Application of the Silicon Photomultiplier for Fluorescence Photobleaching Measurement

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Abstract
In the paper a measurement method of fluorescence intensity reduction (called photobleaching) caused by excitation light was presented. Intensity of fluorescence light was measured by silicon photomultiplier (SiPM) – sensor which allows single photons detection. It has more compact dimensions and lower bias voltage in comparison to photomultiplier tube, presently used in many laboratory devices. Standard photometric cuvettes with a capacity of 1.6 ml and optical path length of 10 mm were used for the measurements. Sodium fluoresceinate dissolved in 10 mM TRIS buffer at pH 8.5 was used as the fluorescent dye. The solution was tested at a concentration of 100 µg per ml with constant excitation light from LED source over the time of measurement.

Key words: silicon photomultiplier, fluorescence photobelaching, sodium fluoresceinate

Introduction

Fluorescence is a phenomenon often used in various analytical methods, especially in biotechnology. It is a fast photophysical process in which a fluorescent dye molecule (a fluorophore) absorbs the energy of a photon falling on it during ~10^{-15} s. This results in the passage of the molecule to the excited state of the electron (singlet state), and then in a very short time (on the order of 10^{-8} s) to go into equilibrium state. At this transition, a fluorescence photon emission occurs. The condition for a radiant transition is the transfer of energy in the form of a photon, not by collision with other molecules. It is necessary to provide energy for the generation of fluorescent radiation, the sources of which are most often the photons of the excitation light. Under the influence of this light, the problem of bleaching (fading) of fluorescent dye molecules appears, due to their photochemical damaging [1–3]. With fixed conditions, such as intensity of excitation light, temperature and concentration of the dye, it is possible to determine the dependence of fluorescence on the exposure time of the sample. For this purpose, it is necessary to use sensitive equipment that allows registration of differences at the level of photons. In the paper we propose the silicon photomultiplier which enables measurement with the resolution of single photons [4]. It is an element composed of a matrix of parallel-connected avalanche photodiodes, placed on a common substrate and working in Geiger mode. Equivalent circuit of the SiPM is shown in Figure 1. Each individual photodiode has a polarizing resistor connected in series, so that each cell (called pixel) works independently of the others. This resistor called quenching resistor is also responsible for extinguishing avalanche so that the cell is ready to receive another photon.

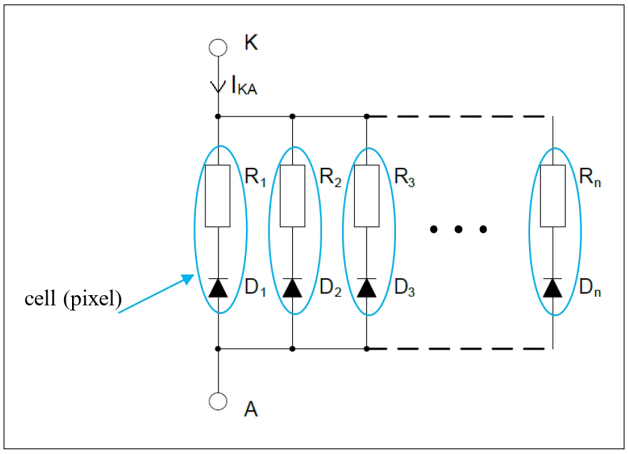

Figure 1. Electrical schematic of the silicon photomultiplier

The I_{KA} current flowing between the SiPM electrodes is equal to the sum of the generated currents in each of the cells and directly proportional to the electric charge resulting from the avalanche effect. Readout electronics systems dedicated to acquiring the signal from the silicon photomultiplier are based on the 3 basic parameters of the signal (Figure 2):

- amplitude
- pulse time duration
- charge generated in the current pulse

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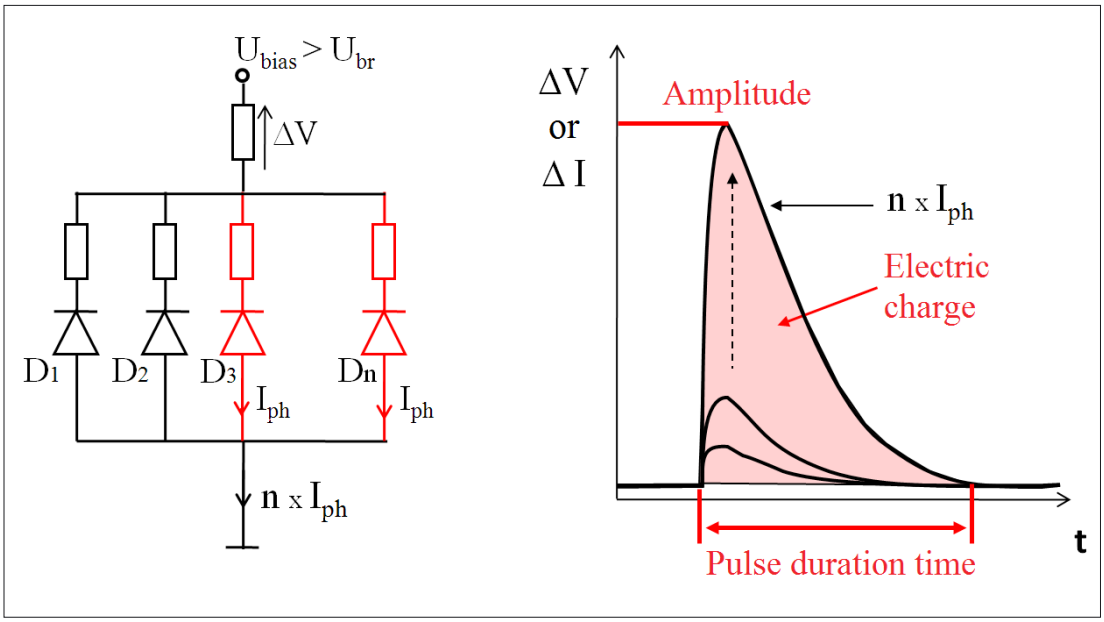

Figure 2. Parameters of the silicon photomultiplier signal from the point of view of acquisition

Devices that converts amplitude to digital form use peak detectors (Peak Detector and Hold) to memorize the pulse heights, for conversion time in an analog-digital converter. The higher is amplitude of pulse from the silicon photomultiplier then the longer its duration. To measure the duration of this impulse, TDC (Time to Digital Converter) converters are used. The simplest implementation of this converter is a comparator with a properly set threshold. On its output there will be pulses with a length directly proportional to the duration of the compared pulse from SiPM. The charge generated in the silicon photomultiplier is directly proportional to the current flowing between its electrodes. Charge to digital converters (QDC) are used to measure this charge. In this type of devices, the executive element is a capacitor, which charges with SiPM current. As a result, the voltage on the capacitor is directly proportional to the charge generated by the photons falling on the detector. This method of measuring signal from the silicon photomultiplier was used during fluorescence intensity measurements.

Instruments and Methods

Measurement system was built with 2 parts: optical and electronic. Optical part is responsible for absorption and emission of light by fluorescence dye. Electronic part based on silicon photomultiplier is intended to measure intensity of fluorescence light.

Optical part

Fluorescence intensity measurements were performed in a photometric cuvette made of polymethyl methacrylate (PMMA) with capacity of 1.6 ml. The test solution was prepared from sodium fluoresceinate dissolved in a TRIS 10mM buffer with a pH equal to 8.5. Measure solution with concentration of 100 µg/ml was prepared by diluting a stock solution (1 mg/ml) derived from a dry sample weighed with a precision weight with resolution 10 µg. The sample was prepared at room temperature. To stimulate the dye molecules, a blue LED S500LLB4G-H + was used, supplied with a constant current of 12.7 mA. Its spectral characteristics are shown in Figure 3 (normalized to range 0 ÷ 1).

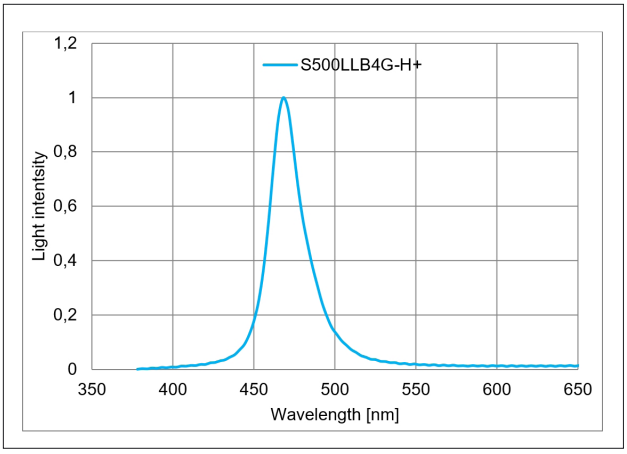

Figure 3. The optical spectrum of the LED diode used in the research

Because the light from the diode propagates at a certain angle, it was necessary to use an optical channel with diode diameter for collimation of the beam. In this way, a uniform volume of the solution with cylindrical shape and 5 mm diameter was obtained. Figure 4 shows the way in which fluorescent particles were stimulated to glow in a PMMA cuvette.

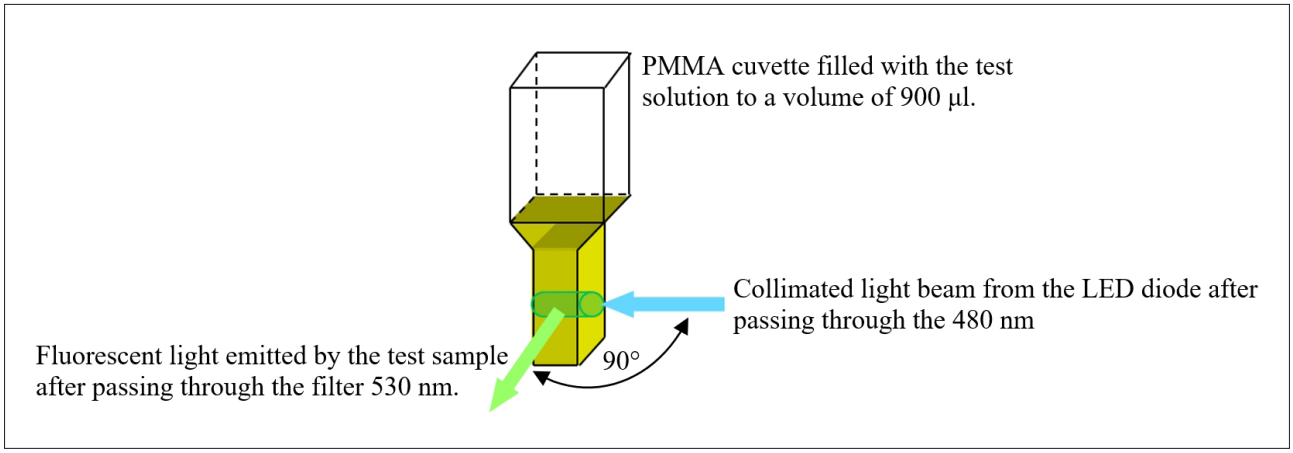

Figure 4. Stimulation of dye molecules for emission of fluorescent light in solution

The optical path length in the solution at a given height is 4 mm. With a diameter of 5 mm, the volume of the excitated solution by light can be calculated from the formula for the volume of the cylinder: $V_C = \Pi r^2 \times d \approx 78{,}5$ µl.

For the absorption and emission bands of sodium fluoresceinate, it is necessary to select the appropriate optical interference filters: primary side (light absorbed) and secondary (light emitted). The professional Bio-Tek filters used in the Synergy HT spectrophotometer were used for the tests. These filters have a high coefficient of attenuation outside the passband. The characteristics of the filters used in the tests are shown in Figure 5.

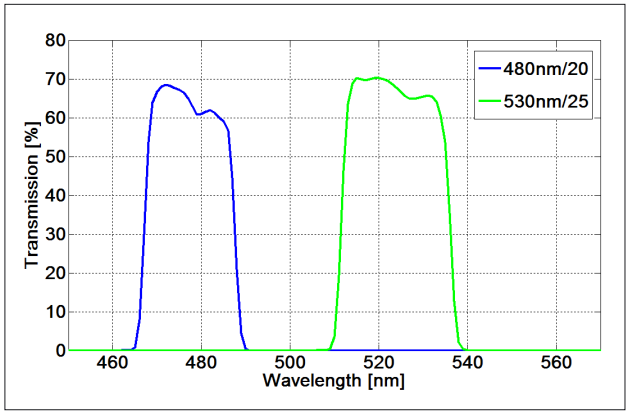

Figure 5. Spectral characteristics of Bio – Tek filters used during tests

The light with a suitably cut-out band reaches the solution of the fluorescent dye, whose particles, in turn absorbing energy, emit fluorescent radiation. This radiation passes through the emission filter, followed by a special collimator coupled with a quartz optical fiber. At the other end of the optical fiber there is a SiPM head, to which the photons emitted by the sample reach. Below is a photograph of the optical block for cuvettes (Fig. 6) and a drawing of the method of combining optical elements in blocks of blackened aluminium (Fig. 7).

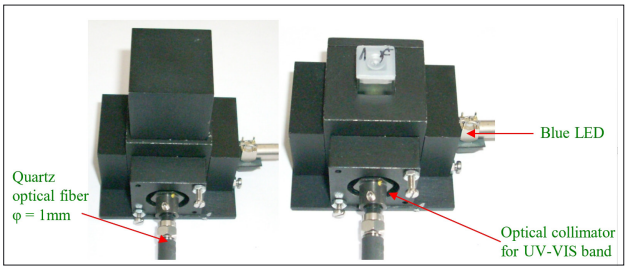

Figure 6. Photographs of optical block for PMMA cuvettes

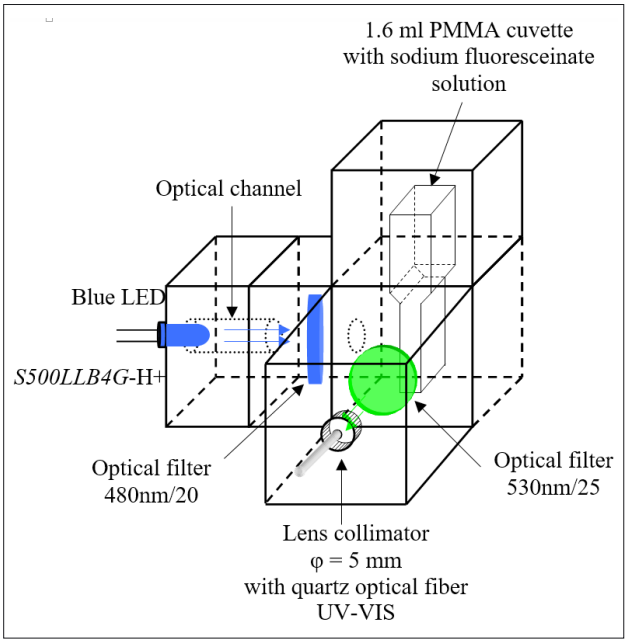

Figure 7. Drawing of the optical part

Electronic part

A block diagram of the electronic part (Fig. 8) provides a general idea of the device structure.

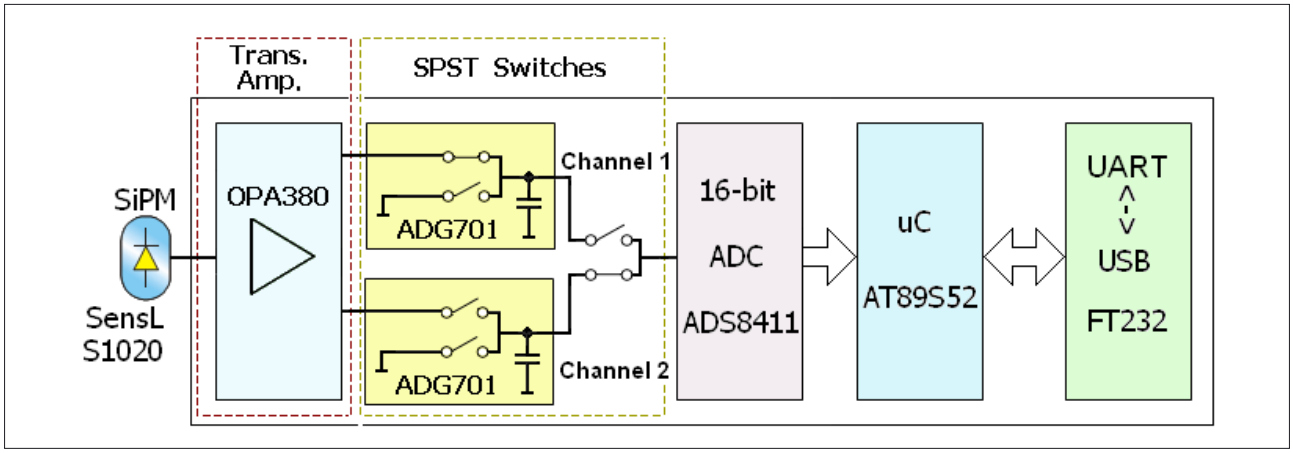

Figure 8. Block diagram of electronic part

Photons of fluorescence light generate a current signal in the silicon photomultiplier. Current is converted by the transimpedance amplifier to a proportional voltage. After the amplifier, a signal is splitted between two independence channels which work in complementary way. By the time when the first channel integrates a charge, a second one converts the charge stored in the previous cycle (as a voltage in a capacitor) to its digital representation (ADC). Each channel has also an SPST (Single Pole Single Throw) switches responsible for change of acquisition phase. Their resistance is at the level of ~4-8 ohms during ON state. A principle of operation of the acquisition unit and operation phases of the system are presented in Fig. 9.

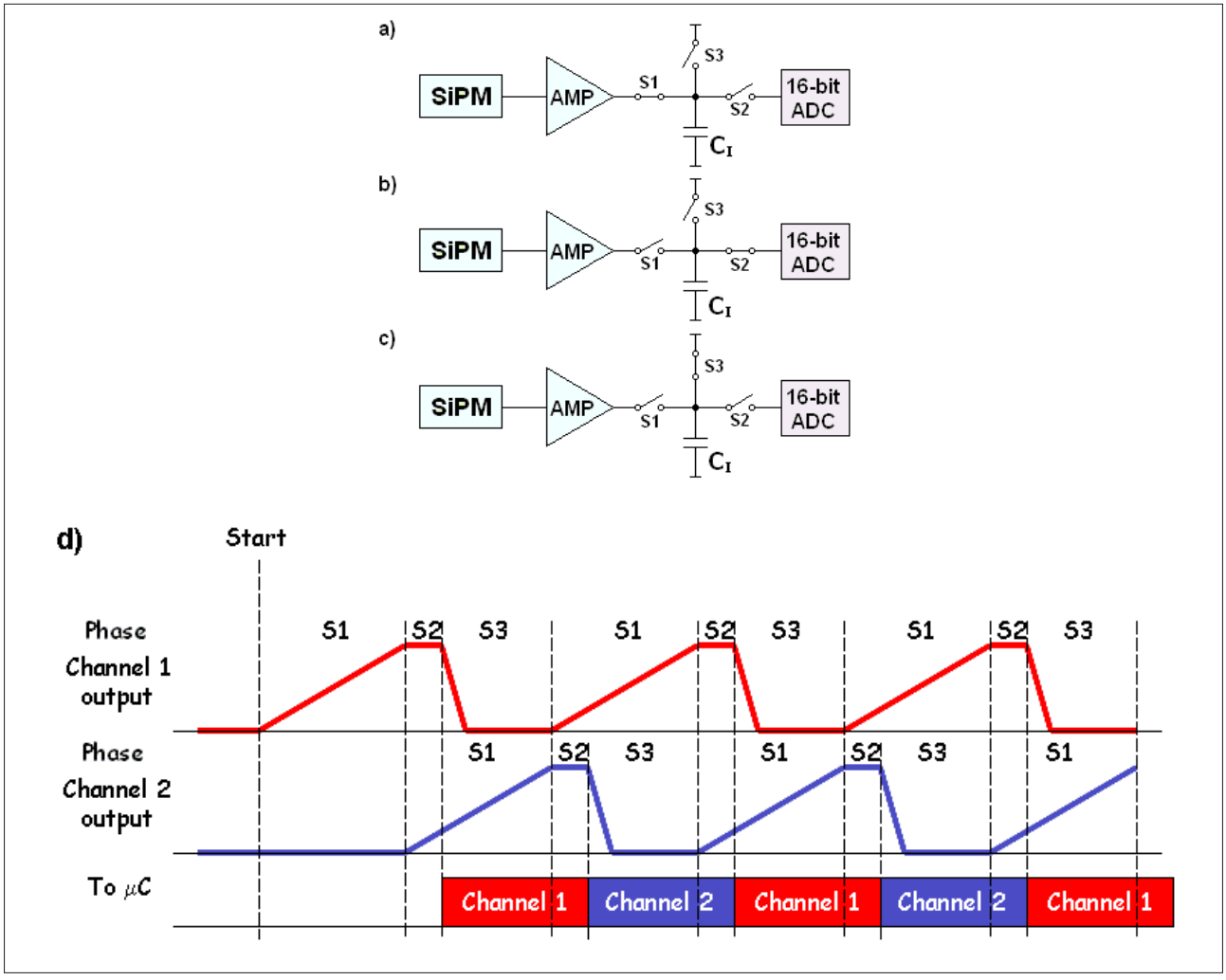

Figure 9. Phases of acquisition: a) S1 – charging, b) S2 - conversion, c) S3 - discharging, d) channels outputs at the time of acquisition

In the first phase (integration), the capacitor is being charged by the current delivered by the amplifier output during defined period of S1. When the S1 phase is finished in one channel, the microcontroller switches the channels and in the same time goes to the second phase: ADC conversion. Capacitor voltage, proportional to the integrated charge is being now converted to its digital form. Digital 16 – bit value is then handed over to the microcontroller, which sends out the data to the computer via the USB interface. In the last phase, the integrating capacitor is discharged to be ready for the next conversion. In the same time the charge in the second channel is still being integrated, and once this process is finished, the microcontroller switches again the channels. The microcontroller algorithm is shown in Figure 10.

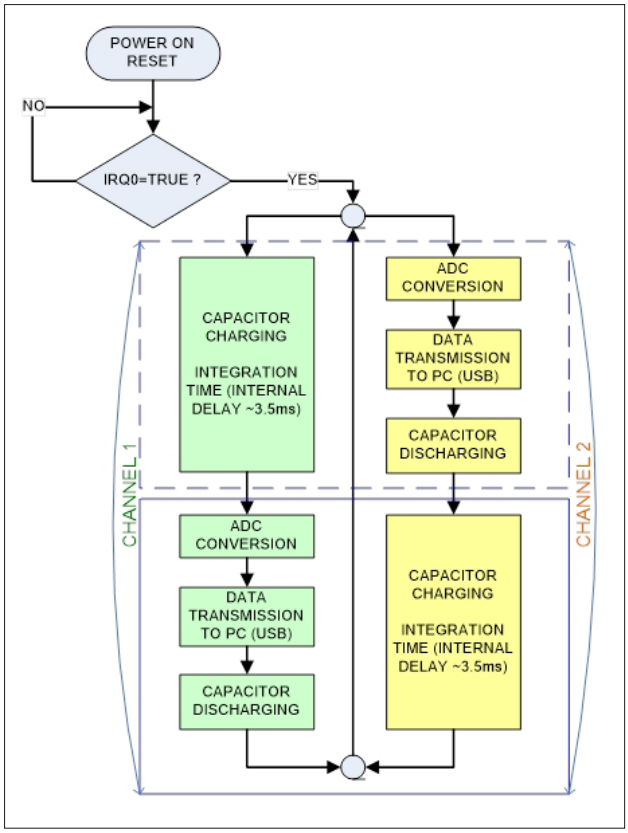

Figure 10. Microcontroller program algorithm

For detection of fluorescence light a silicon photomultiplier from SensL company has been chosen. Model S1020 has a superior Signal to Noise Ratio than standard APDs. The sensor operates in standard scheme from manufacturer's application note. Typically breakdown voltage for this SensL SiPMs is equal 28.4V but it's possible to overrun voltage about 2V. In the final device, breakdown voltage is equal 30.2V.

Results and Discussion

Figure 11 shows the measurement of a sample of sodium fluoresceinate illuminated by continuous light for 30 minutes. During this time, a continuous decrease in the fluorescence intensity was recorded.

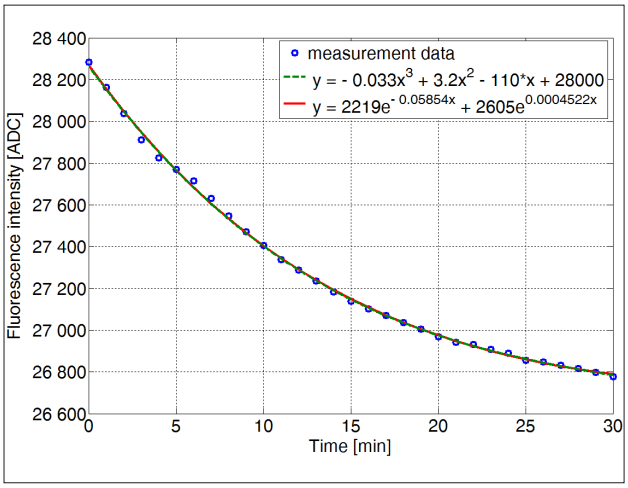

Figure 11. Photobleaching measurement of sodium fluoresceinate sample using SiPM

As can be seen in the Figure 11, the photochemical destruction process of the dye molecules is exponential. This dependence as a function of time can be simplified by writing in the form of a 3-degree polynomial, which greatly facilitates the approximation of measurement data.

The process of destroying the fluorescent properties of the dye molecules is not always undesirable. This phenomenon is closely related to other called Fluorescence Recovery After Photobleaching (FRAP) [5]. During illumination of a certain volume of solution, as shown in Figure 4, degradation of particles occurs in this area. At this time a reduction of the fluorescence intensity is recorded. After turning off the light source, as a result of the diffusion process, the faded particles are mixed with the remaining ones. In this way, in the place of excitation, some of the photochemically damaged dye particles are replaced with others. In molecular biology, FRAP analysis is used to study living cells [6, 7].

Conclusions

Silicon photomultiplier is very high sensitive detector which can be used for measurement of fluorescence light what was confirmed in earlier research [8]. Results of measurements presented in the paper confirms possibilities of application this kind of sensor for fluorescence photobleaching measurement during excitation of fluorophores by continuous light. Data analysis shows that the exponential dependence of fluorescence intensity on time can be written as n^{th} degree polynomial. The study used weak light source which is LED. It is possible to use a strong sources of light in example laser or deuterium arc lamp what will cause photobleaching process will be faster. For the FRAP analysis, mainly pulsed light sources are used. Model of silicon photomultiplier used in this research has a maximum frequency

of operation at the level of tenth MHz. It is therefore possible to measure the time of fluorescence recovery after photobleaching, what will be included in future studies.

References

1. L. Song, E. J. Hennink, I. T. Young, and H. J. Tanke, *Biophysical Journal,* 1995, **68(6)**, 2588–2600.
2. Diaspro, G. Chirico, C. Usai, P. Ramoino and J. Dobrucki, Photobleaching – chapter 39, *Handbook of Biological Confocal Microscopy*, Third Edition, edited by James B. Pawley, 2006, 690–702.
3. D. Wustner, T. Christensen, L. M. Solanko, and D. Sage, *Molecules,* 2014, **19**, 11096–11130.
4. Y. M. Yebras, P. Antoranz and J. M. Miranda, *Journal of the European Optical Society,* 2012, **7**, 12014-1–12014-7.
5. H. Pin Kao, A. S. Verkman, *Biopchysical Chemistry*, 1996, **59**, 203–210.
6. M. E. van Royen, P. Farla, K. A. Mattern, B. Geverts, J. Trapman and A. B. Houtsmuller, *Methods in Molecular Biology*, 2009, **464**, 363–385.
7. N. Kourtis, N. Tavernarakis, Protein Synthesis Rate Assessment by Fluorescence Recovery after Photobleaching (FRAP), *Bio-Protocol*, 2017, **7(5)**, e2156.
8. L. Mik, W. Kucewicz, J. Barszcz, M. Sapor and S. Glab, *Elektronika: konstrukcje, technologie, zastosowania*, 2011, **52(12)**, 61–65.