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QUANTIFICATION OF PROTEINS AND CELLS

Luminometric Nonspecific Particle-Based Methods

by

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“In the field of observation, chance favors only the prepared mind.”
Louis Pasteur

ABSTRACT

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New luminometric particle-based methods were developed to quantify protein and to count cells. The developed methods rely on the interaction of the sample with nano- or microparticles and different principles of detection. In fluorescence quenching, time-resolved luminescence resonance energy transfer (TR-LRET), and two-photon excitation fluorescence (TPX) methods, the sample prevents the adsorption of labeled protein to the particles. Depending on the system, the addition of the analyte increases or decreases the luminescence. In the dissociation method, the adsorbed protein protects the Eu(III) chelate on the surface of the particles from dissociation at a low pH. The experimental setups are user-friendly and rapid and do not require hazardous test compounds and elevated temperatures. The sensitivity of the quantification of protein (from 40 to 500 pg bovine serum albumin in a sample) was 20-500-fold better than in most sensitive commercial methods. The quenching method exhibited low protein-to-protein variability and the dissociation method insensitivity to the assay contaminants commonly found in biological samples. Less than ten eukaryotic cells were detected and quantified with all the developed methods under optimized assay conditions. Furthermore, two applications, the method for detection of the aggregation of protein and the cell viability test, were developed by utilizing the TR-LRET method. The detection of the aggregation of protein was allowed at a more than 10,000 times lower concentration, 30 µg/L, compared to the known methods of UV240 absorbance and dynamic light scattering. The TR-LRET method was combined with a nucleic acid assay with cell-impermeable dye to measure the percentage of dead cells in a single tube test with cell counts below 1000 cells/tube.

Keywords: adsorption of proteins, cell counting, energy transfer, fluorescence, luminescence, quantification of proteins

TIIVISTELMÄ

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Väitöskirjatyössä kehitettiin uusia luminometrisia partikkeliperusteisia menetelmiä proteiini- ja solupitoisuuden määrittämiseen. Kehitetyt menetelmät hyödyntävät näytteen ja nano- tai mikropartikkelien välistä vuorovaikutusta ja erilaisia detektioperiaatteita. Fluoresenssin sammutukseen, aikaerotteiseen luminesenssin resonanssienergiansiirtoon (TR-LRET) ja kaksoisfotonivirittеiseen fluoresenssiin (TPX) perustuvissa menetelmissä näyte estää leimatun proteiinin adsorption partikkelien pinnalle. Analyytin läsnäolon vuoksi luminesenssi joko kasvaa tai laskee systeemistä riippuen. Dissosiaatioon perustuvassa menetelmässä adsorboitunut proteiini suojaa partikkelien pinnalla sijaitsevaa Eu(III)-kelaattia dissosioivalta matalalta pH:lta. Kehitetyt menetelmät ovat käyttäjä-ystävällisiä ja nopeita, eivätkä vaadi vaarallisia reagensseja ja korkeaa lämpötilaa. Proteiinin kvantitoinnin herkkyydet (40-500 pg härän seerumin albumiinia näytteessä) olivat 20-500-kertaisesti paremmat herkimpään kaupalliseen menetelmään nähden. Sammutusmenetelmässä eri proteiinien aikaansaaman vasteen vaihtelu oli pieni ja dissosiaatiomenetelmä sieti biologisissa näytteissä yleisesti esiintyviä häiritseviä aineita. Alle kymmenen eukaryoottisolua näytteessä voitiin havaita ja kvantitoida kaikilla kehitetyillä menetelmillä optimoiduissa määritysolosuhteissa. Lisäksi kehitettiin kaksi TR-LRET-menetelmää hyödyntävää sovellutusta: proteiinin aggregaation havaitseminen ja soluviabiliteettitesti. Proteiiniaggregaatio voitiin havaita pitoisuudella 30 µg/L, joka on yli 10 000 kertaa pienempi kuin tunnettujen UV240-absorbanssi- ja fotonikorrelaatio-spektroskopiamenetelmien vaatima näytteen pitoisuus. TR-LRET-menetelmä yhdistettiin solumembraania läpäisemätöntä nukleiinihappoväriä hyödyntävän menetelmän kanssa, jotta pystyttiin mittaamaan kuolleiden solujen osuus ainoastaan yhden koeputken vaativassa testissä alle 1000 solun näytemäärällä.

Avainsanat: energiansiirto, fluoresenssi, luminesenssi, proteiiniadsorptio, proteiinipitoisuuden määrittäminen, solulaskenta

CONTENTS

ABBREVIATIONS

2PE	two-photon excitation
A	acceptor
Alexa532	Alexa Fluor 532
Alexa680	Alexa Fluor 680
AM	amino-modified
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphate degrading enzyme
BCA	bicinchoninic acid
BF530	dipyrrylmethene-BF_2 530
BrdU	5-bromo-2-deoxyuridine
BSA	albumin from bovine serum
CBQCA	3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde
CFDA-SE	carboxyfluorescein diacetate succinimidyl ester
CFU	colony forming unit
CHO	Chinese Hamster Ovarian
CM	carboxylate-modified
CMC	critical micelle concentration
CV	coefficient of variation
D	donor
DELFIA	dissociation enhanced lanthanide fluorescence immunoassay
DLS	dynamic light scattering
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DNase	deoxyribonucleic acid degrading enzyme
dsDNA	double-stranded deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FRET	fluorescence resonance energy transfer
γG	γ-globulin
HEK	Human Embryonic Kidney
INT	2-*p*-iodophenyl-3-*p*-nitrophenyltetrazolium chloride
LDH	lactate dehydrogenase
LOD	limit of detection
LRET	luminescence resonance energy transfer
LTS	low-throughput screening
LYS	lysozyme
MTS	3-(4,5-dimethyl-2-thiazolyl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
OPA	*o*-phthaldialdehyde
PEG	polyethylene glycol
PBS	phosphate buffered saline
PCV	packed cell volume

pI	isoelectric point
PS	polystyrene
RT	room temperature
TPX	two-photon excitation technique
TR	time-resolved
Tris	tris(hydroxymethyl)aminomethane
TRL	time-resolved luminescence
UV	ultraviolet
UV205	absorbance measurement at 205 nm
UV240	absorbance measurement at 240 nm
UV280	absorbance measurement at 280 nm
VIS	visible

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I Sari Pihlasalo, Jonna Kirjavainen, Pekka Hänninen & Harri Härmä (2009)
An Ultrasensitive Protein Concentration Measurement Based on Particle Adsorption and Fluorescence Quenching.
Analytical Chemistry, **81:** 4995−5000.

II Sari Pihlasalo, Lotta Pellonperä, Eija Martikkala, Pekka Hänninen & Harri Härmä (2010)
Sensitive Fluorometric Nanoparticle Assays for Cell Counting and Viability.
Analytical Chemistry, **82:** 9282–9288.

III Sari Pihlasalo, Jonna Kirjavainen, Pekka Hänninen & Harri Härmä (2011)
High Sensitivity Luminescence Nanoparticle Assay for the Detection of Protein Aggregation.
Analytical Chemistry, **83:** 1163–1166.

IV Sari Pihlasalo, Antti Kulmala, Pekka Hänninen & Harri Härmä (2011)
Sensitive Luminometric Method for Quantification of Proteins Based on Particle Adsorption and Dissociation of Chelated Europium.
Submitted to *Analytical Chemistry*.

In addition, unpublished data is presented.

1 INTRODUCTION

The quantification of total protein and counting of cells are important and basic measurements in biological laboratories on a daily basis. Several methods have been developed to accomplish the task. Generally, the strive for the bioassays has been in developing simple, rapid, sensitive, and versatile systems that are capable of handling high numbers of samples. Thus, an effort has been made towards developing sensitive homogeneous assays, as separation-free assays are typically easier for the end-users than heterogeneous assays. First cuvette and microtiter plate methods for the quantification of proteins emerged from the 1940's to the 1960's. Although the assay sensitivities and experimental techniques have been improved, they still are limiting factors.

Recently, the most commonly used techniques to determine protein concentration utilize absorbance or fluorescence detection. Bradford (Bradford, 1976), Lowry (Lowry et al., 1951), and BCA (Smith et al., 1985) are popular absorbance-based methods. However, they have limited sensitivity and dynamic range, and contaminants commonly found in biological samples interfere with all methods, and both Lowry and BCA methods require an impractical heating step. The Bradford method has high protein-to-protein variability, which prevents the precise quantification of unknown proteins or protein mixtures. Fluorescence-based methods, such as OPA (Lorenzen & Kennedy, 1993; Roth, 1971), CBQCA (You et al., 1997), and NanoOrange (Jones et al., 2003), are generally more sensitive than absorbance-based methods. However, even higher sensitivities and more user-friendly setups are desired, as these methods contain heating steps, long incubations, and/or toxic reagents. Furthermore, the methods are sensitive to contaminants. All commercially available methods are based on the specific identification of protein chemical groups (Liu et al., 1991; You et al., 1997), or the interaction of the protein surface with dye (Bradford, 1976; Lowry et al., 1951; Peterson, 1977; Smith et al., 1985) and/or detergent molecules (Jones et al., 2003; Lee et al., 2003), making them susceptible to protein-to-protein variability. Relatively sensitive absorbance methods utilizing the adsorption of protein to particles have been developed and reported in the literature (Ciesiolka & Gabius, 1988; Stoscheck, 1987). The adsorption is rather independent from the number of the functional groups in proteins and thus, it suggests that the development of the luminometric adsorption-based method could provide high sensitivity and low protein-to-protein variability.

Cells are generally counted using a light microscope in a manual counting chamber, which is a laborious, subjective, and inaccurate method. Automated cell counters improve reliability, precision, and speed, but a relatively high number of cells and costly instruments are required. A number of microtiter plate methods, such as the detection of cellular metabolic activity (Corey et al., 1997; Karmazsin et al., 1979; Kwah, 2001; Yang et al., 1996) and total nucleic acid (Gillies et al., 1986; Jones et al., 2001; Stewart et al., 2000), are also available. However, they have limitations in sensitivity, experimental setup, and responses to different cells. Due to the increasing need for cell biology research at very low cell concentrations, the sensitive counting of cells is essential. The adenosine triphosphate (ATP) assay is the only commercially available method detecting less than 10 cells (Van Lune & Ter, 2003). However, this assay has disadvantages: ATP content varies between cells, only viable cells are quantified, and the assay components are contaminated easily.

New methods are needed for both the quantification of proteins and counting of cells. This thesis reports on the development of several simple and sensitive luminometric particle-based methods. All developed methods are based on the nonspecific interaction between nano- or microparticles and the analyte. Fluorescence quenching, two-photon excitation fluorescence, time-resolved luminescence, and time-resolved luminescence resonance energy transfer are exploited in the detection.

2 REVIEW OF LITERATURE

This chapter gives an overview on absorbance and luminescence phenomena, their use in the detection of molecules and development of different methods, a short theory on the factors affecting adsorption of proteins, and a review of the available methods for the quantification of proteins, counting of cells, and assessment of viability.

2.1 ABSORBANCE

In biological and biochemical research, absorbance measurements are typically made in buffer solutions, in which, besides water and buffer components, biological and organic compounds absorb light. The energies of the photons in ultraviolet (UV) and visible (VIS) regions are sufficiently high for transferring the electrons to the higher electron levels of the molecules. Due to the quantized energy states of electrons in molecules, the distribution of the energies of absorbed photons is discrete. As each electronic transition has a defined energy and a specific wavelength, the corresponding wavelength spectrum is characteristic of the structure of the compound and may be obtained from quantum-mechanics. Due to arduous exact calculations, the interpretation of the absorption spectra of the complex structures has been simplified by explaining the observed bands by the substructures or fragments of the entire molecule. The type of the valence electrons involved in chemical bonds and corresponding molecular orbitals (σ, π, and *n*) are responsible for the features of the UV-VIS spectra. The different positions, orbital shapes, and energy levels of the orbitals and the atoms present affect the absorption of light (Albani, 2004; Harris & Bashford, 1987; Thomas & Burgess, 2007; Woźniak & Dera, 2007).

In the ground state of a molecule, the relative energies of the valence electrons are determined by their participation in the chemical bond (Figure 1). The energy of *n* electron is the highest (zero), as it does not participate in the chemical bond. The energy of σ electron is lower than the energy of π electron, as mobile π electrons form less stable bonds than σ electrons. Upon the absorption of light, the molecular orbitals split and form excited antibonding orbitals (σ* or π*), whose energies are higher than the energy of the nonbonding state *n*. Possible transitions of electrons are shown in Figure 1. The transition energies correspond to the wavelengths of light in the order: $\lambda(\sigma \rightarrow \sigma^*) < \lambda(n \rightarrow \sigma^*) < \lambda(\pi \rightarrow \pi^*) < \lambda(n \rightarrow \pi^*)$. This order may be seen in the absorption spectra of different molecules. The $\sigma \rightarrow \sigma^*$ transitions occur typically in the far UV region (~100-150 nm) and are characteristic of saturated organic compounds (single bonds). The $n \rightarrow \sigma^*$ and $\pi \rightarrow \pi^*$ transitions occur in the middle UV region (~150-250 nm). The $n \rightarrow \sigma^*$ transitions are characteristic of compounds containing atoms with lone electron pairs (e.g. oxygen, nitrogen, and sulfur) and the $\pi \rightarrow \pi^*$ transitions of unsaturated organic compounds (double or triple bonds). The $n \rightarrow \pi^*$ transitions occur in the near UV and VIS regions (> 250 nm) and are characteristic of compounds containing both atoms with lone electron pairs and double or triple bonds. The $\pi \rightarrow \pi^*$ transitions dominate in the middle and near UV regions, as the absorption bands are the most intense (Thomas & Burgess, 2007; Woźniak & Dera, 2007).

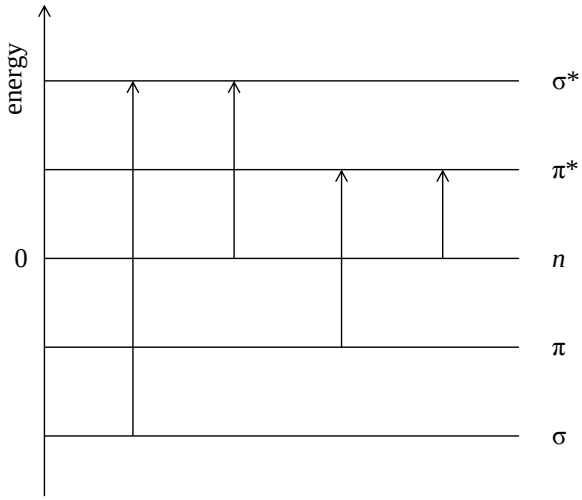


Figure 1. Energy states for σ, π, and *n* electrons and transitions between molecular orbitals. Only the lowest excited σ* and π* energy states are shown. Modified and redrawn from Woźniak & Dera, 2007.

The conjugation of double bonds enhances the delocalization of the π-electrons. The π-electrons can be transferred along the whole conjugated chain. Due to the conjugation, the π and π* energy levels are split and adjacent π and π* levels approach each other. For the linear molecules, the increasing conjugation increases the wavelength of the longest-wave and the probability of the transition. There is also an increased probability of the transition for shorter-wave bands in addition to the longest-wave band. For cyclic molecules, similar observations can be made in the series of polyaromatic rings, e.g. from benzene to tetracene. Furthermore, the addition of a hetero-atom with nonbonding *n* electrons to the structure of conjugated π bonds increases the wavelength of the longest-wave. The spectra of cyclic polyenes may be even more complicated and extinction coefficients higher compared to linear polyenes. As aromatic hydrocarbons absorb UV and VIS light strongly, they can be detected at low concentrations by the absorption of light (Thomas & Burgess, 2007; Woźniak & Dera, 2007).

The properties of the surrounding medium of the molecule affect its absorption spectrum. Changes in temperature, pH, ionic strength, solvent purity and polarity, and concentration of the molecule, and the interaction with another molecule are possible reasons for observed perturbations. The electronic transitions discussed above should be observed as single lines in spectra. However, more or less wide bands at specified wavelengths are observed. The maximum of the band corresponds to the electronic transition and the rest of the band is the result of the rotational and vibrational transitions. An increase in the number of electrons in the higher rotational and vibrational levels upon a rise in the temperature, widens the absorption band. In polar solvents, the absorption peak is typically shifted towards shorter wavelengths due to the electrostatic interactions requiring high energy for the absorption. Instead, in nonpolar solvents the absorption band is situated at longer wavelengths. The binding of an absorbing molecule to another molecule changes also the environment of the absorbing molecule. For instance, in proteins, the binding sites are typically more hydrophobic than the solvent (water) and the molecules interact due to hydrophobic interaction. This interaction may shift the

absorption maximum towards a longer wavelength compared to the free molecule. Molecules, which change color upon a change in the pH, upon reduction/oxidation, or upon the binding of metal ions, are useful as pH, redox, and complexometry indicators (Albani, 2004; Thomas & Burgess, 2007).

Absorbance is exploited in different quantitative methods for the measurement of the concentration of the analyte molecule in a sample. The quantification is based on the use of the well-known Beer–Lambert law, which describes the linear relation between the concentration of the absorbing molecule, the absorbance coefficient of the absorbing molecule, optical path length, and absorbance. Absorbance measurements require no specific skills, are quite simple and fast. However, the quantification from absorbance may have several drawbacks. Scattering or reflection causes deviations from the Beer–Lambert law. The linearity between absorbance and the concentration of the absorbing molecule may be lost at high concentrations due to the saturation of the photomultiplier, stray light, and the aggregation of the absorbing molecule. At high analyte concentrations, the absorbance can alternatively be read, for instance, in the shoulder of the band, but it will lead to imprecision in positioning the wavelength. The interaction of the absorbent with other molecules may also change the spectrum. Perturbations may also be observed, if the temperature does not remain constant between the measurements, the matrix of the samples is different from the one of the standards, or the light beam does not hit at a normal incidence (Albani, 2004; Harris & Bashford, 1987; Thomas & Burgess, 2007).

2.2 LUMINESCENCE

Luminescence has become an important research tool in clinical chemistry, biochemistry, biophysics, medicine, and biology. Luminescence is widely used, since it is versatile and sensitive, as well as (environmentally) safe. Especially in clinical diagnostics, several assays have been developed for ions and molecules in the blood and immunochemical detection of antigens and drugs (Lakowicz, 2006).

Luminescence detection enables the quantification of different analytes at low concentrations. Luminescent analytes may be quantified directly by detecting inherent luminescence. On the other hand, non-luminescent analytes are quantified indirectly with luminescent compounds. Advanced techniques, such as luminescence resonance energy transfer (LRET), luminescence polarization, and two-photon excitation (TPX), are suitable for the detection of analytes with specific and nonspecific interactions. The sensitivity of absorbance-based assays is typically in the micromolar concentration range, while luminescence-based assays reach sensitivity at a nanomolar range or even at a picomolar range. The reason for this is the different measurement geometries and therefore, the relative change of the signal is larger in the luminescence than in absorbance measurement. The change in luminescence at a low luminescence background level is relatively larger than the small change in transmittance at a high transmittance level (Valeur, 2002).

Luminescence is divided into fluorescence and phosphorescence. In fluorescence, the return of the excited electron from a singlet excited state to the ground state is spin allowed. Thus, the fluorescence lifetime is short, typically near 10 ns. In phosphorescence, the return occurs from the triplet excited state and is forbidden transition. Thus, the phosphorescence lifetime is relatively long, typically 1-1000 ms (Albani, 2004; Hemmilä, 1991; Lakowicz, 2006).

2.2.1 Conventional Fluorometry

Fluorescence is the emission of light, which involves the singlet−singlet transition of an electron in a molecule (Figure 2). The generation of fluorescence begins from the excitation, in which the molecule absorbs light and one of its electrons is raised from the lowest ground level S_0 to one of the excited levels S_1-S_n. The absorption of light is mostly followed by rapid internal conversion, in which the electron relaxes to the lowest vibrational level of S_1 and the excess vibrational energy is dissipated in the surrounding environment. Fluorescence is a process, where the electron returns initially to a higher vibrational ground level and the absorbed energy is dissipated as light. Fluorescence has different competitive processes: the energy absorbed can be dissipated in the solvent as heat, the energy absorbed can be given to the nearby molecules (collisional quenching or energy transfer processes), or the excited electron passes to the excited triplet state. In the final step, the electron returns quickly back to the lowest vibrational ground level. Due to the internal conversion, the fluorescence emission maximum locates at a longer wavelength than the absorbance maximum in the spectra of a fluorescent molecule (Albani, 2004; Harris & Bashford, 1987; Lakowicz, 2006; Stokes, 1852; Valeur, 2002).

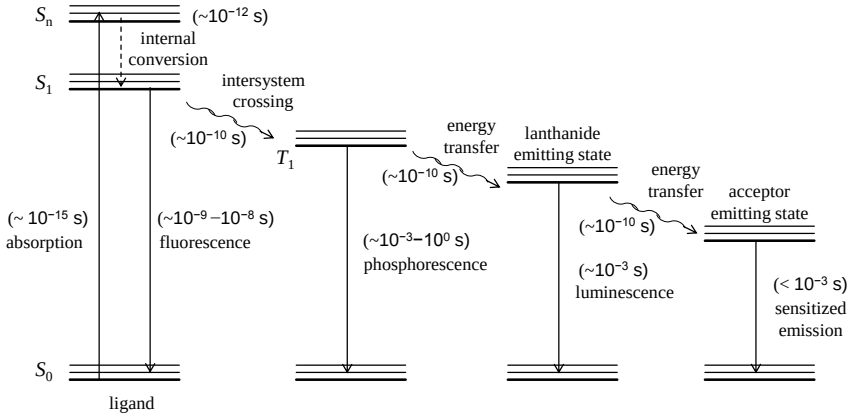


Figure 2. Simplified Jabłoński diagram for the processes: absorption, emission (fluorescence or phosphorescence), time-resolved luminescence (lanthanide), and energy transfer (Albani, 2004; Diamandis, 1988; Lakowicz, 2006).

In fluorescence, the electron in the excited state has an opposite spin to the electron in the ground state. Thus, the return of the electron is spin-allowed and typically fast with fluorescence lifetimes of approximately 1-10 ns in conventional fluorometry. Only few organic dyes, like pyrene and coronene, have a fluorescence lifetime longer than 100 ns. Organic compounds, such as coumarin, fluorescein, Nile red, BODIPY, resazurin, and DNA intercalators, containing aromatic ring structures are typical extrinsic dyes that are coupled to a molecule. Intrinsic dyes, which are part of a molecule, also exist. In proteins, usually the fluorescence of the amino acid tryptophan dominates, while the fluorescence of tyrosine and phenylalanine is weaker due to the quenching by the surrounding structure. In nucleic acids, puric and pyrymidic bases are weakly fluorescent.

The dyes are especially characterized not only with a fluorescence lifetime, which is an average of the time for the excited state, but also with a quantum yield, which is the ratio

between the number of photons emitted and the number absorbed. The fluorescence lifetime determines the average time at an excited state to gather information on the interaction between the dye molecule and the environment. Instead, the quantum yield specifies the brightness of the fluorescence (Albani, 2004; Harris, & Bashford, 1987; Lakowicz, 2006).

Fluorescent compounds have their own specific fluorescence properties. Thus, they can be identified from their fluorescence emission spectra. The fluorescence of the dyes depends on their environment, polarity of the solvent, ions, pH, and other physical and chemical properties. Thus, the fluorescent dyes may be suitable for instance in pH and ion sensing or investigating the biological systems via the surrounding environment of the dye. The fluorescence of the dye may also change upon binding to a biomolecule. This property has been used in assays for the quantification of proteins and DNA. The merocyanine dye in the NanoOrange method has weak fluorescence in the absence of protein, but the fluorescence signal is increased, when the dye interacts with detergent-coated protein. Similarly, the fluorescence of DNA intercalating dyes, such as ethidium bromide, increases upon binding to DNA (Albani, 2004; Lakowicz, 2006; Valeur, 2002).

Organic dye molecules have several advantages over many of the available labels. They are smaller in size than enzyme labels and do not require any substrate. Thus, the interference with the performance of the assay and nonspecific binding are assumed to be lower with organic dye molecules than enzyme labels (Hemmilä, 1991). Radioactive probes have waste, storage, and safety concerns. Moreover, the application of electronic paramagnetic resonance probes is limited mainly to molecular mobility studies. Instead, the field of application of fluorescent dyes is wider (Valeur, 2002).

Generally, the disadvantage of organic dyes is generally short Stokes' shifts, which is the energy or wavelength difference between the band maxima of the absorbed and emitted light (Albani, 2004; Lakowicz, 2006). If the absorption or emission spectra possess more than one band, the Stokes' shift is the difference between the most intense bands (Albani, 2004). A short Stokes' shift may cause self-quenching, i.e. fluorescence resonance energy transfer (FRET) between chemically identical molecules (Albani, 2004; Lakowicz, 2006). Self-quenching occurs for instance at high dye concentrations and in highly labeled target molecules. Therefore, for example fluorescein-labeled antibodies with a labeling degree of four, have the most intense fluorescence (Lakowicz, 2006). The excitation and emission maxima at the UV region of the dyes may cause problems in different applications due to personnel safety, interfering autofluorescence, and photobleaching (Kasten, 1993). Other factors reducing the use of conventional fluorescence relates to the detection of short fluorescence lifetime emissions, as the signal is affected by different processes or sources: Rayleigh and Raman scattering, luminescence originating from the instrument and the microtiter plates, and luminescent impurities and biological species in a sample may increase the fluorescence background and decrease the sensitivity of the detection (Hemmilä & Harju, 1994; Valeur, 2002). In conventional fluorometry, the sensitivity is at least 1000-fold lower than in time-resolved luminometry, in which the background signal is largely eliminated (Diamandis, 1991). Moreover, the hydrophobicity of the dye interferes with the quenching assays or the labeling, in which solubility in water is required (Qu et al., 2004; Zheng et al., 2007). Although the label compared to the protein is generally small, the label may change the chemical, binding, and adsorption characteristics of the protein (Ball et al., 2003; Hemmilä, 1991).

2.2.2 Time-Resolved Luminometry

In time-resolved luminometry (TRL), the sample is exposed to a light pulse and the decay of the emission is followed. The TRL measurement provides information about the occurring processes or interaction of the dye with its environment during fluorescence emission lifetimes as short as 1 ns. In addition, the TRL measurement is encountered in developing assays with long-luminescence lifetime probes. They enable high sensitivity due to the elimination of the background autofluorescence (Figure 3). Autofluorescence originates from biological material in samples and decays usually in nanoseconds. The long luminescence lifetime enables the measurement after a time delay when the autofluorescence has decreased significantly and long-luminescence lifetime probes continue to emit. The detection is time-gated: the emission is measured over a time window after the delay and integrated (Lakowicz, 2006).

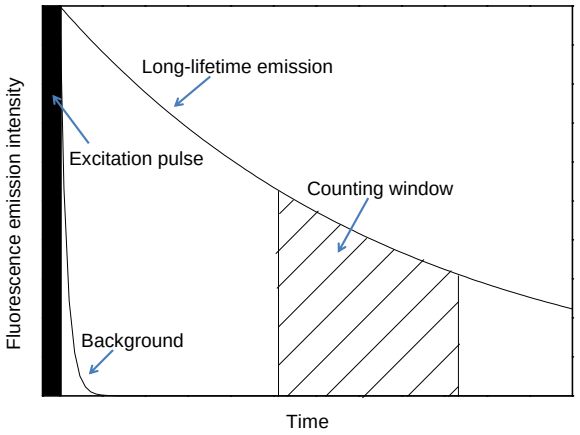


Figure 3. The principle of time-resolved luminometry. Modified and redrawn from Hemmilä & Harju, 1994.

Transition metal-ligand complexes have luminescence lifetimes of 0.01-10 µs. The transition from a metal-to-ligand charge-transfer state is partially forbidden prolonging the luminescence lifetime (Lakowicz, 2006). Phosphorescent compounds, such as tryptophan and alkaline phosphatase, have luminescence lifetimes typically from milliseconds to seconds due to the intersystem crossing to the triplet excited state. For practical work, the luminescence lifetimes of microseconds are not sufficiently long and the phosphorescence is deactivated quickly in liquids (Hemmilä & Harju, 1994; Lakowicz, 2006).

Lanthanide metals, such as europium and terbium, are popularly used in long-luminescence lifetime probes. The luminescence lifetimes are typically 0.5-3 ms (Lakowicz, 2006). Due to the weak absorption of the lanthanide ions, the metals are chelated to easily excitable organic ligands, transferring their excitation energy to the lanthanide ion (Figure 2) (Selvin, 2002). The long luminescence emission lifetime is the consequence of forbidden transitions to 4*f* orbitals shielded from the solvent by higher filled orbitals (Lakowicz, 2006; Selvin, 2002). Due to the shielding, the lanthanide chelates have narrow emission bands. The lanthanide chelates exhibit also long Stokes'

shifts, which decreases the propensity for self-quenching (Hemmilä & Harju, 1994; Selvin, 2002).

The criteria for choosing a suitable lanthanide chelate for assays are mainly universal, but the specific research application may determine the more specific property requirements for the chelate. High luminescence, high stability, and easy labeling are required. A high signal is gained if the chelate protects the ion from quenching by water, absorbs light, and transfers the energy efficiently to the ion. The chelate should be stable, but in certain applications and conditions the release of the ion is desired. The coupling of a chelate to proteins, such as enzymes or antibodies, should be simple and stable and should not affect significantly the binding, adsorption, and other functional properties. The hydrophilicity of the chelates, like organic labels, is needed to avoid the precipitation of the protein−chelate conjugate in water. The chelates consist of mainly three parts: the amine reactive functional group, the excitation energy absorbing part, and ion chelating groups. Examples of the stable chelating and energy directing groups are pyridine, polycarboxylate, and polycyclic structures (Hemmilä & Harju, 1994).

The lanthanides as luminescent labels have also drawbacks. Applications usually require both stable and highly luminescent chelates in assay conditions. If both requirements are not achievable for a single chelate, two-step assays may be needed. In two step setups, the ions are dissociated from the protein conjugates with the essentially non-luminescent complexes, such as Eu^{3+}-EDTA (ethylenediaminetetraacetic acid). The Eu^{3+} ion is chelated again with a large excess of 1,1,1-trifluoro-4-(2-naphthyl)-butane-2,4-dione and trioctylphosphine oxide chelates in the presence of detergent micelles and an acidic buffer (Hemmilä & Harju, 1994; Lakowicz, 2006). For instance, dissociation enhanced lanthanide luminescence immunoassay (DELFIA) technology utilizes this principle (Hemmilä & Harju, 1994). However, due to the long synthesis, both stable and highly luminescent chelates, such as terpyridine-bis(methylenamine)tetraacetic acid (TMT) isothiocyanate and 4-[2-(4-isothiocyanatophenyl)ethynyl]-2,6,-bis{[*N*,*N*-bis(carboxy-methyl)amino]methyl}pyridine (TEKES), are costly. In developing commercial assays, the requirement for TRL detection is important to be considered, as the fluorometers with the time-gating mode are not widely available for researchers. Instead, conventional fluorometers are more commonly in use.

2.2.3 Applications of Luminescence

Luminescence Resonance Energy Transfer

Luminescence resonance energy transfer (LRET) is a process between two molecules, a donor and an acceptor (Figure 2). It is a non-radiative process and does not include the absorption of a donor-emitted photon by the acceptor. The excited donor transfers its energy to the acceptor and typically, the emission spectrum of the donor and the absorption spectrum of the acceptor overlap (Förster, 1948; Selvin & Hearst, 1994). In the energy transfer by resonance, the electron of the donor induces an oscillating electric field that excites an electron of the acceptor (Albani, 2004). LRET can occur with two different mechanisms: Förster's or Dexter's mechanism. Förster's mechanism requires Coulombic interactions, which involve long-range dipole−dipole and short-range multipolar interactions. Instead, Dexter's mechanism requires an intermolecular orbital overlap between the donor and acceptor and consists of short-range electron exchange and charge resonance interactions. LRET with Dexter's mechanism occurs at

donor−acceptor distances shorter than 1 nm (Albani, 2004; Valeur, 2002). Thus, LRET with Förster's mechanism may be longer range than LRET with Dexter's mechanism (Albani, 2004; Förster, 1948; Lakowicz, 2006).

Due to the interaction requirements, LRET efficiency is strongly distance-dependent (Figure 4) and inversely proportional to the distance between the donor and the acceptor raised to the power of six. The distance of 50% efficiency is called the Förster distance. Förster distances are usually 2-9 nm, which are similar to the dimensions of proteins. The efficiency approaches 100%, when the distance between the donor and acceptor approaches 0 nm and at the distance of twice the Förster distance, the efficiency is already only 2%. Alternatively, a non-luminescent acceptor can be paired with a donor leading to a detection scheme, where the energy transfer is detected as a decrease of the donor emission. The relative orientation of the dipoles of acceptor and of donor also affects the efficiency of LRET (Albani, 2004; Förster, 1948; Lakowicz, 2006).

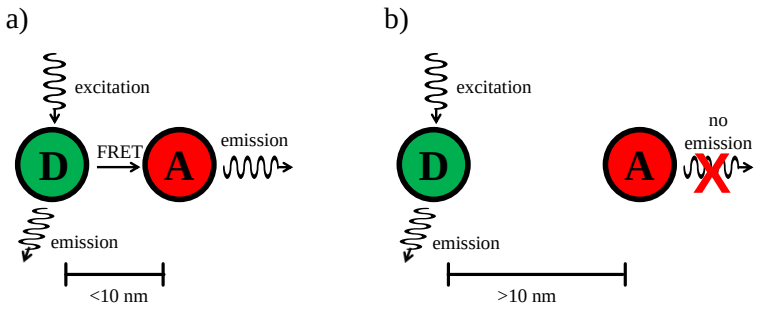


Figure 4. The dependence of luminescence resonance energy transfer (LRET) efficiency on the distance between the donor (D) and acceptor (A): a) short distances approximately Förster distance and b) long distances much longer than Förster distance (Förster, 1948; Lakowicz, 2006).

Due to the dependence of the signal on the donor−acceptor distance, the common application of LRET is to measure short distances between different sites on a macromolecule (Cheung, 1991; Lakowicz, 2006). Other applications include the study of receptor−ligand interactions, protein folding, aggregation, conformational changes, association, flexibility of DNA, and the location of different lipids in membranes (Cheung, 1991; Valeur, 2002). A more complete list of the LRET applications is given in Valeur, 2002. Moreover, the distance dependency enables the development of competitive and noncompetitive assays to measure the concentration of analytes. For instance, in LRET immunoassays the sample antigen brings the donor- and acceptor-labeled antibodies together, and the LRET signal is related to the formed complexes and the concentration of the antigen. Another typical example is the measurement of enzyme activity, in which the enzyme cleaves the substrate labeled with LRET pair and separates the donor−acceptor pair.

Luminescence Quenching

Luminescence quenching is a group of processes, in which the luminescence of a substance decreases and can occur by several different mechanisms. The quenching mechanism is determined especially by the concentration of the quencher, the interaction

between the quencher and dye, and the excited-state luminescence lifetime. At high quencher concentrations, the probability is adequately high that the quencher and dye are close enough during the luminescence lifetime for efficient interaction. This typically leads to static quenching. Moreover, static quenching occurs, if non-luminescent ground state complexes are formed between quenchers and dyes or the quencher stays at an effective distance from the dye (the sphere of effective quenching). At lower quencher concentrations, the extent of quenching and the mechanism depends on the diffusion rate during the excited-state luminescence lifetime. If the dye and quencher can get close to each other during the luminescence lifetime and they collide, the mechanism is called dynamic (also called collisional in the literature) quenching. If the dye and quencher cannot approach during the luminescence lifetime, the emission of the dye is decreased only due to long-range LRET (Albani, 2004; Valeur, 2002). For instance, heavy atoms quench with this mechanism and cause spin−orbit coupling and intersystem crossing to the triplet state. The emission is slowed down due to the spin forbidden triplet−singlet transition. Excluding high quencher concentrations, collisional quenching or long-range LRET generally occurs, when there is no specific interaction between the dye and quencher in the solution, but the specific binding interactions between them can lead to static quenching. The emission of the dye may decrease also due to the attenuation of the incident light by dyes or absorbing molecules. In collisional quenching and long-range LRET, the luminescence lifetime is dependent on the quencher concentration. Whereas in static quenching, the addition of the quencher does not change the luminescence lifetime. Moreover, collisional quenching and long-range LRET do not affect the absorption spectrum of the dye, but in static quenching the spectrum is shifted (Albani, 2004; Lakowicz, 2006).

Two-Photon Excitation Fluorescence

In two-photon excitation (2PE), two photons are absorbed simultaneously by a fluorescent dye molecule. An intensive light source provides a high density of photons required to excite the same dye molecule with two photons. The energy absorbed is two times the energy of a single photon. The probability for the absorption of two photons is a square of the probability for the absorption of a single photon, and thus more restricted than for a single photon. Therefore, the fluorescence occurs only in the immediate vicinity of the focal point, whose volume is in the order of 1 fl (Soini et al., 2000; Soini et al., 2002; Tirri et al., 2005). A homogeneous TPX assay technique and instrumentation utilizing 2PE fluorescence has been described in the literature (Hänninen et al., 2000; Soini et al., 2002; Waris et al., 2002). 2PE fluorescence is measured from the surface of individual microparticles moving in and out of the focal volume due to diffusion or the movement of liquid. These microparticles have typically been coated with antibodies (or antigens) and the binding of the analyte (antibody or antigen) has been traced with a labeled antibody or antigen (Koskinen et al., 2004; Koskinen et al., 2006; Waris et al., 2002). A high intensity laser with a 1064-nm wavelength of light in the instrument is suitable to excite dye molecules having an absorption band at a wavelength of approximately half of the one of the laser. After the excitation, the instrument can detect 2PE fluorescence at 540-700 nm (Waris et al., 2002). The fluorescence from the microparticle surface is related to the degree of binding and the concentration of the analyte in the sample. The fluorescence from the microparticle with a high number of bound labeled molecules can be significantly stronger than from the background reaction solution with single labeled molecules (Soini et al., 2002).

2.3 ASSAY FORMATS

The absorbance or luminescence assays are classified for instance according to the analyte type, detection instrument, separation requirement (heterogeneous or homogeneous), separation technique (for instance washing, filtration, centrifugation, or precipitation), assay technique (for instance luminescence polarization, LRET, or TPX), and assay principle (reagent limited or reagent excess). The basic concepts of separation requirements and assay principles are briefly clarified below, as the assays are simply chategorized into heterogeneous or homogeneous and reagent limited or reagent excess assays, which provide for the simple evaluation of the assays.

Heterogeneous and Homogeneous Assays

Assays requiring a physical separation step, such as washing or centrifugation, are called heterogeneous. The unbound labeled reagent is separated from the bound one before the absorbance or luminescence measurement (Burtis & Geary, 1994). The separation is typically carried on solid-phase, for example particles or a microtiter well surface. Whereas, the homogeneous assays do not require separation. These assays are developed to give a luminescence signal only from the bound labeled reagents or the signal is decreased upon binding. Sometimes homogeneous assays are refered to as assays with single step and molecular size labels, but in this thesis the term homogeneous refers to all separation free techniques. The homogeneous assays are easier to perform and automate than heterogeneous assays and thus, are preferred especially in commercial assays. Homogeneous assays have robust procedures, which are not prone to small variations unlike the experimental setups in heterogeneous assays. Heterogeneous assays are usually suitable for a wide variety of analytes, have higher sensitivities, long dynamic ranges, and interfere less with the different sample matrixes than homogeneous assays (Hemmilä, 1991).

Reagent Limited and Reagent Excess Assays

In reagent limited (also called competitive in the literature) assays, the analyte in the sample competes with the labeled analyte or its analog from the same binding sites or assay reagents. The number of binding sites is limited: At low analyte concentrations, the labeled analyte can bind, but at high analyte concentrations, the analyte occupies all available sites blocking the labeled analyte from binding (Van Grieken & De Bruin, 1994). In reagent excess (also called noncompetitive in the literature) assays, the assay components are added in excess compared to the analyte concentrations. These assays are designed so that the bound analyte creates the change in the signal and the unbound reagent is washed away (heterogeneous) or does not produce a signal (homogeneous) (Demchenko, 2009; Wild et al., 1994). An assay, in which the luminescence of the dye is enhanced, when it binds to the analyte, is one example of a reagent excess assay. The quantification of DNA with intercalating dyes and the quantification of proteins with fluorescamine dye are performed with reagent excess. Sandwich type assays, in which the analyte forms a three layer complex with binding sites, is another example (Demchenko, 2009). This type is typical for immunoassays with large analytes: Sample antigen is bound from one epitope to an antibody attached to a solid surface and a second (labeled) analyte is bound to another epitope (Burtis & Geary, 1994). These types of assays, in which the zero concentration signals are low, are regarded as more sensitive than

corresponding reagent limited assays, in which the zero concentration signals are high. This is due to the larger relative change in the signal (Wild et al., 1994).

2.4 ADSORPTION OF PROTEINS

The adsorption of proteins on solid surfaces is a complex phenomenon. Due to the polymeric structure of proteins, commonly consisting of 20 different amino acids affecting the hydrophobicity and charge, the interaction between protein and solid surfaces is generally strong. The interaction is more varied than for the polysaccharides or nucleic acids with less structural diversity and difficult to interpret solely from the interaction of ions or small rigid molecules with surfaces (Quiquampoix, 2000). Amino acids linked to each other by amide or disulfide bonds (polypeptide chains) form the primary structure of protein, which is thus a complete description of the covalent connections of a protein. A secondary structure refers to the spatial arrangement of nearby amino acid residues of the sequence. It typically results from hydrogen bonds between NH and CO groups, in the same polypeptide chain for α helices and in different polypeptide chains for β pleated sheets. Furthermore, β-turn structures enable the polypeptide chains to reverse direction. A tertiary structure refers to the spatial arrangement of distant amino acid residues of the sequence and is stabilized by several intramolecular interactions, such as hydrogen bonding, ionic interactions, hydrophobic interactions, and covalent disulfide bonds. Finally, a quaternary structure refers to the spatial arrangement of subunits in proteins containing two or more polypeptide chains (Andrade & Hlady, 1986; Stryer, 1988).

Proteins adsorb practically on any surface, hydrophilic and hydrophobic and neutral and charged (Andrade & Hlady, 1986), but the extent of adsorption is affected by many factors, such as protein concentration, pH, and ionic strength of the solution and the properties of a protein. According to the second law of thermodynamics, at a constant pressure and temperature, the change in the Gibbs free energy of the system for spontaneous adsorption must be negative. Both enthalpic and entropic effects acting simultaneously are involved in the adsorption process. The changes in enthalpy are related to intermolecular forces and in entropy to the spatial arrangements of molecules (Quiquampoix, 2000).

Enthalpic Effects

(i) Coulombic interactions. Coulombic forces, that take place between charged molecules or functional groups of macromolecules, are strong and long range forces and the electrical energy is inversely proportional to the distance, *r*, between charges. The binding energies of Coulombic interactions are typically similar to or stronger than hydrogen bonds. Proteins and usually solid surfaces carry electrical charges. The total charge depends on the salt type, pH, and ionic strength in the surrounding solution. For instance, the carboxylic groups are ionized and amino groups deionized, when pH is increased and thus, the amino acid sequence determines the isoelectric point (pI) characteristic for each protein, and the adsorbed ions change the total charge of the protein. Coulombic interactions affect the stability of the proteins, since the electrostatic interactions are stronger in the interior of the protein than in water due to the low conductivity of the protein medium compared to water. However, Coulombic interactions between proteins and the surface are largely decreased by the screening effect of the counterions. Namely, the charged entities are surrounded by an equal number of electrical charges with

opposite signs from the surrounding solution. As a diffuse double layer of ions is formed on proteins and surfaces, the interactions between proteins and surfaces related to the charges could be treated as the overlap of these double layers (Andrade & Hlady, 1986; Höök, 1997; Quiquampoix, 2000; Roth & Lenhoff, 2003).

(ii) Charge−dipole and dipole−dipole interactions. The interaction energies between charged molecules and permanent dipoles are lower and shorter range (decaying as $1/r^2$ without taking thermal averaging into account) than for Coulombic interactions and depend on the orientation between the charge and dipole. However, the charge−dipole interaction is important in water solutions. As strong dipoles, water molecules dissociate the negative and positive ions of the salts and form a hydration shell around charged species, effectively screening the charges. The interaction energy between two dipoles is also dependent on the orientation of the dipoles with respect to each other, are shorter range (decaying as $1/r^3$ without taking thermal averaging into account) than for Coulombic interactions, and generally low. Hydrogen bonding is an exeption since it occurs between an electropositive hydrogen atom bound to a strongly electronegative atom and the lone pairs of the electronegative atom on adjacent molecules. Hydrogen bonding is an important interaction between, for instance, the NH_2 and C=O groups of the protein and water molecules, specific chemical groups of the surface, or the same groups of another or inside the same protein (Höök, 1997; Roth & Lenhoff, 2003).

(iii) Van der Waals interactions. Dipole−dipole interactions can take place, even if one or both molecules do not have a permanent dipole. Such interactions are called van der Waals interactions. These interactions arise from the instantaneous changes in the dipole of atoms, which are caused by the movement of the electrons in the atomic orbitals. These interactions cause brief electrostatic attractions between two molecules. Van der Waals interactions consist of three different types according to the interacting molecules. Dispersion or London forces act between nonpolar molecules, induction or Debye forces between polar and nonpolar molecules, and orientation or Keesom forces between two polar molecules (see above dipole−dipole interaction between permanent dipoles). Each interaction decays as $1/r^6$ in the presence of thermal averaging. Single van der Waals interactions are of short range and generally weak, but can still contribute significantly in macromolecules and solid surfaces (Höök, 1997; Quiquampoix, 2000; Roth & Lenhoff, 2003).

(iv) Steric forces. At short distances, generally below 0.3 nm, the Pauli repulsion inhibits the outer electron clouds of the atom pairs from overlapping. This concept of repulsive interactions can be widened to larger moieties, molecules, or parts of macromolecules and the interaction energy has usually an exponential or power-law (r^{12}) dependence on separation distance. The immediate consequence of the steric forces is that two species of matter cannot be in the same place at the same time (Höök, 1997; Roth & Lenhoff, 2003).

Entropic Effects

(v) Hydrophobic interactions. Hydrophobic hydration forces arise from perturbations in the structure of water in the presence of nonpolar molecules or entities. In the vicinity of hydrophobic molecules or entities of surfaces and macromolecules, water molecules reorganize and form an ordered network structure, which results in a loss of the entropy and therefore an increase in the Gibbs free energy. The most nonpolar amino acids of proteins are actually shielded from water in the core of the protein during the folding

process to minimize the Gibbs energy. The strengths of hydrophobic interactions vary and can be comparable to hydrogen bonds. These interactions are important and sometimes stated even as the primary driving force for protein interaction (Andrade & Hlady, 1986; Höök, 1997; Israelachvili & Pashley, 1982; Quiquampoix, 2000; Rick & Berne, 1997; Roth & Lenhoff, 2003; Subramaniam et al., 1994).

(vi) Modifications in molecular structure. Proteins may change their conformation, essentially observed as increased rotational freedom in the secondary structure of protein, i.e. α-helix and β-sheet content, when they interact strongly with a solid surface. Especially the strong interaction of the protein with hydrophobic or charged surfaces can induce conformational changes in the molecular structure of the protein. These changes are more profound for proteins with low internal stability than for proteins with high internal stability. These conformational changes contribute positively to the entropy as well as enthalpy of adsorption (decreased Gibbs energy), since hydrogen bonds maintaining the amino acid chain in an original conformation are broken due to the rotation and the order in the secondary structure is decreased. The conformational changes enable a stronger interaction of the protein with the hydrophobic surface, as the internal hydrophobic amino acids can reach the surface and the outer more hydrophilic amino acids still shield the core from contact with water (Andrade & Hlady, 1986; Lassen, 1995; Quiquampoix, 2000).

Factors Affecting Adsorption of Proteins

From various types of interactions, both enthalpic and entropic, it can be qualitatively predicted the conditions, in which the adsorption is supposed to and not supposed to take place (Table 1) (Norde et al., 2008). For general predictions, the net charge of the solid surface and the protein, the stability of the protein, and surface hydrophobicity are key factors, when considering the kind of interaction and the extent of adsorption.

Table 1. Prediction of adsorption of proteins in varied conditions. Modified and redrawn from Norde et al., 2008.

			Solid surface			
			Hydrophobic		Hydrophilic	
	Structural stability	Electrical charge	Electrical charge			
			+	–	+	–
Protein	Stable, ”hard”	+	*yes*	*yes*	*no*	*yes*
		–	*yes*	*yes*	*yes*	*no*
	Unstable, ”soft”	+	*yes*	*yes*	*yes*	*yes*
		–	*yes*	*yes*	*yes*	*yes*
			Hydrophobic interactions dominate adsorption		Electrostatic interactions or structural changes dominate adsorption	

Predictions: presence of adsorption, *yes*, and absence of adsorption, *no*.

The adsorption of proteins onto hydrophobic surfaces is a favored process due to its entropic effects. The entropy of the system is increased upon adsorption due to the decreased contact of water with the hydrophobic surface. The structure of water is less disrupted near the adsorbed protein leaving more hydrophilic entities in the vicinity of

water molecules than the bare solid surface. Even if the protein is highly hydrophobic and cannot undergo large conformational changes, the entropy is increased upon adsorption, as the total surface area exposed to water is reduced. The adsorption of a protein onto the hydrophilic surface is more difficult, as both protein and the solid surface tend to retain a hydrated layer between them (Quiquampoix, 2000).

Proteins can be divided into hard, being more stable, and soft, being less stable according to their structural stability. Hard proteins, such as ribonuclease, lysozyme (LYS), α-chymotrypsin, and β-lactoglobulin, have low adiabatic compressibility and are less susceptible to conformational changes upon adsorption. Soft proteins, such as bovine serum albumin (BSA), γ-globulin (γG), hemoglobin, and catalase, having low internal stability, can unfold upon adsorption. Thus, the electrostatic interactions are usually more important for the adsorption of hard proteins, while the adsorption of soft proteins is generally entropically driven. Hard proteins typically do not adsorb onto hydrophilic surfaces, unless there are electrostatic or specific interactions. On the other hand, soft proteins can relatively easily adsorb even on an electrostatically repelling surface. Generally, small proteins are structurally more stable and have a higher proportion of their nonpolar amino acids on the surface of the protein, due to the higher surface-to-volume ratio compared to large proteins. Conversely, large proteins can change their structure more easily. Although this rearrangement is an entropically unfavorable process, the nonpolar amino acids in the core of large proteins are hidden to minimize their contact with water (Andrade & Hlady, 1986; Nakanishi et al., 2001; Quiquampoix, 2000).

The pH of the solution and the pI of the protein affect the adsorption. The maximum adsorbed amount on an electrically charged surface has frequently been found around the pI of the protein. At least three different explanations for this observation have been given in the literature. According to the first one, the lateral electrostatic repulsive interactions between the proteins are minimized at pI due to neutral net charges. Above and below pI, the repulsive lateral interactions are increased, due to the like-charged adsorbed proteins leading to the decreased adsorbed amount. This explanation assumes that the lateral interactions between proteins are more important than the interactions between the protein and surface and that the conformational changes upon adsorption are very limited (Duinhoven et al., 1995a; Duinhoven et al., 1995b). The second explanation considers the structural stability of the protein as a function of pH. At the pI, the structure of the protein is more compact than above or below pI, since the net positive or negative charge leads to repulsion between the like-charged moieties and the decreased stability of the protein. The protein with a net charge is more susceptible to the unfolding upon adsorption than the protein at its pI (Norde & Lyklema, 1978a; Norde & Lyklema, 1978b). The third explanation is related to the significant effect of the interactions between the protein and a solid surface. The reasons for decreased adsorbed amounts are different below and above the pI. Below pI, the positively charged protein unfolds when it adsorbs on a negatively charged surface, due to the strong electrostatic interaction. Above pI, the interaction between the negatively charged protein and the surface is repulsive. The adsorption of proteins is less efficient than at pI in both cases (Quiquampoix & Ratcliffe, 1992).

The ionic strength of the solution has a greater effect on the adsorbed amount, especially when proteins and surfaces are charged. The charges result in a significant screening effect by forming an ion cloud around charged species. The effect is typically negative for systems with oppositely charged surfaces. However, the increase of the ionic strength can enable the adsorption of hard proteins on electrostatically repelling surfaces. Even if the

net charge of the protein is neutral, the counterions are incorporated from the solution to the adsorbed protein layer to reduce the repulsion between the like-charged parts of the protein and the surface by an electrostatic bridging mechanism (Andrade & Hlady, 1986; Quiquampoix, 2000; Andrade, 1985).

The high adsorbed protein mass does not necessarily mean high affinity between the proteins and the surface. One reason is that a strong electrostatic or hydrophobic interaction may result in an unfolding and spreading of the protein on the surface and actually lower the adsorbed amount of protein (Quiquampoix, 2000). Another reason is the configuration of the protein. Considering the shapes of the proteins as rectangles, the proteins may approach the surface with their long axis (end-on configuration) or short axis (side-on configuration) perpendicular to the surface. The configuration reflects on the adsorbed mass, as in the end-on configuration a higher number of protein molecules can fit on the surface than in the side-on configuration. Depending on the protein and the solid surface, mostly proteins may adsorb only with an end-on or side-on configuration, but for instance, fibrinogen may take many different configurations. The protein concentration may also affect the configuration type (Figure 5a). For example, BSA maximizes the number of binding interactions at low concentrations by optimally adapting to the surface by taking a side-on configuration. At high concentrations, the proteins finally take the end-on configuration to maximize the adsorbed amount and minimize the Gibbs energy of the system. Several protein molecular layers have also been observed in certain systems like lysozyme on methylated silica (Andrade & Hlady, 1986; Nakanishi et al., 2001).

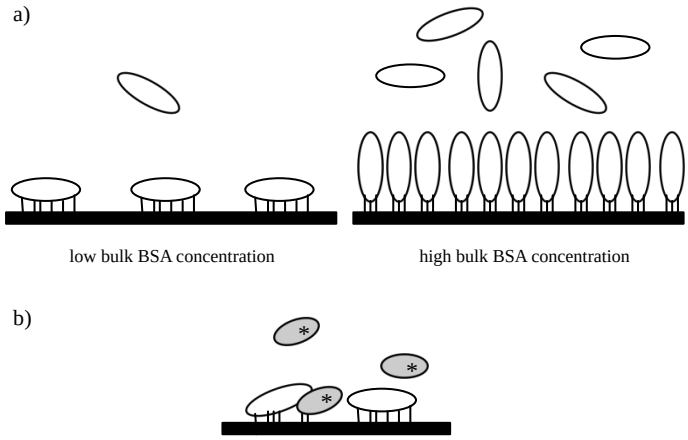


Figure 5. a) The configuration of adsorbed bovine serum albumin (BSA) at high and low BSA concentration (Nakanishi et al., 2001). b) The exchange mechanism for the displacement of the adsorbed protein with a new protein (*) from the solution (Andrade, 1985; Andrade & Hlady, 1986).

The adsorption of proteins is usually an irreversible process, i.e. the desorbed amount of protein after dilution or washing is insignificant. Irreversibility is explained by the high number of interacting points with the surface (Figure 5) or the possible positive change in entropy due to the structural modifications. Few interacting points can detach, but desorption of the protein is statistically improbable, as all interacting points should be lost at the same time. However, exceptions to the irreversible adsorption of proteins have been

observed in the literature. For instance, the adsorption of highly hydrophobic proteins onto highly hydrophilic surfaces is known to be reversible. Another possibility for the detachment of the protein is the exchange of the protein to another protein (Figure 5b). This exchange process for the adsorbed protein is more profound from a hydrophilic than hydrophobic surface (Andrade & Hlady, 1986; Ball et al., 2003; Jennissen, 1981; Nakanishi et al., 2001; Quiquampoix, 2000).

The lateral electrostatic repulsion at a higher degree of coverages, especially for like-charged proteins, decreases the surface coverage from the expected value. Even though there was no repulsion between adsorbed proteins, the coverage is always less than 100%. Assuming the proteins are hard spheres and arrange into the hexagonal close packed structure, the surface coverage is theoretically $\sqrt{3}\pi/6 \approx 91\%$ (Steinhaus, 1999). According to the different semi-empirical models, the proteins leave even more void space between each other upon adsorption than the hexagonal close packing predicts. In the random sequential model applied for the adsorption process, the protein stays near the surface and makes repeated attempts to find a vacant space to adsorb. The solution of the model gives the highest degree of surface coverage, the jamming limit, which is 55% for irreversible adsorption and 82% for equilibrium adsorption (Ramsden, 2003).

2.5 METHODS FOR QUANTIFICATION OF PROTEINS

Sensitive analytical methods for the quantification of protein concentration in solution are important in biological laboratories (You et al., 1997). However, the available methods have limitations in many respects, such as sensitivity, protein-to-protein variability, and dynamic range. Thus, the aim is to develop user-friendly, sensitive, and inexpensive methods to fulfill the needs of the end-users. Well-known or commercially available methods for the quantification of proteins are reviewed in the next sections. The Kjeldahl method is described as a golden standard for validating new methods in the biopharmaceutical and food industries (Wong et al.), but it is not practical and generally avoided nowadays. More current methods are divided into two categories according to detection: absorbance or fluorescence. The available literature methods that are related to the use of the adsorption of proteins for the quantification, or justify the development of new nanoparticle methods, are discussed in Section 2.5.4. To my knowledge, no commercial kits, which exploit adsorption for the quantification of proteins, are available. If possible the sensitivity, dynamic range, incubation time, and sample volumes informed in the text are obtained from the original publications of the methods for the standard protocols. The specifications of the corresponding microtiter plate protocols and commercial modifications are listed in Table 2 in Section 2.5.5.

2.5.1 Kjeldahl Method

The Kjeldahl method (Kjeldahl, 1883; Mann & Saunders, 1975), developed by Johan Kjeldahl in 1883, determines quantitatively the nitrogen content in chemical substances. Proteins contain nitrogen as amino acids and the nitrogen content can be related to the mass of the protein in the sample. Therefore, the Kjeldahl method is applied as the standard method for the quantification of proteins. It is also a universal method in the food industry, as the solid samples require very little sample preparation and the method is regarded as precise and reproducible (McClements, 2003). The nitrogen of the protein is converted into ammonium sulphate by heating with concentrated sulphuric acid and catalysts, such as selenium or mercuric sulphate for approximately 1 h (Figure 6).

Ammonium sulphate is distilled with sodium hydroxide for approximately 5-10 min in a special steam-distillation apparatus to liberate the ammonia. The ammonia is directed into a saturated boric acid solution, in which the ammonia reacts with the acid and ammonium borate is formed. The borate ion is titrated with a hydrochloric acid standard. The pH indicator, with color change near the pH of the saturated boric acid solution (mixed methyl-red/methylene-blue), enables an accurate indication of the end-point of the titration and quantification. The method requires several milligrams of sample. It measures both non-protein and protein nitrogen and thus, all compounds containing nitrogen interfere with the method. The nitrogen content between proteins varies due to the different amino acid sequences and increases protein-to-protein variability. This makes the quantification of unknown proteins or protein mixtures difficult. The concentrated sulphuric acid at high temperatures, large steam-distillation apparatus in a fume hood, and the relatively long testing time are unfavourable for end-users.

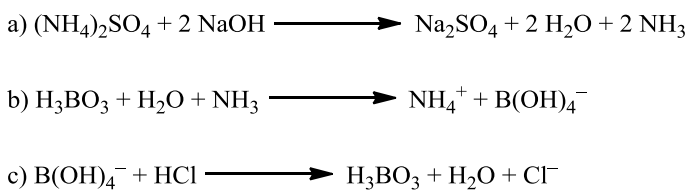


Figure 6. The reactions in the Kjeldahl method: a) the liberation of ammonia from ammonium sulphate with sodium hydroxide, b) the capture of ammonia with boric acid, and c) the titration of the borate ion with hydrochloric acid (Mann & Saunders, 1975).

2.5.2 Absorbance-Based Methods

Ultraviolet Absorbance Methods

The ultraviolet absorbance methods are based on the absorption of ultraviolet (UV) light by proteins in solution. The peak at, approximately 280 nm, results from the amino acids phenylalanine, tryptophan, histidine, tyrosine, cysteine, and cystine containing aromatic rings, and at approximately 200 nm, primarily from peptide bonds and secondarily from tryptophan and tyrosine (Albani, 2004; Pace et al., 1995). The absorbance reading at 280 nm is usually unsuitable in the presence of high contamination with nucleic acid, but a low concentration of nucleic acid is usually corrected by an absorbance reading at 260 nm. The absorbance (assay buffer as a blank) increases linearly for the detection range from 0.02 to 3, when the protein concentration increases. This absorbance range corresponds to 0.03-4 g/L of BSA, calculated by using an absorbance coefficient of 0.667 L g^{-1} cm^{-1} and a 1 cm path length of the quartz cuvette. The reading at 205 nm, where the absorption of nucleic acids is low, is preferred for samples containing excessive contamination by nucleic acids. The measurement at 205 nm is more difficult than at 280 nm, since the 205 nm peak is right on the shoulder of the protein peak. Furthermore, more chemicals, especially compounds containing carbon double bonds, absorb at shorter wavelengths than 280 nm and thus, the measurement is more prone to interference. The methods are fast and convenient, directly from the sample without the addition of any reagents or time-consuming incubations. As the method is nondestructive, the measured protein sample may be used for further experiments. The assays require only a widely used spectrophotometer with a UV lamp. Although these methods are technically simple, they have many disadvantages. The sensitivity is relatively low and the dynamic range

only one order of magnitude. The measurements are commonly performed in quartz or disposable polymer UV cuvettes having lower throughput than microtiter plate measurements (Harris & Bashford, 1987; Stoscheck, 1990).

In the microtiter plate format, expensive quartz microtiter plates can be replaced with polymer plates. However, the optical pathlength varies among samples depending on the curvature of the meniscus and the results obtained with plate readers and spectrophotometers are difficult to compare. Assuming a flat meniscus for dilute samples and a curved meniscus for concentrated samples, the pathlength is 0.32 cm and 0.25 cm for a 100 µL sample in a 96-well microtiter plate, respectively. Thus, the detectable absorbance range of 0.02-3 corresponds to 0.09-20 g/L of BSA (McGown, 1999). Commercially available cuvette-free spectrophotometers need only a 1-2 µL sample volume, but the applicable sample protein concentrations are still as high as for the measurements in cuvettes (NanoDrop, 2009). The operation has low costs, as no plates or cuvettes are used, but an investment in the instrument is required.

Due to the linear relationship of absorbance to protein concentration in cuvette measurements, no protein standards need to be prepared, if the absorbance coefficient is given. However, the protein-to-protein variability is high, i.e. absorbance coefficients vary between proteins and considerable error in concentration can be caused for unknown proteins or mixtures, if the estimate of the coefficient is used. The proteins, which have residues containing only a few aromatic rings, have low absorbance at 280 nm. Therefore, such proteins like gelatin, cannot be quantified sensitively. At 205 nm, the variability between proteins is lower than at longer wavelengths. The substances, such as free aromatic amino acids, nucleic acids, phenolic compounds, and pigments that absorb ultraviolet light, interfere with the assay. Insoluble or colored components left in cell and tissue fractionation samples absorb often in the UV region. Due to the different effect of the secondary, tertiary, and quaternary structure on the absorbance of the protein, other environmental factors, such as pH, ionic strength, detergents, and reducing agents, can alter the absorbance spectrum. However, the ultraviolet absorbance method is commonly used, when a quick estimation is needed and error in protein concentration is not a concern.

Biuret Method

Biuret (Gornall et al., 1949; Robinson & Hogden, 1940) is the oldest method for the quantification of proteins and is still commercially available. It involves only one reagent addition and a single incubation of 20 min. The divalent copper ion in the reagent forms a complex with the amide groups of the protein and is reduced to a monovalent ion under alkaline conditions (Figure 7). The purple complex formed absorbs at 550 nm and the absorbance is directly proportional to the quantity of proteins. Thus, there is no high interference from such compounds as nucleic acids absorbing at short wavelengths (McClements, 2003). The complex is formed between one copper ion and four to six peptide bonds close to each other (Mehl et al., 1949; Pierce Protein Handbook, 2009; Rising & Yang, 1933). Thus, small peptides do not react, instead tripeptides and larger polypeptides or proteins are required to form the complex (Pierce Protein Handbook, 2009). The method has a low sensitivity and short dynamic range of 1-10 g/L in 1 mL sample volume measured in a cuvette with a VIS light spectrophotometer. Biuret is rather insensitive to protein type, as the absorption involves peptide bonds common to all proteins (McClements, 2003). However, proteins with an abnormally high or low

percentage of amino acids with aromatic side groups will give high or low readings, respectively (Layne, 1957). The biological samples contain practically no chemicals, which would interfere with the method significantly (Gornall et al., 1949). Only tris(hydroxymethyl)aminomethane (Tris) (Robson et al., 1968), ammonium salts (Gornall et al., 1949; Harris & Bashford, 1987; Robinson & Hogden, 1940), copper reducing agents (Harris & Bashford, 1987; Pierce Protein Handbook, 2009), and glycerol (Zishka & Nishimura, 1970) have been listed in the literature as agents, which may cause error to the absorbance measurement. The reagent contains sodium hydroxide, since the Biuret reaction is performed in an alkaline media. The corrosive liquid is not practical for end-users. In spite of the disadvantages, the simplicity and accuracy of the method increases the usability, if the required large quantity of protein is not a concern.

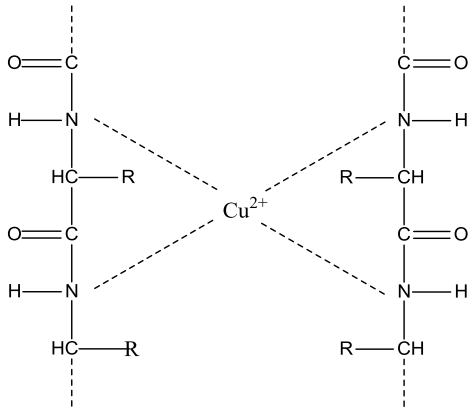


Figure 7. The complex between Cu^{2+} and the nitrogens of the peptide bonds causing the reduction of Cu^{2+} to Cu^{+} in an alkaline solution (Gornall et al., 1949; Harris & Bashford, 1987; Mehl et al., 1949; Rising & Yang, 1933; Robinson & Hogden, 1940).

Lowry Method

Lowry (Lowry et al., 1951), first described in 1951, is the most widely cited colorimetric method. It consists of two steps (Figure 8). The first step is the biuret reaction. In the second step, phosphotungstic and phosphomolybdic acid in a Folin-Ciocalteu reagent react with the radical groups of tyrosine, tryptophan, histidine, cystine, and cysteine in protein and reduce them to molybdenum and tungsten blue (Peterson, 1979). The monovalent copper ion catalyzes the latter reaction (Harris & Bashford, 1987; Legler et al., 1985; Pierce Protein Handbook, 2009; Stonehart et al., 1968). The absorbance proportional to protein concentration is measured near the peak at 750 nm in a cuvette with a VIS light spectrophotometer. The addition of the Folin-Ciocalteu reagent enhances the sensitivity of the biuret reaction. However, the method is cumbersome, as there are two additions of reagents in addition to the preparation of the reagents. The overall incubation time of 40 min is rather long. Furthermore, the vortexing must follow immediately after the addition of the Folin-Ciocalteu reagent to obtain accurate results, due to the instability of this reagent in alkaline conditions. The prepared reagents are stable only for a few weeks or months. Thus, the continual preparation or purchase of new reagents is laborious and expensive. The copper reagent contains corrosive sodium hydroxide like in the Biuret method. It affects the laboratory safety in routine measurements.

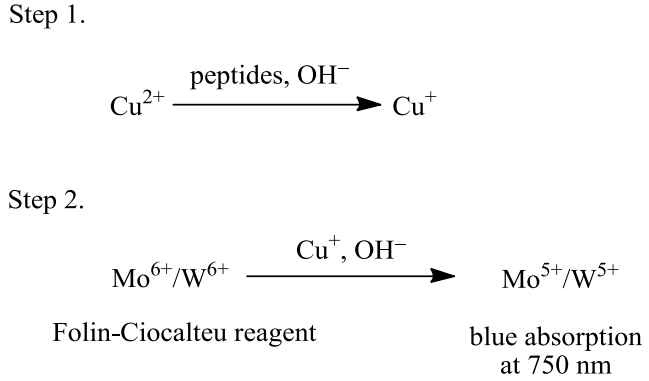


Figure 8. The simplified reaction chemistry of the Lowry method for the quantification of proteins consisting of the biuret reaction (step 1) and the reduction of the Folin-Ciocalteu reagent (step 2) (Harris & Bashford, 1987; Legler et al., 1985; Pierce Protein Handbook, 2009; Stonehart et al., 1968).

The measurable range of protein concentrations for the original method is 25-500 mg/L in a 200 µL sample volume, but different suppliers have been able to extend it in the developed modifications (Bio-Rad DC; Bio-Rad RC DC; Pierce Protein Handbook, 2009). In one modification, the incubation time has also been decreased to 15 min (Bio-Rad DC). A wide variety of contaminants, such as detergents, reducing and chelating agents, ammonium compounds, and strong acids or buffers, interfere with the original Lowry method, since they precipitate with the Folin-Ciocalteu reagent. The response of the assay is affected by the tyrosine and tryptophan content of the protein and the protein-to-protein variability is rather large at room temperature (RT). Heating the protein during the assay is required to equalize the response of different proteins (Dorsey et al., 1977).

Bradford Method

The colorimetric Bradford method (Bradford, 1976) is easy to use, as it has only one reagent addition. It exploits the deprotonation of Coomassie Brilliant Blue G-250 dye upon binding to protein and the increase in absorbance at 595 nm (Figure 9). The protonated form of the dye is stable in solution under strongly acidic conditions and absorbs at 470 nm. Upon the formation of the complex between dye and protein, the deprotonated form of the dye is stabilized and the absorbance maximum shifts to a longer wavelength. The dye interacts with the protein via van der Waals forces between the hydrophobic regions of the protein and the nonpolar region of the dye, and the electrostatic interaction between the negatively charged dye and positively charged amino groups of protein (Georgiou et al., 2008). Mainly arginine, histidine, and lysine residues (electrostatic attraction) and to a lesser extent tyrosine, tryptophan, and phenylalanine residues (hydrophobic interaction) are responsible for the interaction (Compton & Jones, 1985). The amount of formed complex depends on the sample protein concentration and is detected as an increase of absorbance at 595 nm. The protein concentrations of 0.25-1.5 g/L in a 100 µL sample volume can be quantified with a standard protocol, in which 5 mL color reagent is mixed with the sample, incubated for 2 min and the absorbance at 595 nm is read in a 3 mL cuvette with a VIS light spectrophotometer. Aggregates may form and

lead to a loss of signal over time (Held, 2006b). At high protein concentrations, the free dye depletes significantly and the standard curve becomes nonlinear. Taking into account the depletion of the dye by using the ratio of absorbance at 595 and 465 nm improves the linearity (Bearden, 1978).

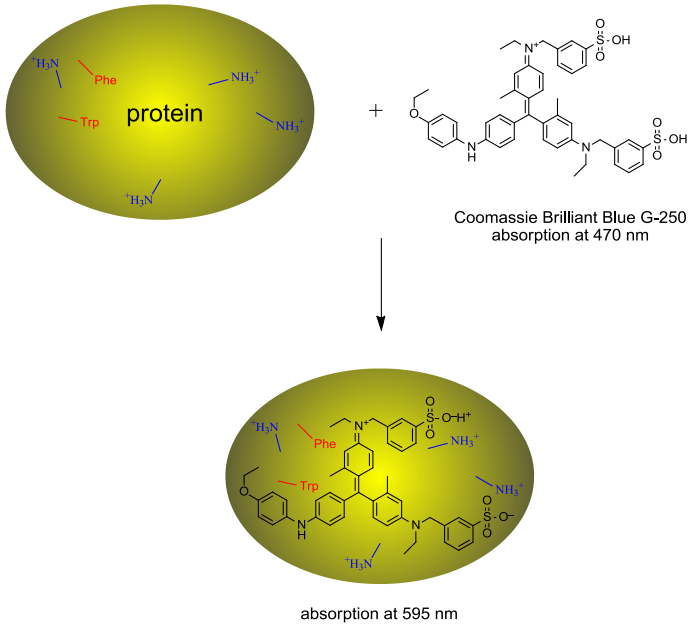


Figure 9. The chemistry of the Bradford method for the quantification of proteins. The deprotonated form of Coomassie Brilliant Blue G-250 dye is stabilized by protein (Georgiou et al., 2008).

The measurement does not require UV light and quartz cuvettes. However, the colored reagent stains the cuvettes and disposable cuvettes are recommended. The method is fast and simple with only one reagent addition at RT. The saturation of the absorbance is complete in two minutes and stable for one hour. The inaccuracy, poor sensitivity, and short dynamic range over a 10-fold concentration range are the limitations for the method. Dilutions or concentrations of the sample are often necessary before measurement. The method is compatible with salts, reducing, chaotropic, and chelating agents, and nucleic acids. As a disadvantage the response is prone to the interference of detergents found in protein extract samples from cell lysates. The detergent binds on protein and covers the binding sites for the dye. The reagent contains also toxic methanol and corrosive phosphoric acid, which increases the risks for a hazard (Pierce Coomassie, 2010). The binding of the Coomassie Brilliant Blue G-250 dye on the protein depends on the amino group content of the protein, and the response can vary even 38% between different proteins (Pierce Protein Handbook, 2009). Free amino acids, peptides, and proteins with low molecular weights do not interact with Coomassie Brilliant Blue G-250 dye. The proteins or peptides below 3 kDa cannot be assayed with the Bradford method (Pierce Protein Handbook, 2009). This can be taken as a disadvantage from the protein-to-protein variability point of view. However, it is an advantage, if interfering substances are concerned.

BCA Method

The BCA (Smith et al., 1985) method combines the biuret reaction with the colorimetric detection of the monovalent copper ion by bicinchoninic acid (BCA) with the intention of improving the sensitivity of the biuret method. After the reduction of the divalent copper ion, Cu^{+} reacts with BCA and forms a complex with two BCA molecules (Figure 10). The complex is colored and absorbs at 562 nm. The absorbance increases linearly with increasing protein concentration. Although the method consists of two reactions, only single reagent addition is required, which is in contrast to the Lowry method. A one-step analysis is enabled due to the stability of the bicinchoninate reagent in basic media and it would allow for an easy-to-use method. However, due to the greater reactivity between the peptide bonds and copper at high temperatures, heating is required to increase absorbance in the presence of protein, improve sensitivity, lower protein-to-protein variability, and shorten incubation times. Although the saturation of the signal takes longer at RT, the absorbance can be measured before the end-point within 2 h. At 37 or 60 °C the samples are incubated for 30 min and after the incubation the samples are cooled to RT before reading. Because the end-point is not reached in a reasonable time frame, all readings must be performed within a short time interval, so that the absorbance results for standards and samples are comparable. Due to heating, a long incubation time, and the measurement before saturation, the usability of the method decreases. Furthermore, the method has the same disadvantage as Biuret and Lowry method: the reagent contains a corrosive agent and is not safe. BSA concentrations of 0.1-1.2 g/L can be assayed with a standard procedure in a 100 µL sample volume using a cuvette. The method tolerates contaminants, especially detergents, better than the Lowry method, as the BCA reagent does not precipitate as easily as the Folin-Ciocalteu reagent in the presence of both non-ionic and ionic detergents. Substances that reduce copper and ammonium compounds interfere with the biuret reaction (first step) in the Lowry method. The formation of Cu^{+}–BCA complex (second step) in the BCA method is influenced by the presence of the same reactive amino acids in the protein as the Lowry method. Thus, the protein-to-protein variability is similar to the Lowry method (Pierce Protein Handbook, 2009). The protein-to-protein variability of 30% for the BCA method is deduced from the literature (Smith et al., 1985).

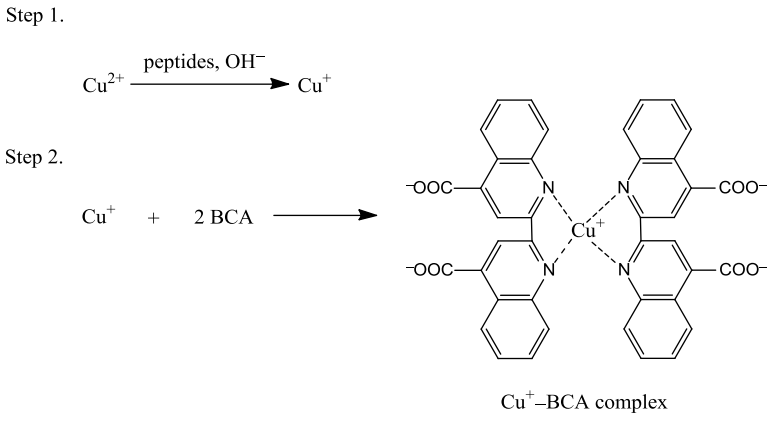


Figure 10. The chemistry of the BCA method for the quantification of proteins consisting of a biuret reaction (step 1) and the formation of the complex between bicinchoninic acid (BCA) and monovalent copper ion (step 2) (Smith et al., 1985).

2.5.3 Fluorescence-Based Methods

Fluorescamine Method

The fluorometric fluorescamine method (Böhlen et al., 1973; Lorenzen & Kennedy, 1993; Udenfriend et al., 1972) utilizes the reaction of the 4-phenylspiro[furan-2(3*H*),1'-phthalan]-3,3'-dione (fluorescamine) dye with the primary amines, ε-amino groups of lysine and terminal amino groups, at a slightly alkaline pH (Böhlen et al., 1973). The dye is non-fluorescent in an aqueous solution, but it conjugates with the primary amino groups of the proteins and a fluorescent derivative is formed (Figure 11). Thus, the method has only one reagent addition, if the pH of the sample buffer is optimal for the reaction. No incubation is required, as the reaction is complete almost instantly at RT (Udenfriend et al., 1972). However, the fluorescence increases slowly during the first 30 min of reaction, and all the measurements are recommended to be performed at a fixed time after the reaction (Böhlen et al., 1973). The fluorescence emission is measured at 475 nm with excitation at 390 nm (Udenfriend et al., 1972). The fluorescence is stable for over several hours (Held, 2006a; Lorenzen & Kennedy, 1993; Udenfriend et al., 1972). The dynamic range is from 10 to over 1000 mg/L in a sample volume of 50 µL measured in a microtiter plate (Lorenzen & Kennedy, 1993). Although the reacted reagent is stable, as a disadvantage, the fluorescamine molecule itself is not stable and is hydrolyzed fast in water (Böhlen et al., 1973; Udenfriend et al., 1972). Therefore, the reagent should be kept free of moisture and be added to the plate while it is being mixed, which further complicates the use of this method (Böhlen et al., 1973; Lorenzen & Kennedy, 1993). The variation between proteins is significant (Lorenzen & Kennedy, 1993), as proteins contain different numbers of amine groups and fluorescamine substituted amino groups may exhibit different quantum yields (Böhlen et al., 1973). This suggests that the quantification of unknown proteins or protein mixtures is not really reliable and BSA is not suitable as a standard (Böhlen et al., 1973). Chemicals, such as Tris or glycine buffer containing primary amines and large amounts of secondary amines containing primary amines as impurities, are contaminants for the assay, as the reagent react with all amines and not only with the amines of the proteins (Böhlen et al., 1973; Lorenzen & Kennedy, 1993). Glycerol may also interfere with the method (Lorenzen & Kennedy, 1993).

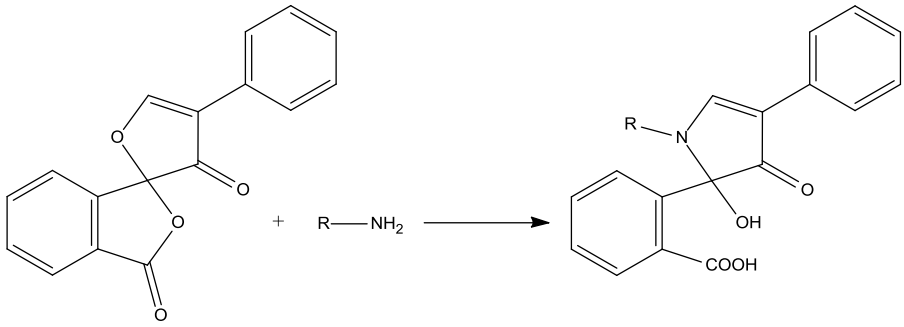


Figure 11. The reaction between 4-phenylspiro[furan-2(3*H*),1'-phthalan]-3,3'-dione (fluorescamine) dye and the primary amino group (Harris & Bashford, 1987; Udenfriend et al., 1972).

OPA Method

The fluorometric OPA (Lorenzen & Kennedy, 1993; Roth, 1971) method is based on the detection of amino groups. *o*-Phthaldialdehyde (OPA) is added to the sample and it reacts with the primary amines in an alkaline medium in the presence of 2-mercaptoethanol as a reducing agent and a fluorescent compound is formed (Figure 12). The method has only one reagent addition. OPA and 2-mercaptoethanol are mixed with the sample in a microtiter plate, incubated 5 min, and the fluorescence is measured at 455 nm excited at 340 nm. No heating is required. The signal, as a function of protein concentration, is linear and a broad range of protein concentrations from 50 to 25,000 µg/L in a sample volume of 200 µL can be quantified (Pierce Protein Handbook, 2009). OPA has better aqueous solubility than fluorescamine (Benson & Hare, 1975; Goodno et al., 1981) and thus, it does not need nonhydroxylic solvents. OPA exhibits also greater fluorescent quantum yield than fluorescamine (Benson & Hare, 1975). However, poisonous 2-mercaptoethanol decreases user-friendliness. Different proteins have varying amounts of lysine residues (Held, 2006b) or the accessibility and availability of lysine residues limit the reactivity and thus, the protein-to-protein variability of the method is not insignificant (Interchim OPA; Pierce Protein Handbook, 2009). Therefore, unknown proteins or protein mixtures cannot be quantified precisely. The amino groups of peptides and buffers, such as Tris and glycine, containing primary amines undergo also the reaction. Whereas, reducing agents, metal chelators, most detergents, and any common buffers do not interfere with the method (Pierce Protein Handbook, 2009). The reagent is unstable and sensitive to storage conditions (Interchim OPA; Pierce Fluoraldehyde, 2010), which increases the work and the costs, if the new reagent solution needs to be prepared for each quantification. The fluorescence increases as a function of time and a maximal signal is reached during an incubation of 5 min. After reaching the maximum, the signal starts to decrease. Thus, the reading is recommended to be performed at the same time interval for all samples to avoid differences originating from the variation in incubation times (Interchim OPA; Pierce Fluoraldehyde OPA, 2009). This may make the method relatively difficult for the end-users.

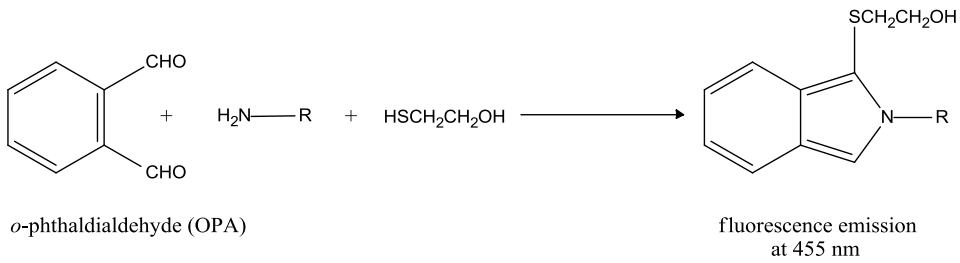


Figure 12. The reaction between *o*-phthaldialdehyde (OPA) and the primary amino group in the presence of 2-mercaptoethanol (García Alvarez-Coque et al., 1989).

CBQCA Method

The fluorometric CBQCA (You et al., 1997) method utilizes the reaction of 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) dye with the primary amines in protein. The dye is virtually non-fluorescent in an aqueous solution. In the presence of cyanide, it conjugates with primary amino groups and forms a highly fluorescent derivative (Figure 13). The conjugate is excited at 430-490 nm and the fluorescence

emission is detected at 525-600 nm. The fluorescence increases linearly as a function of protein concentration for a broad range from 0.1 to 1500 mg/L in a 100 µL sample volume. The assay can be performed either in a cuvette with a fluorometer or in a microtiter plate with a fluorescence microtiter plate reader (You et al., 1997).

The method contains two reagent additions, potassium cyanide and CBQCA, which can be added in succession. The high toxicity and appropriate waste disposal of cyanide complicates the handling. After the reagent additions, a long 90 min-incubation with shaking at RT is required to reach the saturation of the fluorescence signal and high sensitivity. Heating reduces the incubation time to a few minutes. The reaction takes place at a slightly basic pH, pH 7-10.5 being adequate, and optimally 9.3. The sensitivity decreases at a lower pH, since the amines protonate and CBQCA does not react. Detergents and lipids do not interfere significantly with the method. However, the sample must not contain primary amines, such as Tris and glycine, ammonium salts, or high concentrations of thiols, since they interfere with the reaction between CBQCA and the primary amines of the protein. The response of the assay depends on the relative content of primary amino groups available in the protein. Thus, the proteins with a high number of lysine residues can be assayed more sensitively compared to the proteins with low lysine content. The protein-to-protein variability is 30% as deduced from the literature (You et al., 1997).

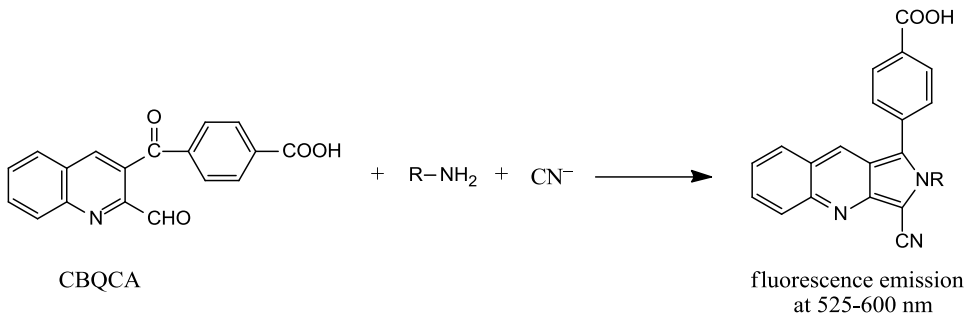


Figure 13. The reaction between 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) dye and the primary amino group (You et al., 1997).

NanoOrange Method

The NanoOrange method (Jones et al., 2003) exploits a merocyanine dye, which binds to detergent-coated proteins. The fluorescence of this environmentally sensitive dye is enhanced upon interaction with proteins. The dependence of the fluorescence signal on the protein concentration creates the grounds for the quantification. The protein assay includes only one reagent addition and measurements can be performed in microtiter wells. However, it requires the heating of the sample at 96 °C for ten minutes and subsequent cooling for 20 min to RT. Therefore, the method needs extra equipment for heating, and the samples should be transferred from test tubes to microtiter wells after they are heated and before the fluorescence is read. The dynamic range of the NanoOrange method is 0.1-100 mg/L in a 200 µL sample volume. The limit of detection (LOD) is better than for many commercial methods, such as BCA, Bradford, and Lowry assay, and the linear dynamic range is long. For the NanoOrange method the protein-to-protein variability of 20%, excluding small proteins, is deduced from the literature and is

among the lowest for the commercial methods (Jones et al., 2003). The method has been tested for plenty of possible contaminants commonly found in protein samples and is found to be sensitive to lipids, salts, and detergents.

2.5.4 Exploitation of Adsorption of Proteins for Quantification of Proteins

Methods for the quantification of proteins, insensitive to amino acid composition and precise for unknown proteins and protein mixtures, should be developed. The methods discussed above or presented in the literature are based on the specific identification of protein chemical groups (Liu et al., 1991; Lowry et al., 1951; You et al., 1997), or the interaction of the protein surface (Bradford, 1976; Lowry et al., 1951; Peterson, 1977; Smith et al., 1985), or the protein−detergent complex (Jones et al., 2003; Lee et al., 2003) with dye molecules. Identified protein chemical groups can be peptide bonds, amino groups, total nitrogen content (Mann & Saunders, 1975; Vogel & Svehla, 1979), and amino acids, such as phenylalanine, tryptophan, histidine, and tyrosine, containing aromatic rings. The dyes may also interact with the proteins or protein−detergent complexes by certain sites, such as amino groups, peptide bonds, phenylalanine, arginine, lysine, tyrosine, tryptophan, histidine, cystine, or cysteine groups, and hydrophobic regions of protein. The response of the assay depends on the number of specific groups and thus, leads to high protein-to-protein variability. Instead, the interaction of the protein with a solid surface does not necessarily require the presence of certain groups or their extent in the protein. The total charge of the protein can be altered as optimal for adsorption by changing the pH of the solution and the protein should bear at least slight hydrophobicity. Supposedly, the interaction of the protein, typically having a large size, with the solid surface is stronger than with a small molecule, since the adsorption has the advantage of a multipoint attachment (Andrade, 1985; Andrade & Hlady, 1986; Nakanishi et al., 2001). The strong interaction might be of benefit to the development of a sensitive method for the quantification of proteins. Smaller molecules do not have as high of an association with a solid surface and are not expected to prevent the protein−solid surface interaction at low concentrations. These aspects create good opportunities for developing an assay for the quantification of proteins exploiting adsorption. The next paragraphs review briefly existing quantification methods based on the adsorption of proteins.

Methods Utilizing Adsorption of Proteins

Rohringer and Holden have applied the nonspecific adsorption of proteins to gold particles for detecting proteins (Rohringer & Holden, 1985). The method relies on the adsorption of protein onto the nitrocellulose membrane and the poor affinity of colloidal gold for nitrocellulose. The method is qualitative and the detection is performed visually. The sample is applied to the nitrocellulose membrane in 1 μL, wetted in water, stained with gold colloid, and washed after a 30 min-incubation. The adsorbed gold particles are observed visually due to their red or blue color. The sensitivity is the highest at a low pH and varies for different proteins from approximately 10 to 100 mg/L. The assay is not user-friendly due to the separation step and quantitative results are not obtained. Moeremans et al. (Moeremans et al., 1985) have reported on a similar approach to Rohringer and Holden's method using either colloidal gold or silver. Nanogram level sensitivity was obtained. However, a long incubation of over 4 h was required to reach the saturation of the staining.

Stoscheck (Stoscheck, 1987) has introduced a method for the quantification of proteins utilizing the adsorption of proteins to the citrate stabilized colloidal gold particles in 1987. The adsorption of protein to the colloidal gold particles at an acidic pH causes a shift in the absorbance maximum of the colloidal gold from 530 nm to 540 nm. The absorbance change is the highest at 595 nm and is proportional to the sample protein concentration. The colloidal gold particles are the only reagent required for this simple method and they may be easily home-made or purchased from manufacturers. The method consists of mixing the sample (10 µL) with colloidal gold (800 µL) and a subsequent 30 min-incubation at RT. The dynamic range is 2-20 mg/L measured in a cuvette with a VIS light spectrophotometer. The protein-to-protein variability of the method seems to be relatively high and the same protein should be used as a standard as the protein being measured. The method has only a few contaminants. The proteins interact well with the colloidal gold at a low pH. Thus, the strong bases interfere with the method. Other substances, such as salts at high concentrations, thiol compounds, and detergents are also contaminants, since they affect the adsorption of proteins.

Ciesiolka and Gabius (Ciesiolka & Gabius, 1988) enhanced the sensitivity of Stoscheck's assay (Stoscheck, 1987) by increasing the gold particle concentration, the addition of a stabilizer polyethylene glycol, optimizing the pH, and using a microtiter plate format. The dynamic range of the improved version is 0.1-20 mg/L in a 10-µL sample. Although the variation between different proteins is improved, it is still high. Furthermore, the method is influenced to a lesser extent by interfering substances than in Stoscheck's assay.

Hunter and Hunter (Hunter & Hunter, 1987) have also presented a method for the quantification of proteins utilizing gold colloid. The method is compatible with a higher number and concentrations of chemicals than the method proposed by Stoscheck (Stoscheck, 1990). However, the method is not equally simple to perform. A sample is dried to nitrocellulose paper in 1 µL and hydrated and washed for 15 min. Colloidal gold is adsorbed to the sample on the washed paper. After an overnight incubation, the paper is washed again and measured by scanning it with a laser densitometer. The washing steps and overnight incubation require too much effort and time from the end-user. Furthermore, the application of the sample is time-consuming and prone to errors and the method requires many sample replicates. The gold particles adsorbed to the protein, which was dried to the nitrocellulose, and the signal scanned increased as a function of protein quantity in the sample applied. The method is sensitive to quantify down to 1.3 ng of protein. However, low concentrations cannot be quantified, as the sample volume is small. The dynamic range is 1-20 mg/L. Nucleic acids, urea, salts, sodium hydroxide, and sugar polymers are compatible with the assay.

The methods reviewed above have absorbance detection. The sensitivity of light absorption spectroscopy is, however, far less than that of fluorescence methods. Recently, a method based on the covalent binding of thiols to the gold particle and fluorescence resonance energy transfer has been developed to determine the concentration of different small molecule thiols. In this assay, thiols displaced an organic dye molecule, Nile red, adsorbed to the gold surface, thus enhancing the fluorescence intensity of the displaced dye (Chen & Chang, 2004). The method was specific only for thiols and was not applied to measure the concentration of other molecules, such as proteins. Rotello et al. have utilized the ability of differently coated gold nanoparticles to quench the fluorescence of conjugated polyelectrolytes to identify different bacteria, nucleic acids, and proteins (Krovi et al., 2006; Phillips et al., 2008). However, the method was optimized for the

separation of the different proteins and not for the quantification of proteins. Thus, the sensitivity of the method was limited to approximately 0.1 mg/L in the quantification of proteins and varied significantly between different proteins (You et al., 2007).

The response for different proteins has not been extensively studied for the homogeneous literature methods reviewed above, but the absorbance-based homogeneous assays (approximately 1-20 ng BSA in a sample) are relatively sensitive. Existing absorbance- and fluorescence-based literature methods utilizing the adsorption of proteins suggest that sensitive and simple methods for the quantification of proteins could be obtainable, if the fluorescence-based method is developed and optimized for the high adsorption of proteins. The development could assure high sensitivity and equal interactions of different proteins with the solid surface, i.e. low protein-to-protein variability.

2.5.5 Summary of Methods for Quantification of Proteins

The features and principles of the methods for the quantification of proteins discussed above are collected in Table 2. The low LOD and low sample volume required is valuable for limited sample amounts, low sample concentrations, and expensive proteins. Sensitivities of 10 ng for the CBQCA (You et al., 1997) and 20 ng for the NanoOrange (Jones et al., 2003) methods having fluorescence detection are the highest that have been achieved for albumin from bovine serum (BSA) with the commercial methods for the quantification of proteins. The methods that have fluorescence detection are usually more sensitive than the methods measuring the changes in absorbance due to different measurement geometries. As a consequence, the change in fluorescence at a low fluorescence background level is relatively larger than the small change in transmittance at a high transmittance level (You et al., 1997). The long dynamic range enables assaying both dilute and concentrated samples without the need of extra steps, like concentration or dilution of the samples, which may be time-consuming and require a large quantity of protein. Dynamic ranges vary from less than one order of magnitude for the Bradford (Bradford, 1976) and modified Lowry (Bio-Rad DC) methods to more than four orders of magnitude for the CBQCA (You et al., 1997) method. Due to the linearity of the calibration curve for most of the discussed methods, the measurement of the whole calibration curve is not necessary. Thus, the quantification is faster and the analysis of the results becomes easier, which is contrary to the nonlinear calibration curves requiring many standard concentrations.

The methods should be easy to perform for the end-users without highly developed hands-on skills and the need to invest in new and expensive instruments. The assays carried out in microtiter plates are simpler and usually 10 times more sensitive than the assays performed in cuvettes using a spectrophotometer or fluorometer (Stoscheck, 1990). The assays are also the better with the fewer steps the assay protocol contains and the shorter the incubation times are. The relatively sensitive CBQCA (You et al., 1997) method requires a 90 min-incubation of the samples at RT, while the absorbance in the Bradford method saturates within 2 min. Due to the elevated temperatures and subsequent cooling steps required to speed up the reaction and to achieve high accuracy and sensitivity, the NanoOrange (Jones et al., 2003), BCA (Smith et al., 1985), and CBQCA (You et al., 1997) methods are not user-friendly. Methods, such as Lowry (Lowry et al., 1951) and its modification (Pierce Protein Handbook, 2009), even require exactly timed reagent additions or the mixing due to the instability of the components participating in the signal development, making the methods impractical.

Furthermore, the instability of the components in the environmental conditions of an assay or storage (Lowry et al., 1951) may decrease the shelf-life of the product, or it becomes laborious if the end-user has to mix the different components before their use. The inexpensive and easily available reagents for the method provide a competitive price for the commercialized kit for the quantification of proteins. The quantification of proteins is a routine determination in biological laboratories. Therefore, the use of the toxic and corrosive chemicals would add to the cost of the waste disposal and risk laboratory safety. For instance, the Biuret (Gornall et al., 1949), Lowry (Lowry et al., 1951), BCA (Smith et al., 1985), and CBQCA (You et al., 1997) methods contain hazardous reagents.

The methods that are insensitive to contaminants commonly found in protein samples are favored, as the removal of the interfering reagents or dilution of the sample is not required. Generally, methods like CBQCA (You et al., 1997), fluorescamine (Böhlen et al., 1973; Lorenzen & Kennedy, 1993; Udenfriend et al., 1972), and OPA (Pierce Protein Handbook, 2009), based on the detection of protein amino groups do not tolerate salts containing amines. The reagent reducing methods, such as Biuret (Gornall et al., 1949; Pierce Protein Handbook, 2009), Lowry (Lowry et al., 1951), and BCA (Smith et al., 1985), do not tolerate reducing agents. Moreover, detergents may reduce the binding of the dye in, for instance, the Bradford (Bradford, 1976) and NanoOrange (Jones et al., 2003) methods.

Unknown proteins or protein mixtures can be quantified by using BSA as a calibrator, only if different proteins give a rather similar response to the measured signal, i.e. protein-to-protein variability is low. The commercially available methods are based on, e.g., the specific identification of protein chemical groups (Liu et al., 1991; Lowry et al., 1951; You et al., 1997), or the interaction of the protein surface (Bradford, 1976; Lowry et al., 1951; Peterson, 1977; Smith et al., 1985), or the protein−detergent complex (Jones et al., 2003; Lee et al., 2003) with dye molecules. Thus, the diverse properties of the proteins cause varying responses.

Table 2. The properties and principles of methods for the quantification of proteins. (See Appendix 1 for reference information.)

Method	Interacting protein sites	Principle	Detection	Incub. time	No. of steps[a]	Temp.	Dyn. range[b] (mg/L)	LOD[b] (µg)	Vol. (µL)	Format	Interferences
Kjeldahl[1]	nitrogen	quantification of nitrogen	acid/base titration	~1.5 h	4	189 °C + 100 °C + RT	-	1000[c]	-[d]	apparatus/ titration	compounds containing nitrogen
UV280[2,3]	aromatic amino acids	absorption of sample	absorbance	0 min	1	RT	30-4000	3	100	quartz cuvette	nucleic acids, aromatic amino acids
							90-20 000	9		96-well	
UV205[2]	peptide bonds	absorption of sample	absorbance	0 min	1	RT	1-100	0.1	100	quartz cuvette	carbon double bonds
Biuret[4-9]	peptide bonds	reduction of copper	absorbance	20 min	2	RT	1000-10 000	1000	1000	cuvette	red. agents, Tris, NH_4^+ salts, glycerol
Lowry[10-13]	peptide bonds/ tyrosine and tryptophan	reduction of copper & Mo^{6+}/W^{6+}	absorbance	40 min	3	RT or 100 °C	25-500	5	200	cuvette	NH_4^+ comp., detergents, red. & chelating agents, strong acids & buffers
				15 min		RT	200-1500/ 5-250	1/0.1	5/20	96-well	chelating agents, thiols, salts, strong acids & buffers
Bradford[14-17]	hydrophobic regions and amino groups	dye−protein complex	absorbance	2 min	2	RT	250-1500	25	100	cuvette/ 96-well	detergents
				10 min			1-25	0.15	150	96-well	
BCA[18,19]	peptide bonds	reduction of copper & Cu−BCA complex	absorbance	30 min	4	37 or 60 °C + RT	100-1200	10	100	cuvette/ 96-well	red. agents, NH_4^+ comp.
				2 h		37 °C + RT	2-40	0.3	150	96-well	red. & chelating agents, NH_4^+ comp., lipids
Fluorescamine[20-22]	primary amino groups	dye−amino group conj.	fluorescence	< 1 s	2	RT	10-1000[e]	0.5	50	96-well	prim. amines, high conc. of sec. & tert. amines, acidic buffers
OPA[23]	primary amino groups	dye−amino group conj.	fluorescence	5 min	2	RT	0.05-25	0.01	200	96-well	prim. amines

Method	Interacting protein sites	Principle	Detection	Incub. time	No. of steps[a]	Temp.	Dyn. range[b] (mg/L)	LOD[b] (µg)	Vol. (µL)	Format	Interferences
CBQCA[24]	primary amino groups	dye–amino group conj.	fluorescence	1.5 h	3	RT	0.1-1500	0.01	100	cuvette/ 96-well	prim. amines, strong acids, thiols
NanoOrange[25]	not available	dye–detergent–protein complex	fluorescence	30 min	3	96 °C + RT	0.1-100	0.02	200	96-well	lipids, salts, detergents
Rohringer & Holden Au[26]	many	protein adsorption	visual	30 min	4	RT	-[f]	0.08[g]	1	nitro-cellulose	not available
Stoscheck Au[27]	many	protein ads. & abs. max. shift of coll. Au	absorbance	30 min	2	RT	2-20	0.02	10	cuvette	salts, thiols, detergents, strong bases
Ciesiolka & Gabius Au[28]	many	protein ads. & abs. max. shift of coll. Au	absorbance	2 min	2	RT	0.1-20	0.001	10	96-well	thiols, detergents, nucleic acids
Hunter & Hunter Au[29]	many	protein adsorption	absorbance	24 h	4	RT	1-20	0.001	1	nitro-cellulose	detergents
Quenching[30]	many	competitive protein adsorption	fluorescence	10 min	3	RT	0.007-2	0.0005	70	96-well	not available
TR-LRET[31,32]	many	competitive protein adsorption	time-resolved luminescence	20 min	3	RT	0.001-1	0.00007	70	96-well	not available
TPX[33]	many	competitive protein adsorption	2PE fluorescence	40 min	3	RT	0.002-9	0.00004	20	384-well	not available
Dissociation[34]	many	competitive protein adsorption	time-resolved luminescence	30 min	3	RT	0.002-0.6	0.0001	70	96-well	ionic detergents strong acids & buffers

[a] includes heating, cooling, and sample and reagent addition steps (reagent and sample preparations and signal detection not counted)
[b] for bovine serum albumin, if possible
[c] estimated as three times the nitrogen content measured for the blank
[d] solid sample
[e] the largest tested concentration 1 g/L in reference 27
[f] qualitative
[g] sensitivity average for 8 proteins

abbreviations:
2PE = two-photon excitation
LOD = limit of detection
RT = room temperature
Tris = tris(hydroxymethyl)aminomethane
Vol. = sample volume

2.6 COUNTING OF CELLS

The counting of cells follows frequently culturing and harvesting cells, since the cell concentration is important for experimentation in biology and biomedicine and in various medical and research applications, for instance, discovering, developing, and testing drugs. Generally, cells are directly counted visually by a hemocytometer. However, it suffers from subjectivity and is unsuitable for low concentrations of cells, is time-consuming and laborious. A number of methods have also been developed to simplify and reduce the work and improve the reliability, precision, sensitivity, and speed of the counting of the cells. Sensitive assays for the counting of cells would enable the concentration determination of low cell concentrations, which is not possible with traditional hematological methods. Low cell counts may be encountered, for instance, in studies with slowly proliferating cells, such as stem cells, and due to the sensitive counting of cells a time-consuming cell culture is avoided. However, adequate sensitivities (Bacterial Enumeration; BD FACSCalibur, 2009; MIDSCI PCV) are not reached with all commercially available methods and the procedures or principles may be complex (Elliott & Auersperg, 1993; Muir et al., 1990) and require expensive equipment or reagents (Campbell, 1988; Coulter, 1953; Schärfe, 2004). The next sections review well-known or commercially available methods for the counting of cells. In this thesis, the methods are divided into two categories: the methods determining the total number of cells, and viability or proliferation assays measuring the number of healthy or dividing cells. Whereas, Patterson (Patterson, 1979) has divided the methods into four categories: 1. visual hematology methods, 2. chemical methods with analytical biochemical procedures, 3. electronic systems with flow-through cells or apertures measuring incorporated dyes or cell numbers, and 4. arrays of miscellaneous procedures. However, the division presented in this thesis is more practical, as the method is chosen according to the information obtained from the samples and not due to procedures and devices involved.

2.6.1 Methods for Counting of Total Cell Numbers

The counting of total cell number does not take into account whether the cells are dead, quiescent, or dividing. The traditional counting of cells with a hemocytometer is subjective and too tedious to be useful in high-throughput screening. Furthermore, it is inaccurate at cell concentrations below 10^5 cells/mL (Allen et al., 1994). Electrical techniques for the counting of cells have been developed to eliminate user-to-user variability and improve the precision and speed of counting. Automated or electronic counting chambers are available from several companies (Beckman Coulter Vi-CELL, 2008; Boisen, 2005; ChemoMetec A/S NucleoCounter NC-100; Digital Bio Adam-MC, 2010; Invitrogen Countess 1, 2009; Invitrogen Countess 2, 2009; New Brunswick Scientific NucleoCounter; Nexcelom Cellometer). The principle is the same as with manual counting by hemocytometer except for the automatic brightfield cell image analysis (Invitrogen Countess 1, 2009; Szabo, 2003). Another possibility is the use of fluorescent nucleic acid binding dyes and the fluorescence detection from individual cells (Boisen, 2005). The sophisticated instruments measure even the sizes of the cells and analyze their morphology. Generally, the counters are simple to use and the count is fast to perform, typically in less than one minute. The disposable chamber reduces the risk of exposure to hazardous samples, such as patient blood samples and virus infected cells, compared to the traditional non-disposable Bürker counting chamber. In addition to the

high instrument upfront costs, the costly chambers and deoxyribonucleic acid (DNA) dyes are constantly used, which increases the amount of waste. Although the typical sample volume of 10 µL is relatively small and the sample concentration has a wider range than in a traditional hemocytometric count, the counters still require high concentrations of cells from tens of thousands to tens of millions of cells per mL, i.e. number of cells ranging from a few hundred to hundreds of thousands of cells in the sample. The automated counting chambers are able to detect cells with a diameter larger than 5 µm disabling the quantification of bacterial cells (Innovatis Cedex XS, 2010; Invitrogen Countess 1, 2009).

Coulter Counting

The Coulter counter (Coulter, 1953; Stewart et al., 2000), invented by Wallace H. Coulter, is a versatile and accurate analyzer for sizing and counting cells. It contains two electrodes, between which an electric current flows. The electrodes situate in a small-sized aperture that cells can only pass through one by one. The cells are drawn through the aperture in a weak electrolyte suspension. The cells cause a change in the electrical conductance momentarily by displacing their own volume of electrolyte. Quantification with the Coulter counter requires high cell concentrations in at least 50 µL sample volume (Beckman Coulter Multisizer 3 Coulter Counter; Harmalker & Lai, 1997; Kubitschek & Friske, 1986; Swanton et al., 1962). The sample should contain at least several thousands of cells per mL. On the other hand, samples with hundreds of thousands of cells per mL increases the probability of two or more cells passing through the aperture simultaneously, and being counted as one cell. This suggests that the count is prone to inaccuracy in the presence of cell aggregates or a large number of dead cells. Cell populations of different sizes require the resetting of the instrument (Phelan & Lawler, 1997). The commercial instruments typically measure volumes and count cells with a diameter ranging from below one micrometer to one millimeter.

Flow Cytometry

In flow cytometry (Givan, 2001; Stewart et al., 2000), the cells are optically detected in a narrow stream of the sample. The stream is produced hydrodynamically by injecting the sample into the center of a sheath flow. The sample surrounded by the flow with a higher velocity reduces the diameter and forces the cells into the center of the stream. The cell entering the laser beam scatters the light forming a signal for the counting of cells (Yang et al., 2006). Most flow cytometers are not able to perform the count from an exact sample volume (Perkel, 2010). Therefore, flow cytometric counting of cells is a relative rather than absolute method. The sample is run with a known concentration of fluorescent beads to which the number of analyzed cells is compared and used in the calculation of sample cell concentration. Another choice is to analyze the exact volume of a sample and calculate the number of cells per volume, if the instrument is designed to draw a precise volume of fluid for counting. Furthermore, the fluorescent labelling of the cells utilized in connection with light scattering in flow cytometry enables the sorting of different cells by their certain characteristics into multiple populations. The sensitivity of hundreds of cells per milliliter is attainable, but optimally hundreds of thousands of cells per milliliter are quantified (BD FACSCalibur, 2009; Hammes & Egli, 2005; Huh et al., 2005). The flow cytometric cell count time depends on sample volume and flow rate and may take from few seconds to hours. For instance, the cell count for a sample in 10 µL volume takes 10 s and in 10 mL 3 h, with a flow rate of 60 µL/min. Although the flow cytometer is versatile

due to its fast cell sorting feature and high accuracy, the operation requires an experienced user, which does not support the daily needs of the routine counting of cells. Other disadvantages include complicated and expensive devices (Huh et al., 2005), maintenance, experimental setup, and complicated sample pretreatment procedures.

Packed Cell Volume

Packed cell volume (PCV) (Sorokin, 1973) is a rapid method to quantify cells. The cells are centrifuged for only one to five minutes within a glass capillary using a bench top centrifuge. The volume of the pelleted cells is related to the number of cells. The tubes for measuring packed cell volume are commercially available (Light Lab PCV; MIDSCI PCV; Sartorius Stedim PCV; Sigma-Aldrich PCV). The method requires a high number of cultured cells and the calibration of the different cells for the relationship between the volume and cell number, since the cell sizes for the cell types vary. The method is easy-to-use compared to a traditional manual or even automated hemocytometric count and offers several distinct advantages. Packed cell volume reduces labor-intensivity and subjectivity, shortens the counting time, and allows the count of multiple samples at once. No special training or expensive instruments are needed compared to the electronic devices for the counting of cells. The presicion is higher, since statistical error is low due to the high number of cells in the pellet and cell aggregates do not influence it. However, a PCV lower than 0.1%, which translates to 200,000 cells in a 500 µL sample, cannot be considered as visually detectable (Light Lab PCV). The sensitivity of approximately ten times lower number of cells in a 100 µL sample is achieved, if PCV is detected with the reader (MIDSCI PCV).

Turbidity

The turbidimetric measurement is a quick method to estimate the concentration of bacteria. The optical density at 600 nm measured with the spectrophotometer is correlated with the cell number, as cells interfere with the passage of the light through the sample. However, millions of bacterial cells are required for the measurement in a cuvette and different cells do not have similar turbidity (Bacterial Enumeration).

CASY Counting

CASY technology (Schärfe, 2004) is an electronic method for the counting of cells. Cell concentrations of less than 100 cells/mL can be detected in a measurement time of 10 seconds with the method. The cell quantification is performed from a 5-100 µL sample (Roche Innovatis CASY TT, 2010; Roche Innovatis CASY TTC, 2010), but small sample volumes would require higher cell concentrations than 100 cells/mL to achieve accurate results. In addition to the counting of cells, the measuring principle enables the determination of the viability and size of the cells. The counting requires investment in the expensive instrument. The cells suspended in an electrolyte solution are exposed to a low voltage field between two platinum electrodes. The sample is scanned with a frequency of 1 MHz in a precision measuring capillary. The cells generate electrical signals, which are analyzed by amplitude, pulse width, course of time, and resulting pulse area, which differs from the Coulter counter, which only measures amplitudes. The aggregation of the cells does not inhibit accurate counting in contrast to the Coulter counter, as the cells do not have to pass through any aperture.

Methylene Blue Assay

Methylene blue dye (Figure 14), which stains negatively charged compounds, such as nucleic acids and proteins, at an alkaline pH (Oliver et al., 1989), has been used in the counting of cells performed in a microtiter plate format (Elliott & Auersperg, 1993). The dynamic range is from a few hundred to more than 3000 cells in a 100 µL sample volume. However, the heterogeneous assay setup containing several steps and incubations of one hour in total is not simple. The cells are fixed onto the microtiter plate surface for at least 30 min. The fixative is washed away and the methylene blue dye is added and incubated for 30 min. After incubation, the plate is washed three times to remove unbound dye and the dye is released from the cells with a mixture of alcohol and hydrochloric acid solution. The absorbance is measured at 630 nm with the microtiter plate reader.

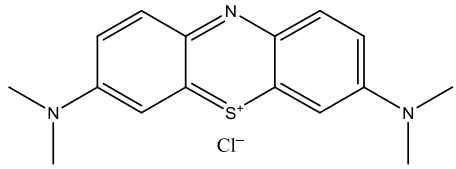


Figure 14. The structure of methylene blue dye.

Total Cellular Protein Assay

Measurement of total cellular protein concentration has been related to the cell count (Patterson, 1979). The methods may be simple, as they do not require any washing steps. However, the total protein concentration varies due to different culture conditions, the age of the culture, and harvesting methods. The protein concentration does not always have a linear relationship to the cell number either as, for example, some cell types like fibroblasts secrete matrix proteins (Laughton, 1984). The assay response varies widely among cell types, as the cells contain different amounts of total protein (Patterson, 1979). The sensitivities of the methods are low. A detection limit of only approximately 1000 cells was reached by Laughton with the Bradford method applied for cell quantification. Furthermore, staining for at least three hours at 37 °C was required to reach this sensitivity (Laughton, 1984).

Acid Phosphatase Assay

The acid phosphatase assay (Connolly et al., 1986; Yang et al., 1996) is based on the hydrolysis of the *p*-nitrophenyl phosphate by intracellular acid phosphatase found in living cells to *p*-nitrophenol. *p*-Nitrophenol is released outside by lysing the cells. The phenolic OH group of *p*-nitrophenol is deprotonated under alkaline conditions and *p*-nitrophenolate is formed (Figure 15). *p*-Nitrophenolate absorbs at 410 nm and the absorbance is proportional to the number of living cells in the sample. The dynamic range of the method is from 1000 to over 100,000 (Yang et al., 1996) or from 100 to 10,000 (Connolly et al., 1986) cells in a 100 µL sample volume, depending on the cell type and the endogenous level of acid phosphatase. The *p*-nitrophenyl phosphate solution is prepared freshly and equilibrated at RT or 37 °C before use (BioVision Acid Phosphatase, 2009; Cayman Acid Phosphatase, 2010; Luchter-Wasylewska, 1996; Sigma-Aldrich Acid Phosphatase, 2009). In the first step, the assay requires incubation at

37 °C for 2 h in the presence of *p*-nitrophenyl phosphate to achieve adequate sensitivity. In the second step, the addition of the sodium hydroxide solution follows in the end of the incubation to stop the reaction. The incubation time is shorter than for assays exploiting the activity of mitochondrial reductase enzyme (see Section 2.6.2), but it is still long and the assay is cumbersome. In fact, the absorbance does not level out even in four hours, which may lead to erroneous results, if all the reactions are not stopped simultaneously. Furthermore, the assay is not suitable or the sensitivity is low for cells, such as human foreskin fibroblasts and mouse hybridoma cells, which have low acid phosphatase activity. The acid phosphatase level of some cell types, for instance canine prostatic epithelial (Connolly et al., 1986) and WI-38 (Vorbrodt et al., 1979) cells, has also been shown to vary during the culture, and the presence of certain chemicals in the culture medium (Bivic & Arsanto, 1987) has altered the phosphatase activity of the cells, causing limitations for the applicability of the method. Fluorometric assays have also been developed for measuring the acid or alkaline phosphatase activity (Abcam Acid Phosphatase; AnaSpec SensoLyte Acid Phosphatase; Huschtscha et al., 1989). They exploit a non-fluorescent reagent, e.g. monofluorophosphate or methylumbelliferyl phosphate disodium salt, whose fluorescence is enhanced upon dephosphorylation by phosphatase. Fluorometric assays are more sensitive than the colorimetric assay, but otherwise they bear the same disadvantages as the colorimetric assay (Abcam Acid Phosphatase).

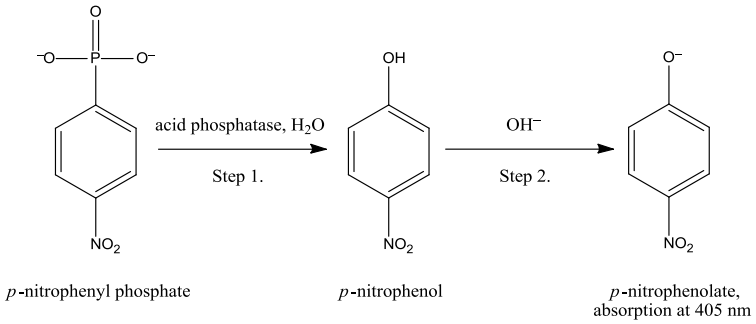


Figure 15. The principle of the acid phosphatase assay. The hydrolysis of the *p*-nitrophenyl phosphate by intracellular acid phosphatase to *p*-nitrophenol (step 1) and the deprotonation of the phenolic OH group under alkaline conditions (step 2).

LDH Assay

The lactate dehydrogenase (LDH) assay (Allen et al., 1994; Guilbault, 1975; Korzeniewski & Callewaert, 1983), used for many years (Nachlas et al., 1960), relates the activity of the lactate dehydrogenase enzyme to the cell number. LDH is ubiquitously present in plant and animal cells, as it is important for a cell, due to the capacity to turn lactate into pyruvate, which again could be transformed into glucose (Caviedes-Bucheli et al., 2006). LDH is released from the cells with a lysis reagent to enable the determinination of the total number of cells in a sample. LDH catalyzes the conversion of lactate to pyruvate (Figure 16). This reaction leads to the concomitant reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH. NADH again interacts with a specific probe to produce a signal. There are two different ways for detection, as a change in fluorescence or absorbance. In the first one, NADH drives the reduction of resazurin (7-hydroxy-10-oxide-3*H*-phenoxazin-3-one) to the fluorescent resorufin product in the

presence of diaphorase as a catalyst (Guilbault, 1975). This reaction is used in CytoTox-ONE (Promega CytoTox-ONE, 2009) and DHL Express (Interchim Cell Biology) kits. In the second one, the NADH reduction is coupled to the reduction of different tetrazolium salts to produce formazans. Tetrazolium salts are used in the method described by Korzeniewski & Callewaert (Korzeniewski & Callewaert, 1983), and in QuantiChrom DLDH-100 (BioAssay Systems QuantiChrom DLDH-100, 2007), CytoTox 96 (Moravec, 1994; Promega CytoTox 96, 2009), and BRSC LDH assay (BRSC LDH) kits as absorbance-based indicators of LDH activity.

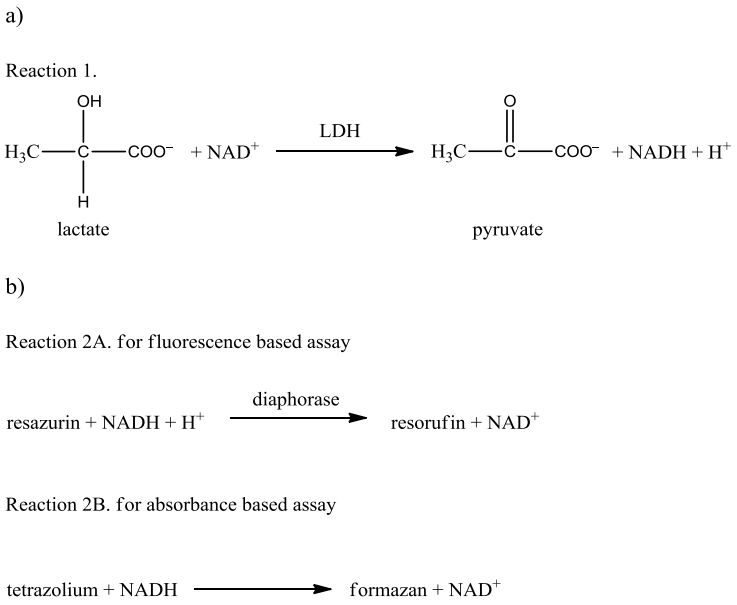


Figure 16. a) The conversion of lactate to pyruvate with the concomitant conversion of NAD^+ to NADH catalyzed by lactate dehydrogenase (LDH) (reaction 1) (Guilbault, 1964). b) The reduction of resazurin (reaction 2A) or tetrazolium salt (reaction 2B) by NADH (Guilbault, 1964; Korzeniewski & Callewaert, 1983; Mosmann, 1983; Scudiero et al., 1988).

The fluorescence-based assay (CytoTox-ONE and DHL Express kits) requires three reagent additions (Promega CytoTox-ONE, 2009; Interchim Cell Biology). The lysis solution is first added to the samples. The plate is incubated, typically for 20-30 min, to reach RT, when the analyte cells were cultured at 37 °C. The reagent containing lactate, NAD^+, diaphorase, and resazurin is added and the plate is incubated, typically 10 min at RT. Lastly, the stop solution is added and the fluorescence emission is recorded at 590 nm using excitation at approximately 540 nm. The method is relatively user-friendly, as it does not contain any heating steps and is not time-consuming. However, it has as many as three steps, making the method laborious. The extra step for the addition of the stop solution is recommended, as the signal does not level out. The assay is based on a kinetic reaction and originally the change in fluorescence with time has been recorded as a function of analyte concentration (Guilbault, 1975). If kinetic mesurements are not performed, identical incubation of each sample must be ensured by the quick addition of the reagents and a brief and efficient mixing. Both ways limit the usefulness and the time-

dependent signal decreases the accuracy of the method. Typically a few hundred cells can be detected in a sample of 100 µL measured in a microtiter plate. At high cell concentrations the substrate becomes rate-limiting, which restricts the dynamic range to hundreds of thousands of cells (Allen et al., 1994). Animal serums that are added into the culture media, are probable interfering agents, as the serum containing LDH can limit the sensitivity (Promega CytoTox-ONE, 2009). Another disadvantage of the method is the dissimilar LHD activity and response for different cell types (Allen et al., 1994; Promega CytoTox 96, 2009). The LDH content of the cell is determined by the size and oxidative activity of the cell (Allen et al., 1994). However, the LDH activity is thought to be similar within the same cell line and a standard curve should be measured for each cell type. Due to the varying LDH activities, the cell mixture samples cannot be quantified either (Allen et al., 1994). The half-life of LDH released from cells into the surrounding medium is approximately 9 h, avoiding the underestimation of the total cell number relatively well (Promega Cell Viability, 2006). However, the addition of lysis solution at the beginning of an experimental exposure period may lead to the decrease of active LDH and result in an erroneous counting result (Promega Cell Viability, 2006).

The colorimetric LDH assays (Allen et al., 1994; Korzeniewski & Callewaert, 1983; Moravec, 1994) use different tetrazolium salts, such as 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (BioAssay Systems QuantiChrom DLDH-100, 2007) and 2-*p*-iodophenyl-3-*p*-nitrophenyltetrazolium chloride (INT) (BRSC LDH; Promega CytoTox 96, 2009). The reduced forms exhibit absorption maxima at different wavelengths (approximately 570 nm for MTT and approximately 490 nm for INT). The absorbance is directly proportional to the enzyme activity. The method has a simple mix-and-measure protocol, although it contains three reagent additions. The cells are first lysed with Triton X-100 and incubated at 37 °C for 30-60 min. The reagent containing NAD^+, lactate, and diaphorase is mixed with the lysed cell sample in a microtiter plate, incubated generally for approximately 30 min at RT or at an elevated temperature 30 or 37 °C. After the incubation, the assay is stopped (optionally) by adding the stop solution and the plate is measured with a plate reader (Allen et al., 1994; Promega CytoTox 96, 2009). As for a fluorescence-based assay, the activity of the enzyme can alternatively be determined from the change in the signal (absorbance) as a function of time without the addition of the stop solution (Korzeniewski & Callewaert, 1983). According to Allen et al. (Allen et al., 1994), the linear dynamic range of the absorbance-based method is from 50 to 5000 cells in a sample of 50 µL measured in a microtiter plate, but may be affected by the cell type.

Glyceraldehyde-3-phosphate Dehydrogenase Assay

The luminometric glyceraldehyde-3-phosphate dehydrogenase (Corey et al., 1997) assay measures the activity of glyceraldehyde-3-phosphate dehydrogenase enzyme released from dead or damaged cells. The cell lysis reagent enables the determinination of the total number of cells in a population. The glycolytic pathway is exploited in the detection. The glyceraldehyde-3-phosphate dehydrogenase enzyme catalyzes the reaction of glyceraldehyde-3-phosphate with NAD^+ in an oxidative phosphorylation reaction, where 1,3-diphosphoglycerate is formed (Figure 17). Another enzyme, phosphoglycerate kinase, enables the transfer of the phosphate group of the phosphoanhydride bond at carbon 1 of 1,3-diphosphoglycerate to adenosine diphosphate (ADP) to obtain 3-phosphoglycerate and adenosine triphosphate (ATP). Formed ATP is detected by means of firefly luciferase in the light-producing reaction. The method is fast, sensitive, and relatively independent

of the cell type studied. The total incubation time is only approximately 10 min and even less than a single cell can be detected in a 5 µL sample volume in a microtiter plate. The dynamic range of the method is at least three orders of magnitude. However, the method requires several reagents and has two steps: the incubation of the sample in the presence of glyceraldehyde-3-phosphate, ADP, and phosphoglycerate kinase to yield ATP, and the addition of luciferase to detect the produced ATP.

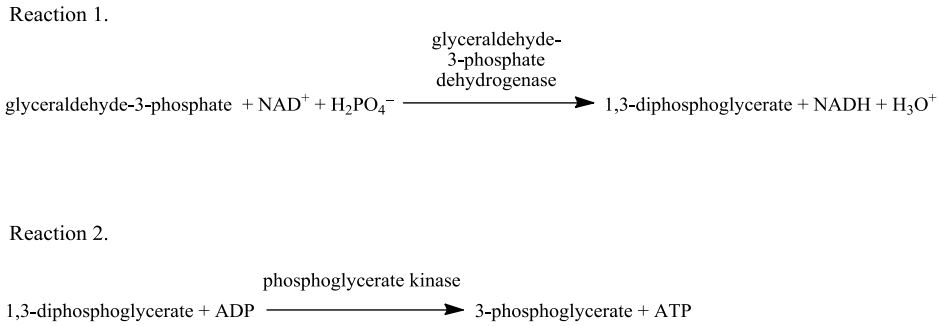


Figure 17. The principle of the glyceraldehyde-3-phosphate dehydrogenase method. The oxidative phosphorylation reaction of glyceraldehyde-3-phosphate (reaction 1) and adenosine triphosphate (ATP) formation (reaction 2) (Corey et al., 1997).

DNA Detection Assay

Fluorescent dyes, such as Hoechst, SYTOX, CyQUANT, SYBR green, diaminophenylindole, and propidium iodide, intercalate DNA and are useful in the counting of cells (Benech et al., 2004; Blaheta et al., 1991; Jansen et al., 2009). The fluorescence of the dyes is enhanced upon binding to DNA and the fluorescence signal is directly proportional to cell number. The cell-impermeable dyes require sample pretreatment, in which the cell membrane is lysed with detergent, ultrasound, papain, or by a freezing−thawing process (Blaheta et al., 1991; Cell Proliferation). Whereas, the assessment with cell-permeable dye does not require any sample pretreatment. The process of cell quantification using DNA dyes is fast and convenient. The assays performed in a microtiter plate format have short incubations of less than 5 min at RT, low cost, and require no washing steps (Adams & Storrie, 1981; Invitrogen CyQUANT, 2006). The method is capable of detecting tens or hundreds of picograms of DNA in a sample, depending on the intrinsic background fluorescence, extinction coefficient, and quantum yield of the dye (Adams & Storrie, 1981; BioTek Hoechst 33258, 2001; Life Technologies DNA Quantitation, 2010). The DNA content is typically pigograms per eukaryotic and femtograms per bacterial cell (Bisseling et al., 1977; Button & Robertson, 2001; Mirsky & Ris, 1951; Rasch et al., 1971). Thus, in a typical sample of 10-200 µL, the sensitivity of the counting of cells is a few tens of eukaryotic cells and approximately a thousand times lower count for bacterial cells. The signal levels out at a concentration of several thousands of eukaryotic cells per a sample (Adams & Storrie, 1981; Gillies et al., 1986; Jansen et al., 2009; Jones et al., 2001; Stewart et al., 2000). The total DNA content per cell is usually independent of cellular metabolism, making the assays relatively accurate. However, the DNA content depends on the cell type and size and, thus, calibration is required for each eukaryotic cell type. Another drawback is the detection of contaminating DNA outside the cells, giving an erroneous high cell count.

2.6.2 Methods for Counting of Viable Cell Numbers

Cell proliferation assays measure the number of dividing cells in a culture and dead cells are not enumerated. Fundamentally, the cell number capable of dividing is quantified by plating the cells or establishing growth curves. Both methods are time-consuming and laborious, as the enumeration by plating the cells consists of growing the cells on the appropriate matrix and counting the colonies formed (colony forming unit, CFU). The cells may divide only a few times and the colonies are not necessarily large enough to be counted. It is not practical for a high number of samples either and suitable only for microbes. A hemacytometric count with Trypan Blue staining enables the quantification of viable cells, since live cells exclude the dye. This method has similar limitations to the counting of total cell number with a hemocytometer. Furthermore, the cells should be counted soon after the staining, due to the toxicity of the dye and uptake of the dye by viable cells during long-term exposure. Trypan Blue stains also serum proteins in a medium and generates a dark background interfering with the counting (Beit-Haemek Trypan Blue Stain).

The microtiter plate assays quantifying viable cell numbers have been developed to speed up the quantification. The proliferating cells have been counted with radioactive methods, such as ^{3}H-thymidine incorporation (Hellman & Ullberg, 1986). The dynamic range of the method is from approximately 1000 to 10,000 cells measured in a microtiter plate (Roche Proliferation/Viability 1). However, radioisotopes bring several disadvantages including toxicity and the dangerous nature of the isotopes for the environment and health, disposal of waste, insufficient labeling of the cells, uptake of radiolabels released from dead cells, and a requirement for sophisticated instrumentation for sample analysis (Cook & Mitchell, 1989; Stewart et al., 2000). These problems have led to the development of nonradioactive methods measuring, for instance, different cellular metabolic activities, ATP content, or the uptake of vital dyes.

Cellular metabolic activity has been related to cell numbers in various fluorescence- or absorbance-based measurements performed in microtiter plates: the detection of mitochondrial enzyme (Kwah, 2001; Morgan, 1998; Mosmann, 1983; Sieuwerts et al., 1995; Twentyman & Luscombe, 1987), intracellular esterase (Karmazsin et al., 1979), the reducing conditions of the cytosol (Page et al., 1993; Voytik-Harbin et al., 1998), or the above mentioned intracellular acid/alkaline phosphatase (Connolly et al., 1986; Huschtscha et al., 1989; Yang et al., 1996) and glyceraldehyde-3-phosphate dehydrogenase (Corey et al., 1997) activities used for the counting of total cell numbers. The most sensitive methods relying on phosphatase activity can detect cell counts down to a hundred cells (Connolly et al., 1986). However, the methods are not always very accurate and fast, as cellular metabolic activities vary from cell to cell, may depend on the environment, and take a long time to produce detectable amounts of the metabolic products (Zhang & Cox, 1996).

BrdU Assay

The traditional method for detecting cell proliferation has long been based on the ^{3}H-thymidine incorporation (Maurer, 1981; Zolnai et al., 1998) into the cell DNA during the synthesis phase of the cell cycle (Dover & Patel, 1994; De Fries & Mitsuhashi, 1995). This method has several limitations, such as the handling and disposal of radioisotopes and the requirement for expensive equipment. Colorimetric, fluorescent, and

chemiluminescent methods, in which 5-bromo-2-deoxyuridine (BrdU) is incorporated as an alternative to ^{3}H-thymidine, have been developed and are commercially available (CHEMICON BrdU, 2005; EMD BrdU, 2009; LABGEN BrdU; Roche Proliferation/Viability 1). The BrdU assay (Hawker, 2003; Maghni et al., 1999; Muir et al., 1990) obviates the requirement for radioisotopes and liquid scintillation counters. The method evaluates cell cycle progression, as proliferating cells incorporate BrdU into the synthesized DNA strands. DNA is denatured, which exposes the incorporated BrdU to immunodetection and the counting of the number of actively DNA synthesizing cells is allowed. The measurement is either colorimetric (Hawker, 2003; Maghni et al., 1999), fluorometric (Savage et al., 1998a; Savage et al., 1998b), or chemiluminescent (Hawker, 2003) depending on the label of the substrate.

The lengthy experimental microtiter plate procedure takes typically 2-3 h (+ BrdU cell labeling) and contains fixing and washing steps. BrdU is added to the sample and incubated for 2-24 h in the tissue culture incubator, when BrdU is incorporated into proliferating cells. The cells are fixed and the DNA is denatured to enable antibody binding to the incorporated BrdU by adding a fixing/denaturing solution (Dinjens et al., 1992). After fixing for 30 min at RT, the wells are washed, anti-BrdU monoclonal antibody is added to locate the incorporated BrdU label, and the plate is incubated for 1 h at RT (Gratzner, 1982). At the end of the incubation, the wells are washed to remove the unbound antibody. The horseradish peroxidase-conjugated goat anti-mouse antibody, which binds to the anti-BrdU monoclonal antibody, is added and the plate is incubated for 30 min at RT. The substrate is added and incubated for 30 min at RT. The reaction is stopped with the addition of 1 M H_2SO_4 as a stop solution (CHEMICON BrdU, 2005; EMD BrdU, 2009; LABGEN BrdU; Roche Proliferation/Viability 1). For instance tetramethylbenzidine (Savage et al., 1998a), *o*-phenylenediamine (Muir et al., 1990; Savage et al., 1998a; Ternynck et al., 1987), or 2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]diammonium salt (Savage et al., 1998a) substrates are suitable for colorimetric detection, a fluorogenic QuantaBlue (Savage et al., 1998a; Savage et al., 1998b) substrate for fluorometric detection, and a chemiluminescent 3-aminophthalhydrazide/4-iodophenol (Hawker, 2003) substrate for luminescent detection. The horseradish peroxidase catalyzes the conversion of the chromogenic substrate tetramethylbenzidine from a colorless solution to a blue solution, which is again turned to a yellow solution after the addition of the stop solution. The QuantaBlue fluorogenic substrate is converted to a blue fluorescent product by horseradish peroxidase (Savage et al., 1998a; Savage et al., 1998b). In the chemiluminescent substrate, 4-iodophenol radicals are formed from 4-iodophenol in the presence of horseradish peroxidase. 3-aminophthalhydrazide then reacts with 4-iodophenol radicals and hydrogen peroxide and dissolved oxygen to yield endoperoxide. The endoperoxide decomposes to an electronically excited 3-aminophthalalate dianion, which relaxes to the ground state and emits light (Kamidate et al., 2009). The absorbance, fluorescence, or luminescence is read with a spectrophotometer or fluorescence or luminescence plate reader and related to the cell number. The higher absorbance/fluorescence/luminescence, the higher number of cells is in the sample.

Two alternative setups have been proposed. In the first one, the addition of the anti-BrdU monoclonal antibody and the horseradish peroxidase conjugated goat anti-mouse antibody have been replaced with the addition of only one reagent, peroxidase-conjugated anti-BrdU antibody (Roche Proliferation/Viability 1). In the latter one, the anti-BrdU monoclonal antibody, the horseradish peroxidase conjugated goat anti-mouse antibody,

and the substrate with the stop solution have been replaced with the anti-BrdU fluorescein conjugate.

The detection limit obtained for the colorimetric, fluorometric, or chemiluminescent assay varies according to the reference (EMD BrdU, 2009; LABGEN BrdU; Muir et al., 1990; Roche Proliferation/Viability 1) from below a hundred cells to a few thousand cells in a sample of 100 µL measured in a microtiter plate, and the signal saturates with 10,000 cells (Roche Proliferation/Viability 1). Higher sensitivity and signal-to-noise ratios are obtained with longer BrdU labeling times. In addition, the sensitivity, dynamic range, and signal-to-noise ratio depend on the substrate and its type (Savage et al., 1998a). Hawker et al. have shown that higher signal-to-noise ratio is obtained with the chemiluminescent detection and 3-aminophthalhydrazide/4-iodophenol substrate compared to the colorimetric detection and tetramethylbenzidine substrate (Hawker, 2003). On the other hand, the fluorogenic QuantaBlue substrate has been shown to give higher signal-to-noise ratio and sensitivity than colorimetric tetramethylbenzidine, *o*-phenylenediamine and 2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]diammonium salt and a commercial chemiluminescent substrate (Savage et al., 1998a).

As a disadvantage, BrdU affects the cell proliferation after its incorporation into DNA. This decreases the reliability of the assay and may decrease the sensitivity or increase the incubation time (Morris, 1991). BrdU also creates a potential health hazard, as it is a teratogen and can cause mutations by replacing thymidine in DNA (Ashman & Davidson, 1981; Bruce & Heddle, 1979) and thus, the user-friendliness of the method is decreased. The fixation of the cells is performed in the assay to prevent the detachment of the cells from the microtiter plate wells (Muir et al., 1990). However, a portion of the cells is still lost during the several experimental processes of the method. The acid denaturation of double-stranded DNA may also decrease the retention of cell numbers, if the fixed cells do not withstand acid denaturation. The method has a long incubation, as immunoreagents should penetrate into the cells to attain a high immunolabeling. Consequently, the antibody binding is time dependent and better sensitivities are only achieved with longer incubations (Muir et al., 1990). However, the BrdU has clear advantages compared to the radiolabeling methods, as it can be used at higher concentrations without toxic effects and it has lower costs, higher speed of counting and higher accuracy, and it is safer to use (Muir et al., 1990).

Neutral Red Assay

With neutral red assay (Elliott & Auersperg, 1993), viable cells are counted by using a supravital dye (Figure 18). Neutral red selectively targets the food breaking lysosomes or vacuoles only in living cells (Dierickx & Van De Vyver, 1991; Elliott & Auersperg, 1993; Fischer et al., 1985; Winckler, 1974), as cell injury decreases the permeability and retention of lysosomal membranes to the dye (Hawkins, 1980). However, the uptake of neutral red is strongly influenced by the metabolic state of the cell (Voytik-Harbin et al., 1998). The theoretical principle of the assay is simple, but the experimental setup is long and contains separation steps. A cell sample of 200 µL in a culture medium is incubated for 3 h in the presence of neutral red in a microtiter plate at 37 °C. After the incubation, two further steps, which require separation, are followed. Firstly, the cells are fixed during 2 min to the wells. Secondly, the neutral red dye is released from the cells during 15 min. The cells are quantified from the absorbance of the dye at 540 nm. The dynamic

range is from approximately 1000 to over 40,000 cells in a sample of 100 µL (Borenfreund & Puerner, 1984; Elliott & Auersperg, 1993; Löwik et al., 1993).

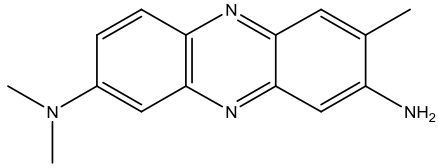


Figure 18. The structure of neutral red dye.

Mitochondrial Enzyme Activity Assays

Mitochondrial enzyme activity is utilized in the colorimetric MTT assay developed by Mossmann (Mosmann, 1983). The living cells produce a mitochondrial reductase enzyme, which reduces a tetrazolium compound 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to 1-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-formazan (Figure 19). Formazan absorbs at 570 nm and the absorbance is proportional to the active cell number. The assay is cost effective, as it allows for a large number of tests to be performed at the same time in the microtiter plate. It requires no washing steps either. However, the method requires prolonged incubation and heating for 4 h at 37 °C. The assay requires the addition of the acidic alcohol solution or organic solvent/detergent at the end of the incubation to dissolve the water insoluble formazan and to negate the interference of the culture medium components with the reading at 570 nm. Thus, this addition increases the number of assay steps and the disposal of organic waste. Furthermore, this last step causes cell debris and protein precipitation, which affects the absorbance reading at 570 nm. The elimination of this effect by reading the absorbance at 630 nm further increases the assay complexity (Chapdelaine). The dynamic range of the method is rather long, from 200 to over 100,000 living cells in a 100 µL sample volume (Mosmann, 1983) and the signal is stable, when the right stop solution is chosen (Chapdelaine; Promega CellTiter 96, 2009).

Cory et al. (Cory et al., 1991), Scudiero et al. (Scudiero et al., 1988), Barltrop et al. (Barltrop et al., 1991), Roehm et al. (Roehm et al., 1991), and the companies selling cell proliferation assays (Roche Proliferation/Viability 1) have developed new compounds to replace MTT and to reduce the steps of the assay. 3-(4,5-dimethyl-2-thiazolyl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS), 2,3,5-triphenyl-2*H*-tetrazolium, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium, and 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate reduce to water-soluble formazans in the presence of the electron coupling reagent, phenazine metho- or ethosulfate in phosphate-buffered saline and no dissolving step is involved. While the MTT assay is an end-point measurement due to the addition of a stop solution, the water-soluble formazans enable the recording of the data during the progress of the cell culture. The water solubility is provided by the negative charge in the structure of the formazans. Furthermore, the components are reduced more efficiently than MTT and the water soluble product is not as toxic to cells as the water insoluble MTT. The sensitivities and dynamic ranges of the assays with novel compounds are comparable to the MTT assay (Buttke et al., 1993; Roehm et al., 1991). As a disadvantage, the reducing agents such as dithiothreitol, ascorbic acid, coenzyme A, glutathione, NADH, and nicotinamide adenine dinucleotide phosphate (NADPH) may

interfere with all of these assays (Rhodes, 1996). The activity of some enzymes, such as dehydrogenases, involved can also persist even when cellular growth is no longer possible and the method may give the wrong information on the number of viable cells (Smith et al., 1982). If agents stimulating metabolicity are present, erroneous results are gained, as the cell proliferation is not increased simultaneously to the same extent as the metabolic activity is enhanced.

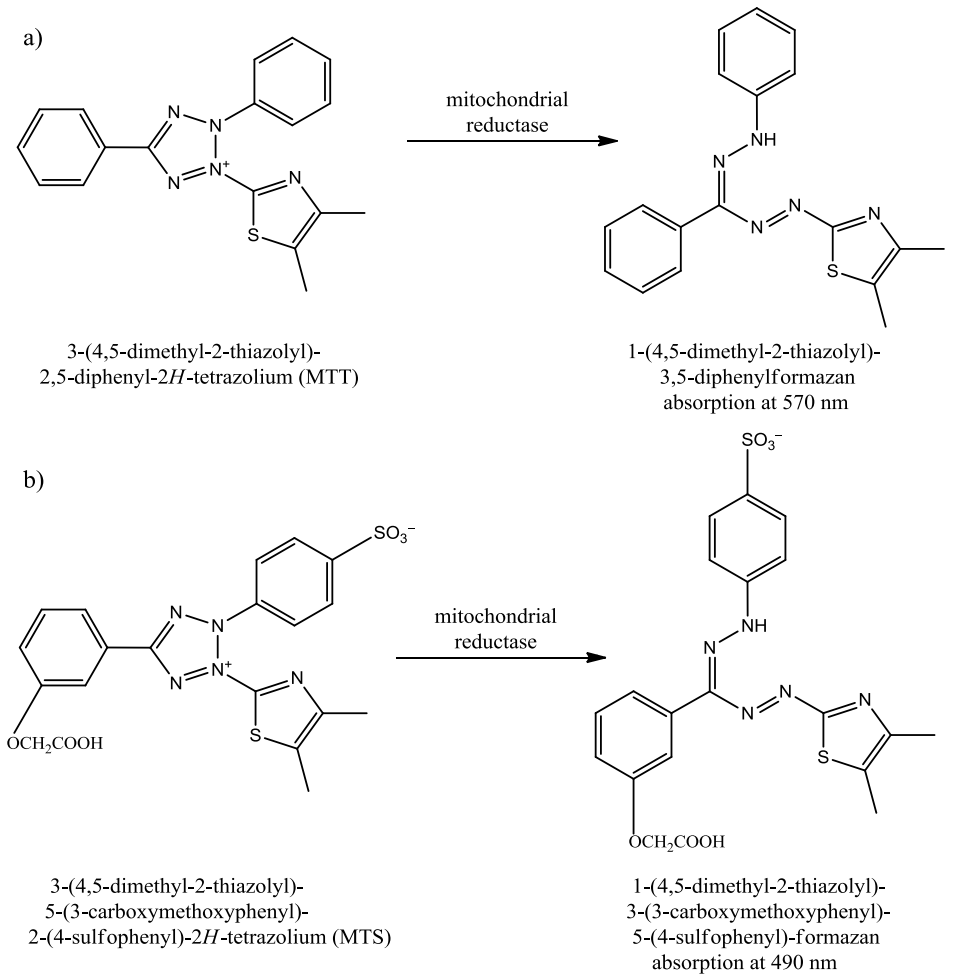


Figure 19. The reduction of a) MTT and b) MTS to corresponding formazans by mitochondrial reductase in living cells (Barltrop et al., 1991; Mosmann, 1983; Scudiero et al., 1988).

AlamarBlue Assay

AlamarBlue (Page et al., 1993; Voytik-Harbin et al., 1998) is a widely used method for the counting living cells and is based on the detection of the cellular metabolic activity. AlamarBlue reagent consists of a non-toxic, cell-permeable dye resazurin as an active ingredient (Figure 20). Resazurin is reduced to resorufin in the cytosol of the living cells,

due to a reducing environment related to the growth of the cell. More specifically reduced forms of flavin mononucleotide and flavin adenine dinucleotides, NADPH, NADH, and cytochromes cause the reduction (Al-Nasiry et al., 2007). Resazurin is indigo blue in color and virtually non-fluorescent, while the reduced form resorufin is pink in color and highly fluorescent (Al-Nasiry et al., 2007). Thus, the viable cell number may be quantified either from an absorbance or fluorescence measurement. Resorufin absorbs at 570 nm and emits at 590 nm (Voytik-Harbin et al., 1998).

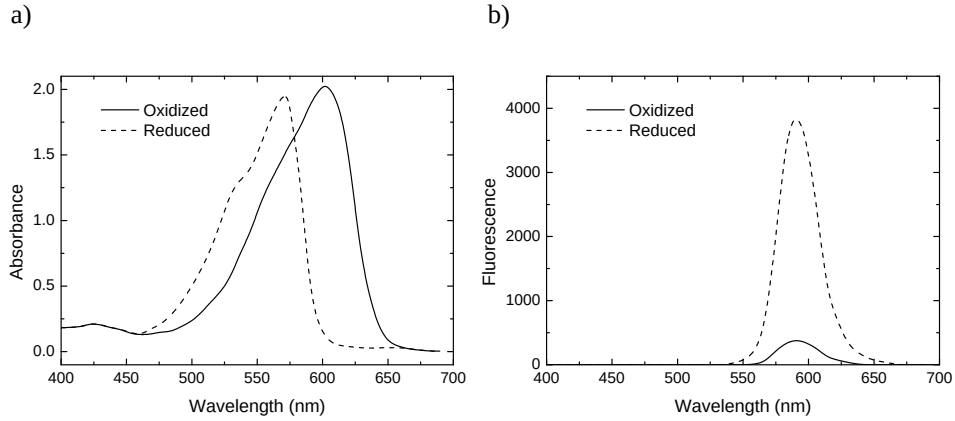


Figure 20. a) Absorption and b) emission spectra of oxidazed (resazurin) and reduced (resorufin) forms of the AlamarBlue reagent. Redrawn from Interchim UptiBlue, 2009.

The method has a simple add-and-read format performed in a microtiter plate, as the ready-to-use reagent is added to the sample in a culture media, and no washing or extraction steps are required. However, the method is time-consuming, as the incubation at 37 ºC following the reagent addition takes over 8 h to reach high sensitivity. Fluorescence-based quantification has a more than ten times higher sensitivity than absorbance-based one (Page et al., 1993). Quantitative differentiation of cell numbers from 100 to 20,000 is possible, based on fluorescence detection in a sample of 200 µL in a microtiter plate (Page et al., 1993; Voytik-Harbin et al., 1998). However, the metabolic activity varies between cells affecting the sensitivity and response of the method for different cell types (Riss & Moravec, 2003). Viable cells continuously convert resazurin to resorufin, which results in the change of fluorescence and absorbance as a function of time until the reagent is exhausted. The reaction and signal generation can be stopped by adding sodium dodecyl sulfate when needed. To speed up the counting, shorter incubation times may be used, although the sensitivity is lower than for long incubations. Conversely, as a disadvantage for long incubations, resorufin can further reduce to colorless hydroresorufin, depending on the cell type, and the dynamic range is reduced (Erb & Ehlers, 1950; O'Brien et al., 2000; Promega CellTiter-Blue, 2009).

The non-toxic reagent does not interfere with the normal metabolism of the cells (Larson et al., 1997), the cells are not destroyed in any step of the assay in contrast to, for instance, radioisotope, MTT (Fields & Lancaster, 1993), and dye uptake (Larson et al., 1997) assays and thus, the method can be applied for monitoring cell proliferation or the effect of drugs on metabolism continuously (Ahmed et al., 1994; Fields & Lancaster, 1993). The adequately stable signal and non-toxicity of the reagent allows the application

of the assay even for prolonged periods of several days (Interchim UptiBlue, 2009). Although the resazurin is generally considered as non-toxic to cells during short cultures with an optimized reagent concentration (Ahmed et al., 1994; Promega CellTiter-Blue, 2009), few studies clearly show that the reagent is harmful for certain cells during longer periods or with a higher resazurin concentration (Gloeckner et al., 2001).

Fetal bovine serum, bovine serum albumin (BSA), and phenol red found in cell culture media interfere with the assay by quenching the fluorescence or affecting absorbance values (Goegan et al., 1995). According to the supplier of the kit, chemicals or test compounds may also have an interference with resazurin reduction or assisting reagents and the assay chemistry should be controlled with positive and negative controls (Riss & Moravec, 2003). The advantage of the method is its low price per assay compared to radioactive methods (Larson et al., 1997) and the more sensitive ATP detection method (Invitrogen AlamarBlue, 2008). The method is safe for the end-users, as it does not contain any hazardous solvents and the reagent is disposable without inconvenient waste treatment.

Intracellular Esterase Assay

The intracellular esterase (Karmazsin et al., 1979) assay exploits the ability of the active esterase enzymes to hydrolyze fluorescein diacetate (Huang et al., 1986; Karmazsin et al., 1979; Persidsky & Baillie, 1977), carboxyfluorescein diacetate (Kaneshiro et al., 1991), or calcein acetoxy methyl ester (Kaneshiro et al., 1993; Kasper et al., 2008; Papadopoulos et al., 1994). These nonpolar and non-fluorescent dyes pass readily through the cell membrane. Inside the living cells their ester bonds are hydrolyzed by esterases, yielding highly fluorescent compounds: fluorescein, carboxyfluorescein, and calcein (Figure 21). The hydrolyzed dyes as polar molecules diffuse out much slower than nonpolar dyes, and accumulate intracellularly leaving the viable cells highly fluorescent. In contrast, the dead cells remain non-fluorescent. The principle can be used in visual hemocytometric counting or flow cytometry (Persidsky & Baillie, 1977), but simple microtiter plate procedures have also been developed. The dye is incubated with the samples at RT for 30-45 min (Invitrogen Viability/Cytotoxicity, 2005). The incubation time is rather short and the assay is relatively simple to perform for the end-user. Calcein acetoxy methyl ester has higher fluorescence, lower bleaching and is not as sensitive to pH changes as fluorescein diacetate and carboxyfluorescein (Kaneshiro et al., 1993). Fluorescein, carboxyfluorescein, and calcein are excited at approximately 490 nm and emit at approximately 520 nm near a neutral pH (Baptista et al., 2003; Cheng et al., 2007; Ralston et al., 1981; Siejak & Frąckowiak, 2005). An assay with calcein acetoxy methyl ester has a dynamic range from a few hundred cells to a million cells in a 100 µL sample volume (Invitrogen Viability/Cytotoxicity, 2005).

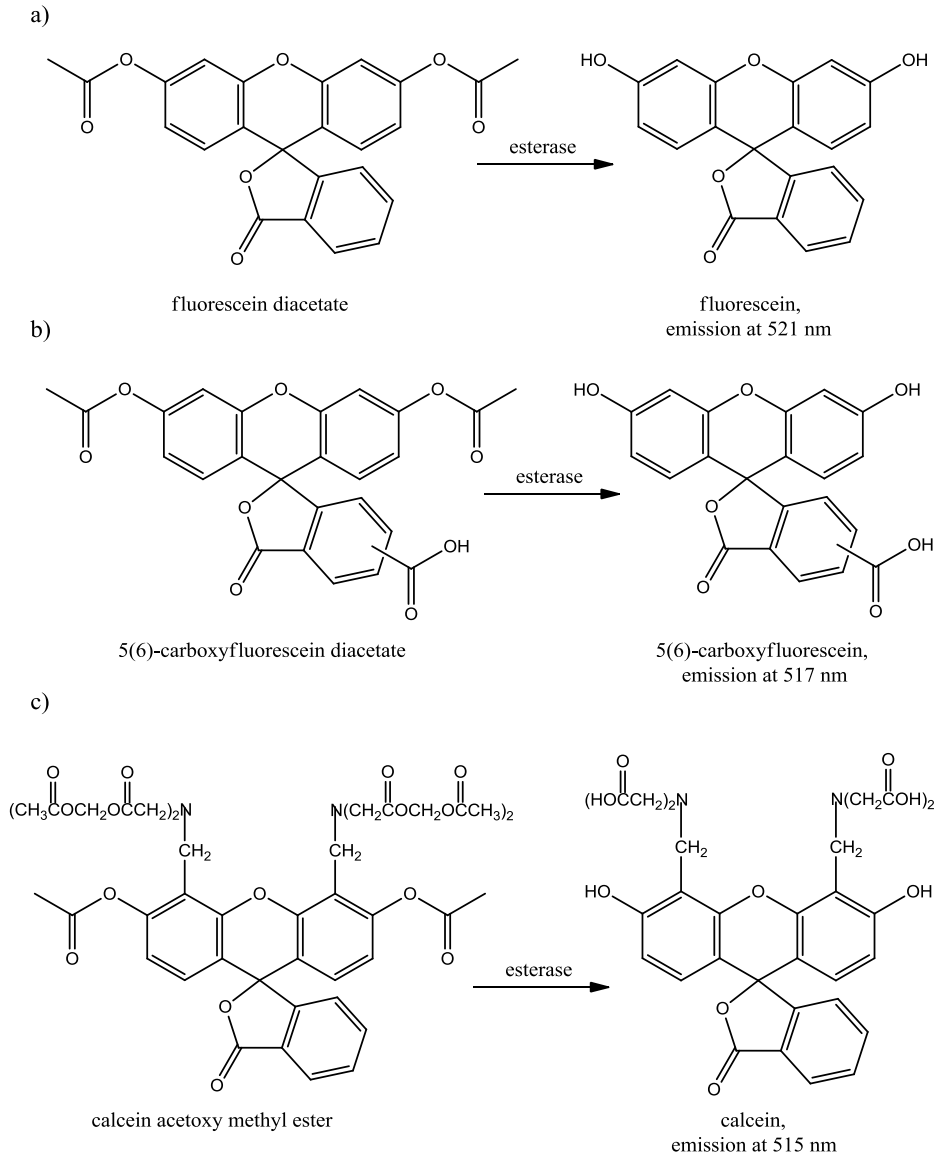


Figure 21. The hydrolyzation of non-fluorescent a) fluorescein diacetate, b) carboxyfluorescein diacetate, and c) calcein acetoxy methyl ester by esterase to highly fluorescent fluorescein, carboxyfluorescein, and calcein, respectively (Huang et al., 1986; Kaneshiro et al., 1991; Karmazsin et al., 1979; Kasper et al., 2008; Papadopoulos et al., 1994; Persidsky & Baillie, 1977; Vitecek et al., 2005).

Conserved Protease Activity Assay

The conserved protease activity as a measure of living cells has been proposed by Niles et al. (Niles et al., 2007). The fluorometric microtiter plate method has only one reagent addition of a fluorogenic and cell-permeable peptide substrate (glycyl-phenylalanyl-

aminofluorocoumarin). The substrate is cleaved by the live-cell protease inside the cell and a fluorescence signal is generated (Figure 22). Dead cells do not contribute to the signal, as this live-cell protease is quickly deactivated upon the breakage of cell membrane and leakage into the surrounding culture medium. After the substrate addition, the plate is incubated at 37 °C for 30 min, or at RT for longer than 30 min, and the fluorescence emission is measured at 505 nm with excitation at 400 nm. As the substrate is enzymatically cleaved, the fluorescence is measured before it levels out. It is impractical for the end-users, as the incubation time should be the same for each sample. The assay sensitivity for HeLa cells is below 50 viable cells in a 100 µL sample volume. However, animal serums may contain protease and decrease the sensitivity (Promega CellTiter-Fluor, 2009).

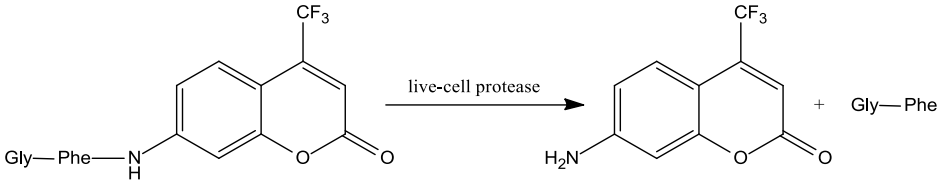


Figure 22. The live-cell protease cleaves the derivatized aminofluorocoumarin substrate to produce the fluorescent compound in a protease activity assay (Niles et al., 2007).

ATP Assay

Quantification of adenosine triphosphate (ATP) is exploited in the counting of cells in a bioluminescence assay with firefly luciferase and its substrate D-luciferin (Campbell, 1988; Crouch et al., 1993). In the presence of ATP and molecular oxygen from the air, D-luciferin is catalytically oxidized by the luciferase (Figure 23). ATP is not the energy source of the reaction, instead, it converts D-luciferin into a form, which is oxidized by the luciferase (Campbell, 1988). In the reaction, oxyluciferin is produced and ATP is dephosphorylated to adenosine monophosphate (AMP). The reaction leads to a light emission at 560 nm, giving a detectable signal, which increases linearly when the sample ATP concentration increases (Campbell, 1988). The plate is read with a luminometer, which precludes compound fluorescence or autofluorescence interference (Olsen, 2009). ATP is present in all metabolically active cells, as it is needed to remain alive and carry out the specialized functions. Thus, the quantification of ATP released from cells with detergent is related to the cell number. When the cells die, the concentration of ATP declines rapidly within minutes, due to the endogenous ATP degrading enzymes (ATPases), and so only living cells are counted.

The method is commercially available as kits with different small modifications from several companies (Interchim Cell Biology; Invitrogen ATP, 2005; Kikkoman PD-10 and LUCIPAC-W, 2001; Lonza ViaLight HS, 2007; PerkinElmer ATPLite, 2002; Promega CellTiter-Glo, 2009). Generally, the method contains two additions of reagents, lysing solution and luciferase/luciferin substrate solution, and no separation steps. CellTiter-Glo has only one reagent addition, as the lysing and substrate solution are first mixed together and the mixture is added to the cells. The assay requires no heating steps, as it can be performed at RT, in which the activity of the luciferase enzyme is the highest (Campbell,

1988). As few as 0.01-0.1 fmol ATP can be detected (Campbell, 1988). Eukaryotic cells contain generally 0.1-10 fmol, and bacterial cells approximately 0.01 fmol ATP per cell (Campbell, 1988). This enables a sensitivity of less than ten or even single cells, and the dynamic range is up to 10^7 cells in a typical 100 µL sample for microtiter plate protocols. High sensitivity is obtained due to the high quantum yield of the chemiluminescent reaction and long-lived luminescence. Approximately 90% of the energy released in the reaction is converted to light (Seliger & McElroy, 1960). Currently, ATP determination seems to be the only commercially available method gaining such a high sensitivity (Maehara et al., 1987; Petty et al., 1995). The method is rapid, as commercial systems have typically a 5 min-incubation after the addition of lysis solution, followed by a 10-15 min-incubation after the addition of a substrate solution, and before the reading of the luminescence. This sensitive, simple, and fast method has been typically exploited for detecting low-level bacterial contamination in samples, such as blood, milk, food, urine, soil, and sludge (Kricka, 1988; Molin et al., 1983; Newmark, 1988; Schifman et al., 1984).

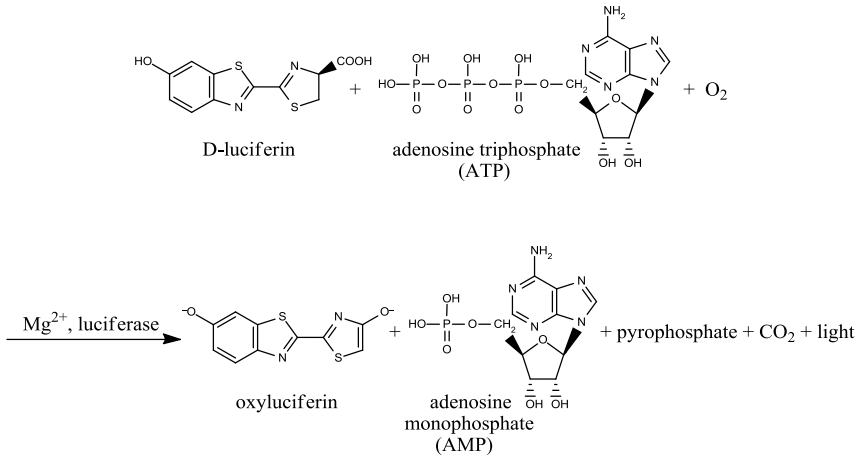


Figure 23. The luciferase catalyzed reaction in an ATP assay (Campbell, 1988).

The optimization of the assay conditions has enabled an adequately stable signal for 4-6 h, at least in ATPLite, CellTiter-Glo, and ViaLight HS kits. Thus, the ATP method can be used to study the effects of drugs and biological compounds on cell proliferation or cytotoxicity (Cree & Andreotti, 1997; Crouch et al., 1993; Kangas et al., 1984; Petty et al., 1995; Storer et al., 1996). However, the use is not straightforward, as these chemicals may be incompatible with the method and can affect its response (Crouch et al., 1993). There are differencies in commercial ATP assay kits, regarding the ability to affect molecules interfering with the method and probably found in cell samples. ATPases can decrease the ATP concentration of the sample and lead to a low response. Moreover, chaotropic agents or detergents found in lysing solutions would normally decrease the activity of the luciferase isolated from *Photinus pyralis* firefly. The effects of these molecules are eliminated in ATPLite or CellTiter-Glo kits by altering the pH, eliminating chaotropic agents/detergents or developing a stable form of luciferase purified from *Photuris pennsylvanica* firefly.

The susceptibility of the method to ATP contamination is a disadvantage. Namely, ATP as an energy source serving molecule, is found everywhere, and thus, it is a high risk for the assay performance and the storage of the assay components. The easy denaturation of the firefly luciferase enzyme hampers the handling and reduces the exploitability from the end-user's point of view, as the samples should be mixed by inverting the tubes and not simply by vortexing (Invitrogen ATP, 2005). Difficulties also arise in preparing the reagents, providing their high purity and establishing the right storage and assay conditions (Campbell, 1988). The response of the assay varies between cells, since the ATP level varies considerably in different cell types (Andreotti et al., 1995; Beckers et al., 1986; Crouch et al., 1993; Lundin et al., 1986; Stanley, 1986). The culture conditions affecting the cytoplasmic volume or physiology of the cells change also the ATP content. For instance, a decrease of intracellular volume, a change in energy metabolism, a cell injury, a substrate deficiency, or oxygen deficiency leads to the decrease of ATP (Crouch et al., 1993; Lundin et al., 1986).

2.6.3 Summary of Methods for Counting of Cells

The characteristics and principles of the methods for the counting of cells discussed above are collected in Table 3. The features affect the choice of the right method for the experimental needs. Depending on the method, the total numbers of cells or only viable cells are counted. For number of applications, such as cell signaling studies, the cell viability is important and the assay quantifying the number of healthy cells should be chosen (Voytik-Harbin et al., 1998). In proliferation assays, such as ^{3}H-thymidine incorporation and BrdU, cells must reproduce and therefore, slowly proliferating cells are difficult or at least time-consuming to count. To quantify viable cells, for instance an AlamarBlue assay, which does not depend on cell division, could be chosen to speed up the counting (Larson et al., 1997). On the other hand, the applications where viable cells are not needed, the viable cell count is an underestimation of the total cell number. With hemocytometer the number of individual cells is directly counted in a sample and the determined cell concentration cannot differ ten- or hundredfold from the correct one. The methods quantifying, for instance, a cellular process or the activity of an enzyme in a cell or a signal related to the cell concentration can lead to a wrong conclusion, as these processes, activities, or signals may vary between individual cells. However, the aggregation, breakup, or division of the cells may lead to an erroneous result with a hemocytometer. Thus, the lysing agents are not suitable with direct count methods, as the lysed cells are not detected.

Further criteria for choosing a method are the ease-of-use, speed-of-count, and ability for sequential sample analysis. Manual hemocytometric count is time-consuming and laborious for several samples and subjective. Automated hemocytometers are more objective and less time-consuming and less laborious (Connolly et al., 1986). However, automated hemocytometers and other automated counters different from hemocytometers are inaccurate at low cell concentrations, require high cell numbers, upfront costs may be high, and several samples cannot be counted simultaneously (Huschtscha et al., 1989). Methods such as ^{3}H-thymidine incorporation and BrdU assays allow for the processing of several samples at once. Due to the long labeling time, the results are usually not obtained until after 24 h, which may prevent the fast continuation of research. These methods are not able to count the cells at true zero time, since the cells may proliferate during the incubation. For other assays like AlamarBlue, MTT, neutral red, and acid phosphatase assays, the incubation times are shorter, but generally several hours are still needed to

obtain the results. The cells can be counted in less than one hour with a DNA intercalator dye, glyceraldehyde-3-phosphate dehydrogenase, LDH, intracellular esterase, conserved protease, and ATP methods discussed in Sections 2.6.1 and 2.6.2.

It is preferred that the assays have simple mix-and-measure protocols with only one reagent addition, no separation steps, and a short or no incubation at ambient temperature. BrdU assay has one of the most difficult experimental procedures containing six reagent additions, several incubations at RT or 37 °C for 4-24 h in total, and two washing steps. In addition, BrdU and LDH assays require the addition of the stop solution, and the MTT assay an organic solvent to dissolve the formed formazan. As the signals of BrdU, LDH, and conserved protease activity assays are measured before reaching the equilibrium, the incubation time must be controlled carefully before the reading of the plate or the addition of stop solution in the end of the assay. PCV, staining, and dye incorporation methods require a separation step. Homogeneous methods are preferred, since the fixing, washing, and centrifugation steps and the instruments performing these steps are not required. The methods requiring incubations at 37 °C or under culture conditions are performed in separate incubators increasing the need for instruments. There are only a few homogeneous assays, e.g. DNA detection (Benech et al., 2004; Blaheta et al., 1991; Jansen et al., 2009) and ATP (Campbell, 1988; Crouch et al., 1993) assays, having short incubation at RT and only one reagent addition. The choice of the detection is determined, for instance, by the following factors: detection environment, available instrumentation, and sensitivity. Assays are mainly colorimetric, fluorometric, luminometric, or radio-isotopic. Radio-isotopic assays are avoided due to the toxicity, storage-related problems, and specific requirements for waste disposal. Fluorometric detection has an advantage over colorimetric detection due to the higher sensitivity.

If an assay has an ability for multiplexing, several parameters are obtained from a single sample providing cost-effectiveness as sample volume, time, and assay components can be reduced. Assays retaining cell viability enable additional assaying. The cell quantification can be combined with, for instance, a genetic reporter, viability, cytotoxicity, apoptosis, or proteasome assay. On the other hand, for example, the reagents for the ATP and LDH assays lyse the cells and limit multiplexing (Promega Cell Viability, 2006).

The dynamic ranges of the methods are not typically the same for all cell types. For instance, the methods based on the detection of cellular metabolic activity result in a different response for different cells, if the activity of the cell types varies. Due to the limited commercial and literature data, the values in Table 3 are not for the same cell types. However, the collected data suggests that the dynamic range is in the same order of magnitude and comparable at least among similar eukaryotic cells. The automated or electronic counting methods have the theoretical sensitivity of one cell in a sample. In practice, the statistical error is so high, that more than a hundred cells in a sample are counted to obtain a lower coefficient of variation. Therefore, the required cell number is estimated differently in the references and for the instruments. Long dynamic range is important, as it avoids the dilution or concentration steps and cumbersome repetitive assaying. The ^{3}H-thymidine incorporation method, having one of the shortest dynamic ranges, has a dynamic range of only one order of magnitude. On the other hand, intracellular esterase and ATP assays have the longest dynamic ranges of over four and six orders of magnitude, respectively.

Table 3. The characteristics and principles of the methods for the counting of cells. (See Appendix 2 for reference information.)

Methods for Counting of Total Cell Numbers										
Method	**Principle**	**Detection**	**Setup**	**Incub. time**	**No. of add.**	**Temp. and cond.**	**Dyn. range (cells)**	**Vol. (µL)**	**Format**	**Interferences & limitations**
Hemocytometry[1,2]	direct count	visual	homogeneous	-	0	RT	> 10^3-	20	count chamber + microscope	lysis agents, laborious, subj. counting, LTS
Automated hemocytometry[3-6]	direct count/ DNA dye	image anal./ fluorometric	homogeneous	-	0 or 1	RT	100-10^5	10-20	count chamber + instrument	lysis agents, small cells (diameter < 5 µm), high upfront costs, LTS
Coulter[7-12]	conductance change	conductance	homogeneous	-	0	RT	> 100-< 10^4	> 50	instrument	lysis agents, cell aggregates, instrument resetting for different cells, LTS
Flow cytometry[4,13-16]	scattering of light	laser light scattering	homogeneous	-	0	RT	10^4-10^7	10-	instrument	experienced user, instrument maintenance, lysis agents, experim. setup, high upfront costs, LTS
PCV[17-19]	total volume of cells	volumetric	heterogeneous	-	0	RT	2×10^5-2×10^8 [a]	500	centrifuge + tube	dependent on cell volume, lysis agents
							> 10^4	100	centrifuge + tube + reader	
Turbidity[20]	interference with passage of light	turbidimetric/ colorimetric	homogeneous	-	0	RT	> 10^6-	100	cuvette	difference in turbidity between cells
CASY[21,22]	electrical signals generated by cells	change in voltage	homogeneous	-	0	RT	10-	5-100	instrument	high upfront costs, LTS
Methylene blue[23,24]	DNA and protein staining	colorimetric	heterogeneous	1 h	3	RT	> 100-> 3000	100	96-well	laborious
Bradford[25-27]	total cellular protein quantif.	colorimetric	homogeneous	3 h	1	37 °C	10^3-	100	96-well	culture cond., culture age, harvesting, cell type, time-consuming
Acid phosphatase[28-31]	enzyme activity	colorimetric	homogeneous	2 h	2	37 °C	> 100-> 10^4	100	96-well	culture conditions, cell type, culture age
LDH[1,32-35]	enzyme activity	fluorometric	homogeneous	30 min	3	RT	> 100-10^5	100	96-well	animal serum, cell type
		colorimetric		60 min		RT/30 °C /37 °C	50-5000	50		cell type
Glyceraldehyde-3-phosphate dehydrogenase[36]	enzyme activity	luminometric	homogeneous	10 min	2	RT	< 1-> 1000	5	96-well	several reagents, reagent impurities
DNA dye[8,37-43]	total DNA quantification	fluorometric	homogeneous	5 min	1 or 2	RT	> 10-> 10^3	10-200	96-well	DNA outside cells, cell type/size, carcinogenic, mutagenic
Quenching[44]	nonspecific interaction	fluorometric	homogeneous	10 min	2	RT	4-300	70	96-well	culture conditions
TR-LRET[44]	nonspecific interaction	luminometric	homogeneous	10 min	2	RT	5-30	70	96-well	culture conditions
TPX[45]	nonspecific interaction	2PE fluorometric	homogeneous	40 min	2	RT	3-200	20	384-well	not available

Methods for Counting of Viable Cell Numbers

Method	Principle	Detection	Setup	Incub. time	No. of add.	Temp. and cond.	Dyn. range (cells)	Vol. (µL)	Format	Interferences & limitations
Standard plate count[20]	cell culture + counting CFUs	visual	homogeneous	24 h-	0	culture cond. (37 °C)	1-	-	culture dish	culture cond./cell type, small colonies, time-consuming, laborious, only for microbes, LTS
Trypan Blue[46]	dye exclusion	visual	homogeneous	-	0	RT	> 10^3-	20	count chamber + microscope	subj. counting
^{3}H-thymidine incorporation[47,48]	DNA dye incorp. during cell culture	radio-isotopic	heterogeneous	2-24 h	1	culture cond. (37 °C)	10^3-10^4	-	96-well	toxicity, disposal of waste, instrumentation, time-consuming
BrdU[48-56]	BrdU incorp. to DNA during cell culture	colorimetric/ fluorometric/ luminometric	heterogeneous	4-24 h	6	culture cond. (37 °C) + RT	> 100-10^4 [b]	100	96-well	teratogenic/mutagenic BrdU, change in cell proliferation, detachment of cells, time-consuming, laborious
Neutral red[24,57-62]	lysosome & vacuole staining	colorimetric	heterogeneous	3 h	3	37 °C	10^3-> 4×10^4	100	96-well	uptake affected by cellular metabolic state, time-consuming, laborious
MTT[63]	mitochondrial reductase enzyme activity	colorimetric	homogeneous	4 h	2	37 °C	200-> 10^5	100	96-well	metabolicity stimulators, red. agents, time-consuming insoluble/toxic formazan, end-point measurement, enzyme activity w/o cell growth,
MTS[64]	mitochondrial reductase enzyme activity	colorimetric	homogeneous	4 h	1	37 °C	200-> 10^5	100	96-well	metabolicity stimulators, red. agents, enzyme activity w/o cell growth, time-consuming
AlamarBlue[57,65,66]	reducing environment in living cells	colorimetric	homogeneous	8-24 h	1	37 °C	3×10^3-10^5	200	96-well	FBS, BSA, phenol red, long-term toxicity, metabolic activity, reduction to hydroresorufin, possible chem. interference, time-consuming
		fluorometric		8 h			100-2×10^4			
Intracellular esterase[67,68]	esterase enzyme activity	fluorometric	homogeneous	30 min	1	RT	> 100-10^6	100	96-well	
Conserved protease[69,70]	protease enzyme activity	fluorometric	homogeneous	30 min	1	37 °C	50-10^4 [c]	100	96-well	animal serum containing protease
ATP[71-77]	D-luciferin oxidation and light prod.	luminometric	homogeneous	15-20 min	1 or 2	RT	1-10^7	100	96-well	ATP, ATP prod./degr. enzymes, nucleotides, arsenate, acidic buffers, low/high temperature quat. NH_4^+ salts, anions, cell type

[a] 0.1-100% PCV

[b] broader dynamic range for chemiluminescent detection

[c] the highest number of cells 10^4 in reference **69**

abbreviations:

2PE = two-photon excitation
BrdU = 5-bromo-2-deoxyuridine
BSA = albumin from bovine serum
CFU = colony forming unit
FBS = fetal bovine serum
LTS = low-throughput screening
No. of add. = number of reagent additions
RT = room temperature
Vol. = sample volume

The sensitivity of the method determines its applicability and suitability for a chosen application. Especially if only few cells, such as slowly proliferating stem cells, are present in a precious sample, only a limited number of methods are applicable. A standard plate count has the sensitivity of one cell in a sample, but only microbes can be counted. The detection of ATP content (Maehara et al., 1987; Petty et al., 1995) seems to be the only commercially available assay reaching nearly a single-cell sensitivity and with the literature method measuring the glyceraldehyde-3-phosphate dehydrogenase enzyme activity, less than single cells can be detected (Corey et al., 1997).

Each method may be interfered with different agents and has limitations dependent on its principle. The common contaminants for the assays are cell lysis agents, reagent impurities, reducing agents, phenol red, and animal serum. The drugs and biological compounds, whose effect on cell proliferation or cytotoxicity is studied, can also interfere with the assays. Parallel time- and sample-consuming tests must be run when such chemicals affect the methods (Zhang & Cox, 1996). If, for instance, the effect of metabolic stimulators on cell growth is studied, metabolic activity-based assays for the counting of cells give the wrong results. Cellular metabolic processes vary also greatly over time, which in turn affects the counting of cells. Chemical agents have also been shown to modulate enzyme activities (Bivic & Arsanto, 1987; Huschtscha et al., 1989). The ATP method has similar problems, as the culture conditions affect the ATP content and the sample may still contain a different number of living cells than the ATP level suggests (Crouch et al., 1993; Lundin et al., 1986).

2.7 VIABILITY TESTS

Most of the methods discussed above count only the total number of cells or the number of viable/living cells and do not state the percentages of viable/living and dead cells in a sample. The cell health is important in establishing optimal cell culture conditions and for obtaining reliable results in cell-based experiments. In properly selected cell culture conditions, the healthy cells proliferate efficiently, providing savings in time, labor, and disposable laboratory ware. In cell-based research, the dead cells can interfere, e.g. by altered antigen expression, binding nonspecifically onto assay components or cellular markers, replacing specific binding sites, uptaking fluorescent probes, and inhibiting the staining or binding of the dyes (Coder, 1997; Frankfurt, 1990; O'Brien & Bolton, 1995). Cell viability is also important in medicine. In cancer research, the cell viability provides information about the effect of the treatment on the cancer cell growth. In the medical treatment of people with autoimmune disease, the evaluation of cell viability helps the physician to determine the progress of the disease and decide on a suitable drug therapy (Ellis-Christensen). On the other hand, the percentage of dead cells can be important in cytotoxicity studies or the handling of hazardous cells (Interchim Cell Biology). Without simple viability tests combining viable and dead cell counts, the number of viable and dead cells should be measured using possibly two to three separate methods requiring their own samples.

The cells may die via two mechanisms: necrosis or apoptosis. Necrosis is an accidental death, which occurs for a group of cells under physically or chemically harsh conditions. Apoptosis is a programmed cell death, which occurs for individual cells, and eliminates unwanted or useless cells under normal biological conditions (Roche Proliferation/Viability 1). Due to the plentiful available viability tests, the methods

distinguishing between necrotic and apoptotic cells are not presented in this thesis for the sake of simplicity.

There are several methods to assess the percentages of viable/living and dead cells. These methods define the living or dead cells differently. Probably, the simplest definition is that viable cells have intact plasma membranes and dead cells broken plasma membranes, which is tested with a dye exclusion (Frankfurt, 1990). Large or charged dye molecules do not permeate intact cell membranes, which provides the differentiation (Coder, 1997). Instead, the dye inclusion test determines that living cells specifically take up a dye molecule. The latter test is more demanding compared to the first one, as the living cell must maintain the dye inside. Methods may also relate the maintenance of pH and ion gradients, morphological changes, the cellular metabolic activity, or the replication of DNA to the physiological state of the cell. The criterion for a viable cell can also be the ability to proliferate under normal culture conditions, although certain cells, such as primary cells that are quiescent and nongrowing can still be healthy (Coder, 1997; Cook & Mitchell, 1989; Roche Proliferation/Viability 2, 2003).

Cook and Mitchell (Cook & Mitchell, 1989) have reviewed the viability measurements that have been used for mammalian cells in the past. Many of these methods measure only the number of dead or viable cells and have already been discussed as applications for the counting of cells (for instance, enzyme activity, LDH release, and quantification of ATP) or they are time-consuming, laborious, or radio-isotopic. It is, however, worth to mention radioisotope release, efflux of Ca^{2+} ion, morphological, colony formation, and growth rate methods. Radioisotope release methods require prelabeling with ^{51}Cr, ^{125}I-UDR, ^{3}H-proline, or ^{75}Se-selenomethionine. They have problems with a high spontaneous release of the label, reincorporation of isotopes released from dead cells by viable cells, chemotoxic/radiotoxic effects, and safety. The ion and amino acid gradients, especially efflux of Ca^{2+} ion, have been related to the cell viability and detected with radio-isotopic, fluorescence, or inductively coupled flame atomic absorption techniques, chromatography, or ion selective electrodes. In addition to the required instrumentation, the sample preparation is often laborious. The detection of morphological changes on the cell surface, in the cellular volume, or in the cytoskeleton are often difficult to interpret and relate to the cell death. In *in vitro* colony formation method, the cells are cultured on cell culture plates in sterile conditions at low cell densities with a known cell number, and the survival and colony formation of individual cells are related to the viability. This method is inconvenient with cell types having low plating efficiencies, unable to grow at a low cell density, forming diffuse or clumpy colonies, and slowly proliferating cells. The *in vivo* colony formation method is even more complicated, as an animal offers the growth environment instead of a cell culture plate and an incubator. Growth rates are generated by total cell count measurements. The method has several drawbacks, for instance cell harvesting into a single cell suspension and several time point measurements are required and the result is not simple to analyze from the growth curve.

The purpose in this thesis is to concentrate on preferably user-friendly and commercially available methods that are in common use nowadays, and capable of assessing the ratio between living and dead cell numbers in the same sample. Different viability tests will be presented below. Each test is a combination of the simultaneous determination of live, dead, or total number of cells. The tests use one or two of the following principles: dye exclusion, dye inclusion, release of cellular compounds, probe of physiological state, and probe of proliferation.

Dye Exclusion Test with Microscopy

In dye exclusion tests, viable cells with intact membranes exclude the dye and dead cells are stained. In addition to the most common Trypan Blue (Beit-Haemek Trypan Blue Stain; Coder, 1997; McGahon et al., 1995), nigrosin (Johnson, 1995), and eosin Y (Interchim Cell Biology) dyes have been used. Cell viability is assessed under a light microscope in a hemocytometer. The sample is simply mixed with the dye, and stained and unstained cells are counted, enabling the calculation of the percentage of viable cells. The test is widely used due to the simple protocol. However, the test is laborious and subjective. Cell culture medium containing serum causes a dark background and hampers the count (Coder, 1997). Moreover, the viable cells start to uptake the dye during continuing exposure and the cells should be counted soon after the staining (Beit-Haemek Trypan Blue Stain; Boisen, 2005). Generally, all dye exclusion tests may also underestimate the number of dead cells, as the intracellular events require time to lead for membrane damage (Cook & Mitchell, 1989).

Dye Exclusion Test with Flow Cytometry, with Fluorescence Microscopy or in Microtiter Plate Reading

Dye exclusion can also be tested using fluorescent DNA intercalator dyes, if cells are counted under a fluorescence microscope. However, the tests can be automated and viable and dead cells differentiated with a flow cytometer (Coder, 1997). Dead cells are identified from viable cells with a cell-impermeable DNA intercalating dye, which enters only cells with broken membranes. Intercalating dyes undergo a fluorescence enhancement upon interaction with DNA. Dyes, such as 7-aminoactinomycin D (Fetterhoff et al., 1993), propidium iodide (Jacobs & Pipho, 1983), acridine orange (Interchim Cell Biology), ethidium bromide (Interchim Cell Biology), or YO-PRO-1 (O'Brien & Bolton, 1995), are mixed with a sample cell suspension and analyzed on a flow cytometer after an incubation of few minutes. The flow cytometer can detect a distinct difference between dead and viable cell populations. A dead cell has a bright fluorescence and a viable cell dim one. The number of cells in both populations is counted providing the percentage of viable cells. The dye exclusion test with flow cytometry is more accurate than with microscopy, as a greater numbers of cells in total are counted. However, the instrument is costly and not widely available and expertise is needed to operate the instrument. Propidium iodide is a popular dye to assess mammalian (Coco-Martin et al., 1992; Jacobs & Pipho, 1983; O'Brien & Bolton, 1995) and bacterial (Nebe-von Caron et al., 1994; Vesey et al., 1994) cell viability. As a disadvantage, DNA dyes might be carcinogenic and mutagenic and pose potential dangers for the health (Cell Viability, 2008).

A membrane permeable DNA dye can be added along with impermeable dye to confirm the test result. The first one, such as propidium iodide, stains dead bacterial cells and the latter one, such as SYTO 9, all bacterial cells (Invitrogen BacLight, 2004). Propidium iodide binds more strongly to DNA than SYTO 9. Thus, the staining of the cell sample yields dead cells stained with propidium iodide and viable cells stained only with SYTO 9. The dyes fluoresce with different colors and cells can be distinguished and counted with a fluorescence microscope or flow cytometer. The test can be performed also as a microtiter plate protocol (Invitrogen BacLight, 2004). The signals for both dyes measured from a well are related to the numbers of viable and dead cells. The microtiter plate protocol is faster, less expensive, and requires no costly instrument with experienced

users, compared to tests with fluorescence microscopy and flow cytometry. Another similar dead/viable dye pair is ethidium homodimer II/SYTO 10, suitable for animal cell viability (Invitrogen Reduced Biohazard, 2001). Propidium iodide/SYTO 16 pair differs from propidium iodide/SYTO 9 and ethidium homodimer II/SYTO 10, as SYTO 16 stains all mammalian cells (Frey, 1995; Luther & Karnentsky, 1996; MBL Live-Dead). Thus, the fluorescing color of dead cells is an overlay of both dyes.

The exclusion of an enzyme or cytoplasmic marker has also been related to cell viability. The protocol of an exclusion test for the DNA degrading enzyme (DNase) (Frankfurt, 1990) is not as simple as the dye exclusion tests. DNA in dead cells is degraded with a DNase treatment and DNase and degraded DNA are removed by centrifugation. DNA dye with a detergent is added to stain viable cells. O'Brien and Bolton have shown the use of labeled actin, cytokeratin, and tubulin as cytoplasmic markers and compared them to other dyes to find an optimal marker (O'Brien & Bolton, 1995).

Dye Exclusion Test with Automated Cell Counting

Automated hemocytometers (Invitrogen Countess 2, 2009; Nexcelom Cellometer) or other electronic instruments for the counting of cells (Szabo, 2003) may exploit dye exclusion to evaluate the percentage of viable cells. The counting of viable cell numbers is generally performed with Trypan Blue. Automated hemocytometers take a digital image and an image analysis program automatically determines the percentage of dead cells in a cell sample. Many methods, for example the Coulter counter, lack a viability measurement (Invitrogen Countess 2, 2009). For advantages and limitations read above.

Dye Inclusion Test with Flow Cytometry

Garner et al. (Garner et al., 1994) have developed DNA dye SYBR-14, which stains only viable cells. SYBR-14 can be used with propidium iodide to stain both viable and dead cells, as the ambiguity and unreliability, which could result from the use of only one dye, may be avoided. The staining mechanism of SYBR-14 is not known, but it has been shown to stain only living cells.

Determination of Physiological State with Fluorescent Probe by Using Flow Cytometry or Fluorescence Microscopy

Cell viability can be assessed with lipid-soluble probes, such as fluorescein diacetate (Coco-Martin et al., 1992), carboxyfluorescein diacetate, calcein acetoxy methyl ester, and rhodamine 123, determining the physiological state (Coder, 1997). Fluorescein diacetate, carboxyfluorescein diacetate, and calcein acetoxy methyl ester can state the viability among eukaryotic cells and tissues, but not microbes (Kaneshiro et al., 1993; Lau et al., 1994; Poole et al., 1993; Vaughan et al., 1995). Rhodamine 123 can assess bacterial (Diaper & Edwards, 1994; Diaper et al., 1992; Nebe-von Caron et al., 1994; Porter et al., 1995) and mammalian (Johnson, 1995) cell viability. These molecules are non-fluorescent and the fluorescence is enhanced inside a normal living and metabolically active cell with intact membranes. The physiological state of the cell indicates also the integrity of cell membranes, as energy is required to maintain ion gradients between cytoplasma and the surrounding medium, a property that is inhibited if the membrane is broken. Fluorescein diacetate, carboxyfluorescein diacetate, and calcein acetoxy methyl ester require cellular esterase activity, and rhodamine 123 the low electrochemical

potential of mitochondrial membranes. Viable cells are fluorescent and dead cells non-fluorescent. The performance of certain probes may be limited by prevailing conditions and individual cells that do not uptake or retain the probe (Shapiro, 1995). On the other hand, the dye can bind to cell membrane upon dying and the staining is retained. Probes are simply mixed with a sample, incubated a few minutes and viable and dead cells are counted with a flow cytometer. The use of the flow cytometer has the same drawbacks as the test above.

Probes determining physiological state can be visualized, not only with a flow cytometer, but also with a fluorescence microscope. The not so common dihydroethidium permeates only the viable cells, whose enzymes are needed to produce fluorescent ethidium monomer, which is retained in the nucleus (Bucana et al., 1986; Wyatt et al., 1991). Dihydroethidium has been suitable to test the viability of neoplastic cells (Bucana et al., 1986), intraerythrocytic protozoan hemoparasites (Wyatt et al., 1991), and sperm cells (Ericsson et al., 1989).

Combined Determination of Physiological State with Fluorescent Probe and Dye Exclusion Test by Using Flow Cytometry, Fluorescence Microscopy, or Microtiter Plate Reading

The determination of the physiological state with a fluorescent probe and dye exclusion tests can be combined together, provided there is a spectral separation of the dyes. The physiological state is determined with probes, such as calcein acetoxymethyl ester, AlamarBlue, or rhodamine 123, labeling viable cells. DNA dyes, such as propidium iodide, SYTOX Green, ethidium bromide, ethidium homodimer I, or ethidium homodimer III, are used to label dead cells (Coder, 1997; Invitrogen Viability/ Cytotoxicity, 2005). The dyes are mixed with a cell sample and incubated for dye uptake and cellular metabolism (Invitrogen Viability/Cytotoxicity, 2005). The tests in this category are not only suitable with a fluorescence microscope or flow cytometer, but also as a microtiter plate protocol (Invitrogen Viability/Cytotoxicity, 2005). The test with calcein acetoxy methyl ester and ethidium homodimer I, ethidium homodimer III, or propidium iodide is applicable to eukaryotic cells and tissues, but not to bacterial cells and yeast (Interchim Cell Biology; Kaneshiro et al., 1993; Lau et al., 1994; Papadopoulos et al., 1994; Poole et al., 1993; Vaughan et al., 1995). The cytotoxicity of compounds that do not affect intracellular esterase activity or the integrity of the plasma membrane is not reliably determined with these tests (Invitrogen Viability/Cytotoxicity, 2005). Alamar-Blue viability counting combined with the dye exclusion test with SYTOX Green is suitable for various yeast, bacterial, and eukaryotic cells (Invitrogen Vitality, 2004; Reinheimer & Demkow, 1990; Visser et al., 1990; White et al., 1996). The individual viable and dead cells are counted with a flow cytometer or the signals measured from a microtiter plate are related to the viable and dead cell numbers. The replacement of resazurin with lipophilic C_{12}-resazurin has enabled the efficient incorporation and retention inside the cell and provided a lower background signal or higher detection limit in the detection methods.

Combined Detection of Release of Cellular Compounds and Determination of Physiological State with Fluorescent Probe by Using Microtiter Plate Reading

Niles et al. (Niles et al., 2007; Promega MultiTox-Fluor, 2010) have developed a microtiter plate method, which detects the amounts of viable and dead cell proteases in

the same sample. A glycyl-phenylalanyl-aminofluorocoumarin substrate permeates the viable cell membrane. It is cleaved by a protease that remains active only inside viable cells, aminofluorocoumarin is released, and a fluorescence signal proportional to the viable cell number is generated. On the other hand, another substrate, bis-alanyl-alanyl-phenylalanyl-rhodamine 110, is cell-impermeable. It is cleaved by a different protease, which is released from dead cells with damaged membranes. The cleavage yields free fluorescent rhodamine 110, whose signal is proportional to the dead cell number. The excitation and emission spectra of aminofluorocoumarin and rhodamine 110 are separated to enable their simultaneous detection. The homogeneous method has one reagent addition and a simple mix-and-measure protocol, but the samples should be incubated at 37 °C for 30 min. It does not damage the cell sample and thus, can be further multiplexed with other methods. The method is also available with luminogenic substrate for the detection of dead cells (Promega MultiTox-Glo, 2009).

Determination of Proliferation with Fluorescent Probe by Using Microtiter Plate Reading or Flow Cytometry

The dye inclusion or exclusion test and determination of the physiological state with a fluorescent probe utilizing viability tests assess plasma membrane integrity and cellular metabolic activity. However, such criteria cannot certainly state that the cell can divide. Probes determining proliferation are required to test cell division. DNA synthesis is used as a measure of proliferation. It can be measured, for example, with DNA intercalator dyes or BrdU incorporation as a microtiter plate test, but the total or dead cell numbers should be counted simultaneously to calculate the percentage of dividing cells (Cell Viability, 2008). These methods also cannot differentiate between cells that have divided once or several times. On contrary, the carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) probe allows for the tracking of multiple cell divisions of individual cells. Intrinsically non-fluorescent CFDA-SE permeates inside the cells and is cleaved by intracellular esterases. The cleavage yields a strongly fluorescent amine-reactive product, which binds to intracellular amino groups and is retained inside cells during cell proliferation. Long-lived fluorescence enables cell activity studies by fluorescent microscopy or flow cytometry (Cell Viability, 2008; MoBiTec Viability/Cytotoxicity, 2007). Two daughter cells are identified from their mother cell by an approximately 50% lower fluorescence intensity. Again, new daughter cells have a 50% lower intensity than in the last generation. Fluorescence intensity stays detectable up to 6-10 cell divisions even during several weeks (Karrer et al., 1992; Weston & Parish, 1990).

3 AIMS

The aim of this study was to develop simple and sensitive methods for the measurement of total protein concentration and the counting of cells based on particle adsorption and luminescence detection. The developed methods were also applied to measure other sample properties, such as the aggregation of protein and cell viability.

The aims were more specifically to develop:

I a fast, inexpensive, and sensitive method for the quantification of proteins based on fluorescence quenching.

II a simple and sensitive method for the counting of total eukaryotic cell numbers based on fluorescence quenching and time-resolved luminescence resonance energy transfer (TR-LRET), and a viability test by combining the developed TR-LRET method for counting the total cell numbers with a known method for counting the dead cell numbers.

III a method to measure the aggregation of protein by applying the developed TR-LRET method for the quantification of proteins.

IV a method for the quantification of proteins having a high tolerance to contaminants and being based on the dissociation of chelated lanthanide.

4 MATERIALS AND METHODS

In this chapter, a short overview of the central reagents, experimental setups, and the principles of the developed methods are presented. More detailed information is described in Publications **I**-**IV**.

4.1 LABELING OF PROTEINS AND PARTICLES WITH LUMINESCENT DYES

Albumin from bovine serum (BSA) and lysozyme from chicken egg white (LYS) were conjugated with dipyrrylmethene-BF_2 530, succinimidyl ester (BF530) (Arctic Diagnostics Oy, Turku, Finland) (**I**,**II**,unpublished). γ-Globulins from bovine blood (γG) was conjugated with Alexa Fluor 680 carboxylic acid, succinimidyl ester (Alexa680) and Alexa Fluor 532 carboxylic acid, succinimidyl ester (Alexa532) (Molecular Probes, Eugene, OR) (**II**,**III**,unpublished). The proteins were labeled in 0.1-0.2 M carbonate buffer, pH 8.3 and separated from the unreacted dyes with gel filtration. Amino-modified polystyrene particles 240 nm in diameter (Spherotech Inc., Libertyville, IL) were labeled with 7-dentate Eu(III) chelate, 2,2’,2’’,2’’’-{[4-[(4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl]bis(methylenenitrilo)}tetrakis(acetato) europium(III) and 9-dentate Eu(III) chelate, {2,2’,2’’,2’’’-{[4’-(4’’’-isothiocyanatophenyl)-2,2’:6,6’’-terpyridine-6,6’’-diyl] bis(methylenenitrilo)}tetrakis(acetato)} europium(III) (**IV**) in 30 mM carbonate buffer, pH 9.9. After overnight incubation, the particles were washed by centrifugation.

4.2 PRINCIPLES AND PROCEDURES OF DEVELOPED METHODS

All of the developed methods presented in this thesis and in Publications **I**-**IV**, are based on the nonspecific interaction of the sample with the nanoparticle surface. The interaction of the sample is detected as the changes in luminescence or LRET signals and the measured signal is related to the sample concentration. Quenching- and TR-LRET-based methods presented in **I**-**III** are reagent limited and the method in **IV** utilizing the instability of the Eu(III) chelate is a reagent excess method.

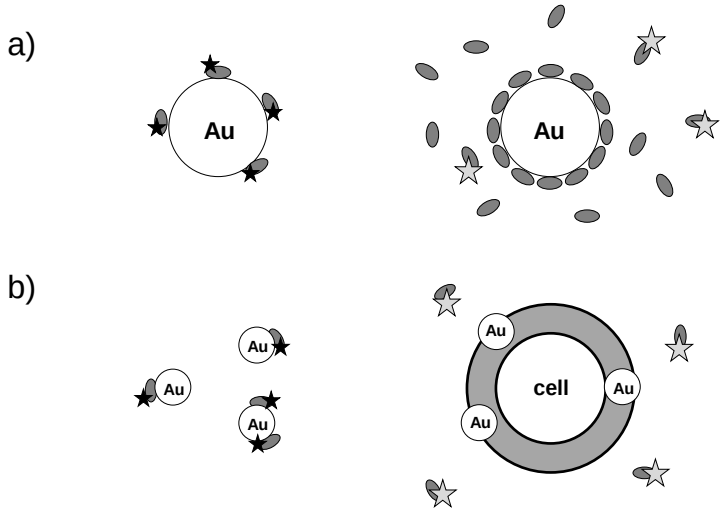
The developed methods are homogeneous and simple to perform. The methods do not require either heating steps nor an incubation time of longer than 40 min. The sample concentration or the degree of aggregation is quantified with quenching and TR-LRET particle assays using a simple mix-and-measure protocol containing the mixing of particles and labeled protein with the sample in Publications **I**-**III**. In the quantification of proteins with a particle assay based on the dissociation of Eu(III) chelate in Publication **IV**, the sample protein is adsorbed at pH 5 to enable an adequate stability for the Eu(III) chelate and high adsorption efficiency, and thereafter, the pH is decreased for the dissociation of the lanthanide ion.

4.2.1 Quantification of Proteins and Counting of Cells with Quenching and TR-LRET Nanoparticle Assays (I-III,unpublished) and Viability Test (II)

Principles

The assays in Publications **I**-**III** and unpublished data rely on the interaction of sample proteins or cells with quencher or donor nanoparticles, and the subsequent reduction of the adsorption of the labeled protein to the particle surface (Figures 24 and 25). In the

quenching assay, the fluorescence of BSA-BF530 is quenched at close proximity to the colloidal gold nanoparticle (20 nm in diameter, British Biocell International, Cardiff, U.K.) surface. The interaction of the sample with the particles reduces the adsorption of the labeled protein and the fluorescence increases. In the TR-LRET assay, the TR-LRET is detected between Eu(III) nanoparticles (Carboxylate-modified europium(III) polystyrene particles 68 or 92 nm in diameter, Seradyn Inc., Indianapolis, IN) and γG-Alexa680 in the absence of the sample. In the presence of the sample, the energy transfer signal is decreased, as the sample occupies the particle surface and prevents the adsorption of γG-Alexa680.



Analyte absent	Analyte present
low signal	high signal

Figure 24. Schematic illustration of the quenching assays (**I**,**II**). Fluorescence quenching is detected by the adsorption of labeled protein onto gold (Au) nanoparticles in the absence of the sample a) protein or b) cells. c) As the analyte interacts with the particle surface, the labeled protein remains in solution and an increase of fluorescence is detected.

Procedures

The methods for the quantification of total protein and the counting of eukaryotic cells with quenching and TR-LRET particle assays were performed in microtiter wells (**I**,**II**). Typically, 70 µL of the sample in buffer was mixed with 10-20 µL of nanoparticles (colloidal gold 20 nm in diameter for quenching and Eu(III) polystyrene particles 68 or 92 nm in diameter for TR-LRET). Labeled protein (BSA-BF530 for quenching and γG-Alexa680 for TR-LRET) was added in 10-20 µL after an incubation of 10 min. Fluorescence emission intensities were measured after an incubation of 10 min. The optimized assay buffers were 5 mM glycine buffer pH 3.0 for the quantification of

proteins with both assays and for the counting of cells with a quenching assay and PBS for the counting of cells with a TR-LRET assay. The final concentrations of particles and labeled protein were optimized for each assay. The number of gold particles in the assay was calculated from the gold chloride concentration and the number of Eu(III) polystyrene particles from the mass percentage of the particles in the stock solution provided by the manufacturers. Depending on the assay, incubation times, the effect of pH and salt concentration, different proteins, particle size, stability of the reagents, different cell types, cell harvesting method, and/or loss of the particles to the wells were tested or optimized.

The total cell count assay based on TR-LRET detection was combined with a known dead cell count assay with cell-impermeable PicoGreen nucleic acid dye to develop a viability test (**II**). The assay was performed in a similar fashion as above and the PicoGreen dsDNA reagent (Molecular Probes, Eugene, OR) was mixed with the sample before the addition of the particles and the labeled protein.

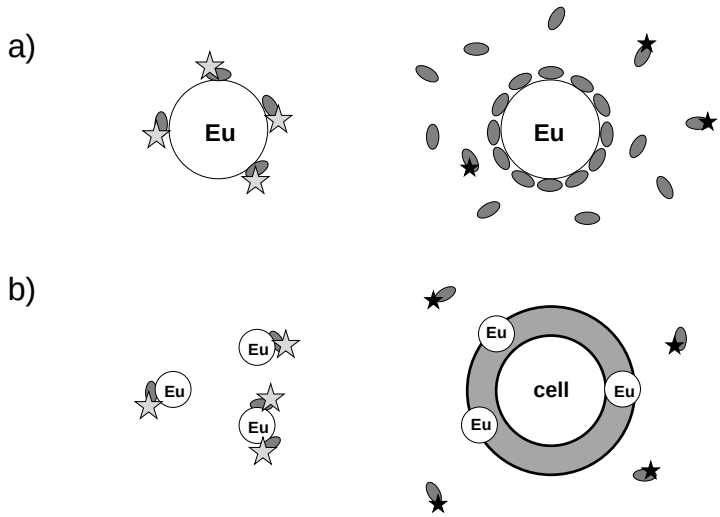


Figure 25. Schematic illustration of the time-resolved luminescence resonance energy transfer (TR-LRET) assays (**II**,unpublished). Luminescence energy transfer is detected by the adsorption of labeled protein onto Eu(III) carboxylate modified polystyrene nanoparticles in the absence of the sample a) protein or b) cells. c) As the analyte interacts with the particle surface, the labeled protein remains in solution and a decrease of luminescence is detected.

4.2.2 Detection of Aggregation of Protein (III)

Principle

The detection of the aggregation of protein was demonstrated with the developed TR-LRET system and heat-aggregated BSA and the developed method is presented in the article **III**. The aggregation is measured at constant BSA concentration. The non-aggregated BSA covers the Eu(III) particles of 92 nm in diameter efficiently and maximal surface coverage is observed. The aggregation of BSA decreases the number of soluble proteins in a solution and the aggregated BSA occupies the particle surface less efficiently. The aggregated BSA leaves a more vacant particle surface available for γG-Alexa680 to adsorb and thus, results in a high TR-LRET signal.

Procedure

The detection of the aggregation of protein was demonstrated with a heat-aggregated BSA and TR-LRET particle assay (**III**). BSA aggregates in 5 mM glycine buffer pH 3.0 were mixed with Eu(III) polystyrene particles 92 nm in diameter in a microtiter well and γG-Alexa680 was added. After mixing, luminescence emission intensity was measured.

4.2.3 Quantification of Proteins and Counting of Cells with Microparticle Assay Exploiting Two-Photon Excitation (unpublished)

Principles

The assays for the quantification of proteins and the counting of cells exploiting a two-photon excitation (TPX) are based on the same basic principle of adsorption competition between sample protein or cells and the labeled protein to the particle surface than the quenching and TR-LRET assays. However, the microparticles (Carboxylate-modified polystyrene particles 3098 nm in diameter, Seradyn Inc., Indianapolis, IN) contain no dye molecules. In the absence of analyte protein or cells, labeled protein adsorbs efficiently to the surface of microparticles (Figure 26). In the presence of analyte, the interaction of the analyte with the particles reduces the adsorption of labeled protein. When no competing sample is present, high two-photon excitation fluorescence is detected from the microparticles due to the adsorbed labeled protein. Sample protein or cells efficiently occupy the particle surface preventing the adsorption of labeled protein to the particles. Thus, labeled protein remains in a solution and the two-photon excitation fluorescence signal is decreased.

Procedures

The methods for the quantification of total protein and the counting of eukaryotic cells with TPX particle assays were performed in microcentrifuge tubes. Typically, 40-80 µL of the sample in buffer was mixed with 7.0-10 µL of polystyrene microparticles 3 µm in diameter. Labeled protein (LYS-BF530 for the quantification of proteins and γG-Alexa532 for the counting of cells) was added in 7.0-10 µL after an incubation of 40 min. 20 µL of this mixture was transferred to the polystyrene microtitration wells with transparent bottoms (384-well plates) and two-photon excitation fluorescence was measured. The optimized assay buffers were 5 mM glycine buffer, pH 2.0, containing 1 mM Triton X-100 for the quantification of proteins and 7 mM tris(hydroxymethyl)-

aminomethane buffer (Tris), pH 6.5, containing 0.7 M NaCl for the counting of cells. The final concentrations of the particles and labeled protein were optimized. The number of polystyrene microparticles in the assay was calculated from the mass percentage of the particles in the stock solution provided by the manufacturer. Depending on the assay, incubation time, assay buffers, and the effect of pH and salt concentration, different sample and labeled proteins, and effects of contaminants were tested or optimized.

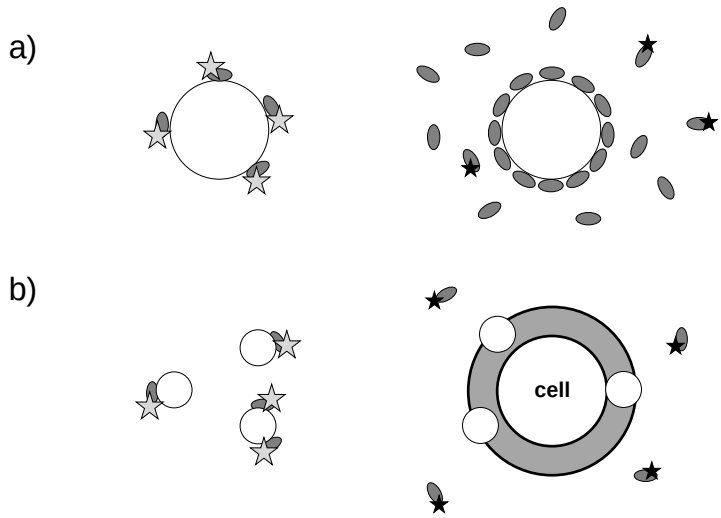


Figure 26. Schematic illustration of the two-photon excitation fluorescence (TPX) assays (unpublished). Two-photon excitation fluorescence is detected by the adsorption of a labeled protein onto carboxylate modified polystyrene microparticles in the absence of the sample a) protein or b) cells. c) As the analyte interacts with the particle surface, the labeled protein remains in the solution and a decrease of fluorescence is detected.

4.2.4 Quantification of Proteins with Nanoparticle Assay Based on Dissociation of Chelated Eu^{3+} (IV)

Principle

Another improved nanoparticle-based method was developed for the quantification of proteins and reported in Publication **IV**. Like in the quenching and LRET methods, the dissociation method is based on the adsorption of the sample protein onto particles. The sample protein is adsorbed to amino-modified polystyrene particles surface-labeled with Eu(III) chelates on the surface (Figure 27). The adsorbed protein protects the Eu^{3+} ion from dissociation at acidic conditions and a high luminescence signal is detected. Whereas, the Eu^{3+} ion is dissociated in the absence of protein and the luminescence is low.

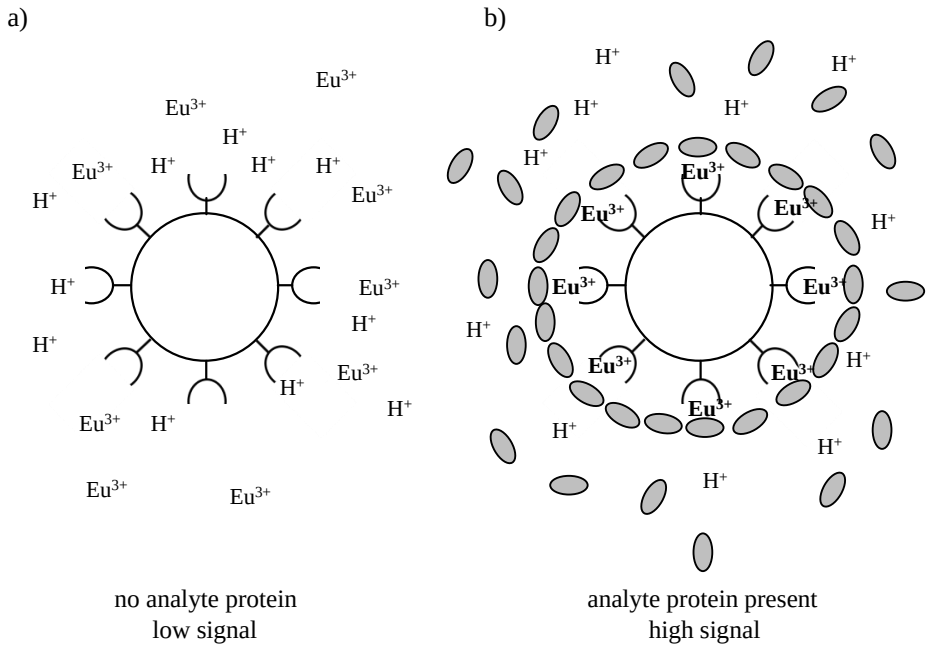


Figure 27. Schematic illustration of the assay utilizing the dissociation of the Eu^{3+} ion (**IV**). a) Under acidic pH, the Eu^{3+} ion is dissociated from the Eu(III) chelate labeled nanoparticles in the absence of sample protein and a low luminescence signal is detected. b) In the presence of sample protein, the dissociation of the Eu(III) chelate is prevented by the adsorbed protein layer and a high luminescence signal is detected.

Procedure

The reagent excess method for the quantification of total protein utilizing the dissociation of Eu(III) chelate was performed in microtiter wells (**IV**). Typically, 70 µL of the sample protein in 3 mM acetate buffer pH 5.0 was mixed with 5.0 µL of the amino-modified particles (240 nm in diameter) labeled with 9-dentate europium(III) chelate. 10 µL of 0.5 M glycine buffer pH 2.0 was added. Luminescence emission intensity was measured after an incubation of 30 min. The method was tested or optimized for particle concentration, incubation times, assay buffers and the effect of pH and salt concentration, Eu(III) chelate type and labeling degree, temperature, different proteins, and the effects of contaminants. The number of Eu(III) chelate labeled nanoparticles in the assay was calculated from the mass percentage of the amino-modified polystyrene particles in the stock solution provided by the manufacturer.

4.3 Instruments and Measurement of Luminescence

The luminescence emission intensities from Eu(III) chelate labeled nanoparticles and the sensitized emission of the fluorescent dye-labeled proteins were measured with TRL, and the fluorescence emission intensities of the fluorescent dyes or labeled proteins with conventional fluorometry using the Victor^2 multilabel counter (Wallac, Perkin Elmer Life and Analytical Sciences, Turku, Finland). The excitation and emission wavelengths of the labels and the delay and measurement times of TRL are listed in Table 4.

Table 4. The excitation and emission wavelengths of the labels and the delay and integration times of the time-resolved luminescence measurement.

Dye	Literature values		Measurement values		Delay time (μs)	Integration time (μs)	Publications
	Absorbance maximum (nm)	Emission maximum (nm)	Excitation wavelength (nm)	Emission wavelength (nm)			
BF530	530[1]	552[1]	530	572			**I**,**II**
Alexa Fluor 680	679[2]	702[2]	340[a]	730	75	50	**II**,**III**
PicoGreen	502[3]	523[3]	485	535			**II**
9-dentate Eu(III) chelate	294[4]	613[4,b]	340	615	400	400	**IV**

[1] ArcDia BF
[2] Invitrogen Alexa Fluor, 2009
[3] Invitrogen PicoGreen, 2003
[4] Hemmilä et al., 1993

[a] excitation wavelength of europium(III) chelate
[b] main peak

The two-photon excitation fluorescence from the surface of individual polymer microspheres (BF530 and Alexa532 dyes) was measured with a TPX Plate Reader (ArcDia International Oy Ltd, Turku, Finland).

5 RESULTS AND DISCUSSION

Summary of the results and discussions of the original publications and unpublished data are presented in this chapter.

5.1 METHODS FOR QUANTIFICATION OF TOTAL PROTEIN (I,III,IV,UNPUBLISHED)

During this thesis work, four different particle assays to measure protein concentration were studied: quenching, time-resolved luminescence resonance energy transfer (TR-LRET), two-photon excitation (TPX), and dissociation-based assays. Quenching, TR-LRET, and TPX assays rely on the adsorption competition between sample protein and labeled protein to the nano- or microparticles (Figures 24-26). The sample adsorption leads to a luminescence signal change and the magnitude of the signal depends on the protein concentration in the sample. Furthermore, depending on the principle of the method, the signal increases or decreases as a function of protein concentration. The method exploiting the instability of the luminescent dye is a reagent excess assay. The adsorbed sample protein prevents the dissociation of the Eu^{3+} ion from the Eu(III) chelate surface-labeled particles at a low pH and leads to a high luminescence signal (Figure 27). In the absence of sample protein, the dissociation at a low pH decreases the luminescence signal.

The primary aim was to develop sensitive methods for the quantification of proteins. High sensitivity for the adsorption-based methods requires efficient surface coverage of a particle with protein. Thus, the adsorption and assay conditions were optimized for each method. When the effect of the pH was studied with bovine serum albumin (BSA), a low pH was found optimal and resulted in the highest signal-to-background ratio with all the investigated particles. The result is explained with the electrostatic interactions between the protein and particle and/or between adsorbed protein molecules for all competitive methods. Citrate (pK_a values: 3.2, 4.8, and 6.4) stabilized gold particles were used in the quenching assay, carboxylate-modified Eu(III) polystyrene ($pK_a(-SO_4H)$ −3, $pK_a(-SO_3H)$ 1.9, and $pK_a(-COOH)$ 4.7) particles in the TR-LRET assay, and carboxylate-modified polystyrene particles in the TPX assay. All particles bear a negative surface charge, which approaches zero at a low pH range, depending on the charged chemical groups on the particles. The isoelectric point determines the overall charge of the protein. Generally, the total charge of protein molecules is positive at a low pH, the repulsive interactions are reduced and the interaction with the particles is enabled. The highest signal-to-background ratio with competitive methods was reached at pH 2-3. The Eu(III) chelate instability at a low pH reduced the signal-to-background ratio in the dissociation exploiting reagent excess assay. Optimal pH 5 tested with three proteins: BSA (pI 4.7) (Peng et al., 2004), γ-globulin (γG, pI 6.4-8.8) (Condie, 1981), and histone (pI 10.8) (Hayashida & Uchiyama, 2006) was higher compared to the competitive methods. All three proteins adsorbed at a pH below their pI. Proteins are positively charged below their pI and thus, they have electrostatic attraction towards the Eu(III) chelate labeled amino-modified polystyrene particles bearing a negative total charge.

Ions shelter the protein with a cloud of charges decreasing both repulsive and attractive electrostatic interactions between the particle and proteins. This depends on the surface charges of the proteins and particles. The highest sensitivity was reached at a relatively low buffer concentration for the quenching method. However, the detection limit of the

dissociation method was not significantly affected by the increased salt concentration up to 200 mM. In competitive methods, the number of particles also affected the sensitivity. A decreasing number of particles enables high sensitivity as long as the assay variation is low at a low signal level. In these assays, the sample protein was first adsorbed to the particles, after which the labeled protein was added. This resulted in a higher sensitivity compared to the simultaneous adsorption of sample and labeled protein to the particle. Thus, the concentration of labeled protein was observed to have an insignificant effect on the sensitivity (except in the quenching method). In contrast, the dissociation method is a reagent excess method and theoretically, the number of particles (as long as the assay variation does not change) is not expected to affect the sensitivity. However, at a high particle concentration the signal at zero BSA concentration increased and sensitivity decreased.

The characteristics of the developed methods are summarized in Table 5. The methods have several advantages compared to the existing methods. All developed methods have two reagent additions. The incubation times (10-40 min) are relatively short compared to the commercially available methods. None of the developed methods require heating to reach high sensitivity and proteins are quantified at RT. The effect of temperature was tested for the dissociation method by adsorbing BSA to the particles at varying temperatures, ranging from room temperature (RT) to 100 °C. Elevated temperature was not found to improve the sensitivity. Sensitivities deduced from the calibration curve data for the four different methods presented in Figure 28, range from 40 to 500 pg BSA in a sample and are approximately 20-500-fold better than in most sensitive commercial OPA (Pierce Fluoraldehyde OPA, 2009), CBQCA (You et al., 1997), and NanoOrange (Jones et al., 2003) methods. On the other hand, the detection limits were 300-4000 times lower than for the well-known and commercial Bradford method (Pierce Protein Handbook, 2009). The coefficients of variation (CV) are low (4-13%) for all assays.

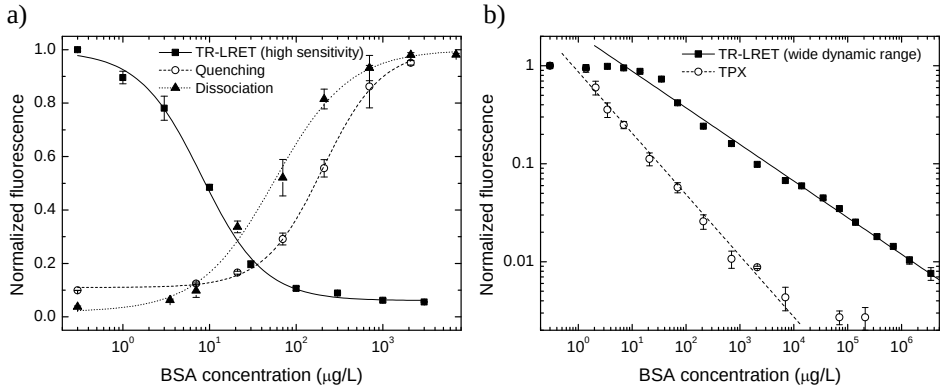


Figure 28. Bovine serum albumin (BSA) calibration curves measured with different particle assays: a) TR-LRET (high sensitivity protocol, particles 92 nm in diameter), quenching, and dissociation (Eu(III) chelate instability) and b) TR-LRET (wide dynamic range protocol, particles 68 nm in diameter) and TPX (**I**,**III**,**IV**,unpublished). The data was fitted to the logistic (TR-LRET high sensitivity) or modified Hill (quenching and dissociation) function (Seber & Wild, 1989) or the curves are linear fits of the data (TR-LRET wide dynamic range and TPX).

Table 5. Characteristics of the developed methods for the quantification of proteins.

	Assay				
	Quenching	TR-LRET (high sens.)	TR-LRET (wide range)	TPX	Dissociation
1. reagent Particle	20 nm Au	92 nm Eu(III) CM-PS	68 nm Eu(III) CM-PS	3098 nm PS	240 nm Eu(III) AM-PS
2. reagent	BSA-BF530	γG-Alexa680	γG-Alexa680	LYS-BF530	0.50 M glycine pH 2.0
Assay format	Homogeneous Reagent limited	Homogeneous Reagent limited	Homogeneous Reagent limited	Homogeneous Reagent limited	Homogeneous Reagent excess
Incubation time (min)	10	20[1]	20[1]	40	30
Dynamic range (mg/L)	0.007-2	0.001-1	0.008-4000	0.002-9	0.002-0.6
Sensitivity (pg)	500	70	600	40	100
Sample volume (µL)	70	70	70	20	70
CV (%)	4	4	4	13	8
Plate	96-well	96-well	96-well	384-well	96-well
Protein-to-protein variability (%)	15	n.d.	n.d.	n.d.	29
Tolerated chemicals	n.d.	n.d.	n.d.	non-ionic detergents, dithiothreitol, salt	non-ionic detergents, solvents, thiols, polyethylene glycol, salt
Publications	**I**	**III**	unpublished	unpublished	**IV**

[1] Valanne et al., 2009

abbreviations: CM = carboxylate-modified n.d. = not determined
AM = amino-modified CV = coefficient of variation PS = polystyrene

For the developed particle-based assays, a high signal at zero protein concentration and a low signal at high protein concentration for a decreasing curve (or vice versa for an increasing curve) enable a high signal-to-background ratio. The advantage of the high signal-to-background ratio can be the wide dynamic range. As an example, the signal-to-background ratio of 100 may lead to a wider dynamic range than the ratio of 10, if the slope of the calibration curve does not change. The TPX assay with microparticles enabled a high signal at zero BSA concentration. Due to the low background signal, the signal-to-background ratio was high and the dynamic range of the assay was approximately four orders of magnitude (Figure 28b and Table 5). With a high concentration of particles and labeled protein (wide dynamic range protocol) in the TR-LRET assay, a high LRET signal at zero BSA concentration was obtained. Smaller particles were used to decrease background luminescence at a high BSA concentration originating from the Eu(III) donors. The thus obtained high signal-to-background ratio led to a widened dynamic range of approximately six orders of magnitude (Figure 28b and Table 5). The sensitivity of the assay depends on the number of particles and thus, the sensitivity was decreased from that of the high sensitivity protocol for the wide dynamic range protocol, but was still improved compared to the most sensitive commercial methods (Jones et al., 2003; Pierce Fluoraldehyde OPA, 2009; You et al., 1997). The extra dilution step or remeasurements are avoided, when the dynamic range is wide. The

logarithm of the signal vs. the logarithm of the BSA concentration for both the TPX and TR-LRET (wide dynamic range protocol) assays are linear. Linearity reduces the number of measured standards to determine the concentration of the sample.

The effect of the particle size was tested in the quenching and TR-LRET assays. With an optimized number of gold particles (approximately the same total surface area) in the quenching assay, the sensitivity and signal response did not vary with the particle size of 20-100 nm in diameter. The least amount of gold (mass) was required in the assay with 20 nm particles and it was applied in the further development of the assay. With an optimized number of Eu(III) particles (approximately the same total surface area) in the TR-LRET assay, the sensitivity and the LRET signal at zero BSA concentration was not affected by the size of the particles. However, the smaller the particles the higher the signal-to-background ratio and the larger the dynamic range were obtained.

The stability of the signal and the labeled BSA was tested in the quenching assay. After the addition of labeled BSA, an equilibrium was reached in 10 min. After the signal leveled out, the fluorescence was essentially unchanged over 5 h. Thus, the protein adsorbed irreversibly and the adsorbed sample protein was not exchanged for the labeled protein. The diluted labeled BSA was stored at a concentration corresponding to the end-user storage condition. Only a minor decrease in the fluorescence signal was detected at a low salt concentration and pH 7.4, and the calibration curve of the assay remained unchanged over eight days. This is an advantage compared to the assays, whose reagent stabilities are limited by storage time and/or conditions (Jones et al., 2003; Lowry et al., 1951; You et al., 1997). According to the manufacturer, the gold colloid suspension is stable for at least one year and therefore, its stability was not studied.

It is desirable that the assay response is equal to all the proteins and proteins can be quantified using, for instance, BSA as a standard to measure unknown proteins or protein mixtures. The protein-to-protein variabilities of the new assays are investigated by running the calibration curves of several proteins. However, it is worthful noting that, even if the methods are tested for several different proteins and variability is low, similar sensitivity for all unknown proteins cannot be granted. The response of the quenching and dissociation assay was studied for different proteins. The tested proteins had different properties: a molecular weight from 14 to 670 kDa, an isoelectric point from 1.0 to 11, and varying dimensions and shapes. According to the literature, the adsorption efficiency to the negatively charged particles, such as citrate-stabilized gold and amino-modified polystyrene particles, is favoured at the isoelectric point of the protein or below (Duinhoven et al., 1995a; Duinhoven et al., 1995b; Geoghegan & Ackerman, 1977; Goodman et al., 1979; Norde & Lyklema, 1978a; Norde & Lyklema, 1978b; Quiquampoix & Ratcliffe, 1992). However, an acidic protein pepsin having pI 1.0 (Chern et al., 2004) was assayed with the quenching assay exploiting gold particles with equal efficacy compared to the other tested proteins. The quenching assay had a low protein-to-protein-variability of 15%. Only ferritin showed a lower response than other proteins, probably due to its unusually high thermal and chemical stability (De Domenico et al., 2006; Martsev et al., 1998; Santambrogio et al., 1992) decreasing its denaturation at the surface and the surface coverage. In the dissociation-based assay, the adsorption of proteins was maximal at a pH range below their pI, but a pH below 3 cannot be exploited due to chelate instability. Thus, all the tested proteins (pI above 5) exhibited maximal adsorption at the assay pH 5.0. However, the response of the assay was not similar to all proteins. Myoglobin and lysozyme resulted in the lowest response. Myoglobin contains

heme groups with Fe^{2+} ions (Ordway & Garry, 2004), which probably quenched the fluorescence signal (Albani, 2004; Si et al., 1997). On the other hand, lysozyme can form several molecular layers (Lee et al., 2005; Lundin et al., 2010; Nakanishi et al., 2001), which may lead to the low surface coverage. The protein-to-protein variability of 29% for the dissociation method was still comparable to the commercially available methods with variabilities of 10-50% (Jones et al., 2003; Pierce Protein Handbook, 2009; Smith et al., 1985; You et al., 1997).

The performance of the TPX and dissociation methods were examined in the presence of various contaminants commonly encountered in biological samples. The interference of contaminants was not extensively tested for the TR-LRET and quenching methods, but the sensitive methods for the quantification of detergents developed by us (Härmä et al., 2010a; Härmä et al., 2010b) suggest that they are highly sensitive to detergents. Already a small detergent molecule decreases the amount of adsorbed labeled protein and increases the distance between the particle and label, decreasing the LRET signal or inhibiting the fluorescence quenching. However, several different buffer components at a pH range of 1.5-4.5 were tested in the quenching assay and nearly identical signals were obtained, suggesting that the buffer components had an insignificant effect on the adsorption of proteins. The distance between particle and labeled protein is not critical for detecting a high TPX signal. Although non-ionic detergents at a concentration above their critical micelle concentration (CMC) decrease the adsorbed labeled protein mass, the TPX signal is still high. Actually, the non-ionic detergents had a positive effect on the sensitivity of the BSA quantification. The protein covered with the micelle occupies a larger area on the particle surface compared to the bare protein molecule. The BSA standard curve was shifted to approximately tenfold lower concentrations in the presence of Triton X-100 at concentrations above CMC, compared to the curve measured without detergent. Other non-ionic detergents, salt, and dithiothreitol, an agent containing thiol, did not overly interfere with the TPX method either. However, the ionic detergents interfered with the assay and prevented the adsorption of the labeled protein and decreased the TPX signal already at concentrations much lower than CMC. In the dissociation method, the quantification of proteins is not based on the adsorption competition. Due to the different detection principle, the signal generation was not as susceptible to the contaminants. The non-ionic detergents did not protect the Eu(III) chelate labeled particles as efficiently as proteins or detergent-coated proteins and non-ionic detergents were tolerated at high concentration levels above their CMC. Especially non-ionic detergents are suitable in the extraction of proteins from cell preparations near their CMC, and are thus found in the biological samples. Instead, ionic detergents prevented the adsorption of proteins already at a low concentration. However, ionic detergents, as they denature proteins and interfere with the purification protocols, are not typically found in biological samples. Furthermore, chelating agent ethylenediaminetetraacetic acid (EDTA), common organic solvents (dimethylformamide (DMF) and ethanol), polymer polyethylene glycol (PEG 3000), 2-mercaptoethanol, agent containing thiol, and reducing agent/sugar glucose interfered only at high concentrations in the dissociation method. Obviously, the suitability of probable interfering agents not investigated in this work cannot be warranted.

5.2 Methods for Counting of Total Cell Numbers (II,unpublished)

During this thesis work, three different particle assays for the counting of eukaryotic cells were studied. Quenching, time-resolved luminescence resonance energy transfer (TR-LRET), and two-photon excitation (TPX) assays rely on the prevention of the adsorption

of the labeled protein to the nano- or microparticles by the sample cells (Figures 24-26). The presence of cells in the sample leads to a change in the luminescence signal, depending on the cell number similarly to the corresponding methods for the quantification of proteins.

The sensitivity and simplicity were the main aims in the development process of the methods for the counting of cells. As eukaryotic cell surfaces contain charged proteins and lipids and their membrane integrity depends on the solution conditions, pH and salt concentrations were important parameters to investigate. The methods were optimized with Chinese Hamster Ovarian (CHO) cells harvested by scraping.

The adsorption of the BF530 labeled BSA (pI 4.7) (Peng et al., 2004) determines the pH for the quenching method. As measured for the quantification of proteins, the signal-to-background ratio was the highest at a low pH. The low adsorption of the labeled protein at a higher pH range prevented the experimental testing of the pH effect on the adsorption of cells. Thus, the calibration curve was measured and the highest sensitivity was reached at the same low buffer concentration and pH than for the quantification of proteins. Labeled γG (pI ranging from 6.4 to 8.8 (Condie, 1981)) was chosen for the TR-LRET method, because an efficient adsorption was achieved at a neutral pH in addition to the low pH. The performance of the TR-LRET method was tested with two buffers: a 5 mM glycine buffer, pH 3.0 and PBS. The sensitivity was higher, when the calibration curve was measured in PBS than in the glycine buffer. Therefore, PBS was chosen as a buffer also, because cells are generally harvested from the culture to physiological pH and salt concentrations using PBS. The sensitivity is measurable in the sample without the need for the dilution of the sample, as the assay buffer is the same as the harvesting buffer. For the TPX method, labeled γG was adsorbed to the particles at a low pH and neutral pH to obtain a high TPX signal. Similar to the TR-LRET method, the optimal interaction between the cells and the particle and the highest sensitivity was reached near pH 7. Increasing NaCl concentration improved the sensitivity up to the concentration of 1 M. In addition, the signal response was equal in four different buffers tested: 5 mM universal, 7 mM phosphate, 7 mM Tris, and 7 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid all of them containing 0.3 M NaCl.

The diameter of a CHO cell is 15 µm (Wei et al., 2006). The quencher gold (20 nm in diameter) and donor Eu(III) nanoparticles (68 nm in diameter) are much smaller in size, and a number of nanoparticles can interact with a cell. In fact, the cell number leading to the saturation of the fluorescence signal corresponded to the calculated number of intact cells required to adsorb the particles on the cell surface for both nanoparticle methods. This suggests that the adsorption process is related to the particle−cell interaction and the total surface area of the particles covered with cells seems to relate to the intact whole cells. However, the microparticles (3 µm) in TPX are in the same size range with the cells and the interaction may have a different origin. On the other hand, the optimal assay conditions, acidic pH for the quenching method (exploitable also for TR-LRET) and high salt concentration (hypertonic solution) for the TPX method, may lead to a compromised cell membrane and the release of the cellular contents. Therefore, the particles may interact with the components of the lysed cells complicating the interpretation of the results and the principle. Nevertheless, cells inhibited the adsorption of the labeled protein and a high sensitivity was reached regardless of the particle size. To verify whether the methods detect viable, dead, or all cells, the calibration curve was measured with the TR-LRET method for the cells initially suspended in water, and was compared to the curve

measured for the cells initially suspended in PBS. The calibration curves measured in PBS overlapped, suggesting that both dead and viable cells give a similar response and the total number of cells is counted.

The concentration of particles and labeled protein was optimized for each method. The concentration of particles affected the sensitivity and was reasonably minimized in all the methods. The concentration of labeled protein could not be varied without the increase of the fluorescence signal at zero cell concentration or the decrease at high cell concentration in the quenching method. Thus, the concentration of labeled protein was optimized according to the number of particles, so that labeled protein adsorbed efficiently and the fluorescence of the label optimally quenched at a zero cell number. The increase of the labeled protein concentration had a positive effect on the cell calibration curves measured with the TR-LRET and TPX methods, as the luminescence at a zero cell concentration and signal-to-background ratio increased, but no effect was detected on the shape of the calibration curves at low cell numbers. Instead, the signals at high cell concentrations increased and the dynamic range widened, as the concentration of labeled protein increased. In the TPX method, three different labeled proteins, LYS-BF530, γG-Alexa532, and avidin-BF530, were studied and the highest sensitivity was obtained with γG-Alexa532. Probably, the different results between proteins are related to their adsorption efficiency and the cells compete in the interaction with the particles better with γG than with LYS or avidin.

The counting of two different eukaryotic cell types (Chinese Hamster Ovarian (CHO) and Human Embryonic Kidney (HEK) cells harvested by scraping) was performed with the quenching and TR-LRET methods. The calibration curves overlapped with both methods. Each developed particle-based method was tested for the detection of bacterial cells. Although the assay conditions, temperature, salt concentration, pH, incubation time, bacterial strain, surface of the particle, bacterial culture conditions, and growing state were varied or the cells were lysed, the sensitivity of the methods was not higher than a few tens of thousands of bacteria in a sample. These results suggest that the adsorption properties of the bacterial surface and cellular contents differ significantly from those in eukaryotic cells. This provides a clear advantage for the developed method for the counting of eukaryotic cells, since the bacterial contamination in the sample does not interfere. However, the bacterial contamination may be a significant problem with methods, such as the detection of DNA or ATP. The effect of the harvesting method was tested with the quenching and TR-LRET methods. The calibration curves for cells harvested by either scraping or trypsinazing overlapped. Thus, it seems that the interaction of the particles and cells is not dominated by the cell surface proteins. Although the developed cell counting assays were investigated for two eukaryotic cells with different harvesting methods in the optimized assay buffer, it is possible that other cells or media gives unpredictable results.

The characteristics of the developed methods are summarized in Table 6. The CHO cell calibration curves in Figure 29 were measured in optimized buffers: the 5 mM glycine buffer, pH 3.0 for the quenching method, PBS for the TR-LRET method, and the 7 mM Tris buffer, pH 6.5, containing 0.7 M NaCl for the TPX method. The methods have several advantages compared to the existing methods. The sensitivity of the methods was nearly at the single cell level: four cells for the quenching method, five cells for the TR-LRET method, and three cells for the TPX method. These sensitivities are comparable to the method based on the detection of ATP. Among commercially available methods, it is

the only assay for the counting of cells capable of detecting less than 10 cells, and even single cells in a sample (Campbell, 1988). The CASY cell counter (Roche Innovatis CASY TT, 2010; Roche Innovatis CASY TTC, 2010; Schärfe, 2004) has a detection limit of ten cells, but the counting requires a special instrument. Although standard plate count (Bacterial Enumeration) theoretically detects one viable cell in a sample, it is impractical and only applicable for bacteria. A luminometric assay based on the measurement of the glyceraldehyde-3-phosphate dehydrogenase activity (Corey et al., 1997) is able to detect less than a single cell, but is not commercially available as a kit. The achieved sensitivities correspond to the cell concentrations of 60 cells per mL for the quenching method, 70 cells per mL for the TR-LRET method, and 200 cells per mL for the TPX method. The dynamic range is the widest and approximately two orders of magnitude for the quenching and TPX methods, but only one order of magnitude for the TR-LRET method. Many of the existing methods presented in Table 3 (Section 2.6.3) have comparable dynamic ranges to those of the developed methods.

The developed particle-based methods have simple experimental setups: no washing steps and equipment are required for these homogeneous methods, luminescence is measured with microtiter plate reader commonly found (except for the TPX method) in biological laboratories, the methods have two reagent additions, and they do not need elevated temperature and incubation in the cell culture conditions. Many of the available assays listed in Table 3 (Section 2.6.3) require an incubation step at 37 °C. Most of the assays have one or two reagent additions, but for example BrdU (Hawker, 2003; Maghni et al., 1999; Muir et al., 1990) requires even six addition steps. The incubation time is 10 min for the quenching and TR-LRET methods and 40 min for the TPX method. The existing methods generally have incubation times significantly longer than 40 min and only a few of the methods listed in Table 3 need an incubation time shorter than 10 min.

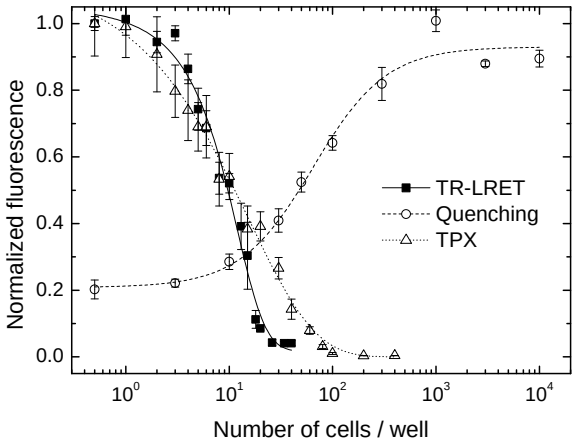


Figure 29. The CHO cell calibration curves measured with different particle assays: TR-LRET (particles 68 nm in diameter), quenching, and TPX (**II**,unpublished). The data was fitted to the Boltzmann (TR-LRET), logistic (quenching), or modified Weibull (TPX) function (Seber & Wild, 1989).

Table 6. Characteristics of the developed methods for the counting of cells.

	Assay		
	Quenching	TR-LRET	TPX
1. reagent Particle	20 nm Au	68 nm Eu(III) CM-PS	3098 nm PS
2. reagent	BSA-BF530	γG-Alexa680	γG-Alexa532
Assay format	Homogeneous Reagent limited	Homogeneous Reagent limited	Homogeneous Reagent limited
Viable/total count	Total	Total	Total
Incubation time (min)	10	10	40
Dynamic range (cells)	4-300	5-30	3-200
Sample volume (µL)	70	70	20
CV (%)	6	12	15
Plate	96-well	96-well	384-well
Publications	**II**	**II**	unpublished

abbreviations: CV = coefficient of variation
CM = carboxylate-modified PS = polystyrene

5.3 CELL VIABILITY TEST (II)

The cell viability test was developed by combining the method for the counting of total cell numbers based on time-resolved luminescence resonance energy transfer (TR-LRET) detection with a known dead cell count method, a nucleic acid (DNA) staining with a fluorescent membrane-impermeable DNA dye (Figure 30). The principle of the TR-LRET method is presented in Figure 25 (Section 4.2.1). The PicoGreen dye is non-fluorescent in the absence of DNA. The fluorescence is increased when bound to double stranded DNA. PicoGreen is membrane-impermeable and thus, it can only pass into the cells with compromised membranes.

The purpose was to develop a simple viability test, in which both total and dead cell count are performed simultaneously from a single cell sample. The combination of the counting of the total number of cells with the TPX method and the PicoGreen dead cell count would have needed two measurements with different instruments. Instead, the total count with the quenching method could not be performed at physiological pH due to the low adsorption of the labeled protein, BSA-BF530. The TR-LRET method as a total count provided the simplest solution, as the labeled protein γG-Alexa680 adsorbed onto the Eu(III) polystyrene particles also at physiological pH and the total count could be performed in PBS. In the viability test, PicoGreen dye, Eu(III) particles, and γG-Alexa680 were added to the cell suspension sample in PBS and the luminescence signals were measured in a single well after mixing all of the assay components. The luminescence signal of TR-LRET was related to the total cell count and the fluorescence from PicoGreen to the dead cell count. Furthermore, the Eu(III) chelate/Alexa680 LRET pair (excitation 340 nm and emission 730 nm) and PicoGreen (excitation 485 nm and emission 535 nm) possess non-overlapping excitation and emission bands.

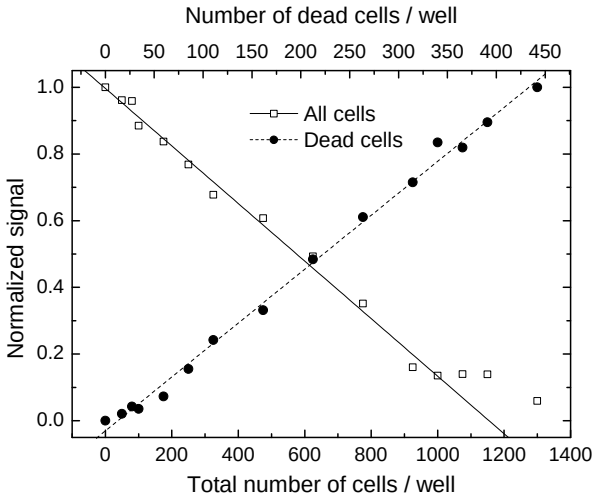


Figure 30. The calibration curves for the total number of CHO cells counted with the TR-LRET particle assay and for dead CHO cells counted with the cell-impermeable nucleic acid dye PicoGreen simultaneously in PBS (**II**). The curves are linear fits of the data. Reproduced in part with permission from Pihlasalo, S., Pellonperä, L., Martikkala, E., Hänninen, P. & Härmä, H. (2010) Sensitive Fluorometric Nanoparticle Assays for Cell Counting and Viability. *Anal. Chem.*, **82:** 9282–9288. Copyright 2010 American Chemical Society.

The TR-LRET total cell count method discussed in Section 5.2 was optimized for the highest sensitivity. However, the dynamic range from 5 to 30 cells in a sample is not suitable for the viability test when dead cells are counted from the same sample. With an increase in the number of assay components, the method can be readily extended to cover higher cell counts. The dynamic range was adjusted with the increased concentration of the LRET pair to match the sensitivity of the PicoGreen DNA dye method. The sensitivity of the total cell count was set to 100 cells. The combined total and dead cell count was demonstrated using the dilutions of CHO cell stock suspension with no pretreatment, such as cell lysis (Figure 30). The number of dead cells in the cell suspension was calibrated with the use of the trypan blue exclusion method and the percentage of dead cells in the cell stock was 34%. In the developed viability test, the detection limit of 90 dead cells was reached in the dead cell count with PicoGreen.

The number of cells required for the combined method is determined by the percentage of the dead cells in the sample and the desired precision. It is, however, comparable to the most sensitive commercial methods or methods found in the literature (Burghardt et al., 1994). The total and dead cell count in the developed viability test is performed from a single sample saving time and a precious cell sample. In contrast, many methods found in the literature or commercial tests, such as cell-impermeable DNA dyes applied for the counting of dead and total number of cells, require two separate tests: the total cell count in one well and the dead cell count in another well. The developed total cell count reached sensitivity as high as 5 cells in a sample. Therefore, the dead cell count with the DNA dye was the main sensitivity limiting factor for the combined viability test. A viability test requiring a lower number of cells may potentially be developed, if the total cell count method is combined to a more sensitive method, such as the measurement of

ATP from viable cells. However, the known viability tests generally are experimentally more difficult than the developed method. The use of dye exclusion, dye inclusion, and dyes determining physiological state and the counting of the cells with a flow cytometer, light microscope, fluorescence microscope, and automated cell counters are laborious and/or require expensive instruments. Suitable membrane permeable DNA dyes (Frey, 1995; Invitrogen Reduced Biohazard, 2001; Luther & Karnentsky, 1996; MBL Live-Dead) or probes determining physiological state (Coder, 1997; Invitrogen Viability/ Cytotoxicity, 2005) have been combined with impermeable DNA dyes to develop viability tests in a microtiter plate. The method detecting viable and dead cell proteases using fluorescent substrates is also commercially available (Niles et al., 2007; Promega MultiTox-Fluor, 2010). However, the known methods require generally from several thousand to hundreds of thousands of cells. Furthermore, the probes determining physiological state may need a long incubation to reach high sensitivity. Instead, each membrane permeable DNA dye is specific to certain cell types. The DNA dyes are also more or less carcinogenic or mutagenic (Cell Viability, 2008).

5.4 Method for Detection of Aggregation of Protein (III)

The method to detect the aggregation of protein was developed with the TR-LRET Eu(III) nanoparticle method (Figure 25 in Section 4.2.1) used for the quantification of proteins and the counting of cells in the previous sections. The method is based on the adsorption competition between sample species and labeled protein and thus, measures changes in the sample. The non-aggregated proteins adsorb efficiently to the surface of the particles and reduce the adsorption of labeled protein leading to the decrease of the TR-LRET signal. If the protein concentration is kept constant, the aggregation of protein leads to the decreased occupancy of the particle surface and an increased TR-LRET signal. The explanation for the decreased occupancy of the particle surface may be, for instance, the increased size (leaving more empty space on the particle), reduced adsorption, or the decreased adsorption rate of the sample species.

The method was demonstrated with heat-aggregated BSA. A solution containing 10 g/L of BSA in 100 mM tris(hydroxymethyl)aminomethane, pH 8, was heated at 100 °C up to 45 min. Samples with different heating times were studied with the developed TR-LRET nanoparticle method, and method comparisons were performed with absorbance measurements at 240 nm (UV240) and dynamic light scattering (DLS). In the TR-LRET method, protein aggregate samples, diluted to the concentration of 30 µg/L BSA in a 5 mM glycine buffer, pH 3.0, were mixed with Eu(III) nanoparticles and γG-Alexa680 in a microtiter plate well, and time-resolved luminescence was detected with a plate reader. The UV240 absorbance was measured at a BSA concentration of 1.0 g/L and DLS was applied at BSA concentrations ranging from 0.10-10 g/L depending on the size of the aggregate.

Calibration curves of BSA and its aggregates were measured for each sample with the TR-LRET method. At a constant BSA concentration, the aggregation of protein led to a less efficient surface coverage and the TR-LRET signal increased. Thus, the particle surface saturation and calibration curve were shifted to a higher total BSA concentration. Both high sensitivity and wide dynamic range for the detection of aggregation was observed at a BSA concentration of 30 µg/L. These signals were correlated to the average size of the aggregate measured with DLS to find out the measurable aggregate size range of the TR-LRET method (Figure 31). The dynamic range of the DLS instrument was

from 10 to 3000 nm covering the size range of the studied aggregates. Therefore, single BSA molecules with the dimensions of 3.4×14 nm^2 (Champagne et al., 1958) are at the limit of the technology. The TR-LRET and UV240 methods detected a similar size range of aggregates from approximately 10 to 1000 nm and are equally simple to perform. However, more than 10,000 times lower optimal protein concentrations for TR-LRET compared to UV240 and DLS, indicates the high potential of the nanoparticle concept for aggregation studies at very low concentrations. Existing methods for studying the aggregation of protein, DLS (Demeester et al., 2005; Schmitz, 1990), size-exclusion chromatography (Roufik et al., 2005), analytical ultracentrifugation (Butler & Kühlbrandt, 1988; Casassa & Eisenberg, 1964), ellipsometry (Ortega-Vinuesa et al., 1998; Santos et al., 2006), atomic force microscopy (Santos et al., 2006), and optical waveguide lightmode spectroscopy (Ramsden & Prenosil, 1994) require high sample concentrations preventing their routine use, as high protein concentrations are not readily available. The last three methods are useful mainly for protein adsorption studies, but may also provide information on the protein surface-aggregation process. Furthermore, many of these methods require trained personnel with specific hands-on skills and expensive instrumentation, are time-consuming, and have low-throughput. Commercially available high-throughput microtiter plate assays, such as ThT, ProteoStat, and PSA200K (BMG LABTECH FLUOstar, 2005; Enzo Proteostat, 2010; ProFoldin PSA200K), require also high protein concentrations ranging from 10 to 1000 mg/L and are sensitive to the protein type. TR-LRET is not highly dependent on the surface characteristics of proteins or protein aggregates due to the nonspecific adsorption (Pihlasalo et al., 2009; Valanne et al., 2009). It also has a rapid and simple, mix-and-measure protocol, which makes it amenable to high-throughput assay formats.

The quantification of proteins and the detection of the aggregation of protein with the same TR-LRET assay principle, make the method versatile, as the same simple system can be used for two or even more different purposes. However, the detection of the aggregation is a disadvantage in the quantification of protein, as the total protein concentration is underestimated, if the analyte proteins in the sample are highly aggregated. The assays developed in this work are not exceptional. To my knowledge, the effect of aggregation on the quantification of proteins for literature and commercial methods has not been extensively studied. The aggregation of proteins is supposed to have similar problems also with the available methods for the quantification of protein. Seemingly, in addition to the UV absorbance methods, assays utilizing the binding of the dye to the proteins or detergent-coated proteins are disturbed from the aggregation, because the dyes cannot bind as efficiently onto the protein aggregates as onto the single protein molecules. Most probably the proteins to be quantified are not highly aggregated, as the non-aggregated proteins are preferred by the end-users and thus, the problem is insignificant.

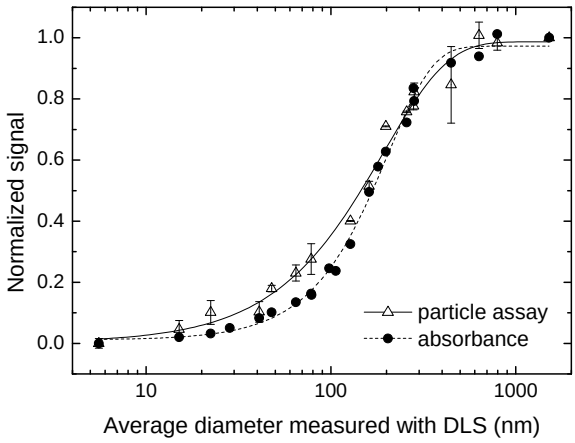


Figure 31. Detection of heat-aggregated bovine serum albumin (BSA) using the TR-LRET assay and UV240 absorbance: background subtracted normalized time-resolved luminescence (30 µg/L BSA for particle assay) or absorbance signals (1.0 g/L BSA for absorption) as a function of the average aggregate diameter measured with dynamic light scattering (0.10-10 g/L BSA for DLS) (**III**). The data was fitted to the type 2 sigmoidal Weibull function (Seber & Wild, 1989). Reproduced in part with permission from Pihlasalo, S., Kirjavainen, J., Hänninen, P. & Härmä, H. (2011) High Sensitivity Luminescence Nanoparticle Assay for the Detection of Protein Aggregation. *Anal. Chem.*, **83:** 1163–1166. Copyright 2011 American Chemical Society.

6 SUMMARY AND CONCLUSIONS

Several nonspecific and luminometric nano- and microparticle-based methods were developed. The developed methods had advantages over the existing methods in sensitivity, dynamic range, simplicity, speed, and costs. The work conducted for this thesis together with other methods developed in the Laboratory of Biophysics show the versatility of the principle. These particle-based methods with varying principles can be used to detect the aggregation of protein and measure the concentration of protein, cells, detergent (Härmä et al., 2010a; Härmä et al., 2010b), and other small molecules (unpublished data). Moreover, other principles of the methods and applications, such as charge and size analysis, exploiting particles and luminescence, are under development in the Laboratory of Biophysics, further increasing the versatility of this simple methodology.

The summaries and conclusions of the methods developed in this thesis are:

Methods for Quantification of Total Protein (**I**,**III**,**IV**,unpublished)

Four different particle-based methods (fluorescence quenching, TR-LRET, TPX, and the dissociation of the lanthanide ion) were developed for the quantification of proteins. These methods have extremely simple mix-and-measure concepts in a high-throughput microtiter plate format. None of the methods require heating and subsequent cooling steps to reach high sensitivity like the available commercial NanoOrange (Jones et al., 2003), BCA (Smith et al., 1985), and CBQCA (You et al., 1997) methods. All the developed methods are more sensitive than any of the commercial ones. Depending on the method, the sensitivity is 40-500 pg BSA in a sample. This sensitivity range is 20-500-fold better than in most sensitive commercial OPA (Pierce Fluoraldehyde OPA, 2009), CBQCA (You et al., 1997), and NanoOrange (Jones et al., 2003) methods, and 300-4000-fold better than in the well-known Bradford method (Pierce Protein Handbook, 2009). A high particle surface area together with a high signal-to-background ratio enables a linear relationship at logarithmic scales and wide dynamic range of approximately four orders of magnitude for the TPX method and approximately six orders of magnitude for the TR-LRET method. The protein-to-protein variability of 29% was measured for the dissociation method and 15% for the quenching method. These variabilities are similar or lower compared to the available methods having protein-to-protein variabilities between 10 and 50% (Jones et al., 2003; Pierce Protein Handbook, 2009; Smith et al., 1985; You et al., 1997). Mainly electrostatic interaction was responsible for the efficiency of the adsorption of proteins, and the low protein-to-protein variability was achieved at a low pH, where most of the proteins have a positive total charge and the particles a negative surface charge, favoring an attractive interaction. The method based on the nonspecific adsorption of proteins is advantageous in contrast to methods relying on, e.g., specific identification of protein chemical groups (Liu et al., 1991; You et al., 1997), or the interaction of the protein surface with dye (Bradford, 1976; Lowry et al., 1951; Peterson, 1977; Smith et al., 1985) or detergent molecules (Jones et al., 2003; Lee et al., 2003). As a consequence, the low protein-to-protein variability potentially results in a more reliable quantification of unknown proteins or protein mixtures without a need for the calibration of each protein. The contaminants, such as non-ionic detergents, salt, and agent containing thiol (dithiothreitol), commonly found in biological samples, were well tolerated by the TPX method and non-ionic detergents, chelating agent (EDTA), organic

solvents (DMF and ethanol), polymer (PEG 3000), agent containing thiol (2-mercaptoethanol), and reducing agent/sugar glucose by the dissociation method. The TPX method requires an instrument, which is not widely available for the end-users. Therefore, single-photon excitation luminescence methods, such as quenching, TR-LRET, and dissociation methods find a potentially larger acceptance. Furthermore, the quenching method with conventional fluorescence detection is more applicable to end-users, compared to the TR-LRET and dissociation methods, as plate fluorometers without the time-gating mode are more commonly in use. Thus, further development of the dissociation-based system could provide a new method, which exploits a dye for conventional fluorometry, tolerates well assay contaminants, and has high sensitivity.

Methods for Counting of Total Cell Numbers (**II**,unpublished)

Three different particle-based methods (quenching, TR-LRET, and TPX) were developed for the counting of cells. The developed particle assays measure the total number of cells, unlike methods measuring ATP-production or mitochondrial reductase or intracellular esterase exploiting cellular metabolic activity. The developed methods are experimentally easy to conduct, as they are homogeneous, have simple mix-and-measure procedures, and contain short incubation steps at RT. The results are gained much faster compared to the traditional hemacytometric counting under the light microscope, as the protocols are simple with short incubations allowing high-throughput. All methods have the limit of detection clearly below 10 cells in a sample. The developed methods are typically more sensitive than the commercial assays or methods found in the literature. Only two microtiter plate type methods have similar sensitivities. The detection of ATP (Campbell, 1988) and the glyceraldehyde-3-phosphate dehydrogenase activity (Corey et al., 1997) are capable of detecting less than 10 cells. The dynamic ranges approximately one or two orders of magnitude for the developed methods are similar to those of the available methods. The simple and sensitive methods enable work with low sample concentrations, saving time, costs, and precious cell samples.

Cell Viability Test (**II**)

The cell viability test was developed by successfully combining the TR-LRET total cell count method with a DNA staining method using a membrane-impermeable PicoGreen dye. This test is also based on a simple mix-and-measure procedure. The test measures both the total and dead number of cells for low cell counts from a single sample, providing savings in sample, time, and assay components. Not only the counting of cells but also the assessment of cell viability is important in selecting proper cell culture conditions, different cell-based experiments, cell-based research, and medicine. The developed test provides a new, sensitive, and rapid method to determine both simultaneously. However, an even more sensitive test may eventually be developed in the future, if the total cell count method is combined with a more sensitive dead or viable cell count method than the PicoGreen staining.

Method for Detection of Aggregation of Protein (**III**)

The method for detecting the aggregation of protein was developed by utilizing the TR-LRET method for the quantification of proteins. The method has several advantages over the existing methods. Due to the high sensitivity the developed method is applicable at the protein concentration of micrograms per liter level. The requirement for high sample

concentrations often prevents the use of the existing methods, such as DLS (Demeester et al., 2005; Schmitz, 1990), size-exclusion chromatography (Roufik et al., 2005), analytical ultracentrifugation (Butler & Kühlbrandt, 1988; Casassa & Eisenberg, 1964), and UV/VIS spectroscopy (Kelner et al., 2003), as high protein concentrations may not be available. DLS (Demeester et al., 2005; Schmitz, 1990), size-exclusion chromatography (Roufik et al., 2005), and analytical ultracentrifugation (Butler & Kühlbrandt, 1988; Casassa & Eisenberg, 1964) also require expensive instrumentation and trained personnel. Commercially available high-throughput microtiter plate assays also require high protein concentrations ranging from 10 to 1000 mg/L and are sensitive to the protein type (BMG LABTECH FLUOstar, 2005; Enzo Proteostat, 2010; ProFoldin PSA200K). However, the developed method does not need special hands-on skills, is not highly dependent on the surface characteristics of proteins or protein aggregates due to nonspecific adsorption (Pihlasalo et al., 2009; Valanne et al., 2009), and is amenable to a high-throughput format due to its rapid and simple mix-and-measure protocol. It detected protein aggregates at low protein concentrations in a size range from approximately 10 to 1000 nm, which suggests that the method could be applicable, for instance, in the quality assessment of protein products in the industry.

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