MASARYK UNIVERSITY

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Analysis of biologically relevant substances in individual cells

Doctoral thesis

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Abstract

This dissertation work presents novel strategy of analyses of biologically important enzymes inside the living cells. Exact analyses and correct determination of the relevant substances inside individual cells are essential for correct interpretation and understanding of physiological, diagnostic, and therapeutic events. This work is focused on single-cell analyses, which are important for determination and quantification of natural cellular heterogeneity. Our research was focused on the bioluminiscence and fluorescence microscopy analyses of biologically active molecules, the proteolytic active caspases. Caspases play important roles in cell signaling regulations during normal and diseased states of the organism and are therefore attractive targets for clinical diagnosis and medical therapy.

The first part of the dissertation work describes an original highly sensitive and selective instrumentation and methodology based on homogeneous single-step bioluminescence assay to quantify caspases and evaluate their heterogeneity in individual HeLa cells. Individual suspended cells were selected under microscope and reliably individually transferred into the detection vials by a micromanipulator. The high sensitivity of the method is given by implementation of photomultiplying tube with a cooled photocathode working in the photon counting mode. Thus, the first goal of the research work was optimization and implementation of the instrumentation and methodology for determining content of caspase-3/7 in individual apoptotic and even non-apoptotic HeLa cells.

The second part of the dissertation thesis is focused on synthesis and testing a novel molecular probe based on Förster resonance energy transfer (FRET) between a highly luminescent quantum dot (QD) as a donor and a fluorescence quencher as an acceptor linked by a specific peptide. In basic principle, QD luminescence, effectively dissipated in the probe structure, is switched on after the cleavage of the peptide by a protease and the release of the quencher. Thus, the second goal of the dissertation work was to synthesize and test a new QD luminescent probe for a long-time monitoring of active caspase-3/7 distribution in apoptotic osteoblastic MC3T3-E1 cells treated with Camptothecin. The novel synthesis strategy of a probe is based on the two-step synthesis consists of: (i) Conjugation of CdTe QDs functionalized by -COOH groups of succinic acid on the nanoparticle surface with the

designed specific peptide (GTADVEDTSC) using a ligand-exchange approach; (ii) A fast, high-yield reaction of amine-reactive succinimidyl group on the BHQ-2 quencher with N-terminal of the peptide. Product yield of the first step of synthesis was analyzed by laboratory made capillary electrophoresis with laser induced fluorescence (CE-LIF). Analyses proved a high reaction yield and nearly no occurrence of unreacted QDs. Concentration of the final product of the synthesis was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES). Kinetic parameters of the reaction of the synthetized probe with caspase recombinant enzyme were determined by time course of fluorescence and interpreted by Michaelis-Menten description of enzymatic kinetics. As a result of comparison with the similar commercial available product, our synthetized luminescent probe provides longer imaging times of caspases in living cells than commercial product.

Abstrakt

Tato disertační práce představuje novou strategii analýz biologicky důležitých enzymů uvnitř živých buněk. Pro správnou interpretaci a pochopení fyziologických, diagnostických a terapeutických dějů jsou nezbytné přesné analýzy a správné stanovení příslušných látek uvnitř jednotlivých buněk. Tato práce je zaměřena na analýzy uvnitř jednotlivých buněk, které jsou důležité pro stanovení a kvantifikaci přirozené buněčné heterogenity. Náš výzkum byl zaměřen na analýzu biologicky aktivních molekul, proteolyticky aktivních kaspáz pomocí bioluminiscenční analýzy a fluorescenční mikroskopie. Kaspázy hrají důležitou roli v regulaci buněčné signalizace v průběhu normálních a patologických stavů organismu a jsou proto atraktivními cíli pro klinickou diagnostiku a léčebnou terapii.

První část disertační práce popisuje originální vysoce citlivou a selektivní instrumentaci a metodologii založenou na homogenním jednokrokovém bioluminiscenčním testu pro kvantifikaci kaspáz a hodnocení jejich heterogenity v jednotlivých HeLa buňkách. Jednotlivé suspendované buňky byly vybrány pod mikroskopem a mikromanipulátorem spolehlivě přeneseny do detekčních vialek. Vysoká citlivost metody je dána implementací fotonásobiče s chlazenou fotokatodou pracující v režimu počítání fotonů. Prvním cílem výzkumné práce byla optimalizace a testování instrumentace a metodiky pro stanovení obsahu kaspázy-3/7 v jednotlivých apoptotických a neapoptotických HeLa buňkách.

Druhá část disertační práce je zaměřena na syntézu a testování nové molekulární sondy založené na Försterově rezonančním přenosu energie (FRET) mezi vysoce luminiscenční kvantovou tečkou (QD) jako donorem a fluorescenčním zhášečem jako akceptorem propojenými specifickým peptidem. V základním principu je QD luminiscence, účinně dissipovaná ve struktuře sondy, uvolněna po štěpení peptidu proteázou a uvolnění zhášeče. Druhým cílem disertační práce bylo tedy syntetizovat novou QD luminiscenční sondu pro dlouhodobé sledování distribuce aktivní kaspázy-3/7 v apoptotických osteoblastických buňkách MC3T3-E1 ošetřených Kamptotecinem. Nová strategie syntézy sondy je založena na dvoufázové syntéze sestávající z: (i) Konjugace CdTe QD funkcionalizovaných -COOH skupinami kyseliny jantarové na povrchu nanočástic s navrženým specifickým peptidem (GTADVEDTSC) pomocí přístupu výměny-ligandu; (ii) Rychlá reakce amin-reaktivní sukcinimidylové skupiny na BHQ-2 zhášeči s N-koncem peptidu s vysokým výtěžkem.

Produkt a výtěžek první syntézy byly analyzovány laboratorně sestavenou kapilární elektroforézou s laserem indukovanou fluorescencí (CE-LIF). Analýzy prokázaly vysoký reakční výtěžek a téměř žádný výskyt nezreagovaných QD. Koncentrace konečného produktu syntézy byla stanovena optickou emisní spektroskopií s indukčně vázaným plazmatem (ICP-OES). Kinetické parametry reakce syntetizované sondy s rekombinantním enzymem kaspázou byly stanoveny pomocí změny fluorescence v čase a interpretovány popisem enzymatické kinetiky podle Michaelis-Mentenové. Výsledkem srovnání s podobným komerčně dostupným produktem je, že naše syntetizovaná luminiscenční sonda poskytuje fluorescenční signál pro zobrazování kaspáz v živých buňkách po delší dobu než komerční produkt.

Dissertation assignment

Research focus

Analysis of biologically relevant substances in individual cells.

Assignment

Continuation in research and development of highly sensitive methods, bioluminescence, laser-induced fluorescence, fluorescence microscopy, correlative light and electron microscopy and capillary electrophoresis (CE-LIF) for the analysis of important molecules in cell populations and individual cells. Methods of micro- and nanotechnologies will be used for the production of elements of analytical devices and the synthesis of luminiscent probes and sensors.

Declaration

I hereby declare that this thesis with the title **Analysis of biologically relevant substances in individual cells** I submit for assessment is entirely my own work and has not been taken from the work of others save to the extent that such work has been cited and acknowledged within the text of mine. I hereby declare that I have completed my work independently under the supervision of the thesis supervisor using the sources and information cited in the thesis. I declare that I have processed the thesis for the dissertation independently under the guidance of Ing. Karel Klepárník, CSc. using the mentioned literature and experimental results measured at the Department of Bioanalytical Instrumentation in the Institute of Analytical Chemistry of the Academy of Sciences of the Czech Republic.

In Brno 26. 4. 2023 …..……

Mgr. Markéta Procházková

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1. Introduction

All the processes in living organism like as growth, development, and maintenance of multicellular organisms depend not only on the production of cells but also on mechanisms to control and destroy them. The maintenance during development, carefully orchestrated patterns of cell death help to control and determine the size and shape of limbs and other tissues. Mechanism of regulation processes are very important in cases when cells become damaged or infected, ensuring that they are removed before they threaten the health of the organism. In these and most other cases, cell death is not a random process but occurs by a programmed sequence of molecular events, in which the cell systematically destroys itself from within and is then eaten by other cells, leaving no trace. In most cases, this programmed cell death occurs by a process called apoptosis what is from the Greek word meaning “falling off,”as leaves from a tree [1].

Processes like apoptosis and maturation (differentiation of the cells) are therefore essential processes for the maintenance of organism homeostasis. Proteases play an essential role as regulators of various biological pathways in living organisms. Functional characterization of proteases in a biological system has traditionally involved determining the substrate specificity and generating a specific probe or inhibitor especially in case of cancer research [2-4]. Due to the coexistence of a large number of proteases and their diverse and specific roles in many biological pathways, technological advances in single cell analysis are needed to fully define and understand their functions. Various functions of proteases are essential for the regulation of the molecular mechanisms in cells and are crucial for the molecular mechanisms of both healthy and diseased states. It follows that proteases are promising therapeutic targets for a multitude of disease indications such as inflammation and various disease conditions including metastasis and viral infection [5, 6].

Among proteases, caspases are major mediators of cell death during apoptosis [7-9]. Caspases mediate apoptosis and inflammation through aspartate-specific cleavage of a wide number of cellular substrates. However, the most current evidence, points to broader functions of caspases including proliferation and differentiation [8, 10]. The non-apoptotic

functions of caspases suggest that they may become activated independently of inducing an apoptotic cascade [11]. Novel functions of caspases were discovered in hard tissue formation [12] as the impact of caspases on the expression of osteocalcin [13].

Several research works are focused on reasonable scientific effort associated with the investigation of caspase corresponding biological roles in living cells in a quantitative manner [14-23]. The enzyme activity has been widely investigated by using endogenous fluorescent metabolites or synthetic fluorogenic substrates [17, 18, 24, 25]. To analyze the physiological events inside single cells, the technologies related to fluorescence and luminescence imaging advanced rapidly in the past two decades [17, 26-29]. The most sensitive and selective instrumentations for targeted identifications of relevant molecules in individual cells represent optical methods [22, 25, 30]. Microscopy imaging methods offer the most complex information [26, 27, 31, 32]. Thus, light microscopies based on monitoring of fluorescence, chemiluminescence or surface enhanced Raman scattering, including super-resolution techniques are widely used for a high resolution and sensitivity imaging of biopolymer structures, cells and tissues [26, 27, 31, 33]. While Raman scattering does not need any staining and provides even single-molecule qualitative analyses in some special cases, luminescence methods are usually based on implementations of labels, probes or sensors [34-36]. Recent development of nanotechnologies brings new extraordinary opportunities into the practice of analytical optical methods. The combination of specific fluorescent probes with powerful and stable light sources, advanced optical arrangements and high-sensitivity detectors allows detecting and tracking of even single molecules with nanometer spatial precision and millisecond time resolution in living cells [37]. Therefore, even the single-molecule spectroscopy in live cells becomes fundamental to cell biology [19-22, 32].

2. Objectives of the dissertation theses

The first objective of our research is to develop a technology for the evaluation of the heterogeneity of caspase content inside cells. For the first time, we want to develop and optimize the simple and sensitive bioluminescence method for investigation of the amounts of specific enzyme inside the individual living cells during different processes. Our research is focused on monitoring of the amount caspase 3/7 during apoptosis in the case of HeLa cells and during cell proliferation, differentiation, and cell death of the MC3T3-E1 preosteoblastic cells. The optimized miniaturized bioluminescence instrumentation can potentially serve as a sensitive tool for diagnostics and monitoring of therapeutic, e.g., anti-cancer, strategies, where single-cell analyses are crucial to distinguish tumors from surrounding healthy tissue cells.

The second objective of the dissertation thesis is focused on synthesis and testing a novel molecular probe based on Förster resonance energy transfer (FRET) between a highly luminescent quantum dot (QD) as a donor and a fluorescence quencher as an acceptor linked by a specific peptide. Thus, the second aim of the dissertation work is to optimize synthesis and test a new QD luminescent probe for a long-time monitoring of active caspase-3/7 concentration distribution in apoptotic osteoblastic MC3T3-E1 cells treated with Camptothecin. The stability of the QD-probe is compared to a commercially available product. The specific QD-probe can potentially enable long-term monitoring of various processes inside living cells and monitoring of the concentration of caspase-3/7 distribution on a long-term scale can be crucial in biological applications.

3. Bioluminescence analysis of Caspase 3/7 inside living cells

3.1. Theory of bioluminescence analysis

The analytical systems based on chemiluminescence and bioluminescence detection methods became very important in various fields of analytical and bioanalytical chemistry. Development of new techniques is continuously expanding thanks to latest achievements that have made accessible both new chemical or molecular biology tools and advanced instrumentation. The progress in bioluminiscence and chemiluminescence analysis have been successfully employed in bio-specific assays, exploiting the high detectability of specific enzymes and processes inside the tissues [38]. Moreover possibilities of coupling bioluminiscence or chemiluminescent reactions with other specific enzyme reactions has led to amplified enzyme-based assays suitable for automation and miniaturization using immobilized enzymes and the availability of sensitive and compact light detectors has allowed successful implementation of bioluminiscence and chemiluminescence detections also in portable analytical devices. Finally, non-invasive in vitro chemiluminescence or bioluminescence imaging has enabled advances and new possibilities in methods routinely and fundamentally used for imaging and detection of natural processes inside cells, monitoring reactions and responses to drugs in tissues and cells, improving cancer diagnosis and treatment, as well as improvement in monitoring of many other important processes inside the tissues and living cells [38].

Single-cell analyses are important for quantification of natural cellular heterogeneity, which cannot be exactly evaluated from averaged data of cell population measurements. It has already been proved that variety of up-to-date technologies for the detection and quantitation of proteases in individual cells have enable to study cellular heterogeneity at the single cell level [3, 16, 23, 28, 29, 39, 40]. Determination of the protease assays is based on cleavage reactions with the specific peptide substrates attached to a signal luminescent molecule. Cleavage reaction of the peptide substrate is specific for each type of protease, but basically two types of molecules for luminescence detection are released: i) a molecule with luminescence originally quenched in a special structure [41-45] or (ii) a basic molecule

amenable to bioluminescent reaction [16, 46, 47]. Some other types of assays analyses are primarily based on the advantage of immune reaction with fluorescently labeled antibodies [29, 48] or interactions of fluorescently labeled inhibitors of proteases as affinity ligands to active centers of enzymes [49]. In cases of reaction with labeled inhibitors, the specific inhibitor and suitably chosen sequence of amino acids in the peptide moiety provide the covalent affinity binding and probe specificity. Antibody caspase labeling can be used in separation methods [50] or for application in cancer therapy [51]. Shi et al. described research work focused on founding a significant concentration heterogeneity of caspase-3 detected in single Jurkat cells by combination of capillary electrophoresis separation with confocal laser-induced fluorescence detection [29].

A wide spectrum of methods based on noninvasive reporters have been developed to monitor caspases using a genetically encoded fluorescent biosensor activated by protein cleavage [52-55]. Specific genetically encoded sensors was employed in some kind of assays for long-term fluorescence lifetime imaging [28, 54]. In one research caspase-3 activation during apoptosis was monitored by time-laps confocal microscopy as the translocation of green fluorescent protein from the cytosol to the nuclei [56]. Big challenge for any protease fluorescent reporter is to achieve specificity to resolve signal of one protease over others. Thus, the undesirable property of multiple proteases to cleave a similar substrate is also a major limitation [57, 58].

Fluorescent reporters are unavoidable in microscopy imaging. However, bioluminescent reaction catalyzed by firefly luciferase is one of the most sensitive analytical tools [59]. Under special conditions, chemiluminescence or bioluminescence detection can approach the absolute, i.e. single-molecule limit in sensitivity [60, 61]. Research group O'Brian et al. developed a homogeneous single-step assay based on a luciferin-luciferase bioluminescence system [46, 62]. The detection of active caspases is based on the bioluminescence chemistry commercially available as Caspase-Glo® reagents. Here, luciferin conjugated with a caspase specific peptide is inaccessible for luciferase. However, after the cleavage of the peptide by an active caspase, the free amino luciferin is released and immediately oxidized by firefly luciferase resulting in photon emission [46, 62].

The extraordinary sensitivity of the bioluminescence methods stems from the absence of a scattered excitation light, photobleaching, and autofluorescence. Moreover, a single active caspase provides multiple cleavages of modified luciferin, and thus generates multiple photons. Their slow and long lasted flow is suitable for photon counting measurement of light. Nevertheless, in near future, new semiconductor solid state detectors like single-photon avalanche diodes, silicon multichannel plates, etc. can be expected to overcome sensitivity of PMTs [38, 63]. Even portable devices like mobile phones can be used as handy detectors [64]. Nowadays, however, PMTs working in photon counting mode still represent the best way to detect low light levels of bioluminescence.

Since the down-regulation and decreased activity of caspase-3/7 is a prognostic indicator of different types of tumors, all improvements in detection and quantification of this enzyme in individual cells achieved a diagnostic importance. Indeed, in some cases it can happen that although the average amounts of caspases determined in cell populations show standard levels, the actual values in a few outlying cells can cause fatal development in the organism. Outliers can thus play a very important role in cancer diagnosis and therapy.

In previous research work a miniaturized apparatus for parallel bioluminescence detection of caspases in individual cells was developed [16, 23, 65, 66]. The aim of this dissertation work was to optimize the developed device and reach not only the limit of detection (LOD), but also the limit of quantification (LOQ) of active caspase-3/7. It means that both limits must be lower than the average amounts of active caspases in single cells. From a biological point of view, it was important to develop an instrumentation for reliable evaluation of variability of caspase-3/7 in both individual apoptotic and non-apoptotic cells that can potentially serve as a tool for diagnostics and monitoring of therapeutic or anti-cancer strategies.

3.2. Experimental part

3.2.1. Caspase-3/7 substrate

Caspase activity was measured with commercially available bioluminescence assay Caspase-Glo® 3/7 for cell apoptosis detection (Promega, Madison, WI, USA). It contains a lysis buffer, caspase substrate and bioluminescent reagents. The mixture was sterile prepared according to the manufacture's instruction and stored in -40 °C. In the miniaturized modification a volume of 7 µL of Caspase-Glo® 3/7 substrate for each individual cell in detection vial was used. The microvials were sterilized by washing with ethanol, with UV-light and by fire torch before adding the substrate to avoid a nonsterile contact, and thus a nonspecific increase of the background. Moreover, the microvials filling took place in laminar box with the equipment for single use.

3.2.2. Recombinant caspase-3

The sensitivity of the method was tested within a broad scale of concentrations of active human recombinant cleaved caspase-3 protein (ab52101, Abcam, Cambridge, UK). Portions of 1 µL of recombinant caspase-3 dissolved in 1 x PBS at desired concentrations were gently mixed with 6 µL of Caspase-Glo® 3/7 substrate injected in detection microvials. The signal of Caspase-Glo® 3/7 substrate alone caused by its spontaneous decay was taken as a blank for each microvial. The amount of 100 active units of recombinant caspase-3 was dissolved in 100 µl mixture of PBS buffer with 5% of glycerol solution. Aliquots of 5 µl from the stock solution were prepared and frozen to - 40 °C. Immediately before experiments 1 µl from the aliquot of recombinant caspase-3 solution was diluted to solutions with different amounts of the activity units in the phosphate buffer solution. Prepared solutions of recombinant caspase-3 were used for the measurements of the calibration line in concentration range 10^{-7} to 10^{-3} of the activity units. The amounts of the activity units in solutions were later recalculated to amounts of recombinant caspase-3 in fg. The values of specific activity (300,000 – 400,000 units/mg), molar mass (28 000 g/mol) and purity (>

95% determined by SDS-PAGE), declared in the datasheet of recombinant caspase-3, were used for the recalculations.

3.2.3. Cell culture

Single-cell detection of caspase-3/7 was previously tested with cell line derived from human cervix epitheloid carcinoma HeLa [42, 43, 67]. In our laboratory, the HeLa cells were cultivated in MEM alpha medium (Gibco, USA) enriched by 10% FBS (F2442, Sigma Aldrich, St. Louis, MO, USA), penicillin/streptomycin (100 U/ml, 100µg/ml) and dispersed using 0.25 % Trypsin (ThermoFisher Scientific, Waltham, MA, USA). The cells were re-suspended in fresh PBS buffer at pH~7.4 and 0.1 M, (BioTech, Praha, Czech Republic) for further bioluminescence analyses. The apoptosis was induced by their addition to 1 µM solution of Camptothecin. The cells were treated for 16 hours. Passages 3–5 were used for the experiments.

3.2.4. Cell manipulation and detection

Micromanipulator ICSI (Eppendorf, Hamburg, Germany) mounted on the table of inverted microscope Olympus IX 71 (Olympus, Tokyo, Japan) was used for the collection and transfer of HeLa cells by a holding micropipette (Microtech IVF, Brno, Czech Republic) with i.d. of 20 µm into 10 µL detection microvials filled with 7 µL of Caspase-Glo® 3/7 reagent. Thus, the cells were selected one by one, captured and inserted into the detection vials and there subsequently checked under microscope. The microvials for bioluminescence reaction were made from glass tubes of sizes i.d./o.d. 1.3/1.9 mm (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany) by fusing at one end. After the filling with reagents, all eight vials were placed into a revolving carousel vial holder and the whole assembly was mounted in front of the photomultiplying tube (PMT) detection window.

Application of Camptothecin is well verified and widely used method for apoptosis induction. As a result of Camptothecin penetration into the cells, we observed induced

apoptosis followed by their detaching. As apoptotic, we selected detached cells floating in the media after Camptothecin treatment. Non-apoptotic proliferating alive cells adhered to the Petri dish were rinsed by Trypsin solution to be detached. For the selection of cells, we developed a glass C-holder made of a microscope cover slide, enabling free movement of the holding micropipette and fast, transparent choice of cells in a 1 mm thick layer of cell suspension, **Fig. 1** and **Fig. 2**.

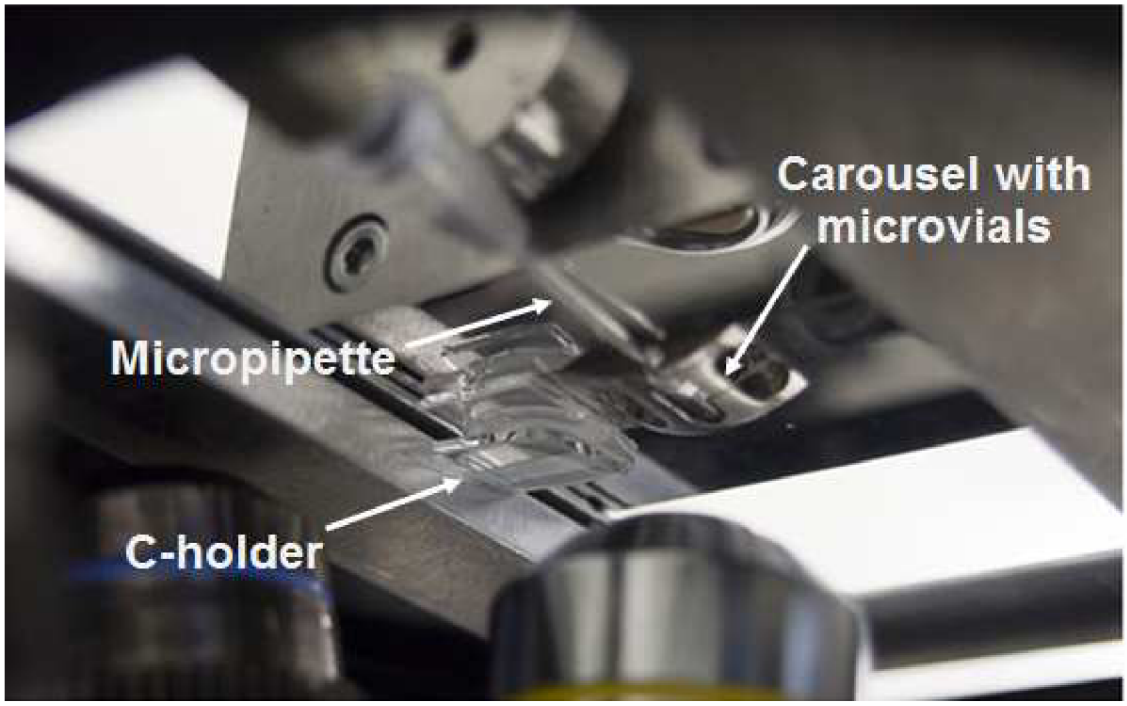

Figure 1 The C-holder with the cell suspension is attached to the carousel next to the microvial holder, and cells are moved immediately and directly into the microvials filled with the substrate.

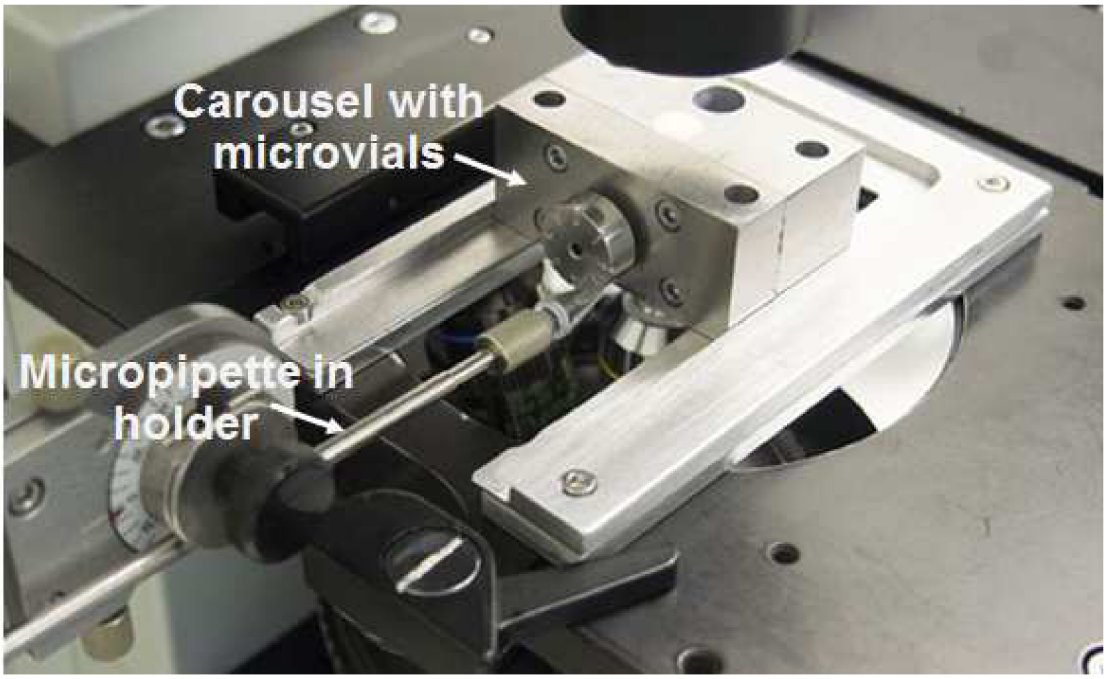

Figure 2 Cell-filling process. The carousel is mounted on the microscope table. The cells are moved from the C-holder into the microvials filled with substrate. The whole transfer process is checked under the microscope.

The advantage of the quantification method developed by Promega is complete cell lysis followed by bioluminescence detection. Thus, unlike in microscopic techniques, the total amount of caspase is reliably quantitated. The bioluminescence emissions leaving individual vials were detected consecutively and repeatedly in 5 min intervals for a period of about 150 minutes until the steady state was reached in all samples.

3.2.5. Vial holder

Detection of caspase-3/7 took place in microvials inserted in a carousel with eight positions. The carousel is closed in a lightproof cassette and rotates around its axis with only a single position facing outside in the direction of PMT detection window, **Fig. 3**.

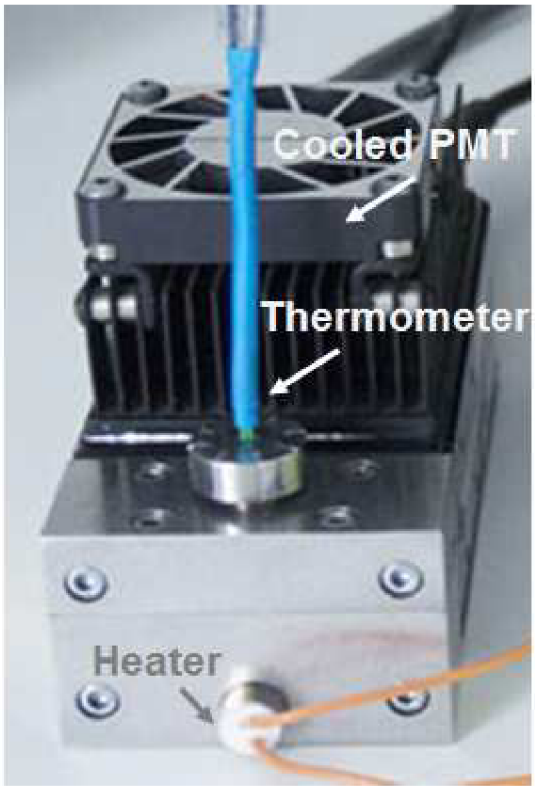

Figure 3 The carousel is heated in front of the body of the PMT detector.

During the measurement, the vials in front of PMT are changed stepwise manually. Thus, bioluminescence leaving individual vials is consecutively detected. No cross talk was observed, even if a vial with an extremely high bioluminescence signal was adjacent to an empty vial, **Fig. 4**.

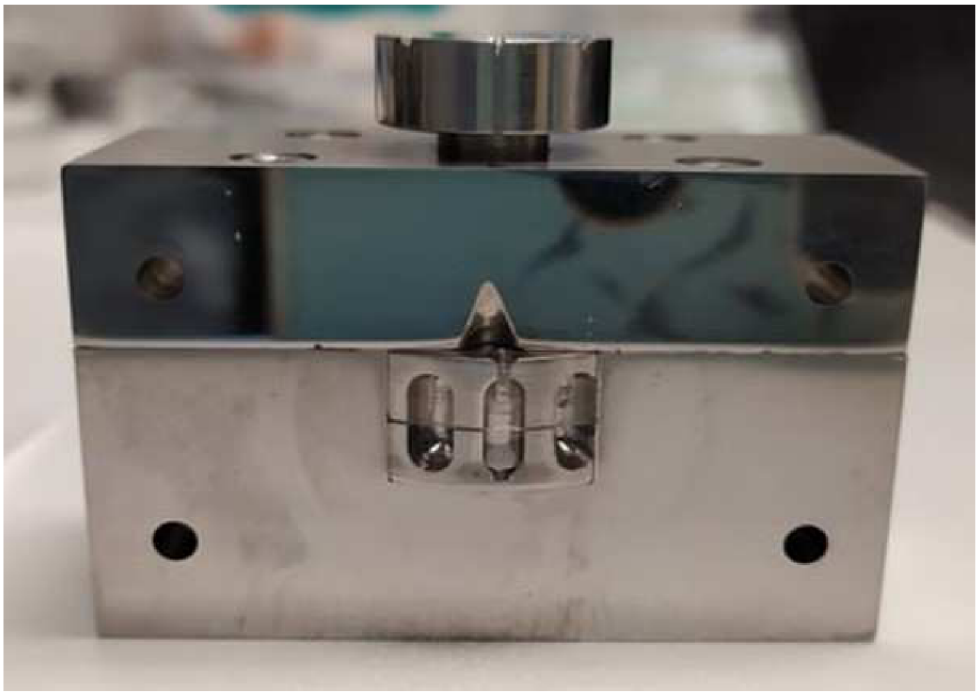

Figure 4 The carousel with eight vials positions for bioluminescence measurement. Vial filled with the substrate is placed in the middle part and next two positions are empty.

To guarantee standard conditions of bioluminescence reactions, the temperature of the cassette is kept at 37 °C by an electric cartridge heater connected to temperature regulator PRO 96-1 (RS Components B.V., AZ Haarlem, Nedherlands). A platinum resistance thermometer Pt100 is inserted directly into the body of the carousel. It was proved by experiment that the heated carousel does not influence the cooling of PMT photocathode. The dark current is constant during the increase of carousel temperature from 22 to 40 °C.

3.2.6. Detector

The detection assembly consists of a photon counting head (H7421-40 series) with a heat sink and fan (A7423), counting unit (C8855-01) and power supply unit with temperature control (M 9011), (all Hamamatsu Photonics, Hamamatsu City, Japan). The photon counting head is furnished with a photocathode cooled to a constant temperature of 0 °C, reducing thermal noise to about 4 counts per second. Thus, a high S/N ratio is ensured even at very low light levels of bioluminescence.

3.3. Results and discussion

All measurements were performed with laboratory-build device for parallel single-cell bioluminescence analyses of active caspases in individual cells (**Fig. 5**) in hand-made vials placed in carousel (**Fig. 6**). New improvements of the device for measuring caspases assays consisted of the introduction of a temperature sensor and attachment of thermoregulation unit which continuously monitors and regulates the temperature inside the carousel during the experiment.

In the case of enzymatic reactions, correct temperature regulation is very important and can significantly affect the speed of ongoing reactions and the ability of the enzyme to cleave the relevant substrate. The carousel was modified so that a thermostat could be introduced into its interior. Temperature inside the carousel was permanently controlled and regulated during all experiments to a set value 37 °C. Another carousel modification – a hole in the middle side over the position of placing vials, allowed the possibility to individual cells could be inserted directly into the detection vials with substrate reagent located inside the carousel positions by using a micromanipulator under an optical microscope [15].

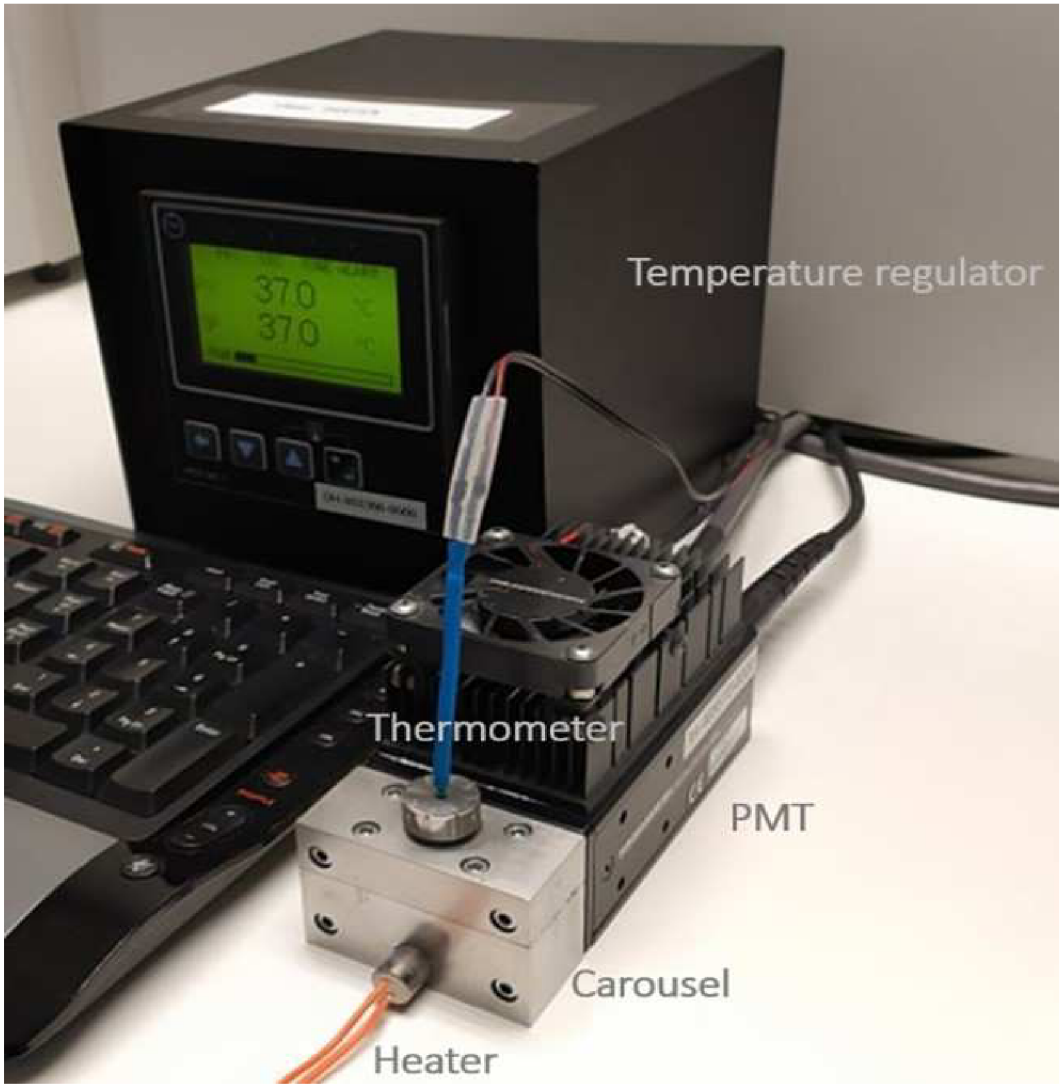

Figure 5 Assembly for bioluminescence measurement.

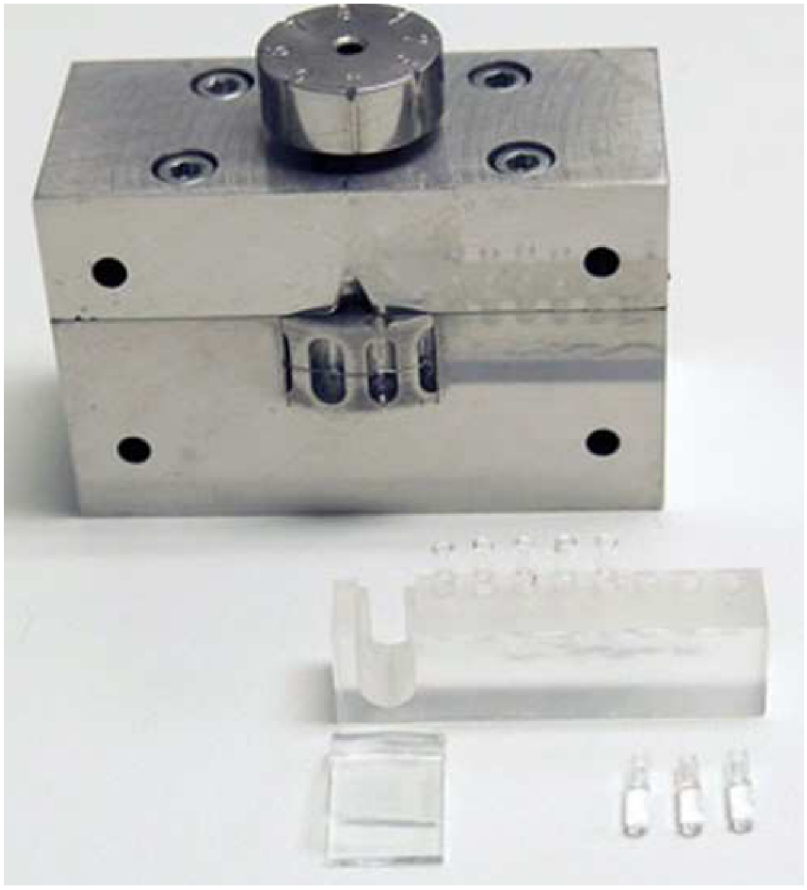

Figure 6 The carousel with eight vials positions for bioluminescence measurement, microvials and C-glass holder.

At the beginning of each experiment, blanks – vials containing only a volume of 7 μL of Caspase-Glo® 3/7 substrate, were measured immediately before adding cells directly to the vials by micromanipulator (**Fig. 7**). The cells were selected one by one, captured and inserted into the detection vials and all the process was checked under microscope throughout. For the selection of cells, we developed a glass holder of the C letter shape made of a microscope cover slide, enabling free movement of the holding micropipette and fast, transparent choice of cells in a 1 mm thick layer of cell suspension [15].

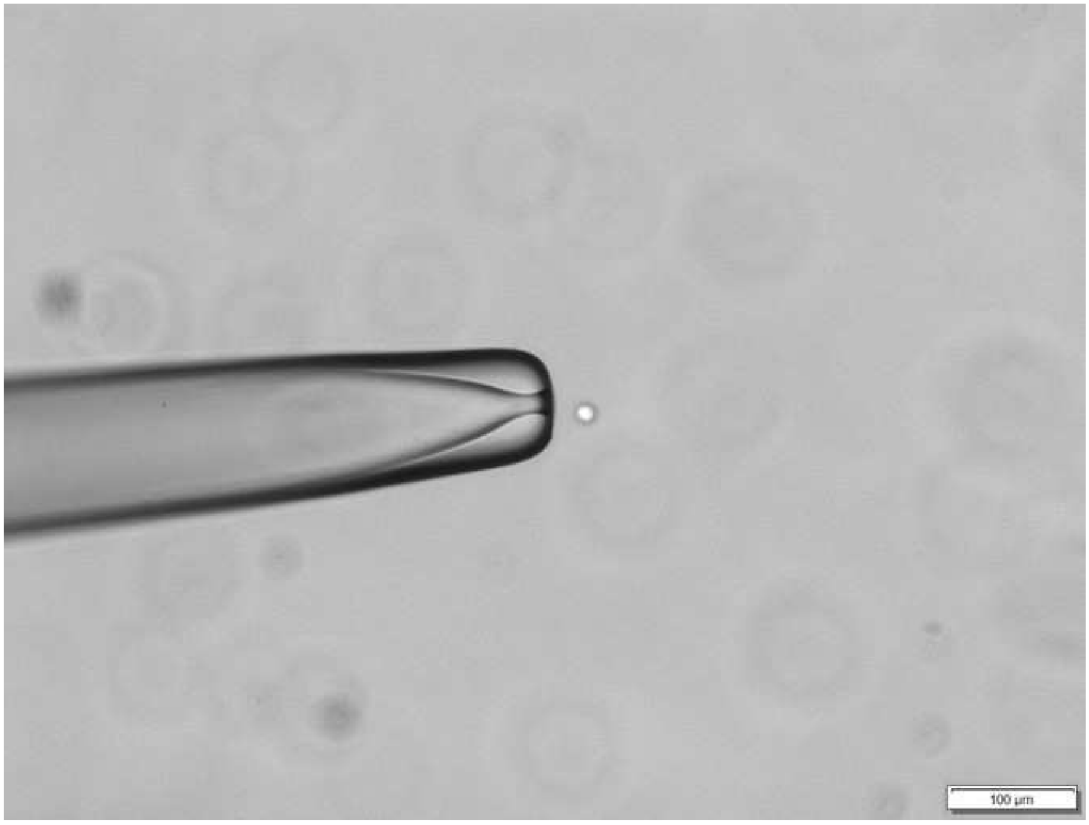

Figure 7 Phase contrast in white light of a collection of the HeLa cell by a micropipette of the micromanipulator.

The Caspase-Glo® 3/7 reagent shows background bioluminescence even without the presence of caspase as demonstrated in **Fig. 8**. The blank signal is caused mainly by a small portion of suddenly released aminoluciferin accessible to luciferase oxidation. It follows that the differences in background signal in individual vials are dependent on the variation of the reagent volume together with the optical properties of detection vials. Hence, the signals of blanks must be exactly subtracted from gross signals of samples to get net bioluminescence initiated by caspase. Therefore, a great effort has been made to optimize the correct determination of the blank signals. The process of making detection vials from glass tubes was optimized to prevent the appearance of microtubules at their fused end. All vials were always checked under a magnifier. Immediately before measurements, vials were washed with ethanol, dried and sterilized by fire torch and also with the UV light. All

manipulations with the vials and their filling with substrate Caspase-Glo® 3/7 reagent were provided in the flow-box sterilized with 65% solution of ethanol and by UV-light. Results obtained from measurements of the blank signals before and after optimization are demonstrated in **Fig. 9**.

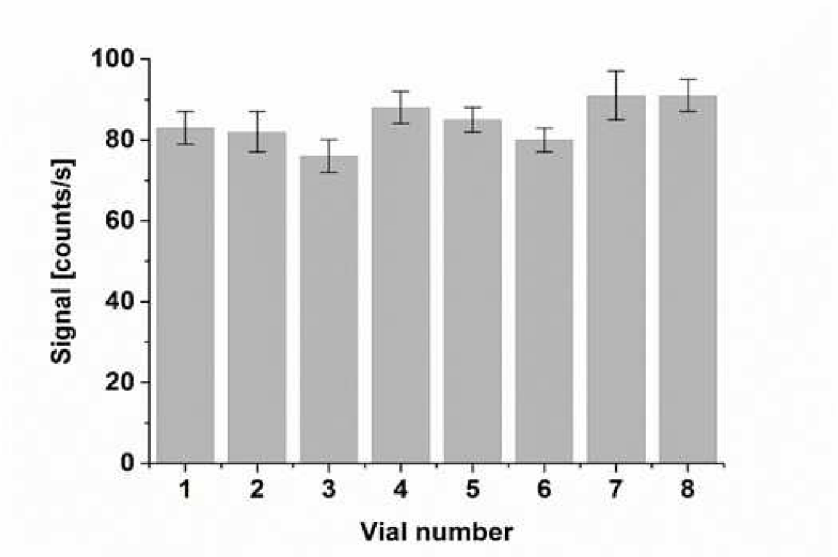

Figure 8 Variability of blank signals expressed in counts per second in eight individual vials. Blank samples contain 7 μL of Caspase-Glo® 3/7 reagent. Pooled mean signal and SD evaluated from 5 measurements of each vial is 84 and 4 counts/s, respectively.

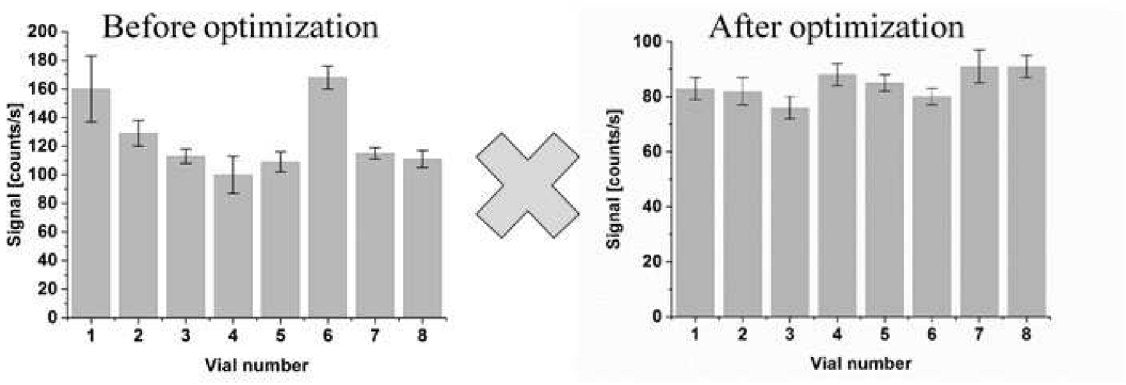

Figure 9 Variability of blank signals expressed in counts per second in eight individual vials. Blank samples contain 7 μL of Caspase-Glo® 3/7 reagent. Results of the mean signal and SD evaluated from 5 measurements of each vial demonstrates signal scatter before and after optimization.

The detection of active caspases is based on the bioluminescence chemistry commercially available as Caspase-Glo® reagents (**Fig. 10**). As was previously mentioned, the principle of the reaction is based on the reaction of luciferin conjugated with a caspase specific peptide with the caspase. At first the substrate is inaccessible for luciferase and after the cleavage of the peptide by an active caspase, the free amino luciferin is released and immediately oxidized by firefly luciferase resulting in photon emission. The ongoing reaction needs an initiation time to reach a steady state, and after the period of time the luminescence signal is stabilized and the photon flux is proportional to the amount of active caspase.

Figure 10 Scheme of homogeneous single-step assay based on bioluminescent Caspase-Glo® reagents.

The instantaneous intensity of bioluminescence is controlled by the kinetics of the diffusion reaction of the Caspase-Glo® 3/7 reagent. In order to be sure that the measured signal is really directly proportional to the concentration of active caspase 3/7, it was necessary to perform model experiments with recombinant enzyme. To determine the initiation time, which is necessary to reach the steady state, the reaction of Caspase-Glo® 3/7 substrate with the enzyme recombinant caspase-3 enzyme was monitored. The dependence of the luminescence emission of this model reaction on different amounts of the recombinant enzyme, is recorded as a course of the signal in time. The results are shown in **Fig. 11**. It is evident from the results that the steady state is attained in about an hour and at lower concentrations it takes more than three hours. Consequently, the signal reading time for the evaluation of a caspase calibration line was set at between 2 and 2.5 hours after mixing of the sample with the substrate.

All measurements were based on the photon counting of light. Since PMT detectors are extraordinary sensitive in the detection of low light levels, a high S/N ratio of bioluminescence signal is ensured. Moreover, there is no interference of a light scattering of an excitation and background light. Thus, the luminescence intensity I at a steady state indicates the sensitivity of the method.

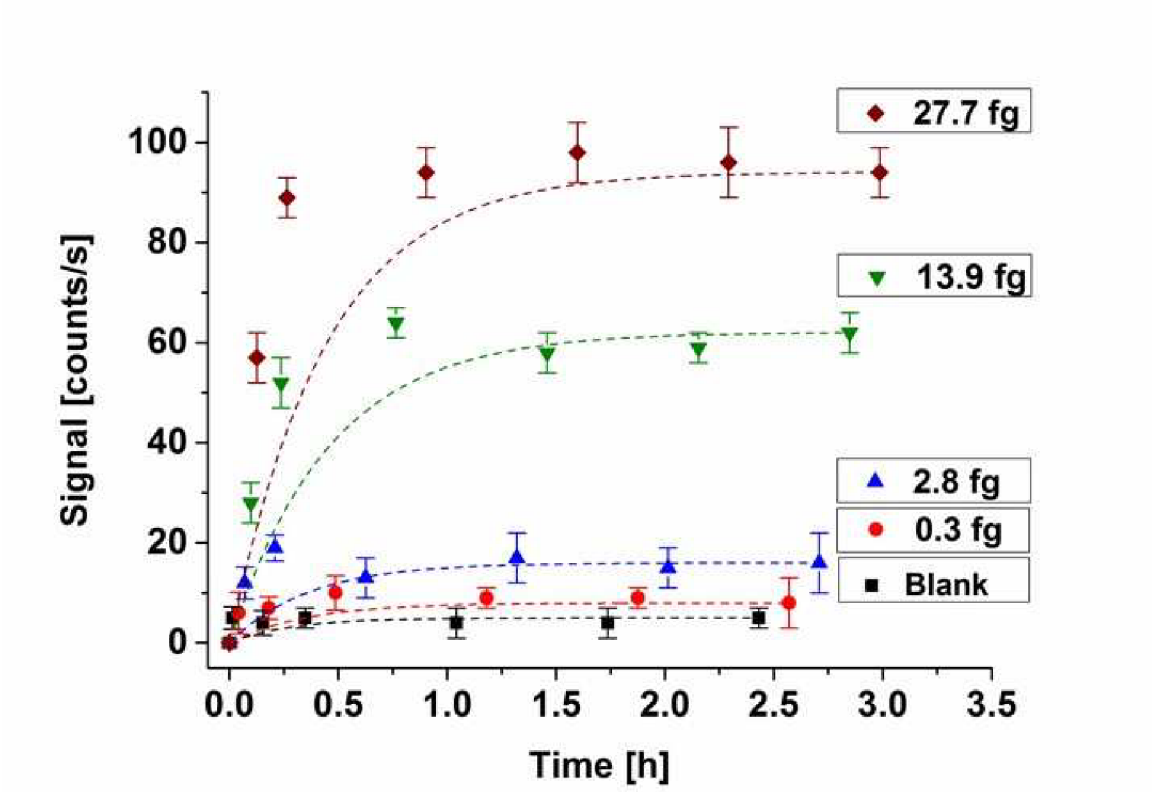

Figure 11 Time course of bioluminescence signal at various amounts of recombinant caspase-3. Recombinant caspase was diluted in PBS buffer and the required amount was added to Caspase-Glo® 3/7 reagent in individual vials. Mean values and SDs were calculated from 5 measurements.

The data from the time course measurements demonstrated in **Fig. 11** were used for the creation of a calibration line showed in **Fig. 12.** Here, the intensities of bioluminescence I are plotted against amounts of recombinant caspase-3 in individual vials. The calibration line was evaluated for five independent measurements of each concentration level. The signal intensities were read at a period of time between 2 and 2.5 hours after mixing of recombinant caspase-3 with Caspase-Glo® 3/7 reagent. Since the values of blanks were subtracted, the bioluminescence intensity at absence of caspase was arbitrary taken as zero. Thus, in the **Fig. 12** the relationship between the bioluminescence signals I (in counts/s) and the recombinant caspase-3 mass m (in fg), $I = 5.67^{*}\ m$ is presented as dash dot line. The calibration equation was estimated by least squares linear regression without intercept. The

grey area represents calibration line with 95% confidence interval. Coefficient of determination (R^2) of the regression model was evaluated to be 0.95.

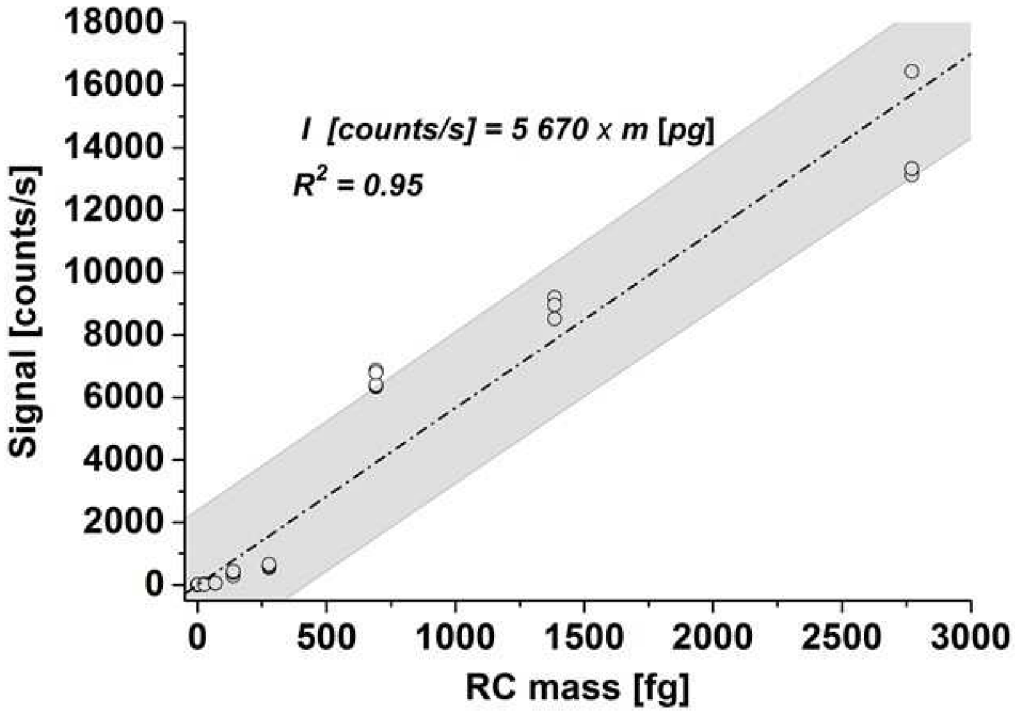

Figure 12 Calibration line (dash dot) - dependence of bioluminescence signal on recombinant caspase-3 mass. Experimental points are represented by hollow circles. Intensities *I* for all masses *m* are read out at 2 hours after mixing. Grey area shows 95% confidence interval of prediction.

The results from calibration analyses were furthermore used for determination of LOD and LOQ of the bioluminescence method. For the better display of the obtained results, the evaluation of both limits on the femtogram level of the calibration line plot is expanded in **Fig. 13**. As a result of numerical calculation and graphical readout, the LOD and LOQ of our system were evaluated to be 2.1 and 7.1 fg of recombinant caspase-3, respectively.

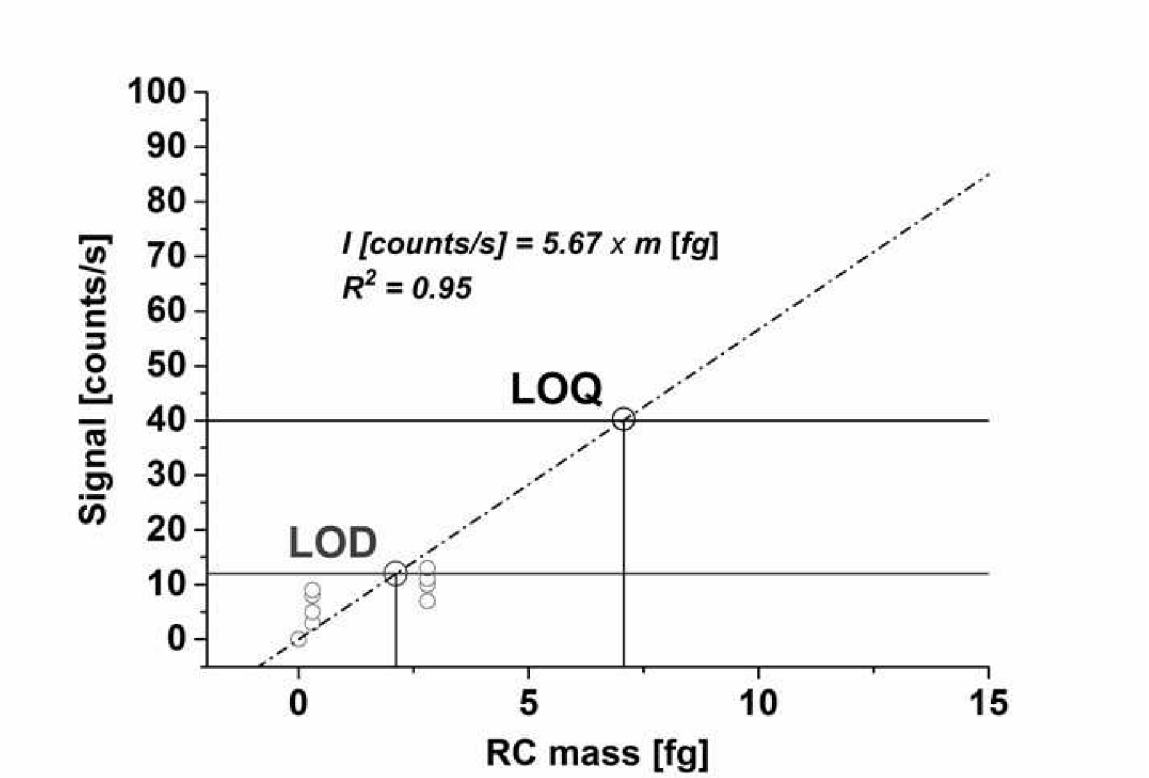

Figure 13 Calibration line (dash dot) is plotted in the range from 0 to 15 fg of recombinant caspase-3. Thus, LOD and LOQ is determined both graphically and numerically to be 2.1 and 7.1 fg, respectively. The limits are determined via IUPAC method as amounts of an analyte providing signal that equals three SDs of blank (4 counts/s) for LOD and ten SDs for LOQ.

Measurements and detection of caspase-3/7 were performed with cell line derived from human cervix epitheloid carcinoma HeLa (**Fig. 14**). In order to check and verify applicability of the improved methodology to a reliable single-cell analysis, masses of caspase-3/7 in individual apoptotic and non-apoptotic HeLa cells were quantitated.

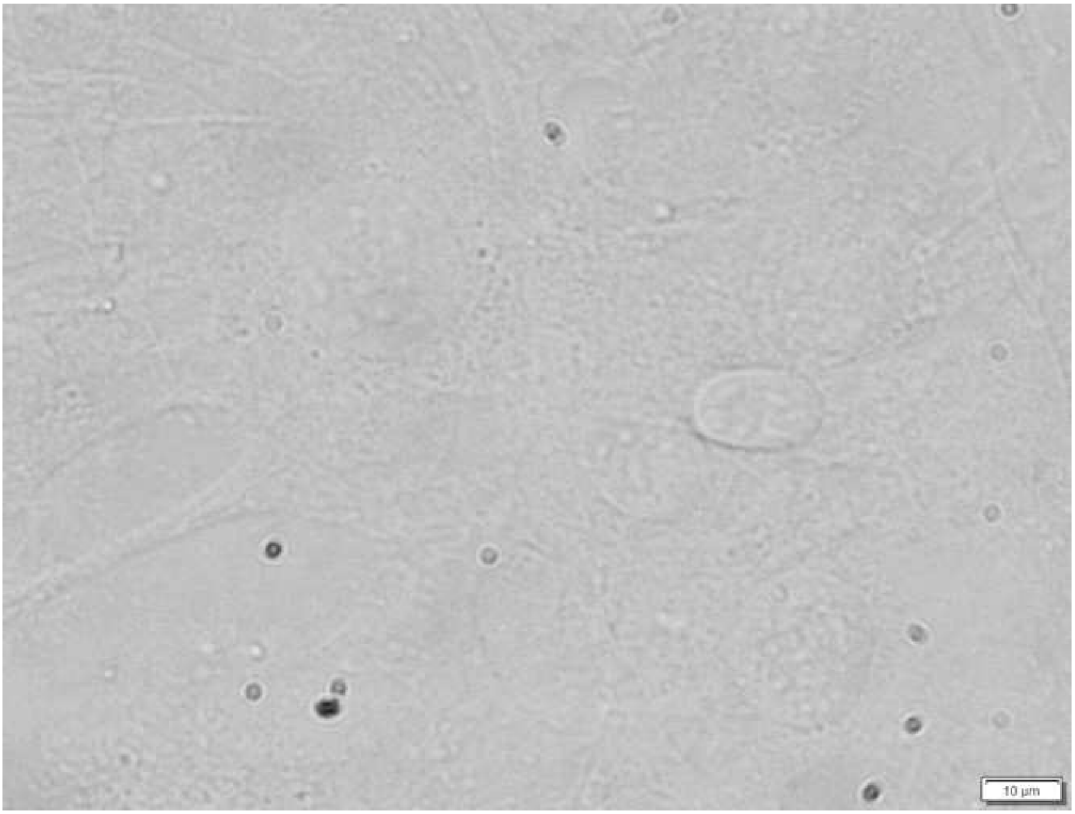

Figure 14 Phase contrast in white light of the HeLa cells in culture in the cultivation medium.

In **Fig. 15,** there are demonstrated the results obtained from the experimental testing of the effect of cytostatics (Camptothecin) on the heterogeneity of caspase- 3/7 amounts in model treated and untreated HeLa cells. Here, the mean masses are evaluated to be 25.2 and 153.5 fg for untreated and treated cells, respectively. These values together with their SDs (error bars) are determined for signals from independent measurements of 16 vials with untreated and 27 vials with treated cells. Mean bioluminescence signals are expressed as mean masses of caspase-3/7 via the calibration line ($m = I/5.67$).

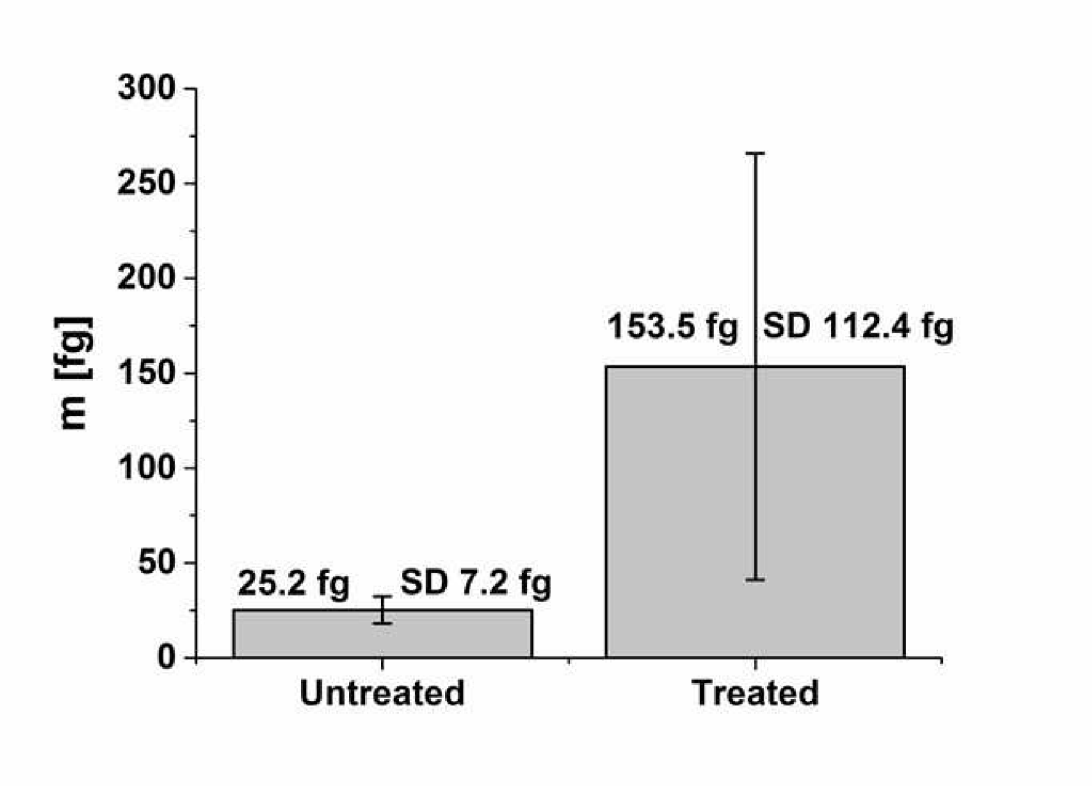

Figure 15 Average amounts of caspase-3/7 in untreated and treated HeLa cells are determined to be 25 and 154 fg, respectively. The mean masses and SDs, expressed as error bars, are evaluated from 16 and 27 independent measurements of untreated and treated cells, respectively.

As was expected by research hypothesis, the mean amount of caspase-3/7 in treated cells is higher than in untreated ones, corresponding to the role of the enzyme in cell death. The surprisingly broad range of masses spans values from 11 to 38 fg in untreated and from 24 to 450 fg in treated cells. Thus, it is highly probable that some of the treated cells did not go through apoptosis. Moreover, a relatively high dispersion of masses of treated cells can be explained by natural variations in cellular physiology, including sizes and phase of cell cycle. It is evident that much larger set of cells must be investigated in practice, to get statistically reliable data. Heterogeneity of caspases in individual apoptotic cells can be essential for adjustment pharmacological treatment of different diseases in the future. The relatively high caspase masses, when compared with LOD and LOQ of our system,

demonstrate that its sensitivity is satisfactory for the detection and quantification of caspase-3/7 in individual HeLa cells. The LOD and LOQ values represent 1.4% and 4.6% of the amount of caspase-3/7 in an average treated cell and 8.4% and 28% of the amount of caspase-3/7 in an average untreated cell. As shown in **Table 1**, caspase-3/7 amounts expressed as numbers of molecules in individual HeLa cells are comparable with data published in literature [67, 68]. The differences are expectable in cells stimulated by various methods.

Table 1 Comparison of caspase-3/7 amounts of bioluminescence signal of treated and untreated HeLa cells with values presented in other published works [67, 68].

Caspase origin	**Mean mass ± SD [fg]**	**Number of molecules min-max range**	**Reference**
LOD (recombinant caspase-3)	2.1	4.52×10^4	This work
LOQ (recombinant caspase)	7.1	1.53×10^5	
HeLa untreated	25.2 ± 7	$(2.34 – 8.20) \times 10^5$ **	
HeLa treated (treated with 1 μM Camptothecin for 24 hours)	153.5 ± 112	$5.06 \times 10^5 – 9.69 \times 10^6$ **	
HeLa (mitochondrial outer membrane permeabilization)		10^4 - 10^5	[68]
HeLa (electrostimulated)	1.06*	2.3×10^4	[67]

**The mass of active caspase was estimated from the published concentration and the volume of the cell with an averaged diameter of 30 μm.*

*** The molecular weight, MW = 28 000 g/mol, used for calculations was taken from the data sheet of the active human recombinant cleaved caspase-3 protein (ab52101, Abcam, Cambridge, UK).*

3.4. Concluding remarks

Through our research work and by provided experiments, we have confirmed that analyses of chemical content of single-cells level request special demands on methodology and instrumentation. In our system, the extraordinary sensitivity and selectivity are given by homogeneous single-step assay based on luciferin-luciferase bioluminescent reaction. Moreover, the bioluminescence signal of individual cells is detected by PMT with a cooled photocathode working in photon counting mode, the most sensitive method of low-light measurements.

Our results of the bioluminescence method testing demonstrate that for a high sensitivity and selectivity of the method, standard analytical conditions and sterile manipulation with the substrates, samples and all equipment must be obeyed. Very important for the reliability of single-cell analyses is to strictly maintain purity of chemicals and sterility of all vessels and instruments.

Based on the optimization of our device for detection and quantitation of caspases, we reached the sensitivity satisfactory for single-cell analyses [14]. We developed instrumentation and methodology for reliable evaluation of the absolute amount of active caspase-3/7 and their variability in cells under various conditions. By keeping the temperature of bioluminescence reaction at 37 °C, improving variations in geometry of detection vials, precise background subtraction from raw signals and minimization of the collection time of cells, the LOD and LOQ of the method were improved substantially to reach the values 2.1 and 7.1 fg of recombinant caspase-3, respectively. These limits are much lower than average mass of caspase-3/7 in apoptotic (153.5 fg) or even non-apoptotic (25.2 fg) cells. The absolute amounts of caspase-3 in HeLa cells are comparable with values published in literature.

Moreover, in our subsequent article, we aimed to determine the heterogeneity of caspase content in HeLa and osteoblastic MC3T3-E1 cells as a part of the investigation of non-apoptotic caspase functions [15]. Our analysis revealed a significant increase of the absolute amount of active caspase-3/7 in differentiated cells compared with proliferating ones with

even higher elevation in apoptotic cells. The exact quantification of the active caspase-3/7 level revealed a three-times-higher amount in differentiated cells (26.73 ± 10 fg) and six times higher in apoptotic cells (51.40 ± 17 fg) when compared with the proliferating ones (8.25 ± 3 fg). These quantitative results indicate the role of caspases as targets for stimulation of cell differentiation in supportive therapies or simulated differentiation of stem cells into request cell lineages.

The presented miniaturized bioluminescence instrumentation can potentially serve as a sensitive tool for diagnostics and monitoring of therapeutic, e.g., anti-cancer, strategies, where single-cell analyses are crucial to distinguish tumors from surrounding healthy tissue cells. Another advantage of this method is that we can select and capture cells with a particular shape and correlate their morphology with measured signals.

The results described in this dissertation thesis indicate a potential of the method to improve detection and increase its throughput in miniaturized devices, e.g. in microtiter plates with 1536 wells, each filled with 5–8 μL of the reagent, essential in clinical practice. As our method is simple and universal, it could be further widely used for other kinds of cellular research.

4. Förster Resonance Energy Transfer probe with quantum dot for a long-time imaging of active caspases inside individual cells

4.1. Theory of quantum dot probes

In a general scale, fluorescent probes allow researchers and biologists to detect individual components and reactions of complex biomolecular structures with excellent sensitivity and selectivity, including monitoring of various processes in living cells [69]. A lot of commonly used fluorescent probes is based on usage of fluorophores or fluorescent dyes and are designed for responding to a specific stimulus or process, or to localize only within a specific region of a biological sample [70]. For the detailed detection and monitoring of many bioanalytical processes and also for bioimaging applications, the use of fluorescent probes is often absolutely unavoidable [71, 72]. Methods for detection and imaging of proteases, like caspases after their activation *in situ* in individual cells very often use specific commercially available fluorescence substrates. As examples of commonly used substrates for caspase analyses, two commercialized assays can be mentioned, FAM-FLICA® and Magic-Red®.

Fluorescently labeled inhibitors of caspases (FLICA) are cell-permeant fluorescent probes that covalently bind to active caspase enzymes via fluoromethyl ketone. The FLICA probes are commonly used for the monitoring of the intracellular locations of active forms of the caspase enzymes inside living cells [49]. In detail the fluorescein- or sulforhodamine-labeled inhibitors contain 3–5 different amino acid sequences as caspase target linked to a fluoromethyl ketone reactive group. In the case of caspases, the ligands that specifically and covalently bind to their active centers are specific carboxy-fluorescein-labeled peptide-fluoromethyl ketone (FMK) inhibitors. These ketone reagents penetrate through the plasma membrane of live cells and the recognition peptide moiety of these reagents provides specificity of the ligand to a particular caspase. Their irreversible binding to the active centers ensures that only the cells with the activated specific caspase enzyme become labeled [49]. Although the primary target for these fluorescently-labeled inhibitors was

originally assumed to be restricted exclusively to active caspase catalytic sites, we now have evidence of their binding to other apoptosis-associated non-caspase enzymes as well [73].

Several other fluorogenic caspase detection systems involve the use of the hydrophobic dye, cresyl violet. This staining, utilized for its metachromatic properties [74], provides the fluorescence light with optimal emission at 628 nm in aqueous solutions, and was subsequently converted to a specific assay substrate for the detection of the enzyme caspase-3/7 [75]. Recently developed Magic Red® (MR) reagent uses the cresyl violet, covalently bonded to the peptide sequence specific for the detection of caspase enzymes. In the Magic Red® Caspase-3/7 Assay Kit, the two copies of the DEVD flanked by other amino acids are covalently bonded to the photostable red fluorophore. The DEVD (peptide sequence: Aspartic acid, Glutamic acid, Valine and Aspartic acid, written in the opposite order from C to N end) is the specific cleavage sequence of caspase-3/7. Upon the cleavage of the DEVD substrate by an active enzyme, free molecules of the cresyl violet are released and provide fluorescence emission. Thus, maximum fluorescence is achieved upon cleavage of both DEVD side chains [75, 76].

Although fluorescent probes based on fluorescent dyes are widely used in unique detection systems and have a lot of advantages like their simplicity, low limit of detection (LOD) of fluorescence emission and especially bioimaging capability, they have one drawback. In the case of long-term monitoring or under conditions of high light intensity of excitation light, irreversible destruction or photobleaching of the fluorescent dye can occurs. These irreversible changes and especially photobleaching will then become the primary factor limiting fluorescence detectability of the used fluorescent probe. For example, the multiple photochemical reaction pathways responsible for photobleaching of the fluorescein have been investigated and described in detail in the literature [77-79]. For this reason, the use of fluorescent probes based on nanomaterials has increased considerably [80-85]. In particular, the use of quantum dots as fluorescent emitters appears very advantageous in many bioanalytical applications and for biological imaging [80, 83-88].

According to their unique photophysical properties, QD nanoparticles have become frequent components of highly luminescent probes with a broad application in bioanalytical

chemistry during the past two decades [17, 82, 89-93]. The usage of QDs in luminescent probes is widely changing the development of new biosensors based on the nanoparticle material [94-97]. The implementation of QDs in the bioanalytical chemistry has already progressed from pure research activities to the routine applications of commercially available fluorescent assays kits [98-100] and nowadays, luminescent semiconductor quantum dots (QDs) are widely applied in different bioanalytical and medical treatment areas [80, 82, 83]. Sensitive and selective biosensors based on QDs are used for detections in a wide range of biomedical applications [35, 87, 101, 102], for detection of the specific cancer markers [103], determination of disease-related biomolecules [83] and as fluorescence probes for cell imaging [104-111]. Compared with commonly used luminescent organic dyes, QDs exhibit excellent photophysical properties: size- and composition-tunable emissions, good quantum yields, wide ranges of excitation wavelengths, narrow and symmetric emission spectra, low photobleaching and relatively long size-dependent luminescence lifetimes [88, 112-115]. Moreover, their high extinction coefficients make them advantageous for absorption and transfer of relatively large amounts of energy [81, 116]. The excellent properties of QDs as donors in FRET brought several significant advantages when implemented in biological analyses [25, 80, 116, 117]. According to the definition, the FRET is a photophysical process by which the energy of the donor luminophore in an excited state is nonradiatively transferred to the acceptor luminophore and then emitted as a longer wavelength photon. The immediate nonradiative process is based on dipole-dipole interactions between a donor and acceptor in the range of 1–10 nm [118-120].

Nevertheless, a further development of preparation and characterization methods of nanoparticles is still required to substantially increase the sensitivity of analyses used in bioanalytical research [121-123]. Indeed, numerous bioanalytical applications of QDs include very specific detection and quantification of biologically relevant molecules, cell imaging, cell tracking, or targeted detection of pathogens, toxins or drugs distribution inside the tissues or living cells [124-126]. Thus, in such biological studies, the sensitivity and selectivity of the fluorescent probe can have a large impact on potential future research or medical treatment. However, for improving selectivity and sensitivity of various biological

applications of the QD based probes, conjugation of QDs with specially selected biomolecules is required [80, 127-130]. Moreover, in most of cases QD particles has to be modified by charged ligands in order to improve their water-dispersion. It follows that capillary electrophoresis with laser induced fluorescence detection (CE-LIF) is an appropriate method of choice for analyses of the QD surface modifications including determination of particle effective charge [113, 131-133].

Since our research was focused on the fluorescence microscopy analyses of the proteolytic active caspases, the aim of the second part of the dissertation thesis is to synthesized and test a novel molecular probe. The synthesis strategy is based on the previous research with the preparation of QD-antibody immunoprobes and FRET probe for DNA analysis [83, 116].

In detail, the function of our probe is based on Förster resonance energy transfer (FRET) between a highly luminescent QD as a donor and fluorescence quencher as an acceptor linked by a specific peptide as a cleavage site for enzyme caspase 3/7. Thus, in principle, our probe consists of a cleavable peptide with caspase-specific amino acid sequence DEVD flanked by QD as a FRET donor and BHQ2 quencher, efficiently dissipating the majority of QD luminescence emission. Thus, the luminiscence of QD is switched on after the cleavage of the peptide by an enzyme and the release of the quencher. The aim of the synthesis is to form a photo-stable fluorescent probe suitable for a long-time sensitive and selective monitoring of the enzymatic activity of peptide-cleaving enzymes in cells under a fluorescence microscope. To demonstrate the capability of our synthesized QD-probe to stain caspases for a long time, its stability monitored as a fluorescence bleaching is compared with commercially available Magic Red (MR-probe), widely used as a standard assay for imaging of caspases *in vitro*.

4.2. Experimental part

4.2.1. Chemicals and instrumentation

The following components of the QD-probe were purchased: CdTe core-type QDs powder (Sigma-Aldrich, USA). The QDs of a diameter of 3.5 nm and molecular mass of 79 000 g mol^{-1}, are -COOH functionalized by succinic acid. Their maximum fluorescence emission λ_{em} is 607 nm [134]. The peptide composed of GTADVEDTSC sequence of amino acids with molecular mass of 996.99 and a purity of 95.44% was synthesized at ProteoGenix (FR). Succinimidyl ester of quencher BHQ-2 is from Biosearch Technologies (USA). Other chemicals for the preparation of background electrolyte (BGE) of CE and sample solvents were Tris(hydroxymethyl)aminomethane (TRIS) and 3-(N-Morpholino)propanesulfonic acid (MOPS) (Sigma-Aldrich, USA), DMSO (p.a.) and acetonitrile (p.a) (Penta, CZ). A fluorescence neutral marker of electro-osmotic flow (EOF), coumarin 334, was purchased from Sigma-Aldrich (USA). Fluorescence probe Magic Red was purchased from ImmunoChemistry Technologies (USA) as Magic Red® Caspase-3/7 Assay Kit enabling to detect and monitor apoptosis *in vitro* over time via intracellular caspase activity.

Cultivation medium DMEM (Dulbecco's Modified Eagle Medium, which contains four times the amino acid and vitamin concentration of original Eagle's Minimal Essential Medium; Termofisher, USA) supplemented with L-glutamine (Biosera, FR), 10% fetal bovine serum (FBS), enriched by penicillin/streptomycin (100 U mL^{-1}, 100 µg), (SigmaAldrich, USA, Biosera, FR). The preosteoblastic cells type MC3T3-E1 (European Collection of Cell Culture, c. n. 99072810) from mouse calva were treated by 0.5 µM solution of Camptothecin in a cell incubator at 37 °C and 5% CO_2 for 24 hours before observations. The treated cells in cultivation site on the 48 well plate were washed with 0.1 M PBS buffer glutamine (Biosera, FR) at pH = 7.4 and covered with the fresh DMEM medium in a volume of 480 µL. The synthesized fluorescent QD-probe was added to the cultivation vial in a volume of 20 µL. The cells were incubated with QD-probe at a concentration of 1 mM, at 37°C and 5% CO_2 for different times and monitored under a fluorescence microscope. Washing of the cells with 0.1 M PBS and changing of the DMEM

media was done once per a week during the long-time experiments. Recombinant caspase-3 protein ab52071 was purchased at Abcam (UK).

The QD-probe cleavage inside the cells was monitored by microscope Olympus IX 71 (JP) with Xe-lamp X-Cite ser. 120 Q (Lumen Dynamics, CA). The fluorescence emission was observed at 600 nm (excitation wavelength of 530 nm). Spectrofluorometer Jasco FP-8500 (Jasco International co., JP) was used for the evaluation of reaction enzymatic kinetics of the QD-probe. Spectrometer Plasma Quant PQ 9000 Elite (Analytik Jena AG, Germany) and Cd standard solution Astasol (Analytika, Czech Republic) were used for ICP-OES measurements.

4.2.2. Capillary electrophoresis

The QDs and their conjugates were analyzed by a laboratory-built CE-LIF system. A laser diode, RLTMLL-405 Series (Roithner Lasertechnik, Austria) with 405/10 nm laser cleanup filter (MaxDiodeTM; Shamrock, USA) was used as the excitation source. The luminescence emission was collected by a microscope objective (40×0.65 NA, Oriel, USA) positioned perpendicularly to the separation capillary and the excitation laser beam. A PMT detector (R647; Hamamatsu, Japan) was used for the monitoring of luminescence emission at 607 ± 35 nm selected by a system of two optical filters including a 405 nm long pass edge filter (EdgeBasicTM; USA) and a 607 ± 35 nm band-pass filter (BrightLine®, USA). The detector signal was recorded at a sampling rate of 25 Hz by the data acquisition and evaluation system CSW 1.6 (Data Apex, Czech Republic). The electrophoresis system was powered by a high power supply (Spellman CE 1000R, USA) at a voltage of +9 kV during injection and analysis. Before each analysis, the bare fused-silica capillary (Polymicro Technologies, USA) of a total and effective length of 50 and 40 cm, respectively, an i.d. of 100 μm, was rinsed with 0.1 M NaOH distilled water, and BGE (50 mM TRIS/MOPS, pH = 9). An electroosmotic flow (EOF) carried the negatively charged QDs towards the detector. The EOF mobility was determined using a neutral coumarin 334 to be 6.84 x 10^{-8} $m^2V^{-1}s^{-1}$.

4.3. Synthesis of FRET QD luminescent probe

This dissertation work propose a novel synthesis strategy of the QDs fluorescent probe. The 3D spectrum of quantum dots, used for synthesis of our fluorescent probe is demonstrated in the **Fig. 16**.

The luminescent probe comprising a specific peptide flanked by a QD on one side and a luminescence quencher on the other side was synthesized in two steps. First, water-soluble CdTe QDs functionalized by two COOH groups of succinic acid at about 14 positions on the surface [132] were conjugated with a specifically designed peptide (GTADVEDTSC) using a ligand-exchange approach. Here, the SH- group of cysteine at the C-terminal of the peptide substitutes the mercaptosuccinic acid at the QD surface by forming a covalent bond with the Cd atom in nanoparticle crystal lattice [86, 135], the reaction scheme is described in the **Fig. 17**.

The reaction yield of QD ligand exchange with peptide was checked by CE-LIF and obtained results are shown in **Fig. 18**.

In the second step of synthesis, the opposite N-terminal amino group of the peptide reacts with the succinimidyl group terminated BHQ-2 quencher, the reaction scheme is demonstrated in **Fig. 19**. This strategy avoids any crosslinking between individual nanoparticles and any nonspecific conjugation bonds.

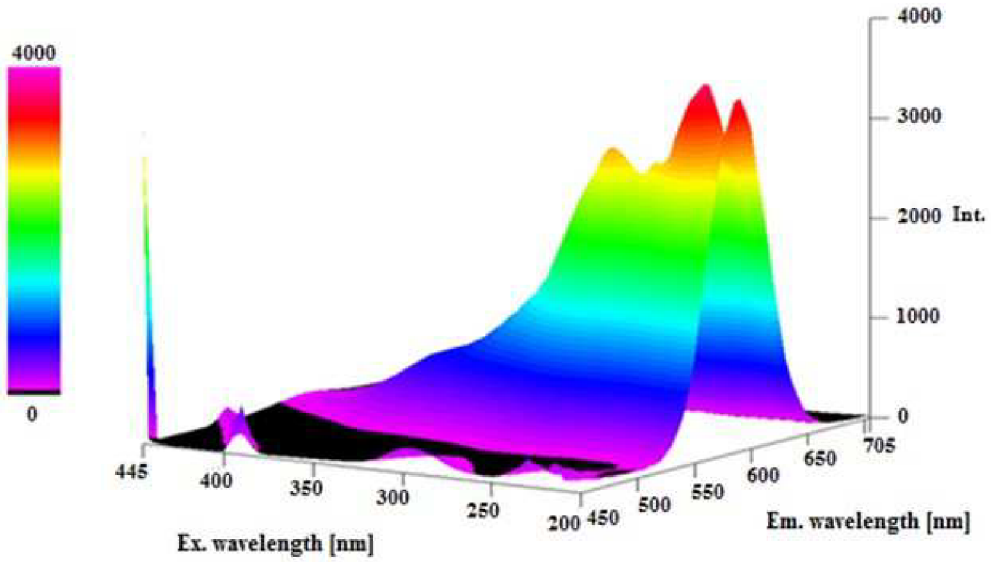

Figure 16 3D spectrum of CdTe core type quantum dots, functionalized by –COOH; fluorescence λ_{em} 610 nm, excitation wavelengths 200-445 nm, emission wavelengths 450-705 nm, QD concentration: 1 mg/L.

4.3.1. First step of synthesis

In more detail, the peptide was dissolved at a concentration of 1 mg mL^{-1} in a mixture of ultrapure water (UPW) and acetonitrile at a volume ratio 1 : 3 to stabilize the peptide against hydrolysis. The addition of acetonitrile to an aqueous solvent is suggested to weaken the hydrophobic interaction and to enhance the peptide-peptide hydrogen bond by π- π stacking and van der Waals interaction [136, 137]. The QDs were dissolved in a UPW at a concentration of 1 mg mL^{-1}. Then, QDs and the peptide were mixed in a 1.5 mL Eppendorf tube. Based on our previous experience, the optimized molar ratio of 1:100 of QD to peptide was chosen for the first step of the synthesis. The molar masses of the QD and the peptide are 79 000 and 996.99 g mol^{-1}, respectively. It follows that the molar ratio 1:100 (QD to peptide) responds to the gravimetric ratio 100:126.6. Thus, 100 µL of the stock solution of QDs (1 mg mL^{-1}) was mixed with 126.6 µL (1 mg mL^{-1}) of the peptide in the first reaction step of synthesis (**Fig. 17**).

Then, the excess of the unreacted peptide was removed by centrifugal filtration in AMICON (Merck, Germany) tubes with a 30 000 g mol^{-1} cut-off centrifugal filter at 6,000 rpm for 10 min. The filtrate was then washed twice with UPW and the product was concentrated by centrifugal filtration at 6,000 rpm for 15 min to get an approximate concentration of 1 mg mL^{-1} in UPW. A volume of 25 µL of the concentrated product was taken for control analysis by CE-LIF to check the yield of the ligand exchange procedure.

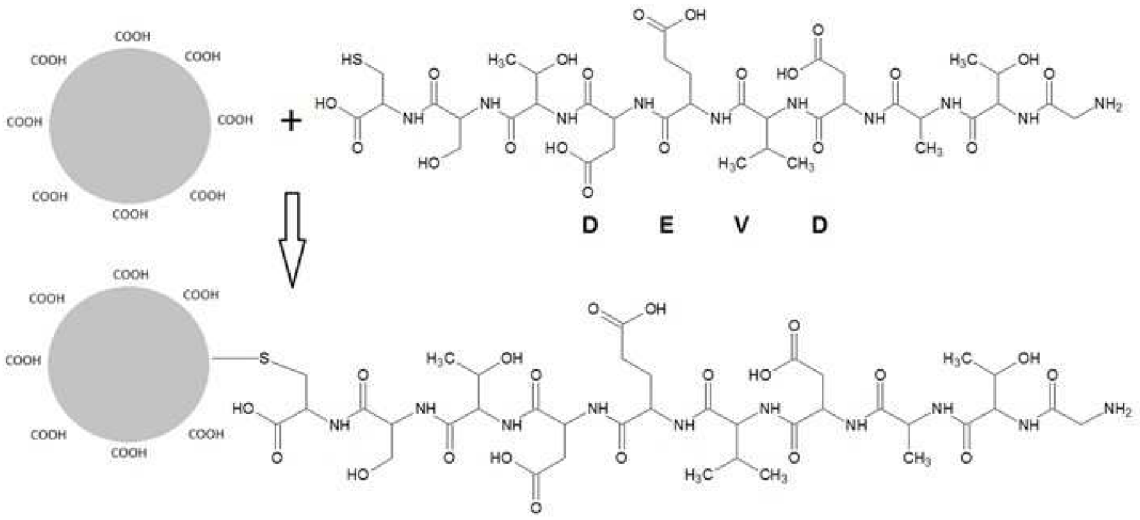

Figure 17 The reaction scheme of the ligand-exchange synthesis on the surface of CdTe QD.

At first, a lot of experiments had to be done to find the suitable conditions and optimize the separation of the native quantum dots and bioconjugation product by laboratory-made CE-LIF. Many different buffers were tested for the separation of analytes: phosphate buffer, electrophoretic buffers - MOPS, TRIS, TAPS and also mixtures of buffers - TRIS/TAPS, TRIS/MOPS at different concentrations - 0.5 M, 0.2 M, 0, 1 M and 0.05 M in the ranges of different pH values 7 -10. The best separation was achieved using buffer TRIS and MOPS mixture with concentration 50 mM and pH = 9 value. Then the different lengths (from 10 to 60 cm) and diameters (50 µm, 75 µm and 100 µm) of the capillaries were tested. The best

results were achieved with the capillary of a total and effective length of 50 and 40 cm, respectively, and i.d. of 100 μm. The best injection conditions were estimated as to be 5 s for coumarin and 20 s for samples of conjugation reaction mixture with the positive voltage of +9kV.

The analysis was performed in bare silica fused capillaries. Here, a strong EOF carried the negatively charged samples toward the detector located at the grounded end of the separation capillary (**Fig. 18**). Thus, the analytes migrate in the order opposite to their ionic mobilities. It means that, the larger conjugates reach the detector faster than smaller native QDs. To test the separation resolution of our methodology and instrumentation system, we mixed the product of the ligand exchange reaction with native QDs and injected it into the capillary. As shown in **Fig. 18**, under the optimum conditions, the mixture of reaction products and native QDs (black record C) can be separated nearly completely. Similarly, the records of our product (red A) and native QDs (blue B) injected independently demonstrate nearly zero impurity of unreacted QDs in our product. A high reaction yield is necessary, because unreacted QDs presented in the probe, could be a reason of an unselective false positive signal. The yield of the first step of synthesis was evaluated by ratio of the peak areas of the product in its zone (A) and in the zone of native QDs (B). The yields of different syntheses vary from 85 to 99% for 40 syntheses. A very low concentration of unreacted products in the zone of native QDs (B) is proof of a high purity of intermediate product of synthesis. All the manipulations with samples were done at room temperature in darkness.

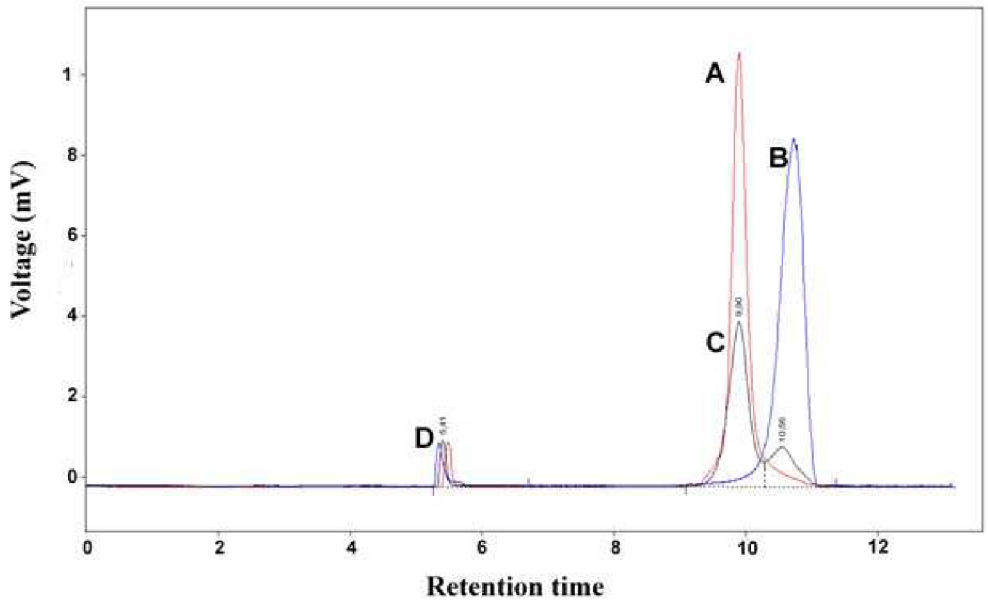

Figure 18 Electropherogram of the QDs conjugated with the specific peptide (A), native QDs (B) and their mixture (C). The EOF mobility was determined using coumarin, a neutral fluorescent marker (peak D) to be 6.86 x 10^{-8} $m^2V^{-1}s^{-1}$. Excitation at 406 nm, emission at 610 nm. BGE: 50 mM TRIS/MOPS buffer, pH = 9. Bare fused silica capillary: total/effective lengths 50/40 cm, i.d. 100 μm. Separation voltage: +9 kV, electrokinetic injection: +9 kV for 20s.

In a previous work analyses in the described system, it was proved that QD conjugates with a different number of ligands are separable by CE [133]. Thus, the appearance of a narrow peak (A) indicates conjugation of only a single peptide to a QD particle.

The principle of a single covalently bonded ligand anchor group on a surface of CdTe nanocrystal was explained by Huang et al [138]. In this review is explained potential change of inorganic nanomaterial surface properties modified by covalent or ion bond with organic materials. A generalized molecule paradigm for organic molecules used for explanation interface modification of inorganic nanomaterials - "anchoring-functionality" design is described. The anchor group binds to nanomaterial surfaces, either through ionic or covalent bond, and the functional group of organic material is a building block that endows the nanomaterials. When an ionic core of the inorganic nanoparticle is shelled by a more covalent lattice, the ionic core is shielded, and ligand exchange is inhibited. Thus, conjugation of quantum dots with peptides can significantly change the surface properties

of inorganic nanoparticles because organic–inorganic interface is a hybrid domain [138]. The formation of a hybrid domain and possible surface modifications of QDs after their conjugation with proteins can significantly affect the properties of the resulting bioconjugation product [138, 139]. For example, protein layers covalently bounded on the inorganic nanomaterial surface can increase the colloidal stability of QDs and have also shown effectiveness at preventing the possible release of toxic metal from QDs [139].

4.3.2. Second step of synthesis

In the second step, the product of the ligand exchange conjugation was washed twice with DMSO and three times with carbonate buffer (0.1 M, pH = 8.5 - 9) in DMSO, and concentrated by centrifugal filtration at 6,000 rpm for 20 min to get an approximate concentration of 1 mg mL^{-1}. The BHQ-2 quencher was dissolved at a concentration of 1 mg mL^{-1} in DMSO. Then, 100 µL of the QD-peptide conjugate was mixed with 20 µL of the solution of the quencher in a molar ratio of peptide to quencher 1 : 1 and was left shaking at 1,000 rpm and room temperature of 23°C in darkness for 1 day. After the reaction (**Fig. 19**), the excess of the quencher was removed using AMICON tube with 30 kDa cut-off membrane. The final product was washed three times with DMSO and eluted in 100 µL to an approximate concentration of 1 mg mL^{-1} and stored at 4 °C upon analysis.

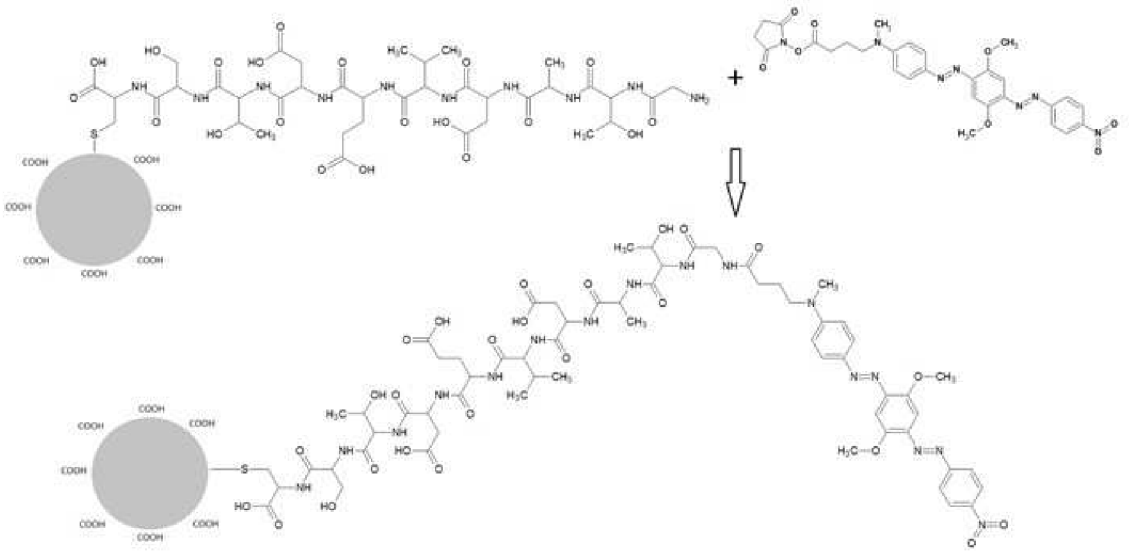

Figure 19 The reaction scheme of the second part of the synthesis. The amine-reactive succinimidyl group terminated the BHQ-2 quencher reacts with N terminal of the specific peptide.

The resulting concentration and yield of the synthesis of the final product was determined using the optical spectrometry with inductively coupled plasma (ICP-OES) method. Since, the QD-probe does not produce any luminescence without enzyme cleavage, the resulting probe concentration in solution was calculated as a result of the analyses of the absolute amount of Cadmium in the quantum dots. Purchased CdTe core-type QDs should contain value CdTe ≥ 50-70 % and molar ratio Cd:Te, 2:1 according to the declarations of the developer [134]. It means, that solution of the native quantum dots should contain 35 – 46 ppb of Cadmium at concentration 0.1 mg/L. All ICP-OES measurements were carried out at the Cd 214.441 nm line using the Ar 420.068 nm line as an internal reference for signal correlation.

First, the calibration solutions of Cd standard were measured in the range 0 – 90 ppb. Thus, in **Fig. 20,** the relationship between the intensity signals J (in AU) and the Cadmium content m (in ppb), $J = 192.17 + 244.95 * c$ is presented as blue line. The calibration equation was estimated by least squares linear regression. The blue area represents calibration line

with 95% confidence interval. Coefficient of determination (R^2) of the regression model was evaluated to be 0.998. The results from calibration analyses were furthermore used for determination of LOD and LOQ of the ICP-OES method. The 2 solutions of the native quantum dots were prepared individually at exact concentrations 0.1 mg/L from solutions of 1 mg/mL by dilution 1 : 10 000 with ultrapure water and measured five times independently. The 8 solutions of the QD-probes were diluted and measured as the same way, after dilution 1 : 10 000 with ultrapure water and measured five times independently. Obtained results are displayed in the **Fig. 20.** For the better display of the obtained results, the evaluation of both limits on the ppb level of the calibration line plot is expanded in **Fig. 21**. The LOD and LOQ of the used method were evaluated as to be 3.15 ppb and 11.81 ppb, respectively.

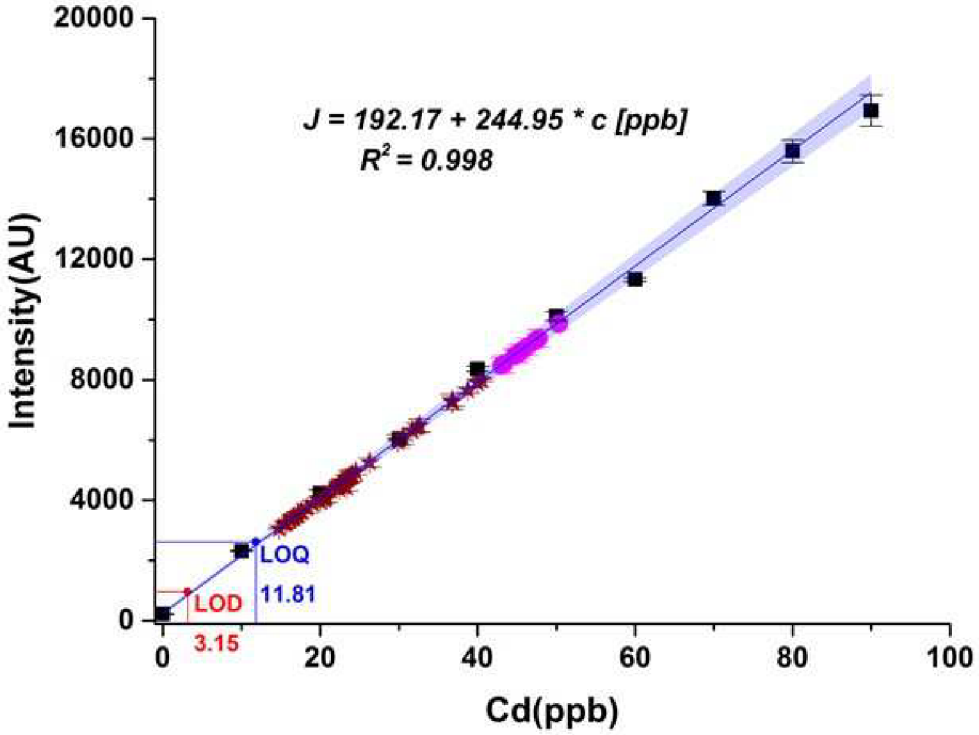

Figure 20 Calibration line (blue line) for Cd standard solutions, experimental points are represented by black squares. Experimental points from determination of Cd amount in solutions of the native QD are represented by magenta circles and QD-probe samples are represented by wine asterisks. Blue area shows 95% confidence interval of prediction.

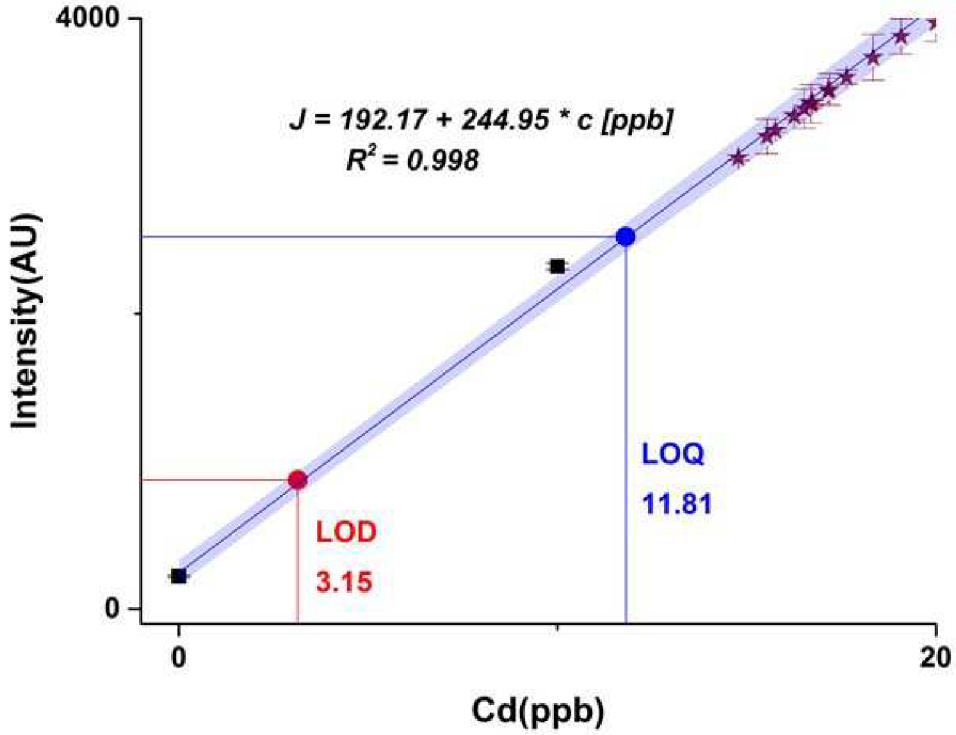

Figure 21 Calibration line (blue line) is plotted in the range from 0 to 20 ppb of Cd amount in solutions. The LOD and LOQ were determined numerically by the software calculation from the calibration line to be 3.15 and 11.81 ppb, respectively.

From the results of ICP-MS analysis, it was determined amount of Cadmium in the solutions of native quantum dots to be 46 ppb at concentration 0.1 mg/L. The same way amount of Cadmium was determined in solutions of QD-probe samples by ICP-MS. Subsequently, the evaluation of the Cadmium content in native QD solutions (concentration 0.1 mg/L, Cadmium content 46 ppb) was used to calculate the resulting QD-probe concentration from the determined amount of Cadmium in the solutions. The determined amounts of Cadmium in QD-probe solutions and the resulting probe concentrations of the final product of synthesis results are shown in **Fig. 22** and **Fig. 23**.

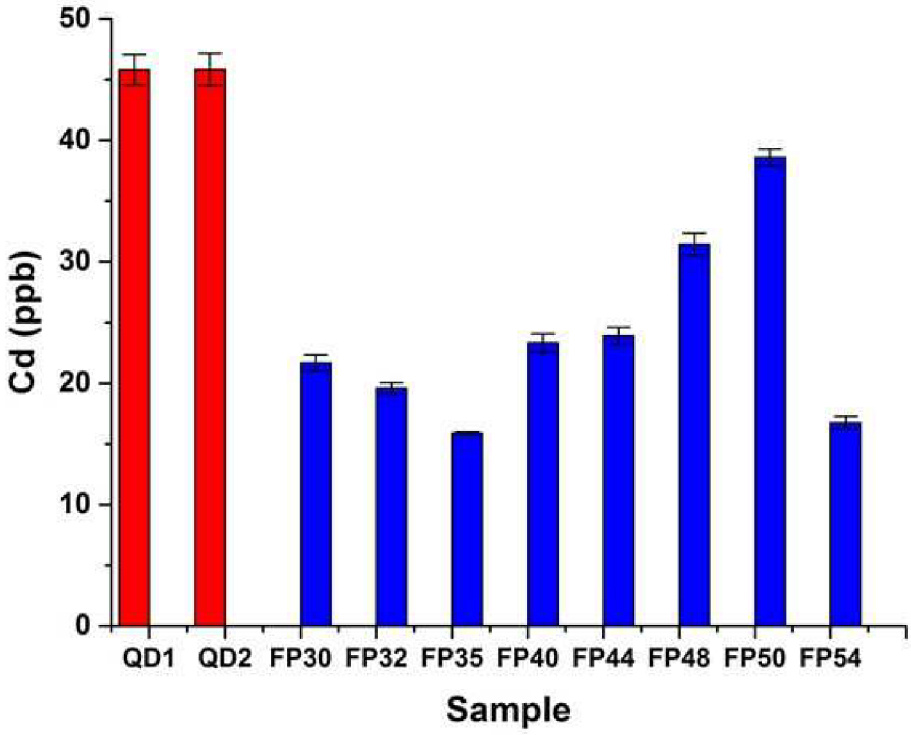

Figure 22 Determination of Cd amount in solutions of the native QD (red) and QD-probe samples (blue) by ICP-OES in ppb values.

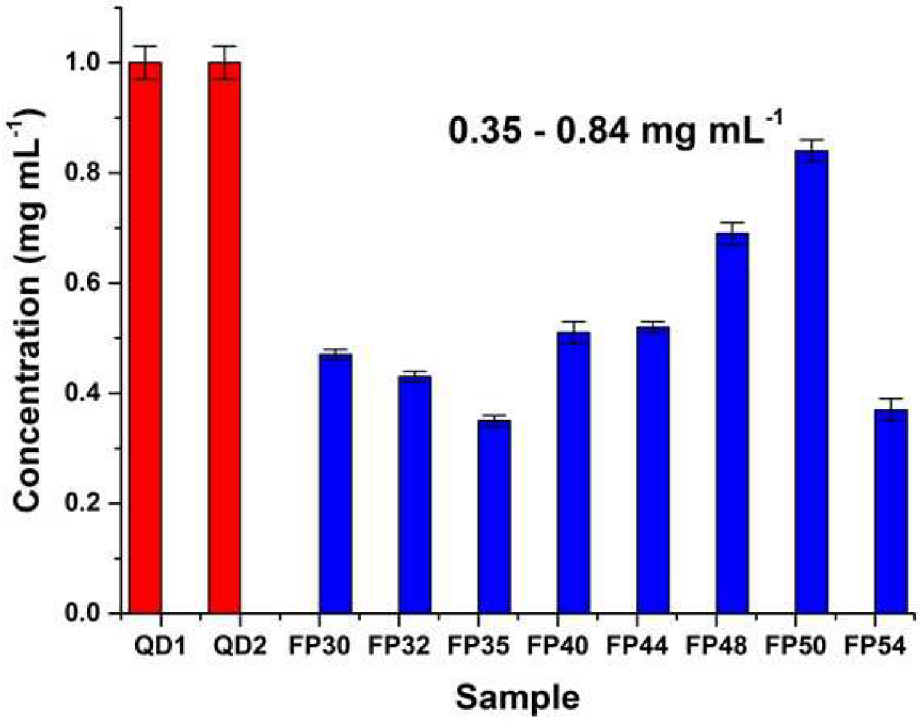

Figure 23 Determination of Cd amount in solutions of the QD native (red) and QD-probe samples (blue) by ICP-OES in mg.mL^{-1} values.

From the obtained results, it is evident, that the concentration of the QD-probe in the solutions is in the range of 0.35 – 0.84 mg mL^{-1}. It follows that the yield of the second step synthesis reaction was 82 %. These results confirmed a good synthesis conditions. Knowledge of the resulting QD-probe concentration is valuable in planning further experiments.

4.4. Results and discussion

As was already mentioned above, the principle of our probe is based on the Förster resonance energy transfer (FRET) between a highly luminescent quantum dot (QD) as a donor and a fluorophore and fluorescence quencher as an acceptor linked by a specific peptide containing DEVD sequence as a cleavage site for enzyme caspase 3/7. Thus, the luminiscence of QD starts immediately after the cleavage of the peptide by an enzyme and the release of the quencher. The principle of the reaction is graphically presented in **Fig. 24**.

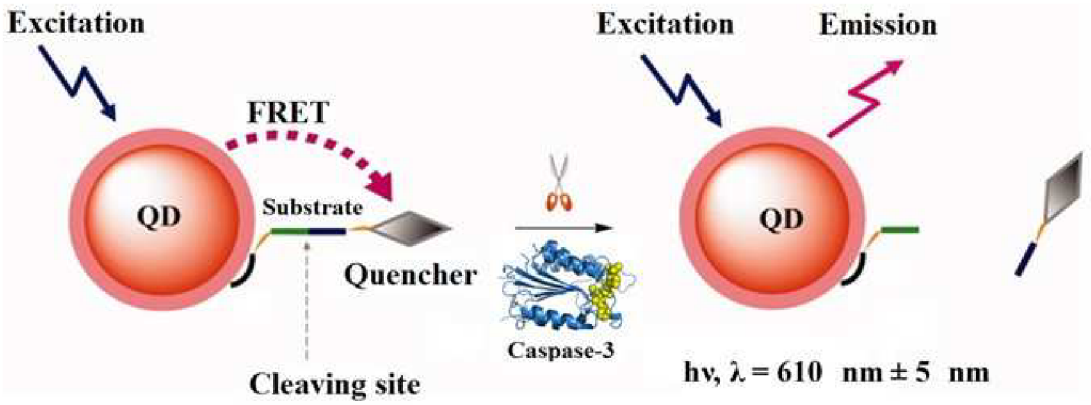

Figure 24 The scheme of the quantum dot FRET-based luminescent probe reaction with cleaving enzyme.

4.4.1. Enzyme kinetics

In order to test the functionality of our synthesized QD-probe, we monitored the model reaction of our QD-probe with a recombinant enzyme. The time course of fluorescence excited at 405 nm and recorded at 590 nm is shown in **Fig. 25**. Here, the quenched luminescence of the probe is stable for the first 10 min, then the fluorescence signal increases rapidly immediately after the addition of recombinant caspase-3 and reaches a limiting value after the cleavage of all substrate molecules. It follows that the cumulative increase in luminescence is caused only by the enzymatic reaction and its maximum value is determined by the amount of QD-probe. As can be seen from the displayed results, the

wavelength of the luminescence maxima does not change during the reaction of the QD-probe with recombinant caspase-3.

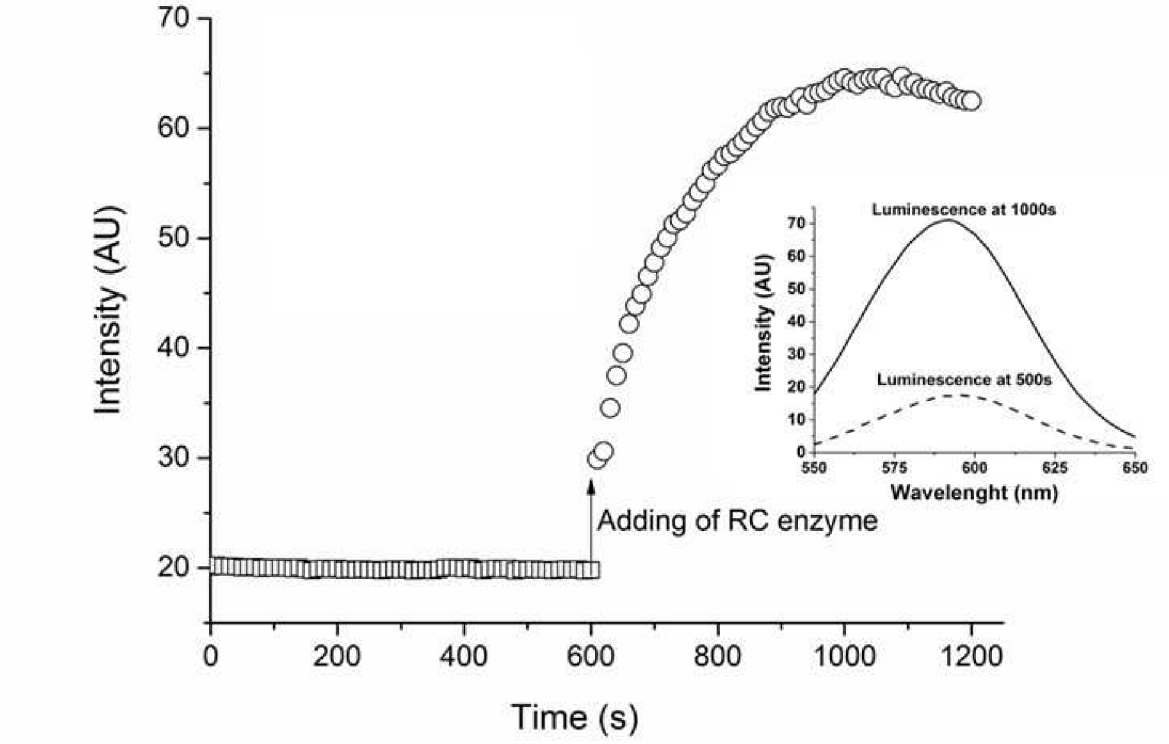

Figure 25 Time course (10 s frequency) of luminescence of 200 µL of QD-probe at a concentration of 10 µg mL^{-1} (0.12 µM) for 600 s and after the addition of 100 µL of recombinant caspase-3 (9.1 pg). Luminescence excited at 405 nm and registered at 590 nm. Inset: luminescence spectra taken at 500 s and 1000 s of the experiment.

To compare the kinetic parameters of our QD-probe with products of other groups, the Michaelis-Menten description of enzymatic kinetics was used for data evaluation. Thus, the initial cleavage velocity v_0 of the recombinant caspase-3 enzyme is plotted against the concentration of our QD-probe substrate in **Fig. 26**. The initial velocities are evaluated as the increase of luminescence with time directly in the spectrofluorometer cell. Here, the caspase enzyme cleaves specifically the DEVD peptide sequence, the BHQ-2 quencher is released, and consequently, increasing intensity of red light luminescence of the QDs is

quantified at 590 nm [134]. The plot is evaluated for nine concentrations of the QD-probe in the range from 0.01 to 3.0 μM mixed with 9.1 pg of recombinant caspase-3 in a volume of 300 μL, all measurements were performed at temperature 33 °C set by external thermostat. The evaluated points represent mean values of three independent measurements for each concentration. Thus, the corresponding Michaelis constant K_M and maximal velocity V_{max} were estimated using the Michaelis-Menten expression

$$v_0 = \frac{V_{max} \cdot S}{K_M + S}$$

where S is the concentration of QD-probe as a substrate. To get the kinetic parameters, we used Lineweaver and Burk linearization method [140].

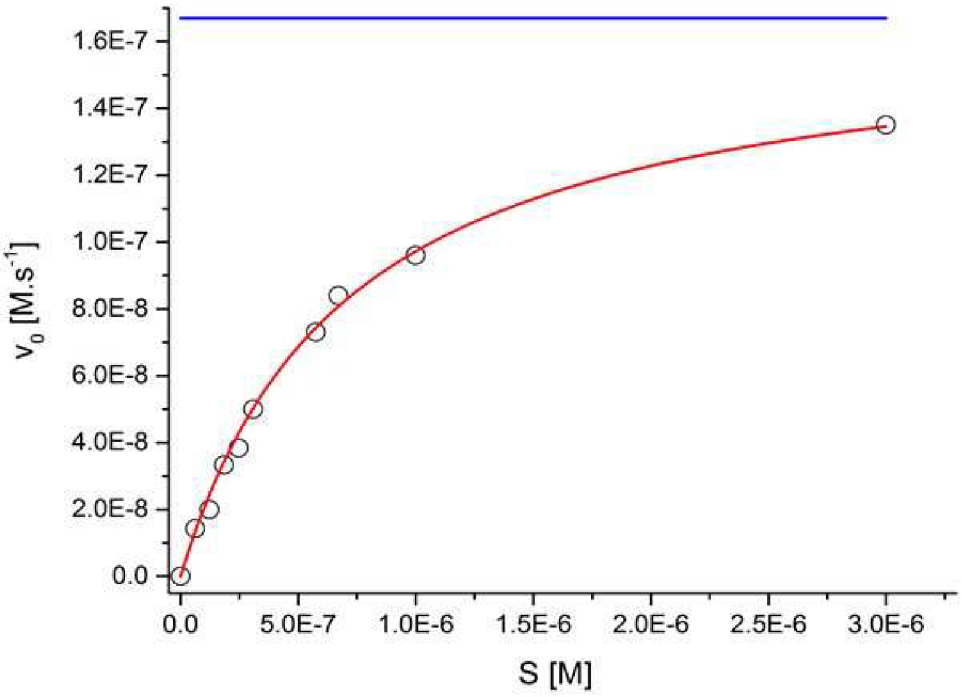

Figure 26 Dependence of the initial cleavage velocity v_0 of recombinant caspase-3 (9.1 pg in a volume of 300 µL) on the concentration S of QD-probe (substrate) in the range from 0.06 to 3.0 µM at 33°C. Red curve: numerical fit of Michaelis-Menten plot. Blue line: concentration limit of initial cleavage velocity. K_M = 3.71 10^{-7} M; V_{max} = 10^{-7} M.s^{-1}.

Only a few data found in literature on the reaction of recombinant caspase-3 and DEVD substrate are compared in **Table 2**. The determined values of K_M = 3.71 10^{-7} M and V_{max} = 10^{-7} M.s^{-1} are slightly lower than the values reported for a similar peptidyl substrate [44, 141], but are comparable with the other work testing reversible caspase-3 inhibitor [142].

Table 2 Comparison of K_M and V_{max} values found in literature for cleavage of DEVD substrate by recombinant caspase 3.

K_M (M)	V_{max} (M.s^{-1})	Reference
$3.71\ 10^{-7}$	$6\ 10^{-6}$	This paper
$(1.8 \pm 0.4)\ 10^{-6}$ $(3.0 \pm 2.5)\ 10^{-6}$	$(30.5 \pm 3.7)\ 10^{-6}$ $(47.0 \pm 30.5)\ 10^{-6}$	[44]
$11\ 10^{-6}$	NA	[141]
10^{-6} - 10^{-8}	NA	[142]

4.4.2. Fluorescence microscopy

a) Comparison of commercial MR-probe with synthesized QD-probe

The luminescence properties of the QD-probe were checked by a long-time monitoring of the enzymatic reaction inside the living cells. In **Fig. 27**, the images of QD-probe staining are compared with those of the MR-probe. Apoptotic osteoblastic precursors from mouse calva, MC3T3-E1 cells, were selected as the experimental model [15]. To induce apoptosis, all preosteoblastic cells were treated by 0.5 μM Camptothecin for 24 hours before observations. The reaction of fluorescent probes inside the cells was monitored by microscope Olympus IX 71 at 600 nm with excitation at 530 nm. The cells were incubated with both probes separately at a concentration of 1 mM, at 37°C and 5% CO_2 for 4, 24 and 48 hours. As demonstrated in **Fig. 28**, the fluorescence images of both probes are similar after the treatment for 4 and 24 hours. An intensive fluorescence is observed in the vicinity of the cell nuclei but not inside (panels B, D, F, H). However, after 48 hours from the application of probes, the QD-probe proved to enter nuclei more readily than MR-probe (compare panels J, L). The QD-probe enters cell nuclei in all observed cells, while the MR-probe induced

fluorescence of the cell nuclei very rarely (see the arrow). Positions of nuclei can be identified in phase contrast images of white light.

A specific issue of our investigation is the toxicity of bare CdTe QDs. We are aware of the fact that our probe itself can induce apoptosis of osteoblastic cells [143]. However, for the monitoring of apoptotic cells treated by Camptothecin, a mild toxicity of the probe is not an obstacle. The cytotoxicity and viability of the cells was tested with a broad range of QD-probe concentrations from 0.5 to 10 mM. Nevertheless, the cells showed high viability (> 90%) in all concentrations of QD-probes. Based on this study, concentration of 1 mM was evaluated as the optimum and used in all experiments.

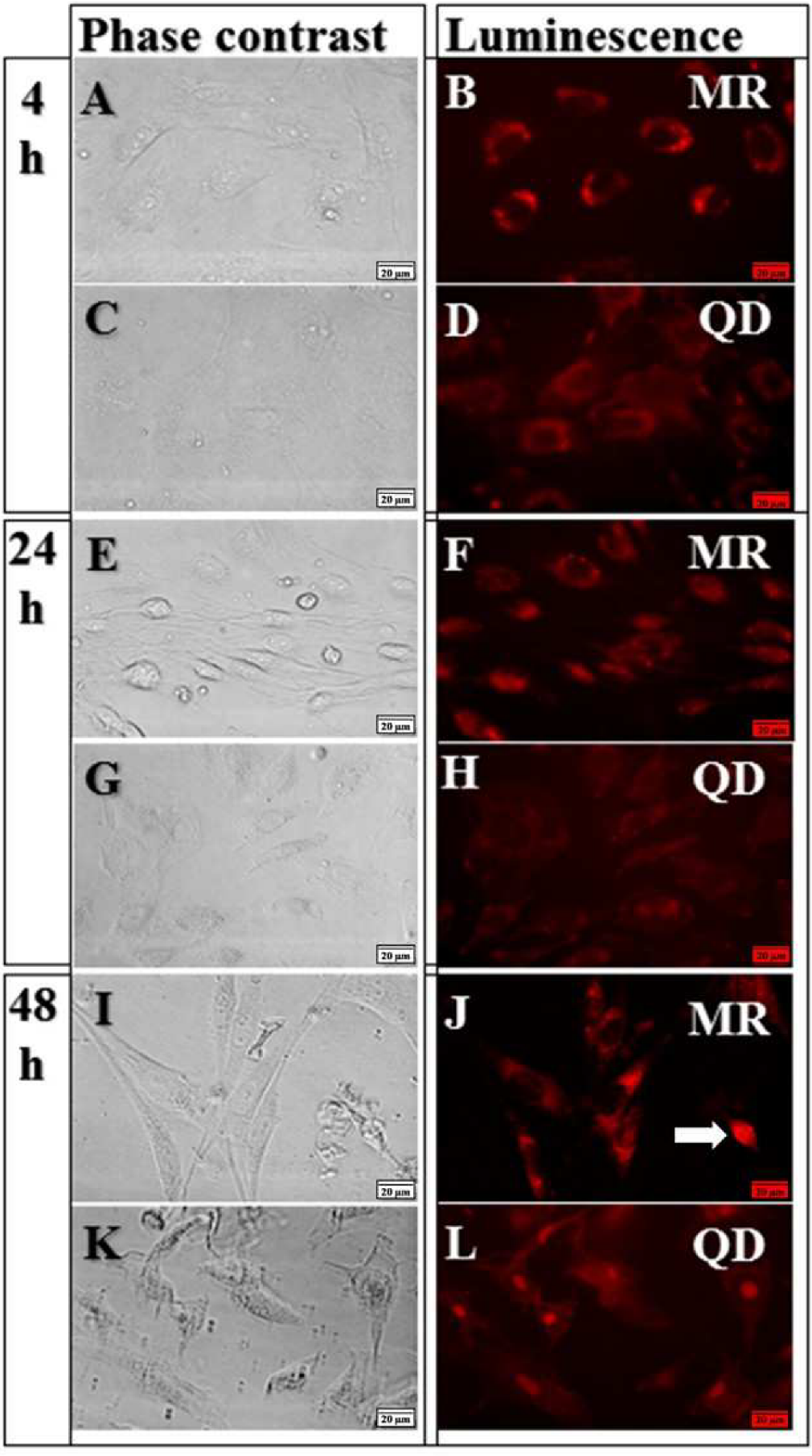
Phase contrast
Luminescence
4 h
A
B
MR
C
D
QD
24 h
E
F
MR
G
H
QD
48 h
I
J
MR
K
L
QD
20 μm

Figure 27 Caspase-3/7 activation in apoptotic MC3T3-E1 cells. Treatment with QD- and MR-probe for 4, 24, 48 hours. Phase contrast in white light of respective samples A, C, E, G, I, K. Luminescence of QD-Probe D, H, L; MR-probe B, F, J. Emission wavelength 600 nm; Excitation wavelength 530 nm; Scale bar = 20 μm.

b) Comparison of MR-probe and QD-probe luminescence in long times

The luminescence of the QD- and MR-probes was checked at 3, 4, 8 and 14 days after their addition to the samples of apoptotic MC3T3-E1 cells. As proved in **Fig. 28,** the cells separately stained by both probes differ. While the QD-probe shows predominant fluorescence in the regions of nuclei and their close proximity at all times (panels D, H, L, P), the MR-probe enters cell nuclei of dead unattached cells only (see the arrows in panels J, N). Moreover, we observed only a very low number of dead cells nuclei stained by MR-probe in the whole population. From a physiological point of view, caspase-3 should be activated in the nuclei of apoptotic cells [144]. Thus, the low luminescence intensity of MR-probes inside nuclei can be explained only by their absence due to a low permeability of the nuclear membrane.

The fact that much larger QD-probes enter nuclei easily, is probable caused by their induced negative charges, responsible for a higher permeability. Several studies reported penetration of negatively charged nanoparticle into cells, despite their interaction with the negatively charged membrane [145]. Likewise, CdTe QDs with surface modifications are known to be transported over plasma membranes into live cells [146]. However, mechanisms of membrane transport are still far from being comprehensively understood [145]. It is difficult to predict the mechanism of transport among: direct transport over membrane, endocytosis, pinocytosis or others [145, 146]. Therefore, we took advantage of our experience and others to synthesize a probe with the capability to penetrate the cellular membranes. High permeability of our QD probe to the cells and cell nuclei was confirmed in other experiments focused only on observation of the behavior of the QD-probe inside the living cells as well. Thus, in all cases the QD-probe shows predominant fluorescence in the regions of nuclei and their close proximity. Results from the subsequent experiments of fluorescence microscopy observations in living cells are presented in **Fig. 29** and **Fig. 30**.

The QD-probes enter the cellular and nuclear membranes as early as 48 hours after the application to the cell population (Panel L, **Fig. 27**) and their luminescence is still bright after more than 14 days in the incubator (Panel P, **Fig. 28**). In the experiments performed during one year, 40 different synthesized QD-probes were tested and their reactions inside living cells confirmed the repeatability of our experiments. The stability of the probe was definitively demonstrated during long-time experiments, where the luminescence of the probe inside the cells was still bright even after more than 14 days in the incubator. This proves imaging capabilities for time-laps investigations of dynamic temporal and spatial cellular events by fluorescence microscopy.

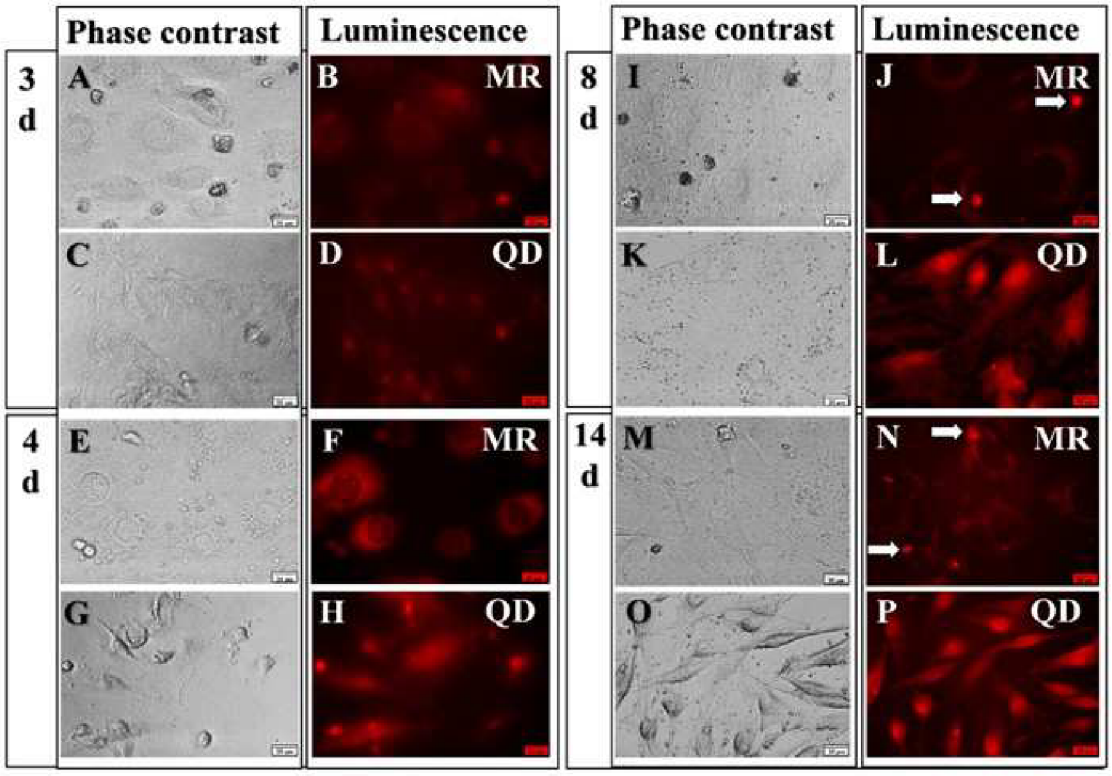

Figure 28 Long-time monitoring of caspase-3/7 activation in apoptotic MC3T3-E1 cells. Treatment with probes for 3, 4, 8 and 14 days. Phase contrast in white light of respective samples in panels A, C, E, G, I, K, M, O. Luminescence of QD-probe D, H, L, P; MR-probe B, F, J, N. Emission wavelength 600 nm; Excitation wavelength 530 nm; Scale bar = 20 μm.

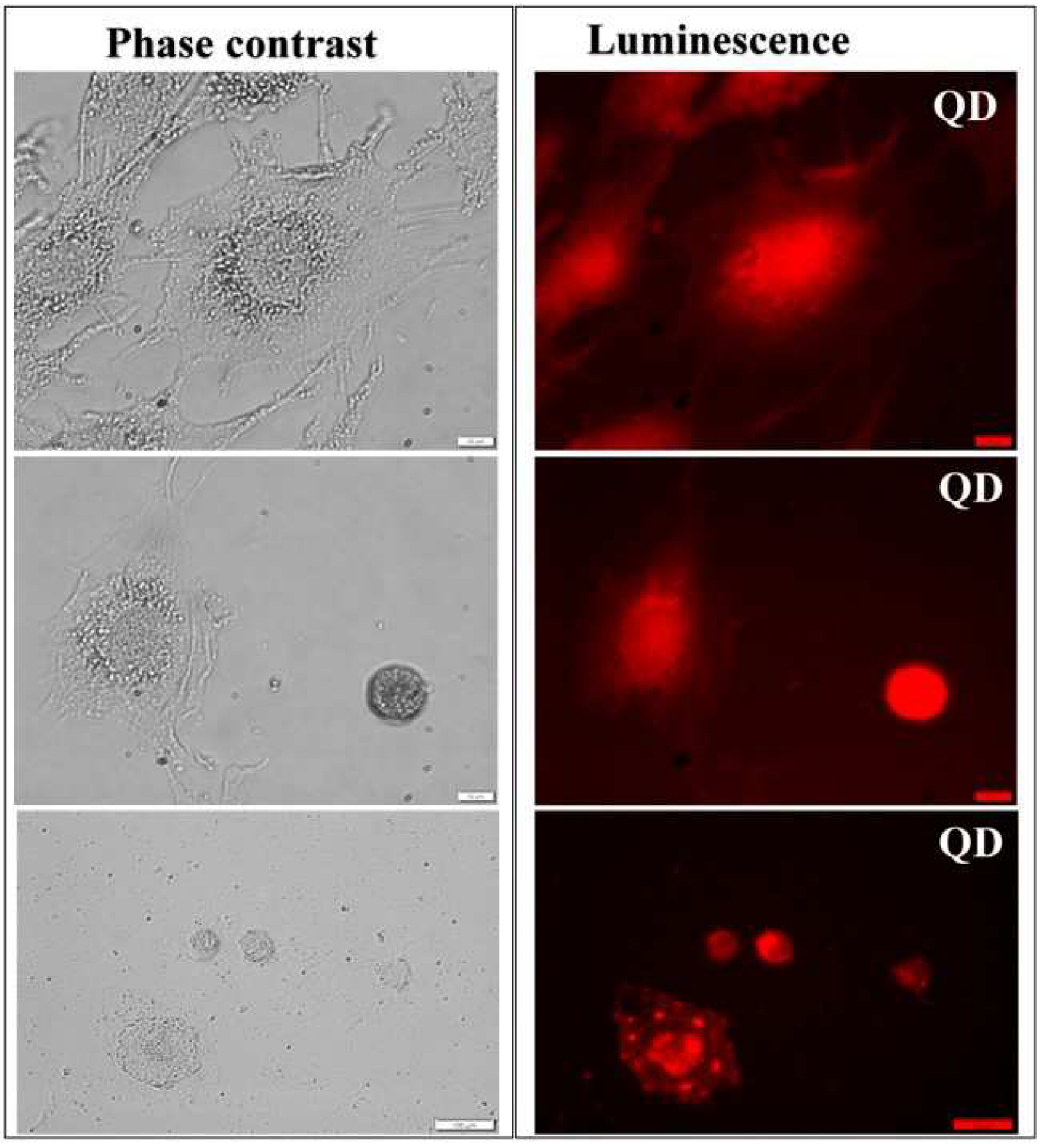

Figure 29 Monitoring of caspase-3/7 activation in apoptotic MC3T3-E1 cells. Treatment with QD-probe for 24 hours. Phase contrast in white light of respective samples and luminescence emission. Emission wavelength 600 nm; Excitation wavelength 530 nm; Scale bar = 20 μm.

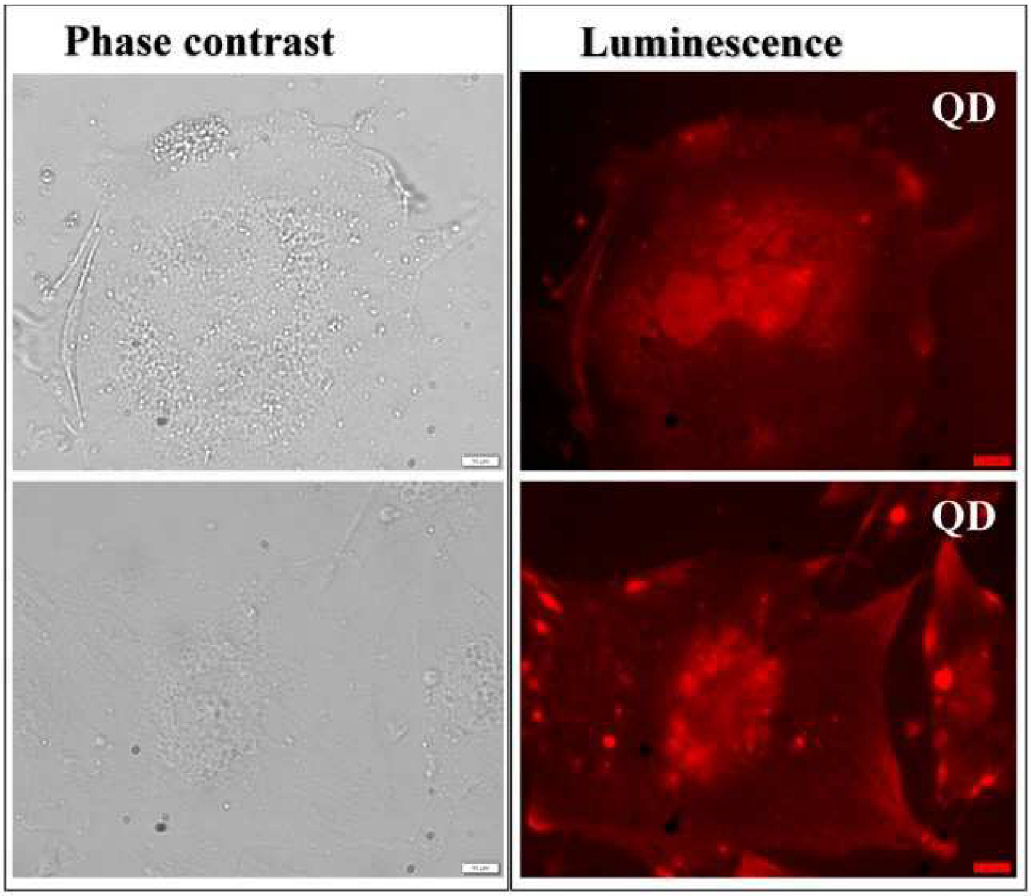

Figure 30 Monitoring of caspase-3/7 activation in apoptotic MC3T3-E1 cells. Treatment with QD-probe for 24 hours. Phase contrast in white light of respective samples and luminescence emission. Emission wavelength 600 nm; Excitation wavelength 530 nm; Scale bar = 20 μm.

c) Photobleaching

Luminescence stabilities of the synthesized QD- and MR-probes cleaved by cellular caspase were studied under the fluorescence microscope as a decrease of luminescence emission at 600 nm during the continual irradiation by the excitation light of 530 nm. In **Figure 31,** the luminescence intensity of a chosen individual cell is collected from the image of the circle area of the same diameter and evaluated by an image processing freeware Fiji every 10 seconds. The intensity of the luminescence emission normalized as a percentage of the initial intensity is plotted against the irradiation time. It is evident that the cell treated

by QD-probe (blue record) shows a slower decrease than the cell treated by MR-probe (red record). In 30 minutes, the luminescence of MR-probe and QD-probe decreased to 32 % and 58 %, respectively. For illustration, the luminescence images of individual cells in circular areas are positioned at the respective points of the plots. The dispersion of luminescence values in both plots is caused mainly by manual focusing of the circles on cells. It must be emphasized, however, that in microscopy practice, the total time of excitation for taking pictures is much shorter than in this experiment.

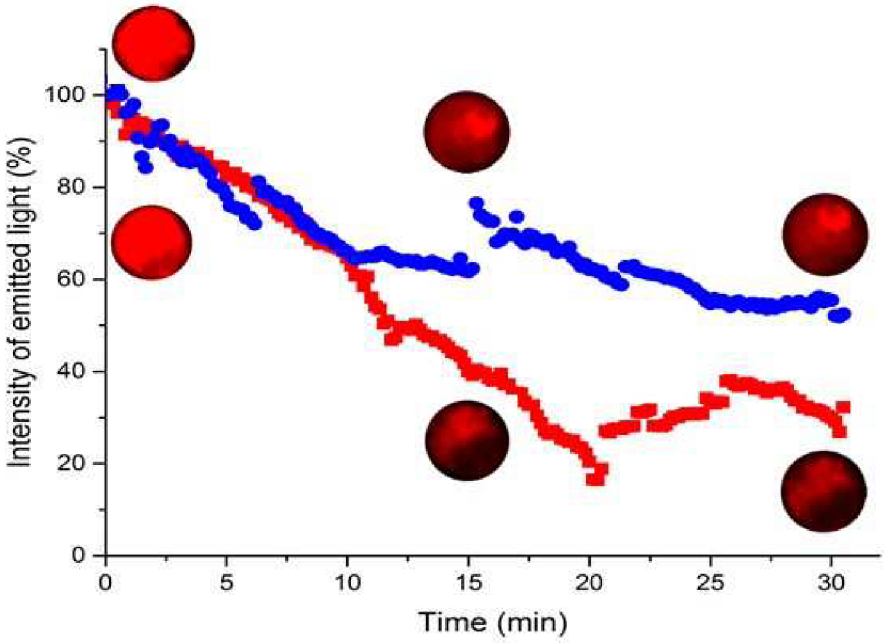

Figure 31 Bleaching of luminescence image of a single cell at 600 nm during a continual excitation by the wavelength of 530 nm. Cells are stained by QD-probe (blue record) and MR-probe (red record). The luminescence intensity of an individual cell collected from the image of the circle area of the same diameter is evaluated by an image processing software Fiji every 10 seconds. Images of individual cells in circular areas show the luminescence of a cell at respective times. The intensity of the emitted light decreased to 32 % (MR-probe) and 58 % (QD-probe) in 30 minutes.

4.5. Concluding remarks

The aim of this research work was the development of a new QD luminescent probe for a long-time quantitative monitoring of active caspase-3/7 inside cells. We have designed and optimized a two-step synthesis of a luminescent probe. The CE-LIF analysis of product of synthesis after the first step proved a high reaction yield and purity of the QD conjugate with the peptide, a prerequisite of the specificity of our luminescent probe. The method ICP-OES analysis was used for determination resulting concentration in solution of our QD-probe.

For the evaluation and comparison of the kinetic parameters of our QD-probe as a substrate with products of other groups, we used Michaelis-Menten description of enzymatic kinetics. Luminescence properties of the QD-probe were checked by monitoring the enzymatic reaction inside the apoptotic osteoblastic MC3T3-E1 cells treated with Camptothecin. The obtained results confirmed functionality of QD-probe. When compared with the commercially available product (the MR-probe), showed better stability of the luminescence signal in time. The QD-probes enter cell nuclei more readily than much smaller MR-probe. A long-time monitoring by fluorescence microscopy proved that QD-probe stains caspase-3/7 in observed cells from several hours to more than 14 days.

5. Conclusion

This dissertation work describes the novel strategies and possibilities of analyses of biologically important enzymes inside the living cells. The work is focused on single-cell bioluminescence analyses and fluorescence microscopy of biologically relevant molecules, the proteolytic active caspases.

The first part of the dissertation thesis deals with the optimization of the highly sensitive and selective instrumentation and methodology based on homogeneous single-step bioluminescence assay to quantify caspase-3/7 and evaluate its heterogeneity in single cells. Individual suspended cells were selected under microscope and reliably transferred into the detection vials by a micromanipulator. Here, the luminescence emission was detected by PMT working in a photon counting regime. The first goal of the research work was successfully achieved. Based on the optimization of the bioluminescence instrumentation and methodology, we reached LOD and LOQ much lower than the average mass of caspase-3/7 in apoptotic or even non-apoptotic cells. The optimized methodology for bioluminescence determination content of caspase-3/7 is routinely applied in our laboratory in analyses of individual HeLa cells and MC3T3-E1 cells.

Moreover, we achieved determination the heterogeneity of caspase content as part of the investigation of non-apoptotic caspase functions. The quantification of the active caspase-3/7 level in differentiated cells, apoptotic cells and the proliferating ones indicate the role of caspases as targets for stimulation of cell differentiation in supportive therapies or simulated differentiation of stem cells into request cell lineages.

The second part of the dissertation thesis was focused on the synthesis and testing a novel molecular probe based on Förster resonance energy transfer (FRET) between a highly luminescent quantum dot (QD) as a donor and fluorescence quencher as an acceptor linked by a specific peptide. Optimal synthesis conditions of the QD-probe were found for two reaction steps. The high reaction yield of the first (ligand exchange) reaction step was checked by CE-LIF system. The final concentration of the synthesized probe was reliably determined by ICP-OES. Thus, the second goal of the dissertation thesis was to prove

potential of new QD luminescent probe for a long-time monitoring of active caspase-3/7 distribution in apoptotic osteoblastic MC3T3-E1 cells treated with Camptothecin. The reaction of the QD-probe with a model recombinant caspase-3 protein confirmed the functionality of the QD-probe. Moreover, the results of the long time monitoring of caspase-3/7 under fluorescence microscope show that our synthetized luminescent QD-probe provides longer imaging times than commercial product Magic Red.

6. List of abbreviations

FRET	Förster resonance energy transfer
HeLa cells	Cell line derived from human cervix epitheloid carcinoma
QD	Quantum dots
CdTe QDs	Cadmium Telluride quantum dots
GTADVEDTSC	Peptide sequence: Glycine, Threonine, Alanine, Aspartic acid, Valine, Glutamic acid, Aspartic acid, Threonine, Serine, Cysteine, written from N to C end
BHQ-2	Black hole quencher succinimidyl ester, dark quencher active ester for coupling to amino groups on peptide substrates
CE-LIF	Capillary electrophoresis with laser induced fluorescence detection
ICP-OES	Optical emission spectroscopy with laser induced plasma
MC3T3-E1 cells	Preosteoblastic cells from mouse calva (European Collection of Cell Culture, c. n. 99072810)
CaspaseGlo®	Caspase Activity Assay for Apoptosis Detection
PMT	Photomutliplier tube
LOD	Limit of detection
LOQ	Limit of quantification
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
MEM α	Minimum Essential Medium alpha
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum

S/N ratio	Signal to noise ration
UV light	Ultraviolet light
SD	Standard deviation
FAM-FLICA®	Carboxyfluorescein fluorescent inhibitor of caspases
Magic-Red®	Red cresyl violet fluorophore for caspase detection
FLICA	Fluorescent inhibitor of caspases
FMK	Fluoromethyl ketone
MR	Magic red
DEVD	Peptide sequence: Aspartic acid, Glutamic acid, Valine and Aspartic acid, written in the opposite order from C to N end
DNA	Deoxyribonucleic acid
QD-probe	Quantum dot probe
MR-probe	Magic Red probe
TRIS	Tris(hydroxymethyl)aminomethane
MOPS	3-(*N*-morpholino)propanesulfonic acid
EOF	Electroosmotic flow
TAPS	[tris(hydroxymethyl)methylamino]propanesulfonic acid
UPW	ultra-pure water
AMICON tube	Centrifugal filter unit
ppb	Part per billion
Fiji	Fiji Is Just ImageJ, image processing software

7. Publications and presentations

7.1. List of publications related to the topic of the dissertation thesis

1. M. Killinger, B. Veselá, **M. Procházková**, E. Matalová, K. Klepárník,: **A single-cell analytical approach to quantify activated caspase-3/7 during osteoblast proliferation, differentiation, and apoptosis**, *Analytical and bioanalytical chemistry*, 413 (**2021**) 5085-5093, doi: 10.1007/s00216-021-03471-9.

   The autor designed and performed model experiments with a recombinant enzyme, evaluated the data from the model experiments and helped with the text corrections of the manuscript.

2. **M. Procházková**, M. Killinger, L. Prokeš, K. Klepárník,: **Miniaturized bioluminescence technology for single-cell quantification of caspase-3/7**, *Journal of pharmaceutical and biomedical analysis*, 209 (**2022**) 114512, doi: 10.1016/j.jpba.2021.114512.

   The autor performed all bioluminiscence and optimization experiments, designed model experiments with a recombinant enzyme, evaluated the data and co-wrote the manuscript.

3. **M. Procházková**, E. Kuchovská, M. Killinger, K. Klepárník,: **Novel Förster Resonance Energy Transfer probe with quantum dot for a long-time imaging of active caspases inside individual cells**, *Analytica Chimica Acta*, (**2023**), In process, Status: Submitted, Required Reviews Completed.

   The autor designed and performed optimization a of the synthesis steps and all experiments - The model experiments with a recombinant enzyme, CE-LIF and ICP-OES experiments, kinetic experiments. The autor performed all experiments of monitoring the QD-probe reactions inside the living cells, evaluated the data and co-wrote the manuscript.

   The results presented in this work are based on this publication.

7.2. List of oral and poster presentations related to the topic of the dissertation thesis

1. **M Procházková**, M. Killinger, K. Klepárník,: **Quantum dot luminescent probe for caspase-3/7 imaging inside cells.** In *17th International Students Conference "Modern Analytical Chemistry", Praha,* **2021**. Oral presentation.

2. **M Procházková**, M. Killinger, K. Klepárník,: **Bioluminescent probe for caspase imaging inside cells based on Förster resonance energy transfer between quantum dot and quencher**. In *XXI. Workshop of Biophysical Chemists and Electrochemists, Brno,* **2021**. Oral presentation.

3. **M Procházková**, K. Klepárník, M. Killinger: **The new quantum dot FRET-based luminiscent probe for Caspase-3/7 imaging inside cells.** In *XXII. Workshop of Biophysical Chemists and Electrochemists, Brno,* **2022**. Poster presentation.

4. **M Procházková**, K. Klepárník,: **Bioluminescent probe for caspase imaging inside cells based on Förster resonance energy transfer between quantum dot and quencher.** In *The Biomania Student Scientific Meeting 2022, Brno,* **2022**. Poster presentation.

5. **M Procházková**, K. Klepárník,: **The new quantum dot luminescent probe for Caspase-3/7 imaging inside cells.** In *Chemistry towards Biology 10 - Instruct, Bratislava,* **2022**. Oral presentation.

7.3. List of other publications

1. Y. Sheena Mary, C. Yohannan Panicker, H. T. Varghese, Ch. Van Alsenoy, **M. Procházková**, R. Ševčík, P. Pazdera,: Acid-base properties, FT-IR, FT-Raman spectroscopy and computational study of 1-(pyrid-4-yl)piperazine. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, **2014**, 121, 436-444, doi:10.1016/j.saa.2013.10.119.

2. D. Němečková, Y. Sheena Mary, C. Yohannan Panicker, H. T. Varghese, Ch. Van Alsenoy, **M. Procházková**, P. Pazdera, A. A. Al-Saadi: 1-Alkyl-1 methylpiperazine-1,4-diium Salts: Synthetic, Acid-Base, XRD-analytical, FT-IR, FT-Raman Spectral and Quantum Chemical Study. *Journal of Molecular Structure*, **2015**, 1094, 210-236, doi:10.1016/j.molstruc.2015.03.051.

3. Š. Koudelka, T. Gelbíčová, **M. Procházková**, R. Karpíšková,: Lineage and Serotype Identification of Listeria monocytogenes by Matrix-assisted Laser Desorption Ionization-time of Flight Mass Spectrometry. *Czech Journal of Food Sciences*, **2018**, 36, 452-485, doi:10.17221/87/2018-CJFS.

7.4. List of patents

1. Process for preparing 1-(pyridin-4-yl)piperazine and 1,1-dialkyl-1-ium derivatives thereof.
   303950, **2013**

2. Phase transfer immobilized catalyst, process of its preparation and use.
   303951, **2013**

3. Ion-immobilized catalysts for activation of electrophiles, process of their preparation and use.
   303987, **2013**

4. Biocatalytic processes for the preparation of Vilanterol.
   WO2017/001907, **2017**

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2007-2008	Study of nonequilibrium kinetics of plasmachemical reactions in atmospheric gases at reduced pressure for applications in analytical chemistry, Czech Science Foundation (GA202/08/1106)

Hobbies

Sport, walking, cycling, reading and handworks.

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