOSENSORES, BASADOS EN FLUORESCENCIA, PARA APLICACIONES AMBIENTALES

RESUMEN

Multilights es un instrumento para la detección de la fluorescencia en aplicaciones bio-sensoriales, el cual permite procesar simultáneamente diferentes tipos de bio-mediadores (como fluoróforos y materiales fotosintéticos). Este artículo describe los diferentes sistemas que conforman el instrumento, como: celdas de medida, acondicionamiento de señales, electrónica digital, gestión de datos, etc. También ilustra las diferentes pruebas realizadas para verificar el funcionamiento del instrumento y sus resultados. Una aplicación real para este instrumento será la detección de contaminantes en el agua (especialmente pesticidas) usando material fotosintético.

Palabras clave: *Biosensores, Biomediator, Fluorescencia, Curvas de Kautsky, Pesticidas, Herbicidas, Contaminación en el Agua.*

1. INTRODUCTION

A biosensor is an analytical device that can measure a biochemical variable using: a biological mediator (like cells, enzymes, plants etc), an associated transducer - electrical, optical, thermal, mass, etc- and an electronics processing system. These types of sensors are characterized by low cost, small size, and -depending of the biological material- good sensitivity and selectivity [1] [2].

Fluorescence consist on the absorption of photons of high energy (larger wavelength) by a material, and the subsequent emission of photons of lower energy (shorter wavelength). When characterizing a fluorescence system must be identified the **absorption spectrum** (the range of light wavelengths that can excite the material) and the **emission spectrum** (the wavelengths of the emitted light) [3].

The fluorescence has been used as transduction method on different applications. By example DNA detection can be done by using fluorescent probes (fluorophores) [4] [5].

Also, a series of biosensors has been developed using the chlorophyll a fluorescence [6]. In this case the measurement is based on the emitted light transient during the first seconds of excitation (also called Kautsky curve [7][8]).

The Kautsky curve is generated when a photosynthetic material is suddenly exposed to an excitation light of the right wavelength after a dark adaptation time. The fluorescence increases from an initial level (called **F0**) to a maximum level (**Fm**) following a series of intermediate steps [9]. The Kautsky curves are used to determinate cell damage and photosynthetic efficiency of plants and algae, by example on space missions[10][11].

This article describes the Multilights instrument. It's a modular fluorimeter for simultaneous measurement of different biomediators. His target application is pre-screening of water samples for pesticide detection, but can be adapted to other applications.

Multilights has been developed to be a small, low cost, easy to use, and portable instrument.

2. THE MULTILIGHTS INSTRUMENT

The instrument consists on four modules of six cells each.

The first module is used for photosynthetic biomediators. This module (module 0) uses red light excitation (680nm) and reads fluorescence on 730nm band.

The other modules are used from fluorophores. Module 1 uses excitation at 370nm and measures at 400nm. Module 2 is 480nm excitation and 500nm measure, and Module 3 is 600nm 640nm.

In the detection using fluorophores the parameter of interest is only the steady state of fluorescence signal. In photosynthetic detection the following parameters must be extrapolated from the kautsky curve [8]:

F0: Initial fluorescence (ideally at t=0 after light pulse), obtained from lineal interpolation of first data samples.

FM: Maximum fluorescence.

Fj: Fluorescence at time 2ms.

Fv: Variable fluorescence (FM – F0).

Fv/FM: Useful on plant vitality analysis [10][11].

Vj: Useful to detect pesticides, acting on D1 pocket binding site

$$V_j = \frac{F_j - F_0}{F_m - F_0} \quad [6]$$

Area: The area above the curve and between F0 and FM is also used on pesticide detection.

2.1 Fluorescence cells

The measurement cells were built in black Delring® by bio-compatibility concerns. These consist of two different blocks: the upper part – detachable, and finished with a polycarbonate window - contains the biological material to be measured and the lower part -fixed to the instrument body- contains the light sources and detectors. Each cell area is only 13mm X 13mm and contains 500uL of biological sample, guaranteeing instrument portability and small size.

In each cell, four light emitting diodes (LED) with a 7,58° inclination produces the excitation light for the biological material. This to take advantage of the

low power consumption, fast response, low cost and spectral precision of led technology.

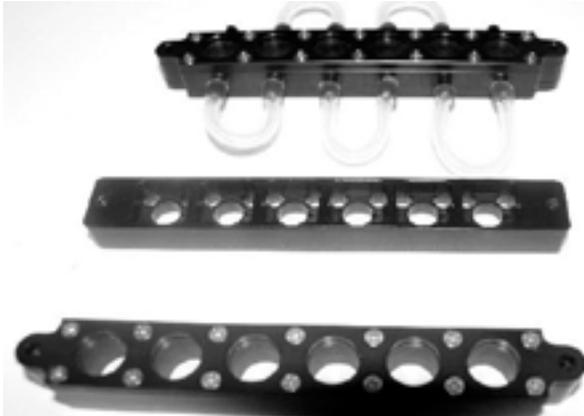


Fig.1 Multilights cells: One part of the cell is fixed to the instrument body (middle image). The other part is detachable and contains the biological material (bottom image and upper image).

Every cell uses one PIN photo-diode as optical detector. In top of it an optical interference filter limits his spectral response to block the led light and transmit the bio-mediator emitted light.

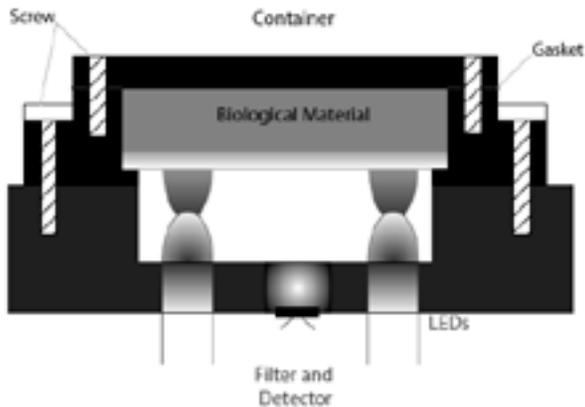


Fig 2. Cell cross section schema, showing light sources, filter and detector

PIN photo-diodes were used because of his better speed (compared against classical PN photo diode) [11], the photo diode spectral sensibility in the 200 to 800 nm range permits his use in all of the cells of instrument. Other type of more sensitive detectors (as photomultipliers or avalanche photodiodes APD) were not suitable to this application by size, cost and complexity concerns[12].

2.2 Analog Signal Conditioning

The photo-diode's current is conditioned using a current to voltage (transresistance) amplifier, designed to guarantee a bandwidth suitable for fast fluorescence measurements (20Khz). This is based on an operational amplifier, and provides a reverse bias of 500mV to the PIN photodetector.

Each module has six transresistance amplifiers (one by cell), their output signals are multiplexed to a single second stage amplifier. Finally, a four to one multiplexer (differential), on a central card (ADC CARD), permits to select the module of interest.

Differential signaling is used between the modules and the central card to reject induced noise.

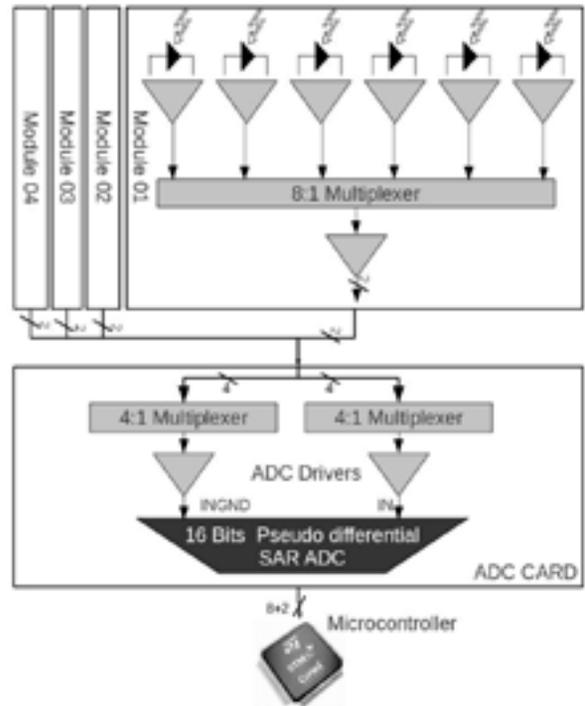


Fig.3 Block diagram of Multilights analog electronics.

The analog to digital conversion is carried out using a 16 bits, 1Msample, SAR, pseudo-differential ADC. Two ADC drivers are used to avoid signal distortion.

2.3 Digital System

The digital system is based on a STM32F103 micro-controller. This MCU has a CortexM3 ARM core running at 72Mhz, providing high speed for

calculation and also a variety of peripherals for system integration. Also 128KB of program flash memory and 64KB of data ram makes this ideal for this application.

Other components of the system, controlled by the MCU are:

A Real Time Clock providing time data information for analysis control and data logging.

A SD card interface for data savings, using the SPI port.

A 128 x 64 pixels monochrome LCD and a 4x4 matrix keyboard for user interface.

Stepper motor driver for automatic fluidics trough peristaltic pump.

Four led drivers (one on each module) allowing to set light luminosity (controlled current) in 127 different levels.

USB interface for computer data transfer and control.

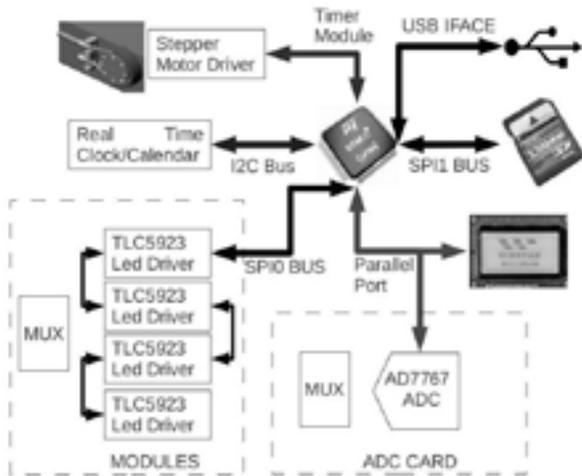


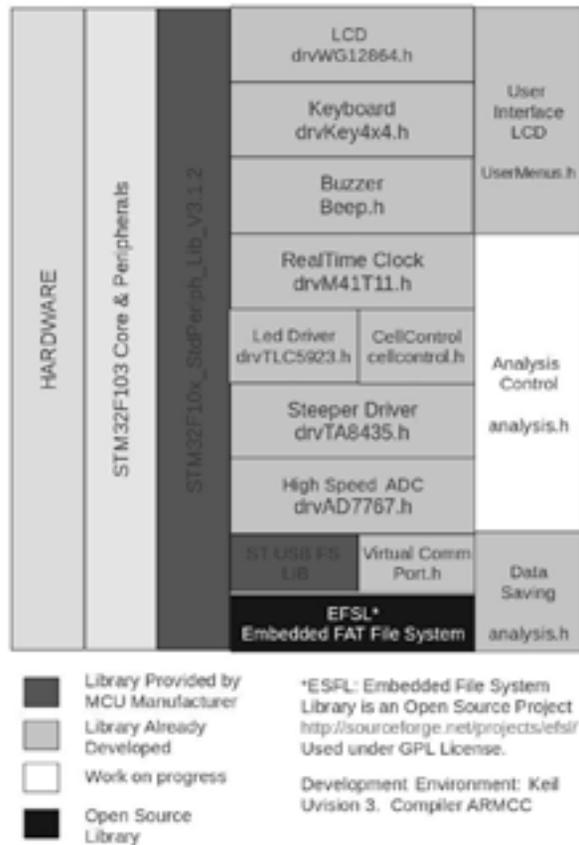
Fig 4. Multilights Digital System, based on STM32F103 micro-controller.

The communication with the Analog to digital converter is done trough a 8 bits parallel interface (shared with LCD). Serial interfaces are also used (SPI to communicate with led drivers, I2C for Real time clock).

2.4 Firmware

The programming for the STM32 micro-controller was developed using C language and the Keil ARMCC® compiler.

The firmware is structured in a set of libraries, classified on three different levels: Micro-controller (Functions related to MCU core and peripherals control), Hardware (drivers to communicate and control the rest of components of the system), and Application (Analysis, data saving and user interface routines). This structure has been chosen



to permit easy code managing, porting and scaling. Fig 5. Firmware library structure.

The libraries for ARM Core and Microcontroller Peripheral Control are provided by ST Microelectronics, together with the USB Full Speed library [13][14].

Also, code from an open source project (Embedded FAT File System Library) [15] was used to manage the SD memory file system.

2.4.1 Analysis

Before of starting a measure the user can configure a series of global parameters (dark time, light time, output method, number of cyclical repeats) and a scan list.

The scan list indicates which cells (from the 24 total cells of the instrument) will be excited and measured, in which order and the light intensity level from every one of those cells.

The measurement process has the following steps:

1. Dark adaptation time: During this time (user configurable from 0 to 15 min), all lights are turned off to permit at the biological material (specially if it's of photosynthetic type) to return to relaxed state.

2. Sampling: On the first cell of scan list, light is turned on (at the light intensity configured by the user) and data is captured on micro-controller's RAM memory (Fluorescence Buffer vector). The light time can be set to a value between 1 and 11 seconds.

3. Data printing / saving: Now the data captured from the current cell are send from the RAM memory to the SD card or the PC (depending on user setting). The Fluorescence Buffer memory is freed.

4. Steps 2 and 3 are repeated from all the cells in the scan list.

5. Steps 1 to four are repeated by the number of cycles indicated by user (from 1 to 999)

2.4.2 Sampling

The signal is sampled at a rate of 100Khz (sample period of 10us), fast enough to capture the transient fluorescence of photosynthetic biomediators (Kautsky curve). Due to the enormous data flow the following schema was adopted (figure 6):

The first one thousand data samples (first ten milliseconds of light) are memorized one by one, only a mobile mean filter is used to smooth the signal.

The following ten thousand samples (10 to 110 milliseconds after light turn on) are compressed memorizing only the mean of the last ten samples (downsampling 1:10). In the same way, samples

between 110ms and 1,11sec are downsampled 1:100.

Finally, from 1,110 seconds to the end of light time, the mean is done every 1000 samples (downsampling 1:1000).

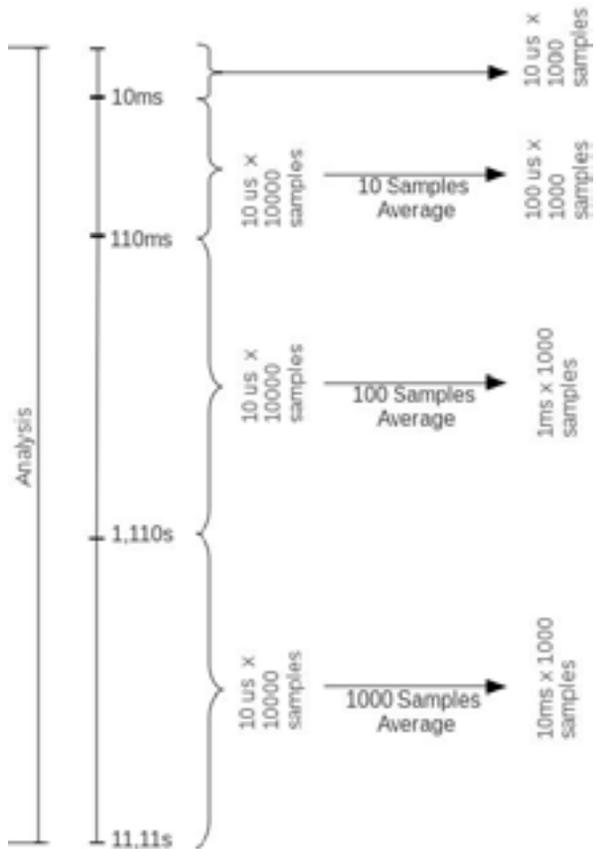


Fig 6. Sampling strategy.

This process is realized on real time (thanks to the high speed of the microcontroller), and avoids the need to use high amounts of RAM memory and / or very fast data transfer/saving systems. Also, permits to retain all the relevant information of the fluorescence transient in a small data package (at maximum 4KB for a full 12 seconds light pulse).

2.4.3 Data saving

The user can choose to send the analysis results to a personal computer via USB interface, or to save those directly on a SD memory card, or both things. When saving to SD card the data from each analysis is saved in his own directory containing the following texts files:

- On the file **settings.txt** are reported the analysis user controlled parameters (dark, light times, scan list, etc), the current data/time and the instrument version.
- The **result files** consist of two columns indicating time (on ms) and fluorescence signal (in arbitrary units). These are named in the format **CxxCyzzz.txt** where **xx** indicates the cell number and **zzz** the cycle number.
- The **stats file**, still on implementation, contains a table with all the curve parameters extracted from the different cells and cycles.

Every analysis directory follows a sequential numeration (001 to 999) and has a parent directory that groups based on the analysis date (named following the convention DDMMYY).

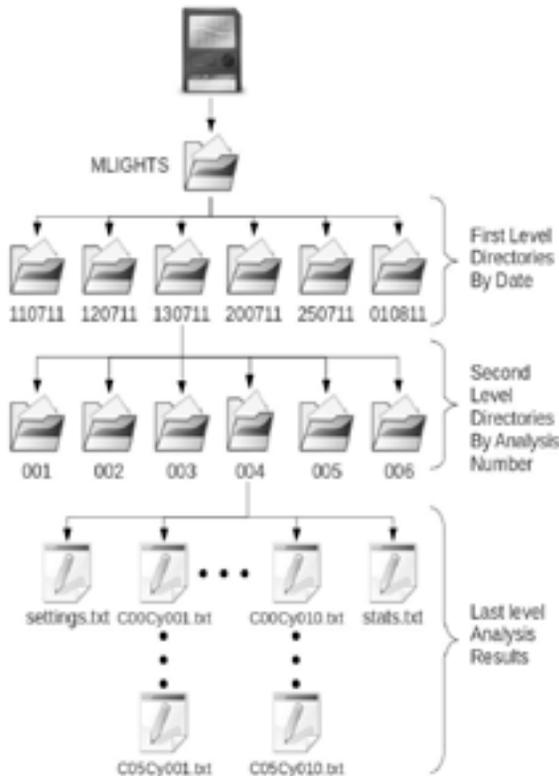


Fig 7. Structure of the SD card directories and files

3. INSTRUMENT VALIDATION

Initial instrument testing has been conducted using Module 0 for photosynthetic material. Test material is *Chlamydomonas reinhardtii* IL Type [16][17][18].

3.1 Excitation light, filtering

This module uses 680 nm red light for algae excitation. Figure 8 shows the led light spectrum (captured using Ocean Optics USB4000 spectrometer).

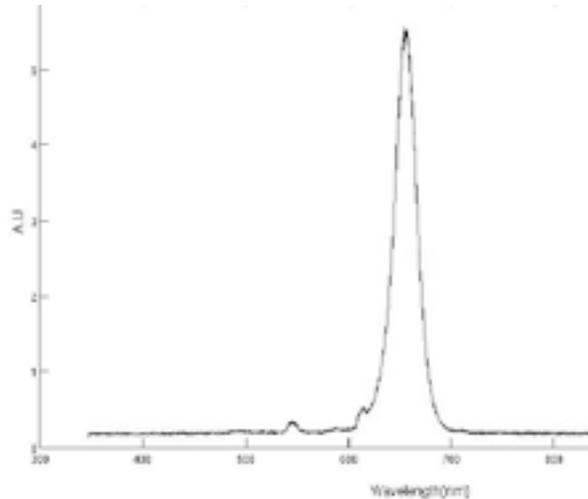


Fig 8. LED spectrum for module 0.

Figure 9 shows the excitation light intensity (measured in $\mu\text{mol/s/m}^2$ with a Quantum Radiometer -Licor-. put in the center of the cell) against different user configured led levels, its evident a linear relationship.

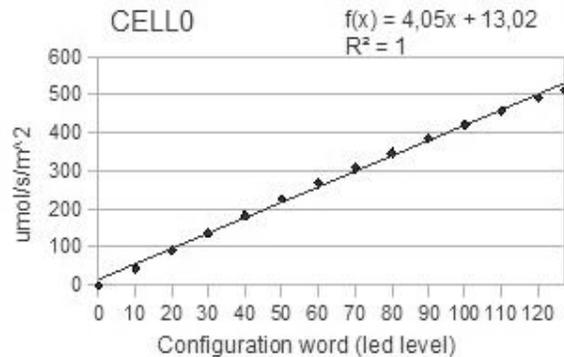


Fig 9. Light intensity vs led level on cell 0, data points and linear regression.

The Figure 10, shows the same experiment of figure 9 repeated on all cells, the error differences on cell light intensity are due by assembling tolerances (specifically on led angles that can be eliminated in production process), also by led and led driver tolerances.

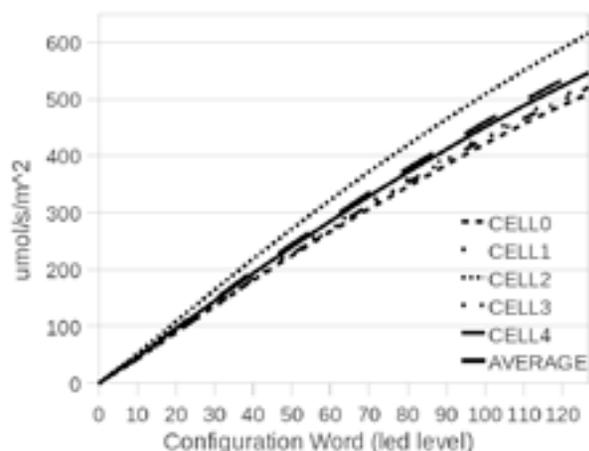


Fig 10. Cell to cell excitation light repeatability.

In this module, a 730nm high pass optical filter is used on all the six cells.

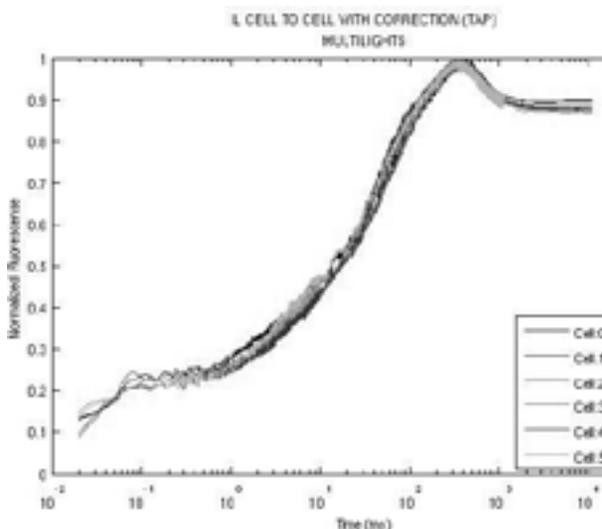


Fig.11. Measurement of the IL mutant (*Chlamydomonas reinhardtii*) using the six cells of module 0. at OD=1, Dark adaptation = 15 min, Light time= 11 secs, Light level=127. Data is normalized to fluorescence maximum.

3.2 Cell testing and repeatability

The same biological material has been measured in all cells, to verify if instrument was able to detect his fluorescence transient and to check the cell to cell instrument repeatability (Fig 11).

The data was processed using Matlab®, also offset has been subtracted using as reference a

measurement done without algae, only with alga's substrate.

As seen, the fluorescence curves overlaps indicating good measurement repeatability, The kautsky curve parameters extracted from the above curves are shown in the table 1.

Table 1: Parameters extracted from cell to cell test.

	Cell 0	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	σ
Fv/Fm	0,764	0,768	0,792	0,787	0,780	0,788	0,009
Vj	0,117	0,090	0,095	0,100	0,085	0,115	0,011

3.3 Testing with pesticides

Preliminary testing has been done using the IL mutant of *Chlamydomonas reinhardtii* under exposition different concentrations of Linuron pesticide.

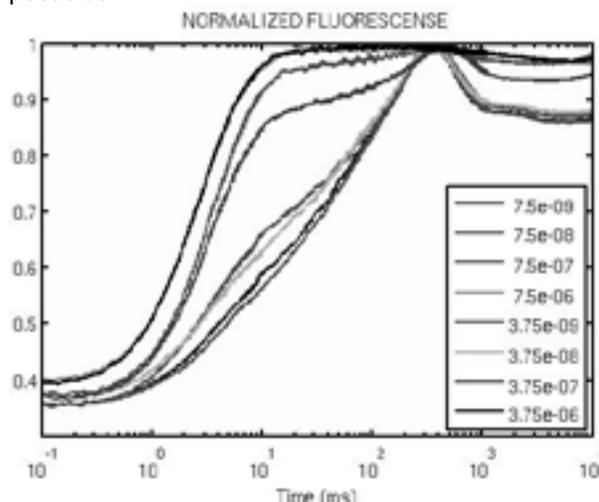


Fig. 12 Kautsky curves of the IL mutant with linuron pesticide at different concentrations(molar), analysis was conducted after dark adaptation time of 15 minutes, light time of 10 seconds, maximum led intensity. Optical density of biological material was 1.

On figure 12, notice how the fluorescence level near 2ms time greatly increases with pesticide concentration. This change on fluorescence transient can be quantified using the Vj parameter (Fig. 13).

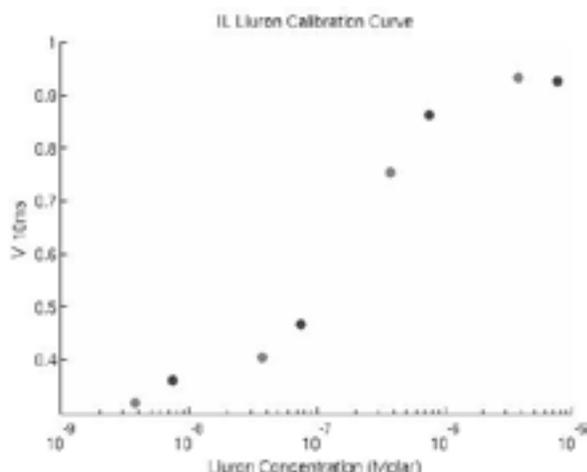


Fig 13. Vj parameter -extracted from curves of fig 12- vs pesticide concentration.

These preliminary results show the right functionality of the instrument. Ongoing work on this type of biological material aims to characterize different mutants of *C. reinhardtii*, in terms of sensibility, selectivity, stability and repeatability for detection of pesticides.

Testing of the other modules is currently on progress and will permit to extend the applications field of the instrument.

4. CONCLUSIONS AND FUTURE WORK

The Multilights instrument has been projected as a platform to work with different types of fluorescence based bio-mediators. This instrument is portable, can be easily adapted for *in situ* analysis, offers a friendly user interface and data saving possibilities.

Testing has shown how Multilights can be used in applications based on Kautksy curves for detection of pesticides, guaranteeing also a good repeatability on cell to cell parameter estimation. Current work is focused on biomediator screening to detect and characterize the most sensitive organisms (*C. reinhardtii* mutants) at the different classes of pesticides.

Thanks to the availability of cells with different configurations, Multilights permits to analyze a sample with different bio-mediators simultaneously. This opens the possibility of using sensor fusion techniques (like neural networks, fuzzy logic, etc) for improving sensors reliability or implementing

some sort of classification (like pesticide type on water samples).

5. ACKNOWLEDGEMENTS

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